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Detection of Non-Halal Plasma Transglutaminase in Selected Surimi-Based Products by using Sandwich ELISA Method

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Abstract: The usage of non-halal plasma transglutaminase to improve the gelling properties of surimi is prohibited for Muslim consumers. The objective of this study is to detect non-halal plasma transglutaminase in surimi products. A total of 12 samples were tested using DEAE, Unosphere Q and BioScale Macroprep High Q columns and further confirmed by Sandwich ELISA method. Three different monoclonal antibody (MAbs) species which were bovine, chicken and porcine were used to observe the reaction against the samples. The reactivity of the antibody against the antigen was defined in a certain range of cutoff value that is very strong, strong, moderate, weak and negative. By using the MAbs of the different species, the result showed S1, S2 and S3 did not contain transglutaminase from bovine while the other samples did. Six samples which were S1, S2, S3, S8, S11 and S12 selected in the ELISA procedure had a very strong reaction with transglutaminase from porcine species. For MAbs of chicken species, S12 has a weak reactivity while other samples showed very strong and strong reaction of transglutaminase. The sandwich ELISA can be a useful method to detect the presence of transglutaminase in surimi-based products, which is derived from blood of different species of mammalian animals. Further study should be done to optimize the specificity of antibody used in the confirmation of TGase in surimi.

Key words: Surimi · Monoclonal antibody · Textural · Sandwich ELISA

INTRODUCTION

Surimi provides the largest source of protein ingredients. It is prepared from mechanically de-boned fish flesh which is washed with water and blended with cryoprotectant. It can be used as a versatile raw material for producing many kinds of value added seafood products [1]. Surimi from various species has been used in different countries for producing surimi based products such as fish cakes, fish balls, fish burgers, fish sausages, fish noodles, imitation crab sticks and imitation shrimp tails [2]. Surimi possesses the functionality including gelling, binding and emulsifying properties and can be used as a functional protein ingredient in several products. It is also a three-dimensional muscle protein network.

A textural characteristic expressed in terms of gel strength is the primary determinant for surimi quality and price [3]. Protein additives have been widely used to maximize the gel strength of surimi. The additives function as proteinase inhibitors and/or as gel enhancers. The most commonly used inhibitors are bovine plasma protein (BPP), egg white and potato starch whey protein concentrate [4, 5]. Benjakul *et al*. [6] reported that the addition of porcine plasma protein (PPP) would increase the gel strength of surimi. Besides, PPP chicken plasma protein (CPP) was able to enhance the gel strength by acting as filler in surimi gel matrix and also as a proteinase inhibitor [7].

Previous researches have reported that blood plasma contain varieties of bioactive compounds including proteinase inhibitor and plasma

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transglutaminase [6, 8]. Jiang and Lee [9] purified transglutaminase (plasma factor XIII) as a possible by-product of pig plasma and demonstrated that the transglutaminase, activated with thrombin and Ca^{2+}, catalyzed covalent crosslinking of MHC and substantially increased the gel strength of minced mackerel. Transglutaminases (TG: EC 2.3.2.13, protein glutamine: amine-glutamyltransferase) are a group of thiol enzymes that catalyses the post-translational modification of proteins mainly by protein to protein cross-linking, but also through the covalent conjugation of polyamines, lipid esterification, or the deamidation of glutamine residues [10-12].

In order to protect the Muslim consumers from non-halal protein additives, the detection of the additive must be conducted. One of the methods which can be used for detection is Enzyme-linked Immunosorbent assay (ELISA). Enzyme-Linked Immunosorbent Assay (ELISA) methods are immunoassay techniques used for detection or quantification of a substance on the basis of specific antigen-antibody reaction [13]. ELISA, as a fundamental and powerful tool, is already widely used in many fields such as medicine, pharmaceutics, environmental sciences and agriculture. Food scientists have successfully applied ELISA methods in meat speciation for more than two decades [14]. This study will focus on the matter of non-halal protein additives by developing method of sandwich ELISA to detect the presence of that non-halal protein additive in surimi based product.

MATERIALS AND METHODS

Samples Preparation: The samples were purchased and selected randomly.

They were prepared by blending and stored in-20°C until needed. The samples were labeled as S1, S2, S3, S4, S5, S6, S7, S8, S9, S10, S11 and S12.

Sample Extraction of Transglutaminase: Preparation of crude Transglutaminase (TGase) was done based on method by Worratao and Yongsawatdigul [15]. The fish samples were homogenized with four volumes of extraction buffer (10 mM NaCl, 5 mM EDTA, 2 mM DTT and 10 mM Tris-HCl, pH 7.5). The homogenate was centrifuged (Allegra 25R Beckman Coulter) at 14,000g for 30 min at 4°C to separate the liquid phase or supernatant from the solid phase. The protein was in the supernatant. Subsequently, the supernatant containing protein which was Transglutaminase was centrifuged at 14,000 g for 60 min at 4° C for desalting. The supernatant was used as crude TGase.

Screening of Tgase Protein from Crude Extract: In this study, purification of Tgase was done based on Worratao and Yongsawatdigul [15]. The purification of protein was done by using Low pressure chromatography (Model 2110, Bio-Rad, Richmond, CA, USA) system according to the peak of the fraction. The fraction that showed the peak was suspected to contain the protein of interest. The fraction of sample was further analyzed by using different columns to determine that the protein was TGase. The crude TGase was applied on three columns including UNOsphere™ Q column (Bio-Rad, Richmond, CA, USA), Macro-Prep® High Q column (Bio-Rad, Richmond, CA, USA) and Macro-Prep® DEAE column (Bio-Rad, Richmond, CA, USA) in order to determine the presence of TGase separately.

The crude TGase was applied onto each columns equilibrated with 10 mM NaCl, 5mM EDTA, 2 mM DTT, 10 mM Tris-HCl, pH 7.5 (buffer A). A flow rate of 1mL/min was maintained. After being washed with two bed volumes of buffer A, the bound components were eluted with a linear gradient of 0-5 M NaCl. Fractions of 3 mL were collected using a fraction collector (Bio-Rad, Richmond, CA, USA). Fractions possessing peak of TGase were pooled.

Total Protein Measurement: Total protein content was measured by using Bradford Method (1976). In this assay, bovine serum albumin (BSA-Sigma) was used as standard. This Bovine serum albumin (BSA) solution at five concentrations including 0 (µg/mL), 32 (µg/mL), 48(µg/mL), 64(µg/mL) and 80 (µg/mL) were prepared by adding certain volume of distilled water to certain amount of BSA powder.The absorbance OD value of these 5 concentration of BSA was read by spectrophotometer and the standard curve was further plotted. The concentration of total protein was evaluated based on the standard curve.

Detection of TGase by Sandwich ELISA Procedure: Detection of TGase was done by using sandwich ELISA method according to MP BioMedicals principles. After screening by using the 3 columns, crude extract containing TGase protein were further analyzed with sandwich ELISA. Several crude extract from other samples

Table 1: Primary antibodies that were used for Sandwich ELISA

Species	Primary antibody
Bovine	Mouse Anti Factor VIII C, Factor VIII C, Monoclonal Antibody, 2A5
Porcine	Mouse Anti Porcine Tubulin Beta (N-Terminal), Tubulin Beta (N-Terminal), Monoclonal Antibody, TU-13
Chicken	Mouse Anti Chicken MHC Class II Monomorphic Monoclonal antibody, 21-1A6

which were selected randomly were loaded into an Econo-Pac Desalting column (Biorad) before being used in the ELISA procedure.

The primary antibodies used were Mouse Anti Chicken MHC Class II Monomorphic, Monoclonal antibody, 21-1A6, Mouse Anti Factor VIII C, Factor VIII C, Monoclonal Antibody, 2A5 and Mouse Anti Porcine Tubulin Beta (N-Terminal), Tubulin Beta (N-Terminal), Monoclonal Antibody, TU-13 for specific species as summarized in Table 1. These antibodies were diluted with the coating buffer (10 mM of phosphate buffer containing 145 mM of NaCl, pH 7.2). A 100 µL of each diluted antibody was added to 96 microtitre plate well (Costar, USA). The antibody should be directed against the antigen to be determined. The plate was covered with plastic film or aluminum foil before being overnight at 4º C.After an overnight incubation, the plate was washed and rinsed with 100 mM of phosphate buffer containing 500 mM of NaCl and 0.1% Tween 20 at pH 7.2 (Buffer B) twice. 100 µL of sample standard was diluted in 100 mM of phosphate buffer containing 500 mM of NaCl and 0.1% Tween 20 at pH 7.2 before being added to each well. The plate was then covered and allowed to stand at room temperature for about 2 hours.

Two secondary antibodies used were (1) Goat Anti Mouse IgG, A, M: HRP, IgG IgA IgM, PolyclonalAntibody, Polyclonal IgG and (2) Rabbit Anti Mouse (H and L), IgG (H and L), Polyclonal Antibody which were conjugated with Horseradish Peroxidase (HRP). After 2 hours incubation, 100 µL of diluted secondary antibody (in 100 mM Citric acid-phosphate buffer at pH 5.0) was further added in each well. These secondary antibodies should be directed against the antigen to be determined. The plate was then covered with plastic film or aluminum foil and allowed to stand at room temperature for about 1 hour before being washed with buffer B.

After the washing step, 100 µL of chromogenic substrate (OPD) was added to each well. The plate was covered and allowed to stand at room temperature for 15 minutes or until color has developed. The plate was protected from light during this period. The reaction was then stopped by adding 150 µL of 1 M sulfuric acid to each well.The optical value was then measured within 3 hours of color development by using ELISA plate reader (Elx 800 BioTek) at 490 nm within 3 hours of color development.

RESULTS AND DISCUSSION

Screening of transglutaminase (TGase) protein from crude extract: The TGase protein was determined based on different column and NaCl gradients. TGase was purified by using three columns including Unospehere Q, Macroprep High Q and Macroprep DEAE these columns have different pore size. The peaks were eluted as applied onto column. Column that eluted peak were anion exchanger column. The pore size of the column that eluted peak was shown in Table 2.

The peak eluted indicated that the samples have anionic properties (Figures 1a, b and c). Unospehere Q, Macroprep High Q, Macro and Macroprep DEAE columns bounded with protein of interest which is TGase. Worratao and Yongsawatdigul [15] obtained a single peak of TGase which is from Tropical tilapia (*Oreochromis niloticus*) by using DEAE-Sephacel column.

In Unospehere Q column, TGase activity were eluted as peaks at fraction 1 and 2, while in Macroprep High Q column peak was eluted at fraction 1. For Macroprep DEAE column, it indicated the eluted peaks which part of them was at fraction 6 and 13. The peaks eluted for Unospehere Q column and Macroprep High Q column were found in sample 12. Meanwhile Macroprep DEAE column eluted peaks in sample 2. These two samples were further analyzed with Sandwich ELISA.

The bound components were eluted with a linear gradient of 0-0.5 M of NaCl. The result showed the peaks were eluted in column Unosphere Q and Macroprep High Q between 0.1-0.25 M of NaCl. For Macroprep High Q column the peak was eluted between 0.4-0.5 M of NaCl. Research done by Worratao and Yongsawastdigul [15] also eluted with a linear gradient of NaCl in purification of Tropical tilapia (*Oreochromis niloticus*) between 0.15 to 0.20 M by using DEAE-Sephacel chromatography.

Table 2: Types of column and the pore size (µm)

Type of column	Pore size (µm)
Unospehere Q	120µm
Macroprep High Q,	50µm
Macroprep DEAE	50µm

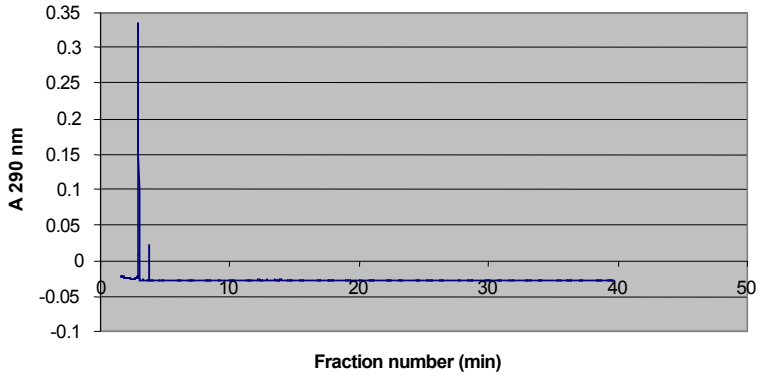

Fig. 1a: Chromatogram of TGase on Unospehere Q column, (X: 0.1-0.25 MNaCl).

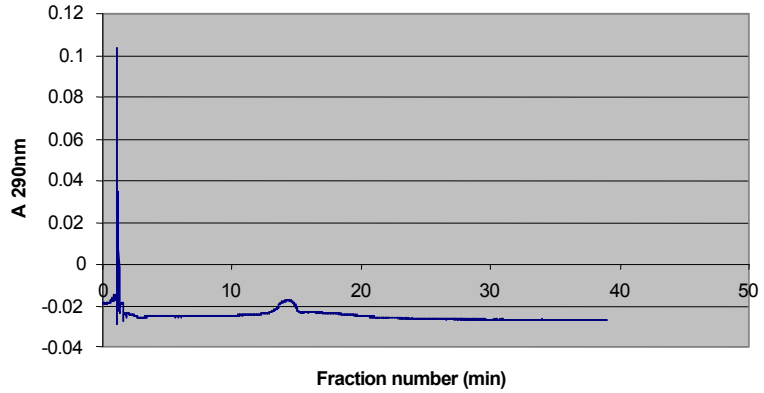

Fig. 1b: Chromatogram of TGase on Macroprep High Q column, (Y: 0.1-0.25 M NaCl)

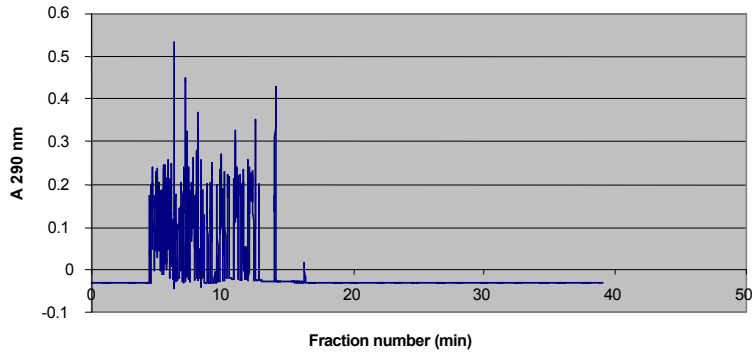

Fig. 1c: Chromatogram of TGase on Macroprep DEAE column. (Z: 0.4 - 0.5 M NaCl)

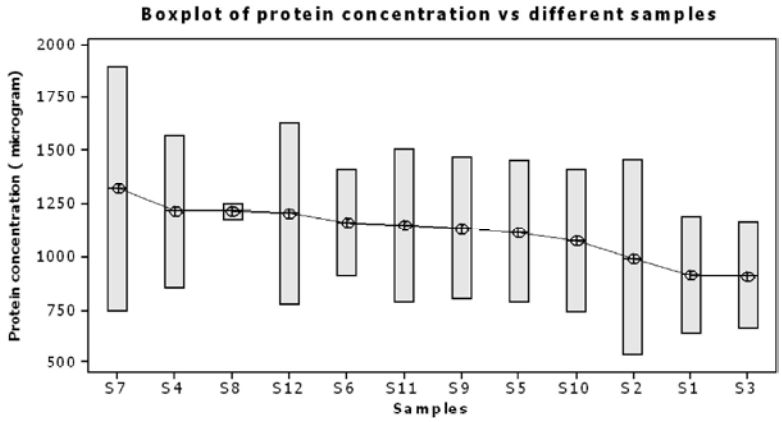

Fig. 2: Total protein of different samples

Table 3: The reactivity of Bovine, Porcine and Chicken

Sample	S1	S2	S3	S8	S9	S11	S12
Bovine	-	-	-	++++	++++	++++	++++
Porcine	++++	++++	++++	++++	+++	++++	++++
Chicken	++++	++++	+++	++++	++++	+++	++

In conclusion, the eluted peaks showed that the presence of the protein of interest, namely TGase was found in sample 12 for Unospehere Q chromatography and Macroprep High Q chromatography. The presence of the protein of interest was obtained in sample 2 for Macroprep DEAE chromatography. To confirm the TGase sources come from blood, the further step of ELISA would be done.All samples showed no difference between the means which stated the non significance different in the concentration of their protein.

Figure 2 indicate that the sample 7 contained the highest protein concentration among the different samples. However, it has the biggest box which means the highest standard deviation with the value is 813.0. There is a lot of overlap between the protein concentrations of the samples. Sample 3 has the lowest protein concentration among the different samples with the standard deviation 357.9. Protein concentration is always related to gelling properties of surimi. A research done by Luo *et al.* [16] showed that protein concentration had a major positive effect on the breaking force and distance on surimi produced from silver carp.

Detection of Tgase by using Sandwich ELISA: The specificity of antibody used is important in detecting the contamination of non-halal Tgase in the samples. The different monoclonal antibodies (MAbs) were used for detecting TGase were from bovine, porcine and chicken. For meat species identification, MAbs of known specificity would provide universal use of a consistent reagent and eventually reduce the cost of the assay [18]. The antibody played role to sandwich the sample that acted as antigen.

The reactivity of the specific bovine, porcine and chicken antibody against the antigen was shown in the Table 3. Any value that was in a certain range obtained was defined. The very strong reactivity indicated that the sample was suspicious to have non-halal plasma according to the specificity of the antibody against the samples and instead.

All the 7 tested surimi showed adulteration with transglutaminase from Porcine and Chicken. Out of 7 samples, 4 tested surimi indicated adulteration with transglutaminase from bovine (Table 3). The result showed that S8, S9, S11 and S12 reactivity were very strong while S1, S2 and S3 reactivity were negatives as bovine antibody reacted to the samples. All samples showed very strong reactivity as porcine antibody was applied to the samples except sample 9 which was just strong reactivity. For chicken antibody, S1, S2, S8 and S9 indicated very strong activity and strong reactivity with S3 and S11. The other sample which was S12 showed weak reactivity against the antibody.

From Table 3, there were samples which showed the same positive result for different species. Large molecules, such as proteins usually have multiple sites for antibody binding. On a given protein, more than 2 antibodies are thus capable of binding. In this case, TGase which acted as antigen might be having many epitopes that could bind to the antibody from different species [19].

CONCLUSION

From this ELISA study, TGase was detected in a few samples with different reactivity due to the range of cutoff. TGse derived from blood as proven by the reactivity of the three species of antigen. By using the MAbs of the different species, the result showed S1, S2 and S3 did not contain TGase from bovine while the other sample did. Six samples which were S1, S2, S3, S8, S11 and S12 selected in the ELISA procedure had very strong reactivity with TGase from porcine species. For MAbs of chicken species, S12 has a weak reactivity while other samples showed very strong and strong reactivity of TGase. ELISA is a reliable rapid technique, highly sensitive and specific for screening transglutaminase. Muslim consumer must alert to the issue of TGase in surimi especially if it was derived from the non-halal sources.

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