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Detection of Blood Transglutaminase Enzyme in Fish Surimi Based Product by Using Polymerase Chain Reaction (PCR) Method

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Abstract: Blood plasma contain transglutaminase (TGase) enzyme - catalyst the reaction of cross-linking between proteins which has a significant impact on properties of protein gel capacity, thermal stability, water holding capacity thereby protein characteristics elasticity, mouth feel, flavour, texture, binding force. The objectives of this study are to design and analyze the specificity of oligonucleotide primers of blood plasma transglutaminase from chicken, bovine and procine blood and to detect the presence of blood plasma transglutaminase in eight samples of fish surimi based products using PCR method. In this study, Polymerase Chain Reaction (PCR) method has been used in detecting the existence of blood transglutaminase enzyme DNA in surimi based products. Specific primers for chicken (*Gallus gallus*), cow (*Bos taurus*) and pig (*Sus scrofa*) blood transglutaminase enzyme were designed for positive detection. Two of the six primers designed for the chicken blood tranglutaminase, G3 and G5 have shown 99 % significant identity to the sequence of *G. gallus* similar to XP-C repair complementing (transglutaminase) and the latter to the hypothetical LOC428804 (transglutaminase) sequence. However, there was no positive result using the six primers designed for *Bos taurus* transglutaminase and the six primers designed for *S. scrofa* transglutaminase. PCR amplification with G3 and G5 primers in surimi based products also showed negative results. Based on this study, G3 primer sequence for chicken showed 99 % of *G. gallus* similar XP-C repair complementing (transglutaminase) followed by G5 primer which also obtained 99 % *G. gallus* hypothetical LOC428804 (transglutaminase) of significant identity. However, for primer of cow and pig, there were no positive results. On the contrary, PCR amplification on surimi based products had not showed positive bands of chicken blood transglutaminase, G3 and G5 in the samples. Further research should be done to verify the consistency of this result and to redesign the primers for cow and pig's blood transglutaminase enzymes in order to increase its specificity which is vital in assuring the reliability of the detection method in food products in accordance to the Halal food guidelines and regulations.

Key words: Transglutaminase · Fish Surimi · Primer Design · Polymerase Chain Reaction

INTRODUCTION

Blood is prohibited to be consumed in Islam. Anything related to blood such as blood plasma is also Haram to consumed [1]. In Malaysia, there are several companies that claim blood has been added to their surimi base product such as fish ball and sausages. This has created controversy issues to the Muslim consumers since surimi base products are one of the main ingredients in their daily cooking.

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Blood plasma contain transglutaminase (TGase) which is an enzyme that catalyze the reaction of cross-linking between proteins which has a significant impact on properties of protein gel capacity, thermal stability, water holding capacity therefore affecting protein characteristics such as elasticity, mouth feel, flavor, texture, binding force.

Chicken plasma protein (CPP) can enhanced the gel strength by acting as filler in surimi gel matrix and also as a proteinase inhibitor [2]. The most commonly used inhibitors are bovine plasma protein (BPP), egg white, potato powder and whey protein concentrate [3,4]. Besides that, the addition of porcine plasma protein (PPP) would increase the gel strength of surimi [5].

Previous studies have reported that blood plasma contains a variety of bioactive compound including proteinase inhibitor and plasma transglutaminase [5,6]. Purified transglutaminase (plasma factor XIII) as a possible by-product of pig plasma demonstrated that the transglutaminase, activated with thrombin and Ca^{2+}, catalysed covalent cross linking of Myosin Heavy Chain (MHC) and substantially increased the gel strength of minced mackerel [7].

The study of blood plasma contamination is important among Muslims, to prevent consumption of added blood ingredients, considered Haram, if it comes from meat not slaughtered in the Syariah way, or Haram and *najs* (deplorable filth) if the source is porcine based. However, study on blood plasma contamination in food is very scarce and limited since blood plasma iscommonly used ingredients of Non-Muslim food preparation. The common method to identify blood plasma in food samples will be Enzyme-linked Immunosorbent Assay (ELISA) [8, 9, 10].

TGase is used as a biomarker in ELISA for detection of blood plasma contamination from non-halal source. For example PCR based detection method could give false positive result because TGase also exists in halal sources (plants, microorganism and marine organism). Therefore, there is a need to study the specificity of biomarker designed for detection of blood plasma. As PCR progress, the DNA generated is used as a template for replication. This sets in motion a chain reaction in which the DNA template is exponentially amplified. In performing PCR, there is a need of finding a primer for detection of blood plasma contamination.

The objective of this study is to design and analyze the specificity of oligonucleotide primers of blood plasma transglutaminase from chicken, bovine and porcine blood as well as to detect the presence of blood plasma transglutaminase in eight samples of fish surimi based products using PCR based detection method.

MATERIALS AND METHODS

Postive Control and Samples Collection: Blood samples for positive control were respectively collected at abbatoirs which are located in the Pedas Halal Park, Negeri Sembilan, (chicken, *Gallus gallus*), Senawang, Negeri Sembilan, (cow, *Bos taurus*) and Ayer Keroh (pig, *Sus scrofa*). All of them have been veterinary inspected to accomplish the health requirement standard before proceeding to slaughtering stage. Fresh blood were collected in centrifuge tube and kept in ice (4°C) during transportation and then stored in -20°C freezer. Besides that, eight samples of surimi based products were randomly purchased from shopping complexes located around Bangi, Nilai and Putrajaya area which then were stored in -20°C prior to analysis. Blood samples that have been used as positive control were labeled as +G for chicken, +B for cow and +S for pig while for all surimi based product were labeled as F1, F2, F3, F4, F5, F6, F7 and F8 (Table 1).

Samples Preparation and DNA Extraction: DNA from Fish surimi based product was extracted using KAPA Express Extraction Kit (Boston, Massachusetts, United States). 10µl of 10X KAPA Express Extract Buffer, 10µl of 1 U/µl KAPA Express Extract Enzyme and 86µl of PCR-grade water were combined with $2mm^3$ fresh samples. The mixture was incubated in thermocyler (Eppendorf, Hamburg, Germany) for 10 minutes at 60°C and 5 minutes at 95°C. Reaction product was vortexed for 2-3 second and centrifuged at 13,400 rpm for 1 minute. DNA containing supernatant was transferred into a fresh tube and only 1µl of the extracted DNA sample was used in the 25µl PCR reaction, without any quantification on its concentration.

Primer Design and PCR Amplification: The primers design was carried out by using primer-blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) based on the transglutaminase sequences obtained from Genbank database (http:www.ncbi.nlm.nih.gov /genbank/). Six primers for each animal were designed and checked for its specificity with NetPrimer (http://www. premierbiosoft.com/NetPrimer. html). PCR master mix reaction were prepared to final volume of 25µl containing 9µl of PCR grade water, 12.5µl 2x KAPA 2G Robust HotStart Readymix, 1.25µl forward and reverse primer and 1µl of samples/positive control. PCR amplification was performed using Mastercycler (Eppendorf, Hamburg,

Table 1: Fish ball sample

Surimi Sample	Name
F1	Fried Fish Ball
F2	Crab Filament Sticks
F3	Crab Flavoured Ball
F4	Kek Ikan Panjang
F5	Bebola Ikan Goreng Besar
F6	Crab Nugget
F7	Crab Nugget Ball
F8	Cili Panjang Ball

Germany) with the following conditions: initial denaturation at 95°C for 3 minutes, denaturation at 95°C for 15sec, annealing at 60°C for 15 seconds and extension at 72°C for 15 seconds for 35 cycles followed by a final extension time of 72°C for 10 minutes 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). The gel was prepared by adding 0.6g of agarose with 40ml 1x TAE buffer pH 8.0 and then heated until it homogenized and dissolved thoroughly. After the agar was cooled to 55°C, the agarose gel tray was transferred into an electrophoresis chamber and 1X TAE buffer was added into the chamber until the gel was barely covered. Then, 10µl of PCR products were mixed with 1µl of loading dye (Promega, Madison USA) and subsequently loaded into wells. 5µl of 100bp DNA ladder (Promega, Madison USA) was used as a marker. Gel was electrophoresed at 100V for 40 minutes and then analyzed using 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 Transglutaminase enzyme together with their respective primers were sent to First Base Laboratories Sdn. Bhd. for sequencing analysis. Similarity of the sequences obtained from sequencing of PCR product was studied using nucleotide-BLAST on National Center for Biotechnology Information website (http://.ncbi.nlm.nih.gov/blast/).

RESULTS AND DISCUSSION

Primer Specificity Analysis: Six of the 20 mer primers from each transglutaminase of different animals, *Gallus gallus* (txid9031), *Bos taurus* (txid9913) and S*us scrofa* (txid9823) were designed using primer-blast based on the transglutaminase sequences obtained from Genbank database. Its specificity was checked using NetPrimer. Based on the result of PCR amplification using designated primers, two of the six primers used in detecting transglutaminase enzyme present in chicken blood were positive as illustrated in Figure 1.

All of the primers used in detecting transglutaminase enzyme present in cow and pig blood showed negative results as illustrated in Figures 2 and 3.

The results are due to the poor quality of primer design and this could be explained by the non-optimal melting temperature for the PCR reaction. In addition, the concentration of primers may un-optimized. Therefore, optimization is required for primer concentration and melting temperature to improve PCR reaction.

Thus, the best of concentration of primer could be selected. Amplicon size also should be considered. Larger amplicon size can cause failure in amplication. At present, the studies which have been done in PCR method are mostly on identifying the species-specific PCR by using species-specific primers for meat species authentication of bovine, porcine and chicken [11]. This method is suitable in detecting the present of bovine, porcine and chicken materials in food samples. However, in detecting blood present in food samples, specific primers of components in blood itself should be used because the DNA detected can be from the whole tissue of porcine, bovine or chicken while using species-specific primers.

Sequencing Analysis: According to the positive result of chicken primers electrophoresis (Figure 1), the two samples of primers of G3 *(Gallus gallus)* similar to XP-C repair completing (transglutaminase) and G5 (*Gallus gallus*) hypothetical LOC428804 (transglutaminase) was purified, sequenced and showed the positive result for sequencing analysis by Nucleotide-Blast. Additional chicken, cow and pig primers show negative results from PCR.

Based on Table 2 which is from Primer Blast and NetPrimer result, melting temperature of the two selected primers is around 55°C-56°C and percentage of GC is 55 %. In addition, G3 does not show the possibility to form protein dimer while G5 has the possibility to form 3 protein dimer at forward primer and 1 at reverse primer. GC content, melting temperature and annealing temperature are strictly dependent on one another [12]. GC percentage is an important characteristic of DNA and provides information about the strength of primer annealing. GC percentage of 50%-60% is the value recommended [13]. Primer dimer is formed by intermolecular interactions

Table 2: Results of two selected Chicken Primers

Primer		Sequence (5'-3')	TM	GC %	Product Size	Protein Dimer	Hairpin
G3	Fw	ACACACAATCGCTGGAGACG	54.97	55	341	0	0
	Rev	TCTGTGCCAACCACTTTCCG	55.02	55		0	0
G5	Fw	TGCTTGCCAGCTATGAGCTG	54.95	55	707	3	4
	Rev	TCTGTGGGCTTTTGGCACTC	54.98	55		1	1

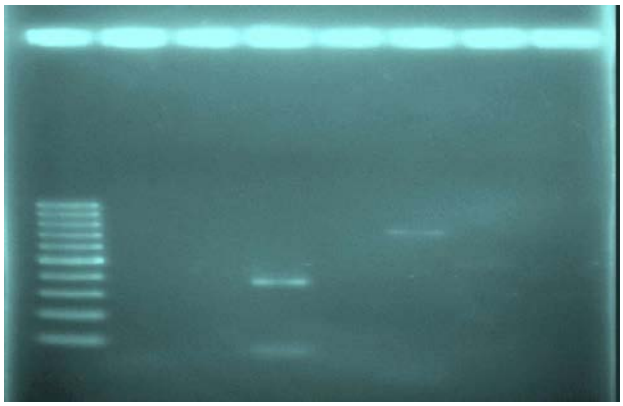

Fig. 1: Photograph of 1.5% agarose gel showing PCR products obtain with six chicken primers. Lanes from left to right: (1) molecular marker (GeneRuler 100bp ladder), (2) G1, (3) G2, (4) G3, (5) G4, (6) G5, (7) G6

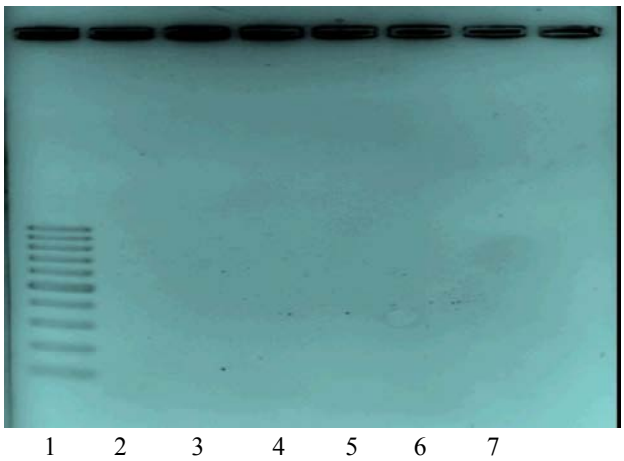

Fig. 2: Photograph of 1.5% agarose gel showing PCR products obtain with six cow primers. Lanes from left to right: (1) molecular marker (GeneRuler 100bp ladder), (2) B1, (3) B2, (4) B3, (5) B4, (6) B5, (7) B6

between the two primers, where the primer is homologous to itself [14]. Intramolecular hairpin can form when a single strand of DNA containing complimentary sequence regions binds to itself.

PCR Amplification with Fish Surimi Based Product: With G3 primer, as expected, positive control gave a positive result by producing a band while negative result showed no band. All of the samples gave no bands and produced dimers as observed on the gels (Figures 4 and 5). Furthermore, amplification using G5 primers revealed that only positive control produced a band while no visible bands were observed for all the samples and negative control. The size of band produced from the positive control was not the expected size.

Bands formed by the surimi samples may indicate the primer-dimer formation based on the extra bands produced. Non-specific product also can be notable as primer dimer. As primers are present at high concentrations, weak interactions can occur between them. Complementary of just one nucleotide between amplimer 3'- ends can give rise to primer dimers after

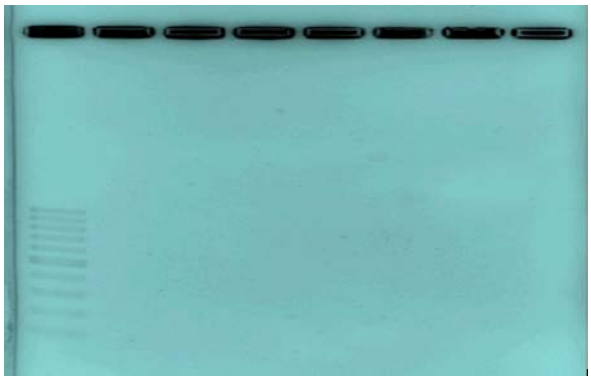

Fig. 3: Photograph of 1.5% agarose gel showing PCR products obtain with six pig primers. Lanes from left to right: (1) molecular marker (GeneRuler 100bp ladder), (2) S1, (3) S2, (4) S3, (5) S4, (6) S5, (7) S6

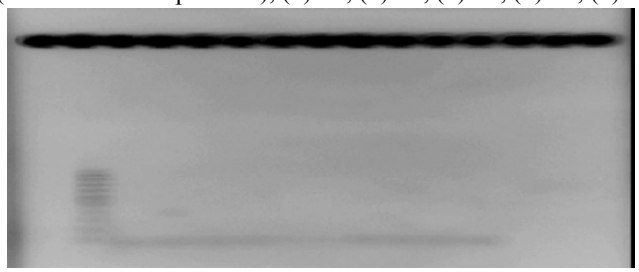

Fig. 4: Photograph of 1.5% agarose gel showing PCR products obtain with chicken primers G3 and DNA prepared from various fishball surimi samples. Lanes from left to right: (1) molecular marker (GeneRuler 100bp ladder), (2) -G: negative control ,(3) +G: positive control , (4) F1: Fried Fish Ball (DODO), (5) F2: Crab Filament Sticks (KAMI), (6) F3: Crab Flavoured Ball (YOKI), (7) F4: Kek Ikan Panjang (UNKNOWN), (8) F5: Bebola Ikan Goreng Besar (ATC), (9) F6: Crab Nugget (MUSHROOM), (10) F7: Crab Nugget Ball (GIANT) , (11) F8: Cili Panjang Ball (GOODFOOD SUPPLY).

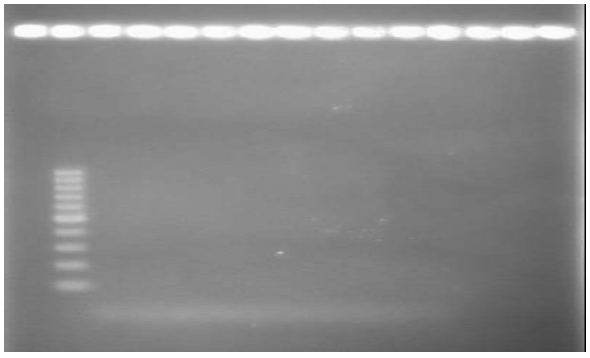

Fig. 5: Photograph of 1.5% agarose gel showing PCR products obtain with chicken primers G5 and DNA prepared from various fishball surimi samples. Lanes from left to right: (1) molecular marker (GeneRuler 100bp ladder), (2) -G: negative control ,(3) +G: positive control , (4) F1: Fried Fish Ball (DODO), (5) F2: Crab Filament Sticks (KAMI), (6) F3: Crab Flavoured Ball (YOKI), (7) F4: *Kek Ikan Panjang* (UNKNOWN), (8) F5: *Bebola Ikan Goreng Besar* (ATC), (9) F6: Crab Nugget (MUSHROOM), (10) F7: Crab Nugget Ball (GIANT) , (11) F8: Cili Panjang Ball (GOODFOOD SUPPLY)

30 cycles [15]. The same study showed that in an analogous PCR, where there was no 3' complementary, primer dimers were still produced. From these findings, Primer dimers derived from inter-primer extension are good substrates for amplification in subsequent cycles of PCR and that intra-primer interactions would produce inefficiently amplified products and so enhance amplification of the 'true targets' [16].

Therefore, in order to reconfirm the specificity of the primers used, the bands produced for PCR product of surimi based products should be sent for sequencing.

Besides that, surimi is minced fish in which all water-soluble proteins in the fish muscle have been washed out and thus contains only 15-16% water insoluble proteins, 75% moisture and 8-9% freezing stabilizers. The water insoluble proteins are elastic and make it possible to form surimi into fish cakes and crab sticks through further processing [17]. A high specificity and sensitivity of the PCR method require a high annealing temperature and selection of primers unable for formation of dimmers, duplexes and hairpin loops structure [18].

At present, researchers detect blood plasma in food products by using different method from DNA method which are sandwich enzyme-linked immunosorbent assay (ELISA) using MAbs 3D6 and 6G12 which recognizes a 60kDa serum protein in bovine blood [19] and a competitive ELISA using MAb 1H9 recognizing a 12kDa cellular protein in ruminant blood [20].

However, it is undeniable that there are several advantages of DNA analysis methods. DNA is relatively stable molecule, thus, allowing analysis of processed and heat treated food products [21]. DNA also carries organism's genetic information and the information content of DNA is greater than protein due to degeneracy of the genetic code as one goes from DNA to protein [21]. Besides that, DNA is remarkably stable molecule allowing its extraction from all kinds of tissue due to the ubiquity of DNA in every type of cell [22, 23].

CONCLUSION

Based on this study, G3 primer sequence for chicken have shown 99% significant identity to the sequence of *G. gallus* similar to the XP-C repair complementing (transglutaminase) followed by G5 primer which also obtained 99% significant identity to *G. gallus* hypothetical LOC428804 (transglutaminase) sequence. However, for primer of cow and pig, there were no positive results. Negative results may cause of the poor quality of primer design. Optimization must be done to get the best design of the primer. On contrary, PCR amplification on surimi based products had not showed positive bands of chicken blood transglutaminase, G3 and G5 in the samples. Therefore, further research need to be done to reconfirm the present of blood transglutaminase enzyme in the samples and comparison with results from other labs will verify the consistency of this result. Adding to that, further analysis on designing very specific primers for blood transglutaminase enzyme of cow and pig need to be done since primer design required a lot of effort, time consuming and well-experienced to produce a very good primer. 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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