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Monoclonal antibody-based enzyme immunoassay for detection of porcine plasma in fish surimi

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ABSTRACT

Detection of porcine plasma using indirect ELISA was developed using mAb B4E1 for the prevention of their usage in human food that creates religious and health conflicts. The immunoassay has a CV < 20% and did not cross-react to other meat and non-meat proteins. The sensitivity of the assay is 0.25% (w/w) of porcine plasma in spiked raw and cooked fish surimi. The assay did not produce a false positive result for any of the commercial fish surimi tested that were not contain porcine plasma. Determination of a 60-kDa antigenic protein of porcine blood using Western blot confirmed its presence in the plasma fraction of the porcine blood. Further proteomic analysis involving liquid chromatography–tandem mass spectrometry (LC-MS/MS) revealed the 60-kDa protein to be porcine serum albumin.

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Porcine plasma; ELISA; monoclonal antibody; antigenic protein; fish surimi

Introduction

Animal blood has been utilised in human food, animal feed and in the laboratory, medical, industrial and fertiliser fields for many years. Currently, advanced technology in animal blood collection and processing techniques have led to the production of numerous blood protein ingredients that could give environmental, nutritional and economic benefits derived from the maximal utilisation of animal blood. The blood can be separated into two parts, a liquid and a cellular portion. Plasma, the liquid portion of blood, is mostly utilised in the food industry because it is tasteless and contains no dark colour associated with the red blood cells. Meanwhile, the cellular portion of blood is not extensively used as compared to the plasma due to its haem component, which possesses an undesirable colour, odour and gives a metallic taste to the end product.

The production of blood products involves the processing of blood into different types of products which can deliver various functional properties. For instance, spray-dried porcine plasma can be used as a proteinase inhibitor to avoid irreversible proteolytic degradation of myofibrillar proteins in surimi caused by

endogenous proteinases that will contribute to gel weakening of surimi and surimi-based foods (Ofori and Hsieh 2012). Bekhit et al. (2014) reviewed several studies about the ability of porcine plasma proteins as proteinase inhibitors in the manufacturing of surimi. In addition, porcine plasma can be used as an egg replacer in bakery products as a foaming, jellification and solubility agent. The plasma and albumin are used as solubility and jellification agents in yoghurt (Álvarez et al. 2012). Besides, fibrinogen and plasma from porcine blood can be added as emulsifiers in cakes and pastries. Thus, the demand for porcine plasma will increase to match the current requirement of bovine plasma in the food industry.

However, Muslims and Jews are prohibited from eating food containing blood as a result of dietary restrictions obligatory of their religion and because of the commands preserved in the Halal and Kosher dietary laws, respectively (Mukhtar and Mohsin Butt 2012). There are also people who avoid blood-containing food as a matter of preference (Ofori and Hsieh 2014). Vegetarians avoid consuming products of animal origin for ethical or religious reasons.

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Prions, or infectious proteins, are extremely resistant to heat, ultraviolet light, ionising radiation, normal sterilisation processes and common disinfectants that normally destroy viruses and bacteria (Huong et al. 2014). Thus, humans are potentially exposed to these infectious agents (abnormal prion proteins) through the consumption of animal blood added intentionally (adulteration) or unintentionally in food because the process involved in the manufacturing of these blood-containing materials does not inactivate the infectious agent as a result of their resistant nature. Even though there is no evidence of natural cases of transmissible spongiform encephalopathy (TSE) in non-ruminants including porcine (Greenlee et al. 2016), the development of a scientific method to detect porcine blood derivatives in food is imperative because although the religious law banning non-Halal or non-Kosher blood has been stated, the addition of porcine blood in food still occurs. This is due to the lack of existing methods to help to enforce the laws while manufacturers may adulterate expensive meats with a high amount of blood proteins fraudulently to increase the protein content in their food products.

Until now, there have been no scientific methods developed to determine the presence of porcine plasma in food. Thus, there is a need to develop a new scientific method which is able to detect porcine plasma in food, especially in processed forms that have undergone heat treatment. In addition, the method should be based on the detection of heated porcine blood as well as plasma and should be able to discriminate between prohibited tissue (blood) of porcine species and allowed tissue (halal meats), as well as discriminating between blood proteins and proteins from other sources (soy protein, whey, potato extracts, egg albumin, etc.) that are commonly present in food.

This study was conducted to develop an indirect non-competitive ELISA for qualitative detection of porcine plasma in fish-based products especially surimi using a monoclonal antibody (mAb) B4E1 developed to be specific to porcine blood. Antigen analysis of porcine plasma was carried out to identify which protein reacts with the mAb. The use of mAb for ELISA offers an extra advantage because the mAb itself is species-specific and differentiates specific animal blood from other blood species, other tissues and common food proteins.

Materials and methods

Preparation of mAb B4E1

The mAb B4E1 used in this work was obtained from the supernatant secreted by B4E1 hybridoma cell by using heat-soluble protein from porcine blood as an antigen, according to the method described by Raja Nhari et al. (2016). The affinity and specificity to porcine blood of the mAb B4E1 has been also validated by a previous study (Raja Nhari et al. 2016).

Production of ascites fluid

mAb B4E1 as described by Raja Nhari et al. (2016) was prepared from ascites fluid generated by a hybridoma. Based on Zhang (2012), BALB/c mice were primed with pristane (P9622, Sigma-Aldrich, St. Louis, USA) 10 days before injection of cells. A cell suspension containing the desired number of viable cells was transferred into a 15-ml conical test tube and was centrifuged at 200g for 5 min. The supernatant was discarded and the cells were resuspended in 10 mM PBS. Each mouse was inoculated with 3.25×10^5 cells/0.5 ml to allow for the hybridoma to grow in the peritoneal cavity area. The mice were then monitored for general health and the accumulation of ascites fluid. Ascites fluid accumulation occurred within 7–21 days following the injection of hybridoma cells. The mice were exsanguinated by carbon dioxide when they showed difficulty in breathing due to the accumulation of ascites fluid. The ascites fluid was drained into the tube by gentle massaging of the abdomen away from the insertion point of a 26-gauge needle. The fluid was centrifuged at 200g for 10 min at 4°C to pellet the cells. The fluid supernatant was aliquoted into volumes to avoid repeated freeze–thaw cycles and was stored at –80°C.

Development of indirect non-competitive ELISA using mAb from ascites fluid

Checkerboard titration

The optimum dilution for ascites fluid and secondary enzyme-conjugated antibody was determined using coating antigen consisted of raw porcine plasma. The plasma was diluted with sodium carbonate–bicarbonate buffer (pH 9.6). An aliquot of 100 µl of 5 µg/ml diluted plasma was added to a 96-well microtitre plate to coat the well surface. The

plate was covered and incubated for 90 min at 37°C. The samples were removed and the plate was washed three times with 300 µl washing buffer. The blocking buffer (200 µl) was added and incubated for 90 min at 37°C. The blocking buffer was then removed and the plate was washed. Aliquots of 100 µl of diluted ascites fluid (1:100–1:204,800) were transferred to the plate and incubated for 90 min at 37°C. The samples were removed and the plate was washed. Then, 100 µl of diluted enzyme-conjugated secondary antibody (anti-mouse IgG [γ chain specific]-peroxidase antibody, 1:2500–1:320,000 in PBS) was added and incubated. The secondary antibody was removed and the plate was washed. This was followed by the addition of ABTS substrate solution (100 µl/well, Roche, Mannheim, Germany). The plate was then incubated in the dark at room temperature (RT) for 60 min until the green colour was developed. The absorbance reading was measured at OD₄₀₅ using a TECAN Infinite® M200 microplate reader.

Cross-reactivity of mAb against meat and non-meat proteins

The cross-reactivity of diluted ascites fluid of mAb B4E1 was determined using ELISA against 0.005 mg/ml of raw and cooked pork, beef, mutton, chicken, fishes, soy protein, whey, potato extract and egg albumin.

Sensitivity of ELISA

The intra- and interassay variabilities of the ELISA were measured with standard dilutions of porcine plasma (40, 200, 1000 and 5000 ng/ml) in triplicate on three different days. The coefficients of variation for intra- and interassays were determined based on the standard deviation and mean for each concentration of standard (Tukiran et al. 2015).

Analysis of spiked fish surimi samples using ELISA

The fish surimi was prepared by mixing approximately 15 g deboned fish mackerel paste, 0.1% (w/w) salt, 5% (w/w) sugar, 1% (w/w) monosodium glutamate (MSG) and starch. The samples were placed in a water bath (45°C) for 20 min and cooked (100°C) for 5 min. Then, the samples were cooled in cold water (4°C) for 5 min. Then, the samples were mixed with 16 ml of 10 mM PBS, homogenised, held, centrifuged and filtered. The protein extract was determined using Bradford assay. Protein extracts from porcine plasma and the fish

surimi were adjusted to achieve a similar concentration. Then, the protein extracts from porcine plasma were added to the fish surimi to produce a spiking level of 10–0.10% (w/w). The spiked samples were subjected to ELISA and the lowest detection level of porcine plasma in the spiked samples was determined.

Qualitative test for detection of porcine plasma in commercial fish surimi

Thirteen commercial fish surimi of various brands were purchased from the local supermarket in Selangor. The fish surimi was minced using a grinder to ensure thoroughness and homogeneity. Ten grams of minced sample from each fish surimi were placed in a 50-ml beaker. The sample was mashed into fine particles using a glass rod. An aliquot of 10 ml of extraction buffer (PBS) was added to each sample. The mixture was homogenised for 3 min at 9500 rpm. The homogenised sample was incubated for 2 h at RT and another 2 h at 4°C. Then, the mixture was centrifuged at 5000 rpm for 60 min at 4°C. Each supernatant was filtered using Whatman No. 1 filter paper and stored at –20°C until further analysis using ELISA. The raw commercial fish surimi was used without any heat treatment.

SDS-PAGE and Western blot

Supernatants from raw, heat-treated porcine blood and commercial porcine plasma (3 µg of protein in 15 µl per lane) were loaded on a 4% stacking gel (pH 6.8) and separated on 12% polyacrylamide gel (pH 8.8). The gel was subjected to electrophoresis at 100 V for 1 h using a Mini-PROTEAN Tetra cell (Bio-Rad, Hercules, CA, USA). Then, the separated proteins were electrophoretically transferred (1 h at 100 V) from the gel to 0.2 µm nitrocellulose membranes using a Mini Trans-Blot unit (Bio-Rad). Western blot was carried out by immersing the membrane in blocking solution (30 g/l gelatine in 1 × Tris-buffered saline) and gently agitated for 1 h at RT. Then, the membrane was washed for 10 min using TTBS washing solution [20 mM Tris, 500 mM sodium chloride, 0.05% (v/v) Tween-20, pH 7.5]. Next, 100 ml of primary antibody solution [1:1000 supernatant of cell culture in 100 ml of 1% (w/v) gelatine, 20 mM Tris, 500 mM sodium chloride, 0.05% (v/v) Tween 20, pH 7.5] was added to the membrane. The membrane was incubated for 2 h. The unbound antibody was

removed by washing the membrane twice in TTBS for 10 min at RT. Then, the enzyme-conjugated antibody solution (33 μ l of goat anti-mouse IgG [H + L] HRP conjugate in 100 ml of 1% [w/v] gelatine, 20 mM Tris, 500 mM sodium chloride, 0.05% [v/v] Tween 20, pH 7.5) was added to the membrane for 2 h of incubation. The antibody was removed and washed twice for 10 min. Just prior to colour development, the membrane was washed in TBS for 5 min to remove residual Tween-20 from the membrane surface. Then, 600 μ l of HRP colour reagent B were added to 100 ml of 1 $\times$ HRP colour development buffer at RT. This solution was added immediately to 20 ml of HRP colour reagent A prior to use. The membrane was immersed in colour development solution within 30 min. The colour development was stopped by immersing the membrane in double-distilled water twice for 10 min.

Identification of antigenic porcine blood protein using liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The targeted 60 kDa protein band was excised from the SDS-PAGE gel and destained with a destaining solution. Then, 100 μ l of 100 mM ammonium bicarbonate/acetonitrile (1:1) was added and incubated with occasional vortexing for 30 min. An aliquot of 500 μ l acetonitrile was added and incubated at RT with occasional vortexing until the gel pieces became white and shrunk and then the acetonitrile was removed. The gel pieces were covered with 50 μ l of trypsin buffer and incubated at 4°C for 2 h. Then, 20 μ l of ammonium bicarbonate buffer was added to cover the gel pieces to keep them wet during enzymatic cleavage. The tubes with gel pieces were placed into an air circulation thermostat and the samples were incubated overnight at 37°C for analysis performed at the limit of the instrument sensitivity, which requires maximal peptide recovery. An aliquot of 100 μ l of extraction buffer was added [1:2 (v/v), 5% formic acid/acetonitrile] to each tube and was incubated for 15 min at 37°C in a shaker.

For further LC-MS/MS analysis, 20 ml of 0.1% (v/v) trifluoroacetic acid was added into the tube, vortexed and incubated for 2–5 min in the sonication bath and centrifuged for 15 min at 10,000 rpm. Aliquots (180 μ l) were withdrawn for further analysis. Dionex UltiMate 3000 RSLCnano (Thermo Fisher Scientific) was used

with mobile phase A (0.1% formic acid in water) and B (0.1% formic acid in ACN). The gradient was set as 5–40% B in 91 min, 2 min to 95% B, 6 min at 95% B, back to 5% B in 2 min and the flow rate was set to 300 nl/min. The separation column used was EASY-Spray Column Acclaim PepMapTM C18 100 A⁰ (2 μ m particle size, 75 μ m id $\times$ 25 cm; column temperature 40°C) and the injection volume was 2 μ l.

An Orbitrap Fusion (Thermo Fisher Scientific) mass spectrometer was used and operated in the data-dependent mode. Full scan spectra were collected (OTMS1) using the following parameters: scan range 310–1800 m/z , resolving power of 120,000, AGC target of 4.0 e^5 (400,000) and a maximum injection time of 50 ms. The method consisted of 3 s Top Speed Mode where precursors were selected for a maximum 3-second cycle. Only precursors with an assigned monoisotopic m/z and a charge state of 2–7 were further analysed for MS2. All precursors were filtered using a 20-second dynamic exclusion window and intensity threshold of 5000. The MS2 spectra were analysed (ITMS2) using the following parameters: rapid scan rate with a resolving power of 60,000, AGC target of 1.0 e^2 (100), 1.6 m/z isolation window, and a maximum injection time of 250 ms. Precursors were fragmented by CID and HCD at normalised collision energy of 30% and 28%.

Statistical analysis

Statistical analyses including mean, standard deviation (SD) and coefficient of variation (CV) calculations were performed using Microsoft Excel 2007.

Results and discussion

Development of mAb B4E1-based enzyme immunoassay for porcine plasma detection

In the previous study we obtained mAb B4E1 with the ability specifically to detect porcine blood. Due to the potential of this antibody in food authentication, its applicability in the development of an enzyme immunoassay for porcine plasma detection was examined in this study. Due to the very low concentration of mAb B4E1 in the supernatant secreted by the hybridoma, ascites fluid containing mAb B4E1 was prepared in this study. Approximately 2.25 ml of ascites fluid was collected from BALB/c mice. Optimum dilutions of mAb

B4E1-containing ascites fluid and secondary enzyme conjugated antibody were then determined. The mAb B4E1-containing ascites fluid underwent several serial dilutions based on the previous study related to the development of mAbs (Hsieh et al. 2007; Wang et al. 2015). Based on Figure 1, the optimum dilutions for mAb B4E1-containing ascites fluid and the secondary enzyme-conjugated antibody were 1:25,600 and 1:2500, respectively. The optimum dilution was based on the absorbance reading of the assay at 405 nm with a value of > 1 . The optimum dilution for both antibodies was then used for further analysis.

Intra- and inter-assay variabilities of the mAb B4E1-based ELISA were then determined in order to assess repeatability and reproducibility of the assay (Table 1). Serial diluted porcine plasma standard solution (40, 2000, 1000 and 5000 ng/ml) was used in the assay. CV (coefficient of variation) is expressed as a percentage of variance to the mean and indicates any inconsistencies and inaccuracies in the assay. A lower CV reflects a good consistency of the assay while larger CV indicates greater inconsistency and error. The intra-assay variation of this mAb B4E1-based ELISA produced CVs in the range of 1.12–19.25%, whereas the inter-assay variation gave CVs of 9.2–16.8%. The CVs of the developed ELISA was $< 20\%$, which is considered acceptable (Tukiran et al. 2015; Kreuz et al. 2012; Xue et al. 2012). The limit of detection (LOD) and limit of quantification

(LOQ) of ELISA were 0.02 μg and 0.1 μg , respectively, which reflect the sensitivity of the antibody when tested with a standard porcine plasma solution. However, the pooled ascites fluids will probably have higher variation when different batches of ascites fluid from different mice are used (Ward et al. 1999). Thus, future research should develop a methodology to efficiently and consistently produce a purified mAb B4E1 through a fermentation process.

The cross-reactivities of mAb B4E1-containing ascites fluid against meat and non-meat proteins (raw and boiled) were determined using the developed assay. The samples involved were raw pork, beef, mutton, chicken, fishes, soy protein, whey, potato extract and egg albumin. The specificity of the assay to porcine plasma was confirmed as evidenced by the absence of cross-reactivity with the raw samples (Figure 2). The absorbance of each sample was lower than the baseline that reflects the absorbance of the control (sodium carbonate–bicarbonate buffer, pH 9.6) and was significantly different as compared to raw porcine plasma ($P < 0.05$). Additionally, cross-reactivity tests of mAb B4E1-containing ascites fluid against boiled pork, beef, mutton, chicken, fishes, soy protein, whey, potato extract and egg albumin also showed that there was no cross-reactivity with the any tested samples as the absorbance of each sample was lower than the control and significantly different as compared to boiled porcine plasma ($P < 0.05$) (Figure 3). This result indicates that mAb B4E1 is a candidate for detection of porcine

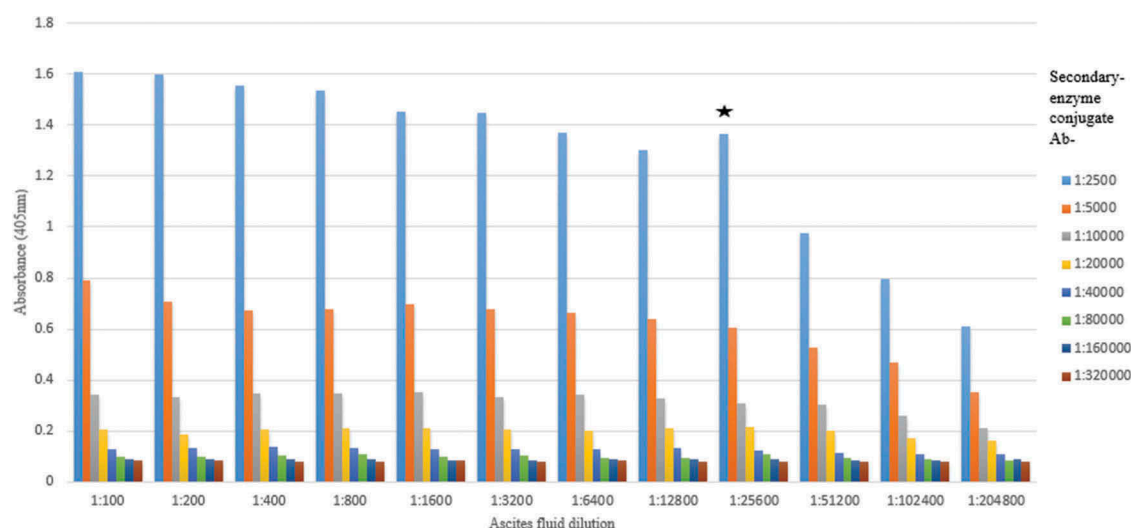


Figure 1. Dilutions of ascites fluid mAb B4E1 (1:100–1:204,800) and secondary enzyme conjugated antibody (1:2500–1:320,000) used in the reaction to raw porcine plasma using ELISA. Dilution of 1:25,600 and 1:2500 (marked with a star) for mAb B4E1 and secondary enzyme conjugate antibody, respectively was chosen. All data were expressed as mean $\pm$ SD; $n = 3$.

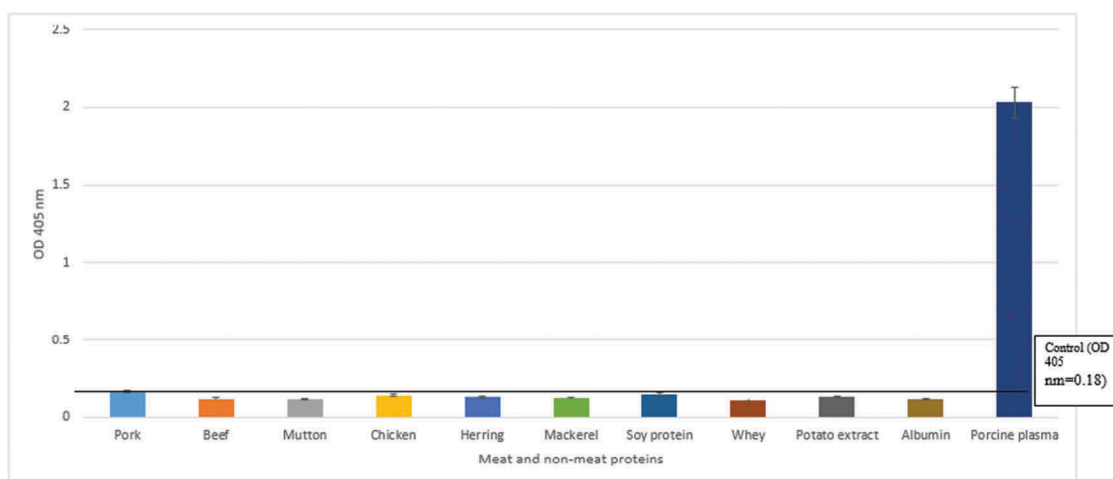


Figure 2. Cross-reactivity of mAb B4E1 against raw meat and non-meat proteins. The absorbance of the raw meat and non-meat proteins was lower than the control. Absorbance for each sample was significantly lower as compared to porcine plasma. The P -value for each sample was 0.0000 ($P < 0.05$). All data were expressed as mean $\pm$ SD; $n = 3$.

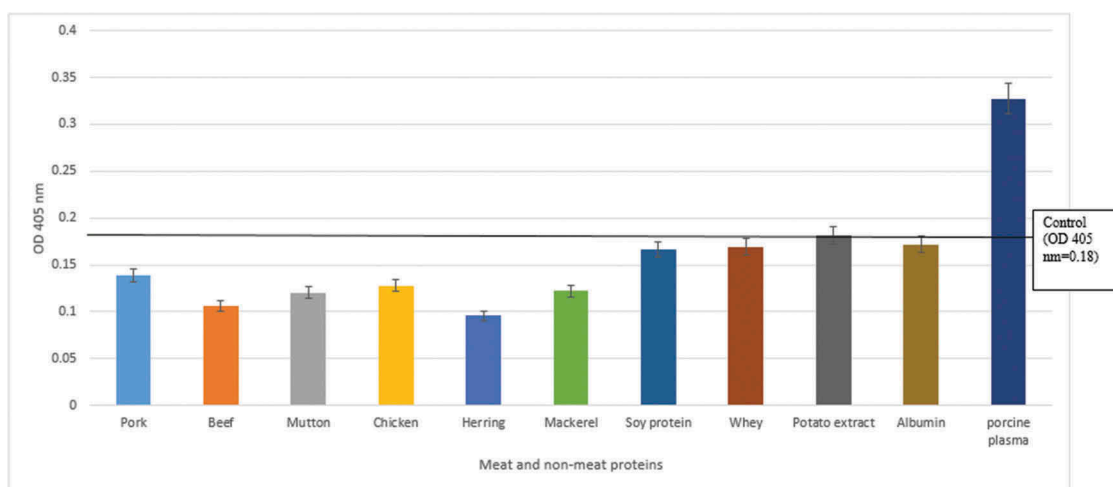


Figure 3. Cross-reactivity of mAb B4E1 against boiled meat and non-meat proteins. The absorbance of the boiled meat and non-meat proteins was lower than the control. Absorbance for each sample were significantly lower ($P < 0.05$) as compared to porcine plasma. The P -value for each sample was 0.000 ($P < 0.05$). All data were expressed as mean $\pm$ SD; $n = 3$.

plasma using ELISA as no reaction occurred between mAb B4E1 and meat and non-meat proteins that are commonly used in food and animal feed. The specificity results obtained in this study are also in agreement with the previous study that used cell supernatant of mAb B4E1 secreted from the hybridoma (Raja Nhari et al. 2016) as the primary antibody.

Analysis of spiked porcine plasma in model fish surimi using mAb B4E1-based enzyme immunoassay

Model fish surimi spiked with different amount of porcine plasma from 0.10% (w/w) to 10% (w/w) were

analysed using ELISA to evaluate the applicability of the mAb B4E1 in detecting porcine plasma in a commercial fish surimi. As evidenced in Figure 4, the developed assay successfully detects as low as 0.25% (w/w) porcine plasma in a model fish surimi. The lowest porcine plasma spiking level, 0.10% (w/w) could not be detected by the assay as reflected by an absorbance reading lower than the control. Porcine plasma spiked at 0.10% (w/w) did not produce an antigenic reaction in the ELISA due to the occurrence of matrix effects between porcine plasma proteins and other components in the fish surimi. Matrix effects or the effects of other substances in the fish surimi might

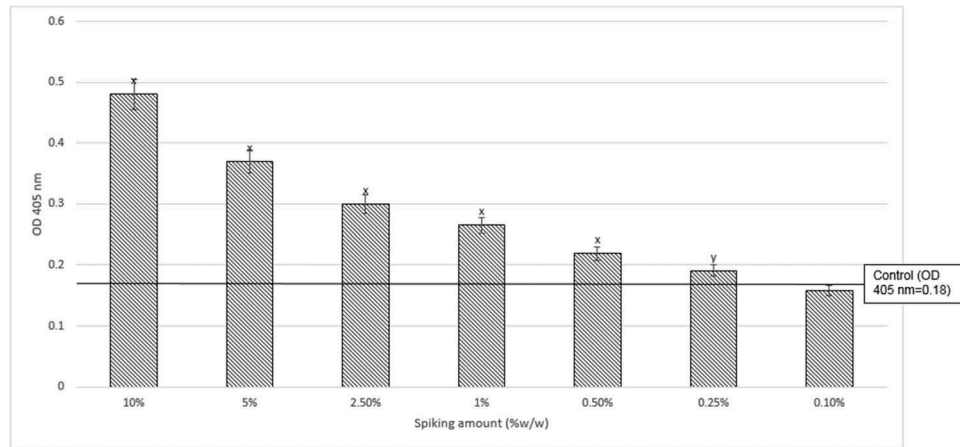


Figure 4. Evaluation of ELISA towards spiked samples of porcine plasma in a fish surimi. Absorbance for all spiked samples was a significantly different ($P < 0.05$) as compared to mean of absorbance for control. Value of P -value: $x < 0.05$ and $y < 0.05$. All data were expressed as mean $\pm$ SD; $n = 3$.

inhibit the antibody–antigen binding. Most matrix effects can be caused by other substances in the sample (phospholipids, carbohydrates and metabolites), pH of the sample, high viscosity, direct interaction between the antigen and other proteins in the sample and salt concentrations (Cai et al. 2013). In this case, the ratio of concentration between fish meat proteins, salt, sugar and MSG at 0.10% (w/w) may be higher than the concentration of porcine plasma proteins. The amount of porcine plasma in fish surimi depends on the type of surimi because the plasma impacts the functional properties of fish surimi. In this study, the ELISA could become a reliable qualitative method for detection of 0.25% (w/w) porcine plasma in fish surimi when the mAb B4E1 reached its sensitivity at 0.25% (w/w) porcine plasma in laboratory model fish surimi. The previous study by Ofori and Hsieh (2007) showed that their ELISA could detect 0.1% (w/w) spray-dried bovine plasma in spray-dried porcine plasma. However, spiking of bovine plasma and porcine plasma into laboratory-prepared food has not yet been tested.

Porcine plasma detection in commercial fish surimi using mAb B4E1-based enzyme immunoassay

The inclusion of porcine plasma in fish surimi is known to inhibit the degradation of myofibrillar proteins caused by endogenous proteases that reduce the textural property of the surimi (Singh and Benjakul 2017). The mAb B4E1-based enzyme immunoassay developed in this study was used to detect the presence of porcine plasma in the commercial fish surimi in both raw and boiled conditions. Commercial fish surimi do not contain porcine plasma listed in their ingredients although brands 1, 6 and 8 have no ingredients information on their packaging (Table 2). Of the 12 samples being analysed, none was found to contain porcine plasma at a concentration of more than 0.25% (w/w) (Figure 5). The absorbance of the commercial fish surimi found by the mAb B4E1-based enzyme immunoassay was lower and significantly different as compared to the control ($P < 0.05$). The validity of the assay was proven by successful detection of raw

Table 1. Intra- and interassay variability of indirect non-competitive ELISA.

Standard (ng/ml)	Day 1		Day 2		Day 3		Intra-assay variation CV (%)			
	Mean	SD	Mean	SD	Mean	SD				
	Absorbance (405 nm)						Day 1	Day 2	Day 3	Inter-assay variation CV (%)
Control	0.141525	0.027253	0.124475	0.00396	0.140186	0.016819	19.25643	3.185827	11.99741	13.06559
40	0.145775	0.020093	0.139083	0.006183	0.144883	0.009103	13.78343	4.445494	6.283146	9.208376
200	0.196400	0.023408	0.222050	0.003695	0.208358	0.016651	11.91851	1.664188	7.991645	9.288068
1000	0.457233	0.028464	0.386042	0.006658	0.362700	0.018723	6.225168	1.724561	5.162079	11.23989
5000	0.695167	0.036141	0.538492	0.006075	0.477000	0.023908	5.198867	1.128217	5.012160	16.89297

The absorbance of mAb B4E1 against raw porcine plasma was recorded for three days. The variability of intra and inter-assay of ELISA were determined based on CV % ($n = 6$).

Table 2. Commercial fish surimi products analysed by ELISA.

Brand	Ingredient	Issuer country of Halal certification
1	N/A	Malaysia
2	Surimi, salt, wheat flour, MSG	Malaysia
3	Itoyori surimi (nemipterus, vigatus, sugar, sorbitol, sodium tripolyphosphate), potato starch, wheat starch, soybean oil, US-certified colour (FD and Yellow No. 5 (tartrazine), FD & C Red No. 3 (erythrosine)	N/A
4	White fish meat (threadfin bream), soya milk, water, potato starch, soybean oil, sugar, salt, egg albumin powder, collagen case, flavour enhancer (E621), emulsifier (E451(i))	Thailand
5	Fish meat, salt, starch, sugar, oil, MSG	Malaysia
6	N/A	Malaysia
7	Surimi, starch, palm oil, sugar, salt	Malaysia
8	N/A	N/A
9	Surimi, starch, palm oil, sugar, salt, flavour enhancer, calcium carbonate, D-xylose, milk, margarine, white pepper, soup powder, dried shitake mushroom	Thailand
10	Fish meat, starch, sugar, salt, oil, MSG	Malaysia
11	Fish meat, corn starch, salt, sugar, MSG	Malaysia
12	Surimi, tapioca starch, salt, sugar, V/o, albumin, seasoning.	Malaysia

Twelve fish surimi products were available in the market with each product certified Halal from Malaysia and Thailand. N/A, not available.

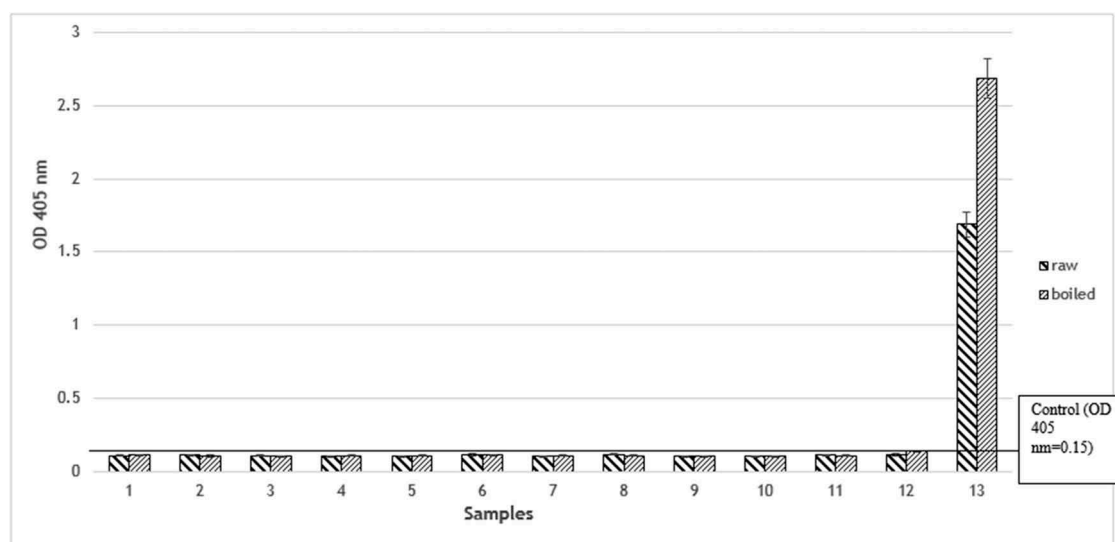


Figure 5. Cross-reactivity of ELISA against commercial fish surimi products (1–12). Label 13 is standard porcine plasma. The mAb did not cross-react with all commercial fish surimi whether raw or after the boiling process. Absorbance for all samples was significantly lower than standard porcine plasma ($P < 0.05$). All data were expressed as mean $\pm$ SD; $n = 3$.

and boiled porcine plasma that have been used as positive control.

From the results, we could also conclude that mAb B4E1 was non-reactive towards any proteins or additives which are ingredients of commercial fish surimi based on its ability to detect the presence of 0.25% (w/w) porcine plasma in laboratory model fish surimi.

Identification of putative antigenic porcine blood proteins against mAb B4E1

The protein extracts from raw and heated porcine blood and porcine plasma were separated based on their molecular weights using SDS-PAGE. As

illustrated in Figure 6, most of the proteins from porcine blood and porcine plasma were degraded after heat treatment (boiling and autoclaving) as a significant reduction in the number and intensity of polypeptide bands were observed. During heat treatment, blood proteins will be denatured and the higher molecular weight proteins will degrade into lower molecular weight peptides. The number of polypeptide bands that can be stained was reduced. Coomassie blue R-250 has a sensitivity of approximately 100 ng. Therefore, the number and intensity of cooked porcine blood proteins were reduced as compared to its native condition. Silver staining may be suitable to confirm the degradation level of the

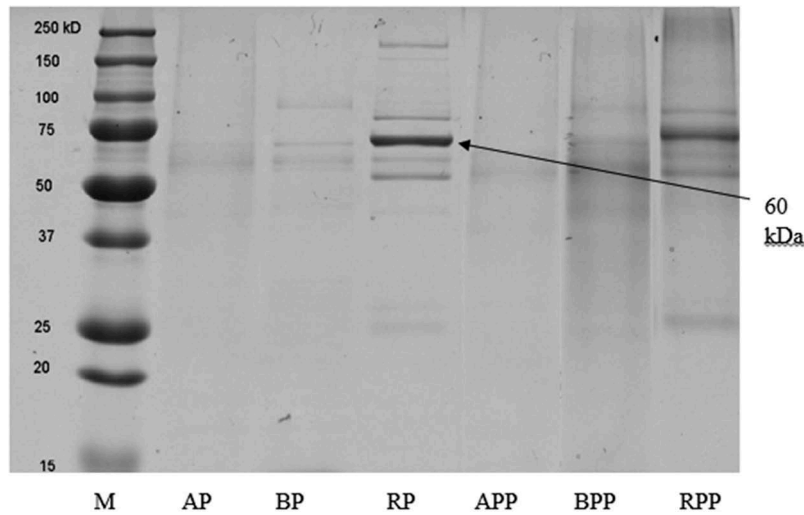


Figure 6. SDS-PAGE of porcine blood and spray-dried porcine plasma. Lane M, Precision Plus Protein™ Standards All Blue; Lane AP, autoclaved porcine blood; lane BP, boiled porcine blood; lane RP, raw porcine blood; lane APP, autoclaved porcine plasma; lane BPP, boiled porcine plasma; lane RPP, raw porcine plasma. The arrows indicate the band of antigenic proteins.

cooked proteins as its sensitivity is approximately 0.25 ng and it will stain lower molecular peptides.

Western blot analysis using mAb B4E1 against proteins of porcine blood and porcine plasma reveals the binding of mAb B4E1 to an approximately 60 kDa protein in porcine blood and plasma (Figure 7). mAb B4E1 was found to be able to detect the putative antigenic protein in raw, boiled and autoclaved porcine blood. The reactivity of mAb B4E1 to autoclaved porcine blood was expected as the latter was used as the immunogen for the production of mAb (Raja Nhari et al. 2016). However, mAb B4E1 was unable to detect antigenic proteins in autoclaved porcine plasma, but was found to be reactive towards the putative antigenic

protein in raw and boiled commercial porcine plasma. The production of commercial porcine plasma involves a severe heat treatment in the spray-drying step, with inlet air temperatures of 200–230°C and outlet air temperatures of > 80°C for at least 2 min. Further autoclaving of commercial porcine plasma may cause irreversible denaturation or degradation of the antigenic protein, which explains the inability of the mAb to bind to the antigenic protein (Boxman et al. 2017).

LC-MS/MS analysis of putative antigenic protein

The polypeptide bands of porcine plasma between 50 and 75 kDa were excised and analysed using LC-MS/

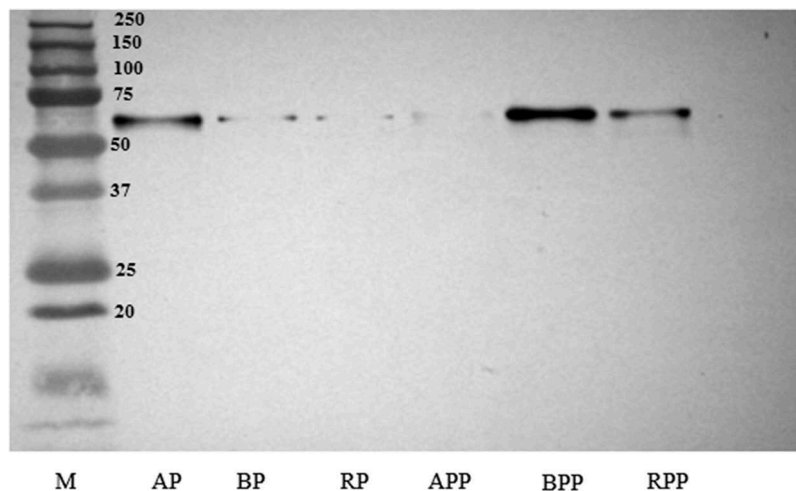


Figure 7. A representative Western blot showing selectivity of mAb to 60 kDa porcine blood. Lane M, Precision Plus Protein™ Standards All Blue; Lane AP, autoclaved porcine blood; lane BP, boiled porcine blood; lane RP, raw porcine blood; lane APP, autoclaved porcine plasma; lane BPP, boiled porcine plasma; lane RPP, raw porcine plasma.

Table 3. List of proteins found in raw and cooked porcine plasma.

Accession no.	Protein	Species	MW (kDa)	Calc. pI	Sum PEP Score (> 200)		Coverage (%)		PSMs		Unique peptides	
					Raw	Cooked	Raw	Cooked	Raw	Cooked	Raw	Cooked
P08835	Serum albumin	<i>Sus scrofa</i>	69.647	6.49	286.48	227.97	71.83	67.05	889	574	53	46
P09571	Serotransferrin	<i>Sus scrofa</i>	76.918	7.14	193.61	161.37	62.36	60.06	280	264	40	36

MS in order to identify the potential antigenic protein. Table 3 shows the two putative antigenic proteins to be identified as porcine serum albumin and porcine serotransferrin. The higher value of protein score, unique peptides, peptide–spectrum matches (PSM) and a higher percentage of coverage of porcine serum albumin was obtained in raw porcine plasma as compared to the cooked porcine plasma. In detail, for the raw porcine plasma, the protein score of porcine serum albumin was 286.4 and the sequence percentage of the porcine serum albumin that is covered by identified peptides of porcine plasma was 71.8%. The PSM was 889, which is the total number of identified peptide spectra of porcine plasma matched with the porcine serum albumin and the number of unique peptide sequences to porcine serum albumin was 53. For cooked porcine plasma, the protein score was 227.9; the sequence percentage of the porcine serum albumin covered by identified peptides of porcine plasma was 67.05%; the number of peptide sequences that are unique to porcine serum albumin were 46, and the number of PSM was 574. The reduction in protein score, sequence coverage, the number of unique peptides and PSM of porcine serum albumin in cooked porcine plasma as compared to raw porcine plasma is a possible indicator of the denaturation or degradation of several peptides of the serum albumin in cooked porcine plasma. In addition, the raw and cooked porcine plasma were positively matched with the porcine serum albumin rather than porcine serotransferrin as the protein possesses the higher value of protein score, unique peptides, PSMs, and a higher percentage of coverage in both raw and cooked. Importantly, this result suggests that the porcine serum albumin could potentially be used as a marker for detection of porcine plasma in raw and cooked food.

Conclusion

This study has developed an indirect non-competitive ELISA for the detection of porcine plasma in a laboratory model of fish surimi and fish surimi products using mAb B4E1, which is specific to blood and able

to discriminate between other blood and tissues including meat and non-meat proteins. The inter- and intra-assays of ELISA showed coefficients of variation (CVs) < 20%. LOD and LOQ of ELISA were 0.2 µg/g and 1 µg/g, respectively. The sensitivity of the ELISA is 0.25% (w/w) porcine plasma in fish surimi. No commercial fish surimi contained porcine plasma when tested using the ELISA. mAb B4E1 binds specifically to a 60-kDa protein of raw and heated porcine blood, found to be porcine serum albumin (Accession no.: P08835; MW = 69.647 kDa, calculated pI = 6.49). We conclude that mAb B4E1 combined with ELISA method could be applied by regulatory agencies as the first quality control tool for detecting porcine plasma in fish surimi.

Disclosure statement

No potential conflict of interest was reported by the authors.

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References

- Álvarez C, García V, Rendueles M, Díaz M. 2012. Functional properties of isolated porcine blood proteins modified by Maillard's reaction. *Food Hydrocoll.* 28:267–274.
- Bekhit AA, Hopkins DL, Geesink G, Franks P. 2014. Exogenous proteases for meat tenderization. *Crit Rev Food Sci Nutr.* 54:1012–1031.
- Boxman ILA, Jansen CCC, Hägele G, Zwartkruis-Nahuis A, Cremer J, Vennema H, Tijsma ASL. 2017. Porcine blood used as ingredient in meat productions may serve as a vehicle for hepatitis E virus transmission. *Int J Food Microbiol.* 257:225–231.
- Cai Q-F, Wang X-C, Liu G-M, Zhang L, Ruan M-M, Liu Y, Cao M-J. 2013. Development of a monoclonal antibody-

- based competitive enzyme linked-immunosorbent assay (c-ELISA) for quantification of silver carp parvalbumin. *Food Control*. 29:241–247.
- Greenlee JJ, Kunkle RA, Smith JD, Greenlee MHW. 2016. Scrapie in swine: a diagnostic challenge. *Food Saf*. 4:110–114.
- Hsieh YHP, Ofori JA, Rao Q, Bridgeman CR. 2007. Monoclonal antibodies specific to thermostable proteins in animal blood. *J Agric Food Chem*. 55:6720–6725.
- Huong VTL, Hoa NT, Horby P, Bryant JE, Kinh NV, Toan TK, Wertheim HFL. 2014. Raw pig blood consumption and potential risk for *Streptococcus suis* infection, Vietnam. *Emerg Infect Dis*. 20:1895–1898.
- Kreuz G, Zagon J, Broll H, Bernhardt C, Linke B, Lampen A. 2012. Immunological detection of osteocalcin in meat and bone meal: a novel heat-stable marker for the investigation of illegal feed adulteration. *Food Addit Contam Part A*. 29:716–726.
- Mukhtar A, Mohsin Butt M. 2012. Intention to choose *Halal* products: the role of religiosity. *J Islam Mark*. 3:108–120.
- Ofori JA, Hsieh YHP. 2007. Sandwich enzyme-linked immunosorbent assay for the detection of bovine blood in animal feed. *J Agric Food Chem*. 55:5919–5924.
- Ofori JA, Hsieh YHP. 2014. Issues related to the use of blood in food and animal feed. *Crit Rev Food Sci Nutr*. 54:687–697.
- Ofori JA, Hsieh YHP. 2012. The use of blood and derived products as food additives. In: El-Samragy Y, editor. *Food additive*. Rijeka: InTech; p. 229–256.
- Raja Nhari RMH, Hamid M, Rasli NM, Omar AR, El Sheikh AF, Mustafa S. 2016. Monoclonal antibodies specific to heat treated porcine blood. *J Sci Food Agric*. 96:2524–2531.
- Singh A, Benjakul S. 2017. Effect of serine protease inhibitor from squid ovary on gel properties of surimi from Indian mackerel. *J Texture Stud*. x:xx.
- Tukiran NA, Ismail A, Mustafa S, Hamid M. 2015. Enzyme immunoassay for the detection of porcine gelatine in edible bird's nests. *Food Addit Contam Part A*. 32:1023–1028.
- Wang Z, Jiyuan Y, Su C, Xinyuan Q, Lijie T, Yijing L. 2015. Development of an antigen capture enzyme-linked immunosorbent assay for virus detection based on porcine epidemic diarrhea virus monoclonal antibodies. *Viral Immunol*. 28:184–189.
- Ward PA, Adams J, Faustman D, Gebhart GF, Geistfeld JG, Imbaratto JW, Scharff MD. 1999. Monoclonal antibody production. Grossblatt, editor. Washington (DC): National Academy Press.
- Xue H, Xing Y, Yin Y, Zhang T, Zhang B, Zhang Y, Song P, Tian X, Xu Y, Wang P, et al. 2012. Application of an enzyme immunoassay for the quantitative determination of azo dye (Orange II) in food products. *Food Addit Contam Part A*. 29:1840–1848.
- Zhang C. 2012. Hybridoma technology for the generation of monoclonal antibodies. In: *Methods in molecular biology*. New York (NY): Humana Press; p. 117–135.