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Analytical Methods

Halal authenticity of gelatin using species-specific PCR

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ABSTRACT

Consumption of food products derived from porcine sources is strictly prohibited in Islam. Gelatin, mostly derived from bovine and porcine sources, has many applications in the food and pharmaceutical industries. To ensure that food products comply with halal regulations, development of valid and reliable analytical methods is very much required. In this study, a species-specific polymerase chain reaction (PCR) assay using conserved regions of mitochondrial DNA (cytochrome b gene) was performed to evaluate the halal authenticity of gelatin. After isolation of DNA from gelatin powders with known origin, conventional PCR using species-specific primers was carried out on the extracted DNA. The amplified expected PCR products of 212 and 271 bp were observed for porcine and bovine gelatin, respectively. The sensitivity of the method was tested on binary gelatin mixtures containing 0.1%, 1%, 10%, and 100% (w/w) of porcine gelatin within bovine gelatin and vice versa. Although most of the DNA is degraded due to the severe processing steps of gelatin production, the minimum level of 0.1% w/w of both porcine and bovine gelatin was detected. Moreover, eight food products labeled as containing bovine gelatin and eight capsule shells were subjected to PCR examination. The results showed that all samples contained bovine gelatin, and the absence of porcine gelatin was verified. This method of species authenticity is very useful to verify whether gelatin and gelatin-containing food products are derived from halal ingredients.

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1. Introduction

At present, gelatin is a very popular ingredient in various food and pharmaceutical products. Gelatin is, by far, most widely used in confectionery products and pharmaceutical capsules around the world (Schrieber & Gareis, 2007). Gelatin is a mixture of polypeptides obtained by the partial hydrolysis of collagen (Cheng et al., 2012). In the large-scale manufacture of gelatin, the primary raw material used is the collagen found in cattle and pig (Schrieber & Gareis, 2007). Commercial forms of gelatin such as sheets, granules, or powders are mainly sourced from bovine bone and hides, pig skin, and, more recently, pig bone (Hermanto & Fatimah, 2013; Jaswir, Mirghani, Mohd Salleh, Hassan, & Yaakob, 2009). Other sources such as fish and poultry are very new and are mostly processed to cater to specific religious consumer groups (Gómez-Estaca, Montero, Fernández-Martín, & Gómez-Guillén, 2009; Karim & Bhat, 2009).

In Islam, consumption of products considered non-halal, such as those containing porcine gelatin, is strictly forbidden (Widyaninggar, Triyana, & Rohman, 2012). Some Islamic countries have established strict regulations for producers and importers to stamp their products with a halal certificate to distinguish them from non-halal products (Hermanto & Fatimah, 2013). In this regard, development of reliable and sensitive analytical methods for evaluating halal authenticity is very critical. Methods that rely on protein analysis or physicochemical properties, such as chemical precipitation (Hidaka & Liu, 2003), Fourier transform infrared spectroscopy (Hashim et al., 2010), electrophoretic analysis (Hermanto & Fatimah, 2013), high-performance liquid chromatography (Nemati, Oveisi, Abdollahi, & Sabzevari, 2004), mass-spectrometer detection (Zhang et al., 2009), and enzyme-linked immunosorbent assay (Venien & Levieux, 2005), have been applied to differentiate bovine gelatin from porcine gelatin. These methods proved to be unsuitable for differentiating a mixture of gelatin as well identifying the origin of gelatin in food products. This is mainly because of the similarities in structure and physicochemical properties of gelatin derived from different sources. Moreover, the presence of highly degraded proteins due to extreme

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Table 1
The characteristics of bovine and porcine primers.

Species	Primer sequences 5′–3′	Product size	Genes	Ann. temp. (°C)	Accession number
Bovine	5′-GCCATATACTCTCCTTGGTGACA-3′ 5′-GTAGGCTTGGGAATAGTACGA-3′	271 bp	Cyt b	58	J01394
Porcine	5′-GCCTAAATCTCCCCTCAATGGTA-3′ 5′-ATGAAAGAGGCAAATAGATTTTCG-3′	212 bp	Cyt b	58	AF039170

temperature and pH treatments during gelatin production render these methods almost unreliable (Tasara, Schumacher, & Stephan, 2005).

Polymerase chain reaction (PCR) can be used as an alternative method for rapid and sensitive detection of porcine DNA in gelatin due to the higher stability of DNA compared to protein (Aida, Man, Yaakob, Raha, & Son, 2007). Earlier reports have also described PCR using species-specific primers in highly processed food products (Calvo, Zaragoza, & Osta, 2001; Ghovvati, Nassiri, Mirhoseini, Moussavi, & Javadmanesh, 2009; Hidaka & Liu, 2003; Jia-qin et al., 2008; Murugaiah, Mustakim, Mohd Noor, & Radu, 2010). There are a limited number of reports available on animal species-specific identification of gelatin, gelatin-containing food, and pharmaceutical products using PCR techniques (Cai, Gu, Scanlan, Ramatlapeng, & Lively, 2012; Demirhan, Ulca, & Senyuva, 2012; Tasara et al., 2005). Real-time PCR is sensitive and specific enough to trace small amounts of target DNA; hence, it is successfully applied in the detection of bovine and porcine DNA in gelatin mixtures, gelatin-containing food products, and capsule shells. However, due to the high cost of real-time equipment and reagents, not all laboratories are able to apply this method. Conventional PCR using species-specific PCR has been shown to be sensitive in the detection of 0.1% of bovine gelatin in porcine gelatin, but it has not been employed in terms of identifying the animal origin of the gelatin used in food and pharmaceutical products.

The present study aimed to perform conventional PCR using species-specific primers for identifying the animal origin of gelatin powders and to investigate the halal authenticity of some gelatin-containing food products and capsule shells.

2. Material and methods

2.1. Sample preparation

Gelatin samples of known species origin, bovine and porcine, were received in powder form from the Food and Drug Organization of Iran and used as reference standards. Binary gelatin mixtures were prepared by adding 0.1%, 1%, 10%, and 100% (w/w) of porcine gelatin into bovine gelatin and vice versa. A total of 16 samples were prepared for the present study. Eight pharmaceutical capsule shells of unknown species origin were collected from pharmacies in Tehran. Food products labeled as containing bovine gelatin including two marshmallows (imported from Turkey), two jellies, two desserts, and two cakes were purchased from supermarkets in Tehran.

2.2. DNA extraction

DNA was extracted from pure gelatin powders, capsule shells, and gelatin-containing food products, using a DNeasy mericon Food Kit (Qiagen, Hilden, Germany), Small Fragment Protocol (2 g), recommended for highly processed foods. Initially, 10 ml of lysis buffer and 25 μl of proteinase K (20 mg) solution were added to 2 g of the sample, vortexed briefly, and incubated for 30 min at 60 °C in a water bath (Memmert, Schwabach, Germany) with constant shaking. After incubation, the solution was cooled to room temperature (15–25 °C) on ice and then centrifuged (Eppendorf, Hamburg, Germany) for 5 min at 2500×*g*. Subsequently, 700 μl of the clear supernatant was transferred to a microcentrifuge tube containing 500 μl of chloroform, vortexed vigorously for 15 s, and centrifuged at 14,000×*g* for 15 min. After centrifugation, 250 μl of the upper aqueous phase was added into a fresh microcentrifuge tube containing 1 ml of binding buffer and mixed thoroughly by vortexing. Then, 600 μl of the mixture was pipetted into a spin column placed in a collection tube, centrifuged at 17,900×*g* for 1 min, and the flow-through was discarded. This step was repeated with the remaining sample. Afterwards, 500 μl of the wash buffer was added to the spin column, followed by centrifugation at 17,900×*g* for 1 min, and the flow-through was discarded. The collection tube was centrifuged again at 17,900×*g* for 1 min to dry the membrane. Finally, the spin column was transferred to a 1.5-ml fresh microcentrifuge tube, and 30 μl of elution buffer was added onto the membrane, incubated for 1 min at room temperature (15–25 °C), and then centrifuged at 17,900×g for 1 min to elute. The purified DNA solution (30 μl) was immediately used.

2.3. Species-specific primers and PCR amplification

The information about two sets of primers for bovine and porcine used for PCR amplification is listed in Table 1. These primers were published by Tartaglia et al. (1998) and Lahiff et al. (2001) for bovine and porcine DNA, respectively. PCR amplification was carried out using a master thermal cycler (Eppendorf, Germany). The 20-μl reaction mixture was prepared in a PCR tube with 2 μl of 10 × PCR buffer (CinnaGen Co, Tehran, Iran), 0.2 mM of deoxyribonucleoside triphosphates (dNTPs) (CinnaGen Co, Iran), 1.5 mM $MgCl_2$ (CinnaGen Co, Iran), 0.05 units of *Taq* DNA polymerase (CinnaGen Co, Iran), 0.5 μM each of forward and reverse primers listed in Table 1 (Eurofins MWG Operon, Germany), 10–100 ng of DNA template, and nuclease-free water to adjust the volume (CinnaGen Co, Iran).

The PCR program used for amplification of the target genes is as follows: initial denaturation at 94 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 40 s, and extension at 72 °C for 30 s. Then final extension was conducted at 72 °C for 5 min. The amplified products of PCR were analyzed using 2% agarose gel in TBE (Tris–borate–ethylenediaminetetraacetic acid) 0.5× buffer stained with DNA safe stain (CinnaGen Co, Iran) as a visualizing agent and run for 45 min at 100 V.

3. Results and discussion

In highly processed food products such as gelatin and gelatin-containing food and pharmaceutical products, DNA is degraded into short fragments. This issue caused some difficulties in PCR amplification as reported by earlier researchers (Mafra, Ferreira, & Oliveira, 2008; Martín et al., 2007). The essential prerequisite for PCR amplification is therefore obtaining sufficient template DNA for analysis. For this reason, DNA isolation was performed using a commercial kit under optimized column-binding conditions adjusted for maximal recovery of short DNA fragments.

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