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Identification polypeptide biomarkers of porcine skin gelatin by two-dimensional electrophoresis

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Abstract

The peptide composition of gelatin is known to vary very common that the electrophoretic pattern of gelatin from one source differs from another source even for the same raw material. Therefore, the present study aimed to use proteomics field to identify gelatin polypeptides biomarker for depending on the condition under which collagen is hydrolyzed. Hence, it is porcine skins. The polypeptides obtained for porcine skin gelatins can be used as reference in future to detect the origins of gelatin added in the processed food. We compared porcine skin gelatin samples obtained from three producers. Total average numbers of polypeptides of porcine skin gelatins from company A, B and C were 303 ± 2.8, 285.5 ± 3.5 and 270.5 ± 4.9 spots respectively. 10 biomarkers were identified and presented in all different companies. We also did a mixture of porcine and bovine skin gelatin to detect the presence of these 10 biomarkers. The level of adulteration that could be detected was as low as 1.0% w/w.

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Introduction

Gelatin is practically been applied in all areas of modern life. Gelatin is the primary product commonly used for quality improvement of foodstuffs and medicaments. In the food industry, gelatin can be found in many products like yogurt, ice cream, desserts and sweets (gummy bears and marshmallows). The pharmaceutical companies use gelatin in soft and hard capsules, for binding in tablets, for treating wounds and as colloid to expand plasma after severe blood losses (Venien and Levieux, 2005).

Gelatin is a high molecular weight polypeptide derived from collagen, the primary protein component of animal connective tissues. The essential constituent of gelatin is protein. The protein content is between 85 and 92% and the remainder being mineral salts and moisture (Schrieber and Gareis, 2007). Gelatin is obtained by partial hydrolysis of the collagen contained in bones and hides mainly from bovine and/or skins from pigs (Venien *et al*., 2005). It can be derived from both acid-treated and alkali-treated process which known as type A and type B respectively. The elevation of aspartic and glutamic acids resulting from deamidation of asparagine and glutamine respectively are caused by alkaline pre-treatment as well as acid pre-treatment, to a lesser extent (Eysturskar *et al*., 2009); Gómez-Guillén, *et al*., 2011) . Thus, type B gelatin will have lower isoelectric points (p*I*) which is between pH 4.7 and 5.6 compared to type A gelatin which is between pH 6 and 9.5 (Schrieber *et al*., 2007).

Two-dimensional electrophoresis (2-DE) is a powerful separation technique, which allows simultaneous resolution of thousands of proteins and the greatest strength of 2-DE is its ability to spot proteins that have undergone post-translational modifications(Beranova-Giorgianni,2003;Rabilloud, 2002). The protein analysis of food and dairy products has been established for several years with discovery of many protein biomarkers. It seems the separation of gelatin polypeptides mixture according to molecular weight (*M*r) and isoelectric point (p*I*) offers possibility to exhibit differences between different gelatin origins. Together with PDQuest software, prominent spots can be identified for each gel of an experiment (Rosengren *et al*., 2003). The experimental data can also be analyzed statistically. Therefore, 2-DE coupled with PDQuest software can be an effective method to characterize complex proteins patterns of gelatin from different sources. These protein profiles can be signatures of different sources of gelatin.

Despite to the wide field of gelatin applications, there are restrictions with regard to consumption of gelatin from certain sources. Some countries with total majority of Muslims and Jews are prohibited the usage of porcine origin due to religious

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concerns. Nowadays, most of available gelatins are manufactured from mammalian resources (porcine and bovine). Furthermore, the gelatin origin of ingredients in processed foods is not always readily apparent. Thus, the present study has established a method of 2-DE to evaluate the polypeptides pattern of gelatin from porcine origins. To the best of our knowledge, there are no studies have been reported on gelatin utilizing 2-DE. Previous studies reported mainly on polypeptide patterns of muscle meat, sesame seed, leaf, root (Han and Wang, 2008; Carbonaro, 2004). Therefore, it is useful to establish methods to identify specific biomarker(s) for porcine gelatin. The objective of this study was to apply 2-DE to identify specific biomarkers for porcine skin gelatin and the percentage of detection limit. The potential biomarkers obtained for pig skin gelatin will be used as references in future to identify the presence of pig skin gelatin in any products.

Materials and Methods

Materials

Porcine skin gelatin (Type A gelatin) was purchased from 3 different companies; Sigma-Aldrich (St. Louis, MO, USA) (PSS), Fluka Chemie GmbH, Buchs (PSF) and PB Gelatin Vilvoorde, België-Belgique (PSP). Bovine skin gelatin (Type A gelatin) was purchased from Restar Gelatin (Xiamen) Co. Ltd. China. Ready immobiline pH gradient (IPG) strip (7cm, linear, pH 3-10), urea, 1.5M Tris-HCl buffer pH 8.8, bio-lyte 3/10 ampholytes and dithiothreitol (DTT) were purchased from Biorad (Hercules, CA, USA). 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), acetone, iodoacetamide and FAST-Silver Staining kit were purchased from Merck (Darmstadt, Germany). Thiourea and bromophenol blue were purchased from GE Healthcare (Buckinghamshire, United Kingdom). Glycerol was purchased from Wako Pure Chemical Industries, Ltd (Tokyo, Japan) while ethanol (absolute) and glacial acetic acid were from RCI Labscan Ltd (Bangkok, Thailand) and Fisher Scientific (Leicestershire, United Kingdom) respectively.

Preparation of samples

Protein from porcine skin gelatin (PSG) was extracted by dissolving 15 mg of each sample gelatin in 0.5 ml of deionized water at 50°C for 1 hr. Then, 1 ml of extraction buffer (7M urea, 2M thiourea, 4% CHAPS, 2% bio-lyte 3/10 ampholytes, 20 mM DTT) was added to extract the dissolved gelatin. The samples were agitated at 2000 rpm for 10 min at room temperature and 100 µl samples were aliquots and stored in at -80°C.

100 µl samples were subjected to precipitation using acetone precipitation. 300 µl of acetone (pre-chilled at -20°C for at least 1 hour) was added to 100 µl samples. The samples were vortexed and left overnight at -20°C. Then, the samples were centrifuged at 10,000 xg (Centrifuge 5810R, Eppendorf, Hamburg, Germany) at 4°C for 30 minutes. The resulting white pellet was then air-dried and solubilized in 200 µl of 7 M urea, 2 M thiourea, 4% CHAPS, 0.5% bio-lyte 3/10 ampholytes and 20 mM DTT. The samples were vortexed and incubated at room temperature for 10 min. The samples were then centrifuged at 12,000 xg for 5 min prior to rehydration or stored at -80°C for further analysis.

The protein content was quantified using the modified Lowry protein assay (Lowry, Rosebrough, Farr & Randall, 1951); reducing agent and detergent compatible (RC-DC) protein assay (BioRad, Hercules, CA, USA) and used bovine serum albumin (BSA) as the standard.

Isoelectric focusing (IEF)

The first dimension was started with IEF using a Protean IEF system (Biorad, Hercules, CA, USA). IEF was performed according to (Görg et al., 2000) with slight modifications. IPG strips were loaded with 125 µl samples and actively rehydrated for 16 h at 50 V. Subsequently, IEF was carried out according to the following program: 150 V for 1 h, 300 V for 1 h, 4000 V for 2 h and 4000 V for 20 000 Vh. Each IPG strips were equilibrated with 2.5 ml equilibration buffer (6M urea, 2% SDS, 50 mM Tris-HCl pH 8.8, 20% glycerol, 2% DTT) for 15 min at room temperature. Then, the IPG strips were then alkylated with 2.5 ml equilibration buffer (6M urea, 2% SDS, 50 mM Tris-HCl pH 8.8, 20% glycerol, 2.5% iodoacetamide) for 15 min.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

12% polyacrylamide gel was made based on 10 ml of gel solution formulation (Bio-Rad Laboratories, Hercules, CA, USA). The IPG strip was positioned vertically on top of the gel. The strip was fixed in place with 0.5% w/v agarose overlay (Bio-Rad Laboratories, Hercules, CA, USA). Gel was run with running buffer (25 mM Tris pH 8.3, 192 mM glycine, 0.1% w/v SDS) using a Mini-PROTEAN® Tetra system (Bio-Rad Laboratories, Hercules, CA, USA) at 10 mA for 15 min and then 20 mA until the bromophenol blue dye reached the bottom of the gel.

Image analysis

Protein spots were stained by FASTsilver™ Gel Staining Kit (Calbiochem Merck, Darmstadt, Germany). Briefly, the gel was fixed in freshly prepared 30% ethanol containing 10% acetic acid for 10 min. Subsequently, the gel was washed twice for 10 min with 10% ethanol. Then the gel was again washed 3 x 10 min with highly purified water. It was soaked in sensitizer for 30 min and subsequently in developer until spots was visible. The development of spots was stopped by soaking in 22% acetic acid for 10 min.

Stained gels were scanned on Densitometer GS 800 (Bio-Rad Laboratories, Hercules, CA, USA). The spots were analyzed using PD Quest Basic software (Bio-Rad Laboratories, Hercules, CA, USA). The analysis involves spots detection, background subtraction, estimation of approximate molecular weight, removal of artifacts (e.g. horizontal and vertical streaks) and detection of spots using Gaussian model.

Adulteration

Experimental samples of raw gelatin were prepared by adding porcine skin gelatin type A (PSS), in a proportion ranging from 5 to 1% (v/v), to bovine skin gelatin type A (BSG) (Table 1). These sample mixture were then subjected to 2-DE.

Table 1. Proportion of porcine gelatin in bovine gelatin

Porcine gelatin (%)	Bovine gelatin (%)
5	95
4	96
3	97
2	98
1	99

Results and Discussion

Potential biomarker of porcine skin gelatins

A characteristic feature of the image obtained was the presence of more than 260 visible protein spots in the region corresponding pI in slightly acidic and basic. 2-DE manages to resolve 303 ± 2.8 spots in PSS, 285.5 ± 3.5 spots in PSF and 270.5 ± 4.9 spots in PSP. The porcine skin gelatins for all three different companies had pI ranged from pH 5 until 8 (Figure 1). This result is in line with the previous report (Schrieber *et al*., 2007) where the pI of acid processed gelatin was between pH 5 and 9.5.

2-DE technique that has been applied for detection of gelatin origin constitutes a considerable challenge. Sample preparation is one of the most crucial steps for high resolution of proteins separation in a 2-DE gel and it is expected to be difficult due to the presence of salts, minerals and other interfering compounds which come from during the processed of gelatin (Schrieber *et al*., 2007; Isola *et al.*, 2011). To overcome this problem, establishment of 2-DE run conditions was accomplished in order to obtain gels with the highest resolution, separation of spots proteins and the highest reproducibility.

Therefore, based on our preliminary studies (data not shown), extraction of protein from gelatin using an extraction buffer followed by protein precipitation using cold acetone gave the best resolution so far for the studied gelatin. However, the quality of gel images relatively poor due to 2-DE intense of horizontal streaking especially at the alkaline region. This streaking is might be due to the depletion of reducing agent in the basic part of the IPG strip and cause formation of intra- and intermolecular disulphide bonds (Acín *et al*., 2009; Bai *et al*., 2005). Furthermore, it also can be caused by water transport to the anode due to a reverse electroendosmotic flow (Cortón *et al.*, 2004). Nevertheless, clear distinct spots can be observed in all gels.

PD-Quest software specifically recognized 10 dominant proteins spots (Table 2) which are consistently present in PSS, PSF and PSB with *M*r and p*I* of approximately 52.3 kDa; 5.0 (spot 1), 52.15 kDa; 5.1 (spot 2), 48.2 kDa; 7.2 (spot 3), 46.6 kDa; 8.4 (spot 4), 41.2 kDa; 5.2 (spot 5), 39.1 kDa; 6.1 (spot 6), 24.08 kDa; 6.0 (spot 7), 15.91 kDa; 6.0 (spot 8), 15.8 kDa; 6.3 (spot 9) and 16.0 kDa; 6.5 (spot 10). These 10 selected protein spots can be used as biomarker to detect the presence of porcine gelatin (Figure 1).

Table 2. The intensity, molecular weight and isoelectric point of 10 proteins spots whih present in PSS, PSF and PSB

Spot	[a]*Mr*	[b]p*I*	[c]PSS	[d]PSF	[e]PSP
1	52.30	5.0	83.40	126.45	110.75
2	52.15	5.1	56.30	103.20	65.55
3	48.20	7.2	232.10	262.55	315.45
4	46.60	8.4	615.90	488.40	403.90
5	41.20	5.2	83.40	126.45	110.75
6	39.10	6.1	303.65	290.45	236.30
7	24.08	6.0	64.80	85.30	76.65
8	15.91	6.0	307.35	335.95	301.50
9	15.80	6.3	112.15	111.35	82.05
10	16.00	6.5	118.55	198.55	145.70

[a] Molecular weight
[b] Isoelectric point
[c] Porcine skin gelatin from sigma
[d] Porcine skin gelatin from Fluka
[e] Porcine skin gelatin from PB Gelatin

Adulteration

Pure versus adulterated samples were compared to see the presence of 10 selected protein spots earlier. Even with thorough observations of each gel, it was neither easily nor clearly differentiated, especially when the percentage of adulterated porcine gelatin

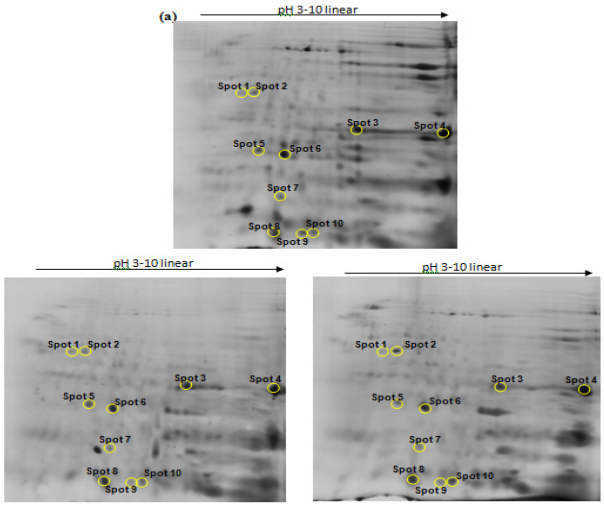

Figure 1. 2-DE gel images of 10 protein spots which present in (a) PSS, (b) PSB and (c) PSF

was too low. It was due to the gel was not reproducible caused by the local geometrical distortion of gel which can lead to the incorrect matching of the protein spots between gels (Jones *et al*, 2004; Aittokallio *et al.*, 2005). However, to overcome this problem, we used PDQuest software to make landmark on some of dominant spots in all gels.

The intensity of protein spots were measured based on the pixel intensity within a given protein spot minus the most frequently occurring pixel intensity of the background intensity (Phongpa-Ngan *et al.*, 2011). There were differences in the intensities of ten biomarker spots between samples with different level of adulteration (Figure 2).

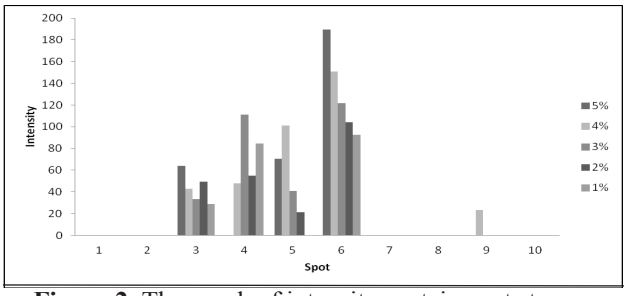

Figure 2. The graph of intensity protein spots to see the presence of any 10 protein biomarkers in 5 to 1% adulteration of pig skin gelatin

However, spot 1, 2, 7, 8 and 10 were not present in all gels. Spot 4 appeared in all level of adulteration and absent in 5% of adulteration while spot 5 appeared until 2% of adulteration. Spot 9 only present in 4% of adulteration. The presence and absentee of protein spots at certain level of adulteration could be due to variation of 2-DE gel experiments that affect the measured protein levels. The variation could be caused from sample preparation, sample loading (pipetting errors, sample loss during gel loading), inconsistent staining time and inconsistent exposure times during scanning (Nishihara and Champion, 2002). However, we had performed normalization step to minimize the some experimental variations.

Conclusion

The study exhibited 10 biomarker gelatin polypeptides of porcine skin were successfully identified by 2-DE. This is first work in applying 2-DE on commercial gelatins. Based on the present study, extraction of protein from gelatin using an extraction buffer followed by protein precipitation using cold acetone gave high resolution of proteins separation in 2-DE gel. In addition 2-DE method has the ability to detect as low as 1% level of adulteration in gelatin samples. The data represented can serve as basis in identifying the sources of gelatin for authentication purposes.

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