

PCR-RFLP Using *BseDI* Enzyme for Pork Authentication in Sausage and Nugget Products

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ABSTRACT

A polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) using *BseDI* restriction enzyme had been applied for identifying the presence of pork in processed meat (beef sausage and chicken nugget) including before and after frying. Pork sample in various levels (1%, 3%, 5%, 10%, and 25 %) was prepared in a mixture with beef and chicken meats and processed for sausage and nugget. The primers CYTb1 and CYTb2 were designed in the mitochondrial cytochrome *b* (*cyt b*) gene and PCR successfully amplified fragments of 359 bp. To distinguish existence of porcine species, the amplified PCR products of mitochondrial DNA were cut by *BseDI* restriction enzyme. The result showed pig mitochondrial DNA was cut into 131 and 228 bp fragments. The PCR-RFLP species identification assay yielded excellent results for identification of porcine species. It is a potentially reliable technique for pork detection in animal food processed products for Halal authentication.

Key words: pork identification, cytochrome *b*, PCR-RFLP, *BseDI* restriction enzyme

INTRODUCTION

Nowadays, many kinds of meat products have been commonly consumed and spread across countries without limitation. The relatively new products such as: nuggets, sausage, burger, hot dog, meat ball, etc are widely accepted by consumers regardless of gender, ethnics, and ages. Further processing of meat has given more economic advantage for the producers and convenience for the consumers. The wide variety of meat products available in the Indonesia market seems favorable but leads to fears and doubts, where almost all population are moslem who are prohibited to consume pork.

Identification of the origin of meat used in processed meat products has always been a concern for a variety of reasons including wholesomeness, adulteration, religious factors, and control of unfair-market competition in the meat industry (Arslan *et al.*, 2006). Methods used for identification of species animal origin in the raw meat include sensory analysis, anatomical differences, histological differentiation of the hair that may

possibly exist in the meat, properties of tissue fat, and level of glycogen in muscle tissue, as well as electrophoresis and hybridization of DNA (Ilhak & Arslan, 2007).

PCR-RFLP allows amplification of a conserved region of DNA sequence using PCR and the detection of genetic variation among species by digestion of the amplified fragment with certain restriction enzymes. This technique has been used for speciation by exploiting DNA sequence variation within the mitochondrial DNA (Sebastio *et al.*, 2001; Lopez-Andreo *et al.*, 2005), 12S rRNA (Fajardo *et al.*, 2006) and 16S rDNA and nuclear markers (Rastogi *et al.*, 2007). Ling-Sun & Chich-Sheng (2003) have also applied PCR-RFLP analytical method based on ribosomal 12S rRNA for identifying porcine, caprine and bovine meats. A similar method using 12S rRNA PCR-RFLP was also applied to the identification of beef, buffalo and chevon meats (Girish *et al.*, 2005). Molecular DNA approach using D-loop mitochondrial DNA was also applied for the pedigree analysis of Indonesian native chicken (Sulandari & Zein, 2009).

Cytochrome *b* gene was used to study inter- and intra species of animals because contains sequence diversity among animal species. Hsieh *et al.* (2001) reported the percentage of sequence diversity between the different species range from 5.97 to 34.83% and the percentage range of sequence diversity between in the same species

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was from 0.25 to 2.74%. Mitochondrial DNA was chosen in this study which the reason that much faster than nuclear DNA and thus contains more sequence diversity compared to nuclear DNA, facilitating the identification of closely related species (Brown *et al.*, 1996; Vavter & Brown, 1986). Bellagamba *et al.* (2001) found that compared to other techniques for species identification by DNA-based methods, PCR-RFLP of mitochondrial DNA has offered the greatest advantage when applied to the analysis of short fragments of degraded DNA in processed food.

Developments in molecular biology have facilitated identification of plant, bacteria, and animal species with high accuracy (Ilhak & Arslan, 2007). Polymerase chain reaction (PCR) follows with restriction fragment length polymorphism (RFLP) for DNA fragmentation techniques have been frequently used for identification of meat species. *BsaI* I restriction enzyme was previously used for the identification of pork, however it was conducted in overnight reaction (Aida *et al.*, 2005) and consequently require long times. *BseDI* is known as isoschizomer of *BsaI*, *BssECI*, dan *SecI*. This enzyme recognizes and cleaves DNA fragment on sequence of CCNNGG. *BseDI* restriction enzyme is produced from *Geobacillus stearothermophilus* bacteria (Fermentas AB) and *Bacillus stearothermophilus* RFL1434 (USBiological), while *BsaI* enzyme was isolated from *G. stearothermophilus* J695 bacteria (New England Biolabs). Both enzyme cleaves the same DNA sequence, however the application of the *BseDI* for the meat species identification was very little. Therefore, the objective of the present study was to identify the presence of pork in mixture cooked meat which includes beef sausage and chicken nugget using *BseDI* restriction enzyme for the RFLP analysis.

MATERIALS AND METHODS

Samples Selection and DNA Extraction

Meat samples of beef cattle, chicken, and pig were obtained from local market in Yogyakarta and were grounded. Sausage product was prepared using beef and pork in various level of pork (1%, 3%, 5%, 10%, and 25%), while chicken nugget was prepared using chicken and pork at various levels. Beef sausage and chicken nugget were fried around 160-170 °C for 5 min. The samples were stored at -20 °C before DNA extraction.

DNA was extracted from 50 to 100 mg of meat products using the *high pure PCR template* protocol for animal tissue provided with the *high pure PCR template kit* (Roche, Germany). Approximately 50-100 mg of meat samples was blended and placed in a 1.5 ml microcentrifuge tube. Three hundred microlitres *tissue buffer* and 40 µl *proteinase K* were added and mixed by vortexing. The mixture was incubated at 55 °C in a water bath to disperse the sample overnight until the tissue was completely lysed. In the following day, 200 µl of *binding buffer* was added and incubated at 70 °C for 10 min. The mixture was mixed by vortexing for 15 sec. One hundred microlitres isopropanol was added to the sample, mixed thoroughly by vortexing, put into the *high filter*

tube in *collection tube*, pour sample in it. All tube were centrifuged at 8,000 g for 1 min. The flow-through and *collection tube* was discarded and the *high filter tube* was placed in a new 2 ml *collection tube*. Five hundred microlitres *wash buffer* was added and spun at 8,000 g for 1 min. The flow-through and *collection tube* was discarded and the *High Filter Tube* was placed in another 2 ml *collection tube*. After throw out the solution, the tube was spun at full speed for 10 sec to dry *high filter tube* and the flow-through and *collection tube* was discarded. The *high filter tube* was placed in a clean 1.5 ml microcentrifuge tube. Two hundred microlitres *prewarmed elution buffer* was added and spun at 8,000 g for 1 min to elute. The DNA solution was then stored at 4 °C.

Oligonucleotide primer. A pair of primers was employed in PCR reaction. The PCR primers used were CYTb1 (5'-CCA TCC AAC ATC TCA GCA TGA TGA AA-3') and CYTb2 (5'-GCC CCT CAG AAT GAT ATT TGT CCT CA-3'), as reported by Kocher *et al.* (1989).

Polymerase Chain Reaction Amplification of Cytochrome b Gene

Amplification of the mt cyt b gene was performed in a final volume of 25 µl containing 250 ng of extracted DNA, 12.5 µl mega-mix royal (optimized mixture of *Taq* polymerase, anti-*Taq* polymerase monoclonal antibodies in 2x reaction buffer (6 mM MgCl₂) with 400 µM dNTPs, stabilizer and blue loading dye) (Microzone Ltd, West Sussex, UK), and 20 pmol of each primer. Amplification was performed with a thermal cycler according to the following PCR step-cycle program: pre-denaturation of 94 °C for 2 min to completely denature the DNA template, followed by 35 cycles of denaturation at 95 °C for 36 sec, annealing at 51 °C for 73 sec, and extension at 72 °C for 84 sec. Final extension at 72 °C was conducted for 3 min followed by the final cycle for complete synthesis of elongated DNA molecules. Two microlitres of PCR products were electrophoresed at constant voltage (50 V) on 2% agarose gel (Promega, Madison, USA) for about an hour in 1x TBE buffer, pH 8.0 and stained by ethidium bromide. A 100 bp DNA ladder (Promega, Madison, USA) was used as size reference. The gel photo was taken using the Syngene gel documentation system.

Restriction Fragment Length Polymorphism

Two units of RE *BseDI* (Fermentas) were applied to 10 µl of amplified DNA in a final volume of 20 µl digestion mixture [containing 1x reaction buffer (10 mM Tris-HCl, 100 mM KCl, 1 mM EDTA, 0.2 mg/ml BSA, 1 mM DTT and 50% glycerol)] and incubated at 55 °C for 3 h for optimal result. Two microlitres of the digested samples were electrophoresed at constant voltage (50 V) on 2% agarose gel (Promega, Madison, USA) for about an hour in 1x TBE buffer, pH 8.0 and stained by ethidium bromide. A 100 bp (Promega, Madison, USA) was used as size reference. The gel photo was taken using the Syngene gel documentation system.

RESULTS AND DISCUSSION

Isolation of DNA and Amplification Using PCR

DNA was extracted from meat products using High Pure PCR Template Kit (Roche, Germany). DNA extraction using the proteinase K 1 mg/ml was done for at least 10 h incubation time. Genomic DNA was used as template for the amplification of PCR with the universal primer. The present study designed to extend PCR-RFLP application for the specific identification of pig, targeting sequences of the *cyt b* mitochondrial DNA gene.

PCR has the potential sensitivity and specificity required to achieve detection of a target sequence from template DNA. The amplicons of the cytochrome *b* gene of the beef sausage and chicken nugget are shown in Figure 1 and 2. Gene of cytochrome *b* was used for the amplification of PCR and resulted DNA fragment of approximately 360 bp for beef sausage before or after frying and chicken nugget. These small amplicons are ideal for use with processed foods where DNA commonly was degraded. This result indicated that pig, beef,

and chicken DNA in processed meat was successfully amplified in PCR reaction. The result of PCR amplification was similar to the result of Aida *et al.* (2005) which reported the presence of 360 bp fragment. This result also indicated that frying of meat products at 100 °C for 5 min did not produced degraded DNA into small size. Tanabe *et al.* (2007) investigate the feasibility of using mitochondrial *cyt b* gene to detect pork in commercial food products from a market and evaluated them for the presence of porcine DNA. Electrophoresis of PCR product clearly detected porcine DNA, while no amplification occurred in others meat sources: cattle, chicken, sheep and horse.

Analysis of PCR-RFLP

PCR product was digested using the *BseDI* restriction enzyme at 2 U/μl for 3 h incubation time. The result of analysis from various level of pork in beef sausage and chicken nugget (Figure 3), indicated that mixture of pork could be digested by *BseDI* restriction enzyme and were detected until level 1%. *BseDI* cleavage bands

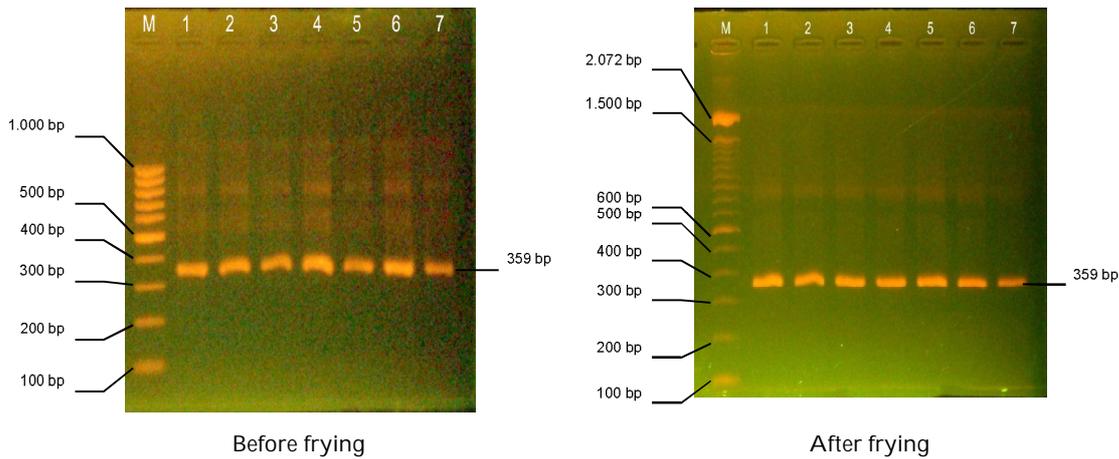


Figure 1. Electrophoretic patterns of PCR product of the 359 bp cytochrome *b* on 2% agarose gel from beef sausage before and after frying with various pork levels. M= marker; 1= pork (100%); 2= (beef 75% : pork 25%); 3= (beef 90% : pork 10%); 4= (beef 95% : pork 5%); 5= (beef 97% : pork 3%); 6= (beef 99% : pork 1%); 7= beef (100%).

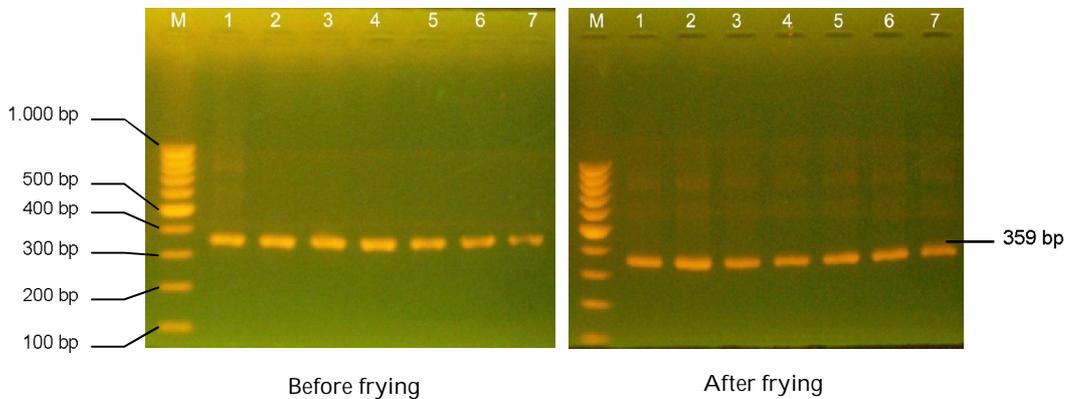


Figure 2. Electrophoretic patterns of PCR product of the 359 bp cytochrome *b* on 2% agarose gel from chicken nugget before and after frying with various Pork levels. M= marker; 1= pork (100%); 2= (chicken 75% : pork 25%); 3= (chicken 90% : pork 10%); 4= (chicken 95% : pork 5%); 5=(chicken 97% : pork 3%); 6= (chicken 99% : pork 1%); 7= chicken (100%).

visualized in the gel were enough and suitable for the discrimination of pork in processed food. *BseDI* endonuclease cleaved the cytochrome b gene products of pig species into two DNA fragments of 228 and 131 bp (Figure 3 lane 1 to 6), and did not cleave cytochrome b gene of beef and chicken as shown in Figure 3 lane 7.

Frying of meat products did not significantly influence the quality of DNA and consequently the PCR amplicon of cytochrome b gene could also be digested by this enzyme. This result was similar to Ong *et al.* (2007) reported that the pork level until 1% can be clearly detected by PCR-RFLP analysis of cytochrome b gene. Aida *et al.* (2005) reported that *BsalI* restriction enzyme was applicable to identify porcine in a mixture of beef, goat and chicken, while Ong *et al.* (2007) used various restriction enzyme of like *AluI*, *BsalI* and *RsaI* to identify pork from different mixture of meat chicken, pig and

cattle in various modifications. Other study by Fajardo *et al.* (2006) reported PCR-RFLP based on the mitochondrial 12S rRNA fragment targeted cannot be applied for species identification in thermally treated meats. In the analysis of food matrices in which thermal action or other processing effects may degrade the DNA present in the food tissues. However this result allow us to reported that PCR-RFLP of the mitochondrial cytochrome b gene is a suitable alternative that can be applied to the detection of pork in processed meat in commercialized products such as sausage and chicken nuggets.

Based on RFLP pattern by using CLC sequencer (Figure 4), *BseDI* is applicable to differentiate pork from beef and chicken species in the meat mixtures. This was supported by the laboratory analysis using the restriction enzyme for PCR product. Assay developed during this work was able to detect 0.1% of their respective target

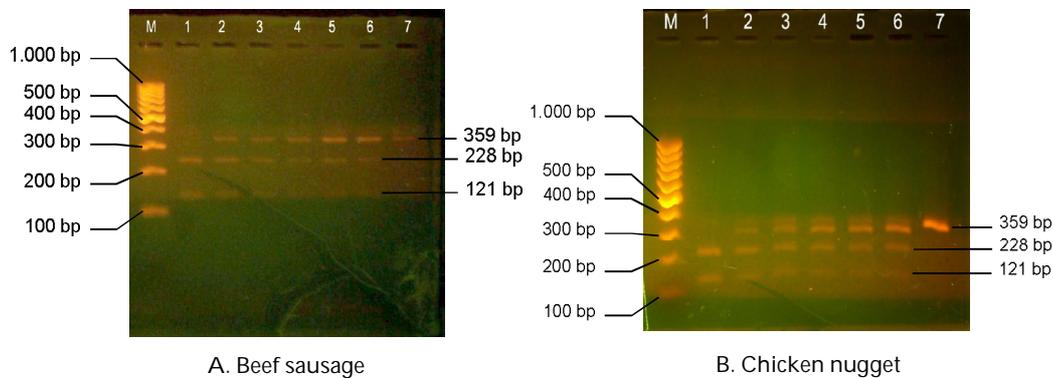


Figure 3. *BseDI* restriction profile of cytochrome b PCR product amplified from samples. (A) M= 100 bp ladder size standard; 1= pork (100%); 2= (beef 75% : pork 25%); 3= (beef 90% : pork 10%); 4= (beef 95% : pork 5%); 5= (beef 97% pork 3%); 6= (beef 99% : pork 1%); 7= beef 100%. (B) M= 100 bp ladder, 1= pork (100%); 2= (chicken 75% : pork 25%); 3= (chicken 90% : pork 10%); 4= (chicken 95% : pork 5%); 5= (chicken 97% : pork 3%); 6= (chicken 99% : pork 1%); 7= chicken (100%).

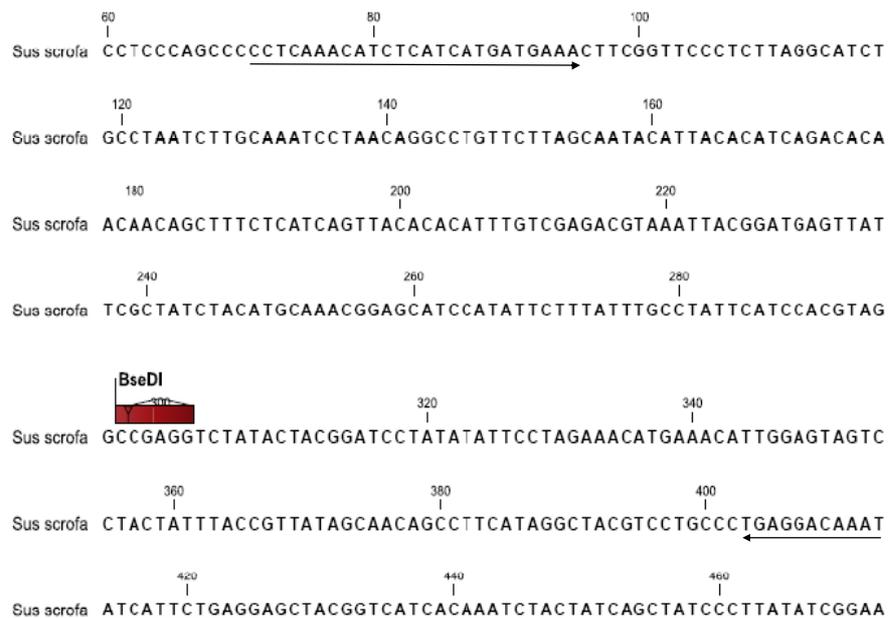


Figure 4. Part the nucleotide sequence of the cytochrome b from *Sus scrofa* (pig) and cutting site of enzyme *BseDI*

species. The small target size of 360 bp of cytochrome b has advantageous for detection of material in processed foods, such as cooked meat products, where extensive DNA degradation has been observed. The digestion of PCR amplicon of 360 bp cytochrome b gene resulted different fragment size, between 131 and 228 bp.

PCR-RFLP represents the appropriate method to identify a certain species in fresh meat and cooked meat. Identification the species through analysis of restriction patterns is quite and practical application. However, this method needs to be validated and detail evaluate to apply for distinguish of porcine from other meats in processed food system.

CONCLUSION

Frying around 160-170 °C for 5 min of processed meat did not affect the porcine DNA degradation and DNA could be isolated and amplified. The results also allow us to conclude that PCR-RFLP using *BseDI* of mitochondrial cyt b DNA gene is a suitable alternative that can be applied to the detection of fraudulent of pig species present in meat mixture products.

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