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Detection of Blood Thrombin Enzyme in Surimi-Based Products by Using Polymerase Chain Reaction (PCR) Method

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Abstract: The detection of blood thrombin enzyme presented in eight samples of surimi based products was investigated by using Polymerase Chain Reaction (PCR) method. Specific primers for chicken (*Gallus gallus*), cow (*Bos taurus*) and pig (*Sus sucrofa*) blood thrombin enzyme were designed for positive detection. Two primers for chicken blood which are Gal2 and Gal3 showed 98% and 99% in significant identity of *G.gallus* coagulation factor II (thrombin) while two primers from cow which are Bos4 and Bos6 showed 100% significant identity of *B. Taurus* coagulation factor II (thrombin). On the contrary, there were no positive results on pig primers with *S. sucrofa* coagulation factor II (thrombin). PCR amplification with Gal2 and Bos4 primers in surimi based products showed several positive results while Sus5 primer showed none. Further research should be done to verify the consistency of this result and redesign a specific primer for pig's blood thrombin enzymes is vital in order to guarantee the quality of food products to comply with Halal food guidelines and regulations.

Key words: Blood thrombin enzyme · Surimi-based product · Polymerase chain reaction

INTRODUCTION

Surimi can be defined as a wet concentrate of fish muscle, that is mechanically deboned, water washed and mixed with cryoprotectant [1]. Surimi usually made from low value fish flesh may be highly exposed to proteolytic activities, thus, reducing the quality of surimi [2]. Protein additives have been widely used in surimi manufacturing such as whey protein, egg white protein, plasma protein and soy protein to enhance the gel strength via inhibition of proteolysis caused by endogenous proteinases [3]. Nowadays, animal blood, by-products of slaughtered animals were known as the cheaper protein additives are used as emulsifiers, stabilizers, clarifiers, or nutritional components to enhance the properties of foods [4]. Blood plasma have been reported to be a protease inhibitor and give good gelling properties that help to achieve the desired textural properties of surimi based products [5].

Blood plasma is a complex mixture with over 100 different proteins being albumin (60%), globulins (35%) and fibrinogen (4%) with serum albuminbeing the main protein implicated in the formation of heat-induced gels. Several of proteins present in blood plasma contribute to increase surimi gel strength, such as factor

XIII which is also known as a type of transglutaminase [6] kininogen [7] and α_2-macroglobulin (α_2 - M) [8]. Another component present in blood plasma is thrombin enzyme which initiates the gelling of fibrinogen by converting fibrinogen to fibrin. Fibrin interacts with collagen enabling the binding of meat pieces, fish, poultry and seafood [9]. However, according to Islam, blood is prohibited to be consumed by the Muslims as it is considered to be filthy and harmful [10]. Certain methods have been develop in detecting blood component present in food such as spectrophotometric [11], chromatographic, electrophoretic [12], immunoassay [13] and DNA-based methods [14]. DNA-based method is

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rapid, inexpensive, simple and helps in producing relatively large numbers of copies of DNA molecules from the minute quantities of DNA even though the DNA is of poor quality [15]. Therefore, the purpose of this study is to design and analyze the specificity of oligonucleotide primers of thrombin enzyme from chicken, bovine and porcine blood.

MATERIALS AND METHODS

Postive Control and Samples Collection: Blood samples for positive control were respectively collected at abbatoirs. Fresh blood were kept in ice (4°C) during transportation while eight samples of surimi based product were randomly purchased at the shopping complex located around Bangi, Nilai and Purajaya area, all the samples were stored in -20°C prior to analysis.

Samples Preparation and DNA Extraction: Blood samples and surimi based products samples were extracted KAPA Express Extraction Kit (Boston, Massachusetts, United States). Then, the mixture were incubated in thermocyler (Eppendorf, Hamburg, Germany) for 10 min at 75°C and 5 min at 95°C. Reaction product were vortexed for 2-3 second and centrifuged at high speed, 13,400 rpm for 1 min. DNA containing supernatant were transferred to a fresh tube before 1µl of DNA extract used directly in 25µl PCR, without quantification.

Primer Design and PCR Amplification: The primers designs were carried out by using primer-blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) based on thrombin sequences obtained from Genbank (http:www.ncbi.nlm.nih.gov/genbank/). 14 primers were designed and were then checked for its specificity with NetPrimer (http://www. premierbiosoft. com/Net Primer. html). PCR master mix reaction were prepared to the final volume of 25µl containing PCR grade water, 2x KAPA 2G Robust HotStart Readymix, forward and reverse primer and samples/positive control. PCR amplification were performed in a Mastercycler (Eppendorf, Hamburg, Germany) with the following conditions: initial denaturation at 95°C for 3 minutes, denaturation at 95°C for 15 sec, annealing at 60°C for 15 sec and extension at 72°C for 15 sec for 35 cycles followed by a final extension time of 72°C for 10 min and cooling 10°C. Negative controls were included in each batch of samples.

Electrophoresis: PCR products from all samples were further analysed by gel electrophoresis using 1.5 % agarose (Promega, Madison, USA). Electrophoresis was run with 120 V for 45 minutes and then analyzed by using a gel documentation system (Bio-Rad Laboratories, Segrate, Italy).

DNA Purification and Analysis of PCR Product by Sequencing: Positive samples from PCR amplification of DNA blood thrombin enzyme together with their respective primers were sent to First Base Laboratories Sdn. Bhd. for sequencing analysis. Similarity of the sequences obtained was studied using nucleotide-BLAST on the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/tools/primer-blast).

RESULTS AND DISCUSSION

PCR Amplification with Primers Designed for Blood Thrombin Enzyme of Chicken, Cow and Pig: All of the primers used in detecting thrombin enzyme present in chicken blood were positive and gave a range of 100-300 bp products as illustrated in Figure 1. The intensity of the band was high; indicating the high concentration of DNA. For the bovine blood, two of the six primers tested produced the band and showed the positive results, gave a range of 100-200 bp products as illustrated in Figure 2. However, some non specific products could be observed on the gels with Bos 1 and Bos 5 (Figure 2). With pig blood, only two out of five primers showed positive results and were gave the size in range below 100 bp products as illustrated in Figure 3. However, the bands showed low intensity bands, which introduced a certain difficulty in visual interpretation.

Sequencing Analysis: Based on the results of sequencing that have been sent (Table 1), only five of the seven positive primer samples showed the positive results. The outcome of Nucleotide- BLAST results, indicated significant identity of the *G.gallus* coagulation factor II (thrombin) with Gal1 primer product, with 92% identity (Accession numbers: NM 204605), plus Gal2 primers presenting 98% identity (NM 001012608) while another primer, Gal3 (NM_001012608) showed 99% of identity. Each of primer has gap within query sequences which labeled as dashed, indicating insertions or deletions of bases. However, for Gal2 primer, the difference was due to the mismatched of 1 microsatellite sequences of C bases.

Table 1: List of positive primers from sequencing analysis

Name of primer	Sequence	Accession Number	Identities (%)
Chicken, *Gallus Gallus*			
Gal1 Fw	5'-CTGTCGGAACCCTGATGCAG-3'	NM_204605	92
Gal1 Rev	5'-AGCTGTCGCAGTAATGCAGG-3'		
Gal2 Fw	5'-CTGGCTGTTTGGGGAAGGTC-3'	NM_001012608	98
Gal2 Rev	5'-ACAGCGGAATGGTTCCCAAC-3'		
Gal3 Fw	5'-ATCCGGGGTTGTGCCTTTTC-3'	NM_001012608	99
Gal3 Rev	5'-GCTCAGATTCCCTCAGCACG-3'		
Cow, *Bos Taurus*			
Bos4 Fw	5'-GCTCAGGAAAGAAGAGGCGG-3'	BC113233	100
Bos4 Rev	5'-TTGAGGGTGGAGAGGCAGAG-3'		
Bos6 Fw	5'-TACGCCTTGCTCACATGTGG-3'	BC134502	100
Bos6 Rev	5'-TGTTGTGGACATCGTGGCAG-3'		

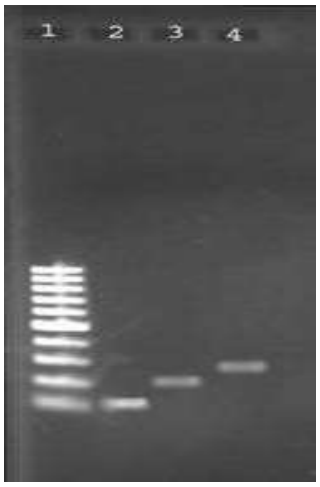

Fig. 1: Photograph of a 1.5 % gel showing PCR products obtained with 3 primers and DNA prepared from chicken blood. Lanes from left to right: 1-Gene Ruler 100bp l adder; 2- Gal 1; 3-Gal 2; 4-Gal 3.

Fig. 2: Photograph of a 1.5 % gel showing PCR products obtained with 6 primers and DNA prepared from cow blood. Lanes from left to right: 1- GeneRuler 100bp ladder; 2- Bos 1; 3-Bos 2; 4- Bos 3; 5- Bos 4; 6-Bos 5; 7-Bos 6.

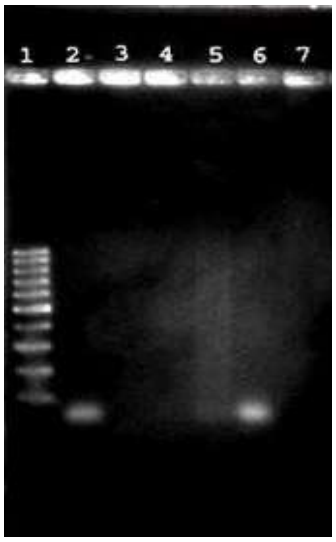

Fig. 3: Photograph of a 1.5 % gel showing PCR products obtained with 5 primers and DNA prepared from pig blood. Lanes from left to right: 1- GeneRuler 100bp ladder; 2- Sus 1; 3-Sus 2; 4- Sus 3; 5- Sus 4; 6-Sus 5.

Besides that, positive primers for cow which are Bos4 (BC113233) and Bos6 (BC134502) showed the good result by producing 100% of significant identity of *B. Taurus* coagulation factor II (thrombin) receptor-like while for the pig primers, sequencing step cannot be further analyzed due to the lack of DNA during purification. Therefore, the bands that have been produced by PCR products can be categorized as non specific products [16, 17].

All of positive primers have very low E-values (much less than 1) which provide an estimate of statistical significance. The databases which have higher Expect-values such as 0.5 or above indicate that these sequences may have been matched by chance alone. In this study, several primers were initially tested and, finally only one primer of each blood animal was selected for the PCR amplification which are Gal2, Bos4 and Sus5 in

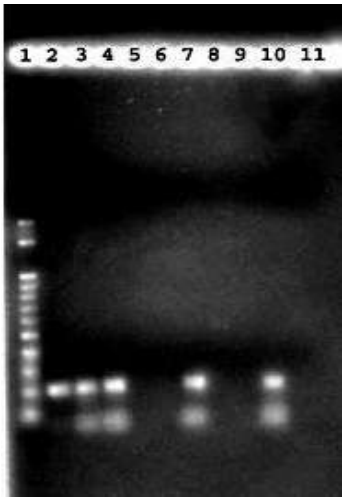

Fig. 4: Photograph of a 1.5% agarose gel showing PCR products obtained with Gal2 primer and DNA prepared from 8 samples of fish surimi based products. Lanes from left to right: 1-molecular marker (GeneRuler 100 bp ladder); 2- positive control; 3-Fried Fish Ball; 4-Crab Filament Sticks; 5-Crab Flavoured Ball; 6-*Kek Ikan Panjang*; 7-*Bebola Ikan Goreng Besar*; 8-Crab Nugget; 9-Crab Nugget Ball; 10-Cili Panjang Ball; 11-negative control.

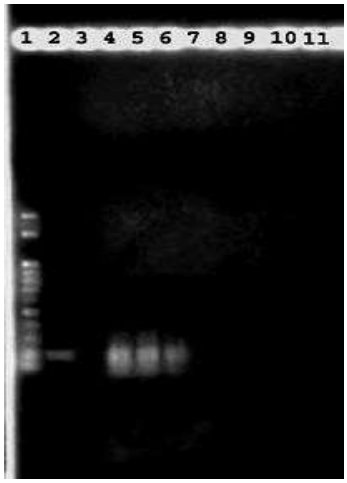

Fig. 5: Photograph of a 1.5% agarose gel showing PCR products obtained with Bos4 primer and DNA prepared from 8 samples of fish surimi based products. Lanes from left to right: 1-molecular marker (GeneRuler 100 bp ladder); 2-positive control; 3-Fried Fish Ball; 4-Crab Filament Sticks; 5-Crab Flavoured Ball; 6-*Kek Ikan Panjang*; 7-*Bebola Ikan Goreng Besar*; 8-Crab Nugget; 9-Crab Nugget Ball; 10-Cili Panjang Ball; 11-negative control.

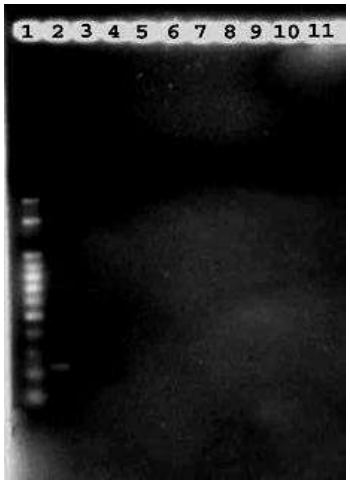

Fig. 6: Photograph of a 1.5% agarose gel with PCR products obtained using Sus5 primer and DNA prepared from 8 samples of fish surimi based products. Lanes from left to right: 1-molecular marker (GeneRuler 100 bp ladder); 2-positive control; 3-Fried Fish Ball; 4-Crab Filament Sticks; 5-Crab Flavoured Ball; 6-*Kek Ikan Panjang*; 7-*Bebola Ikan Goreng Besar*; 8-Crab Nugget; 9-Crab Nugget Ball; 10-Cili Panjang Ball; 11-negative control.

detecting thrombin enzyme present in surimi based products on the basis of the number, intensity and distribution of bands able to clearly distinguish among species.

PCR amplification of Gal2, Bos4 and Sus5 primers in surimi based products: With Gal2 primers, as expected, positive control gave a positive result by producing a band while negative result showed no band produced. Four samples out of 8 which are S1, S2, S5 and S8 were produced the bands, however, all of the bands produced extra bands as could be observed on the gels (Figure 4). Amplification with Bos4 primers gave single and monospecific products for positive result while for S2, S3 and S4 produced the bands which were not obvious and clear. All the bands of those samples could be observed on the gel were like having extra bands. Particularly, there were no visible products obtained with other samples which were S1, S5, S6, S7, S8 and negative control (Figure 5). Amplification of Sus5 primers showed only positive control produced a band while no visible bands were observed for all samples and negative control. However, the band produced for positive control was not as expected as illustrated in Figure 6.

CONCLUSION

Based on this study, specific primer in detecting blood thrombin enzyme for chicken, cow and pig were successfully designed and specificity of the primers were obtained by sequencing analysis. The present of positive chicken's and cow's blood thrombin enzymes in the samples may suggest the possibility of manufacturers applying non-Halal plasma protein as food additive in their products. Another issue of concern may be caused by sharing of the same processing equipments in the production of mammalian meat products and seafood products, which may negatively affect the Halal status of surimi products. However, further research should be done to optimize the specificity of pig primers in the confirmation of pig blood thrombin enzyme and to reconfirm the present of blood thrombin enzyme in the food samples. Comparison of PCR method with ELISA will be very useful to authenticate the reliability of the results thus, can be used by both regulators and manufacturers to detect the presence of blood ingredients in foods are vital in order to guarantee the quality of food products and compliance with Halal food guidelines and regulations.

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