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DEVELOPMENT OF MONOCLONAL ANTIBODIES AGAINST PORCINE BLOOD FOR DETECTION IN FISH-BASED PRODUCTS

RAJA MOHD HAFIDZ BIN RAJA NHARI

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By

RAJA MOHD HAFIDZ BIN RAJA NHARI

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

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June 2017

Chairman : Muhajir Hamid, PhD
Faculty : Biotechnology and Biomolecular Sciences

Animal blood mainly from porcine and bovine have been used in human food as a binder, gelling agent, emulsifier, coloring agent and iron's supplementary in most meat and fish-based products. However, certain communities including Muslims and Jews are strictly prohibited to consume animal blood in food due to prohibition assigned by each religious and related to health and personal matter. The addition of porcine plasma in fish surimi has been highlighted by the local authority in Malaysia. To date, a specific method to detect porcine plasma in food is unavailable. In this study, we have developed monoclonal antibodies (mAbs) against heated soluble proteins (HSPs) of porcine blood using fusion technology for detection of porcine plasma in fish surimi. Specificity of mAbs against blood, non-blood (meat and non-meat) and commercial animal plasma proteins from different species were determined using indirect non-competitive ELISA. Antigenic components of porcine blood were determined using Western blot. The sensitivity of ELISA has been determined to analyze fish surimi that spiked with porcine plasma. After several screening of hybridoma was made, 27 hybridomas produced mAbs were selected. Based on that, fifteen mAbs are specific to raw and heated porcine blood, one mAb only specific to raw porcine blood and another 11 mAbs are cross-reacted with other animal blood. The fifteen mAbs specific to porcine blood are also not cross-reacted with meat and non-meat proteins. Based on specificity to animal plasma, twelve mAbs from 15 mAbs are specific to porcine plasma while other 3 mAbs are cross-reacted to chicken plasma. Western blot showed that 2 mAbs bind 60 kDa, 8 mAbs bind 85 kDa and 2 mAbs bind 250 kDa of the antigenic protein of porcine blood. The mAb labeled as B4E1 was selected to be used for detection of porcine plasma. The developed ELISA has a limit of detection (LOD) and limit of quantification (LOQ) of 0.2 µg/g and 1 µg/g, respectively for a standard solution of porcine plasma. The intra- and inter-assays of ELISA with coefficients of variation (CVs) less than 20% were able to detect at least 0.25% (w/w) porcine plasma in fish surimi. In conclusion, this study has successfully obtained the hybridoma-producing mAbs that are specific to porcine blood and porcine plasma. This study also has developed indirect non-competitive ELISA for

detection of porcine plasma in laboratory model fish ball and fish surimi products using mAb B4E1 with LOD and LOQ of 0.2 µg/g and 1 µg/g, respectively with the sensitivity of the ELISA is 0.25% (w/w) porcine plasma in fish surimi.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

PENGHASILAN ANTIBODI MONOKLON TERHADAP DARAH KHINZIR BAGI PENGESANANNYA DI DALAM PRODUK BERASASKAN IKAN

Oleh

RAJA MOHD HAFIDZ BIN RAJA NHARI

Jun 2017

Pengerusi : Muhajir Hamid, PhD
Fakulti : Bioteknologi dan Sains Biomolekul

Darah haiwan terutamanya daripada khinzir dan lembu telah digunakan dalam makanan manusia sebagai pengikat, agen gel, pengemulsi, pewarna dan penambah zat besi di dalam kebanyakan produk berasaskan daging dan ikan. Namun begitu, sesetengah masyarakat termasuk orang Islam dan Yahudi adalah dilarang sama sekali menggunakan darah haiwan dalam makanan kerana larangan yang ditetapkan dalam agama masing-masing dan berkaitan dengan hal kesihatan dan peribadi. Penambahan plasma khinzir dalam surimi ikan telah diketengahkan oleh pihak berkuasa tempatan di Malaysia. Setakat ini, kaedah khusus bagi mengesan plasma khinzir dalam makanan masih belum diwujudkan. Dalam kajian ini, kami telah membangunkan antibodi monoklon (mAb) terhadap protein darah khinzir terlarut yang dipanaskan (HSPs) menggunakan teknologi lakuran untuk mengesan plasma khinzir dalam surimi ikan. Kespesifikan mAb terhadap protein darah, protein bukan darah (daging dan bukan daging) dan protein plasma haiwan komersil dari spesies yang berbeza telah ditentukan dengan menggunakan ELISA bukan kompetitif tidak langsung. Komponen antigen darah khinzir telah ditentukan menggunakan Western blot. Kepekaan ELISA telah ditentukan untuk menganalisis surimi ikan yang ditambahkan dengan plasma khinzir. Selepas beberapa saringan hibridoma dibuat, 27 hibridoma yang menghasilkan mAb telah dipilih. Berdasarkan itu, lima belas mAbs spesifik untuk darah khinzir mentah dan dipanaskan, satu mAb hanya spesifik untuk darah khinzir mentah dan 11 mAb yang lain bersilang tindak balas dengan darah haiwan lain. Lima belas mAb yang spesifik untuk darah khinzir didapati tidak bersilang tindak balas dengan protein daging dan protein bukan daging. Berdasarkan kespesifikan kepada plasma haiwan, dua belas mAb daripada 15 mAb spesifik untuk plasma khinzir sementara 3 mAb yang lain bersilang tindak balas kepada plasma ayam. Western blot menunjukkan bahawa 2 mAb mengikat 60 kDa, 8 mAb mengikat 85 kDa dan 2 mAb mengikat 250 kDa protein antigen darah khinzir. MAb berlabel B4E1 telah dipilih untuk digunakan bagi mengesan plasma khinzir. ELISA yang dibangunkan mempunyai had pengesanan (LOD) dan had kuantifikasi (LOQ) masing-masing 0.2 μg/g dan 1 μg/g untuk larutan piawai plasma khinzir. Asai intra dan inter ELISA

dengan pekali variasi (CV) adalah kurang daripada 20% dan ia boleh mengesan plasma khinzir sekurang-kurangnya 0.25% (w/w) plasma khinzir dalam surimi ikan. Kesimpulannya, kajian ini telah berjaya meperoleh hibridoma yang menghasilkan yang spesifik kepada darah dan plasma khinzir. Kajian ini juga telah dapat membangunkan ELISA bukan kompetitif tidak langsung untuk pengesanan plasma khinzir dalam model laboratori bebola ikan dan produk surimi ikan menggunakan mAb B4E1 dengan had pengesanan (LOD) dan had kuantifikasi (LOQ) masing-masing 0.2 μg/g dan 1 μg/g dengan sensitiviti ELISA iaitu 0.25% (w/w) plasma khinzir dalam surimi ikan.

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I certify that a Thesis Examination Committee has met on 6 June 2017 to conduct the final examination of Raja Mohd Hafidz b. Raja Nhari on his thesis entitled "Development of Monoclonal Antibodies Against Porcine Blood for Detection in Fish-Based Products" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

Members of the Thesis Examination Committee were as follows:

Nik Mohd Afizan Nik Abdul Rahman, PhD
Senior Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Noorjahan Banu binti Mohammed Alitheen, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Asmah binti Hamid, PhD
Associate Professor
Universiti Kebangsaan Malaysia
Malaysia
(External Examiner)

NOR AINI AB. SHUKOR, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 8 August 2017

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Muhajir Hamid, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Abdul Rahman Omar, PhD
Professor
Institute of Biosciences
Universiti Putra Malaysia
(Member)

Shuhaimi Mustafa, PhD
Professor
Halal Products Research Institute
Universiti Putra Malaysia
(Member)

RUBIAH BINTI YUNUS, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

Declaration by graduate student

I hereby confirm that:

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This is to confirm that:

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Signature: _____
Name of
Chairman of
Supervisory
Committee: Muhajir Hamid

Signature: _____
Name of
Member of
Supervisory
Committee: Abdul Rahman Omar

Signature: _____
Name of
Member of
Supervisory
Committee: Shuhaimi Mustafa

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LIST OF ABBREVIATIONS

ABTS	2,2-azino-bis-3-ethylbenzthiazoline- 6-sulfonic acid
AP	Autoclaved porcine blood
APP	Autoclaved porcine plasma
BP	Boiled porcine blood
BPP	Boiled porcine plasma
C_{H1}, C_{H2} and C_{H3}	Constant domains (heavy chain)
CIF	Complex Initiation Factor
CPL	ClonePix FL
C_L	Constant domain (light chain)
CV	Coefficient of variation
CO_2	Carbon dioxide
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
FITC	Fluorescein isothiocyanate
FU	Fluorescence units
g	Gravitational force
h	Hour
HAT	Hypoxanthine-aminopterin-thymidine
HPRT	Hypoxanthine-phosphoribosyl-transferase
HGPRT	Hypoxanthine-guanine-phosphoribosyl-transferase
HRP	Horseradish peroxidase
HSPs	Heated soluble proteins
HT	Hypoxanthine thymidine
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
JAKIM	Jabatan Kemajuan Islam Malaysia
kDa	Kilo Dalton
LOD	Limit of detection
LOQ	Limit of quantification
LC-MS/MS	Liquid chromatography tandem mass spectrometry
mAb	Monoclonal antibody
min	Minute
mL	Milliliter
mM	Millimolar
MSG	Monosodium glutamate
MWCO	Molecular weight cut-off
OD_{405nm}	Optical reading at 405 nm
PBS	Phosphate buffered saline
PEG	Polyethylene glycol

RNA	Ribonucleic acid
RP	Raw porcine blood
rpm	Revolution per minute
RPMI	Roswell Park Memorial Institute
RPP	Raw porcine plasma
RT	Room temperature
SD	Standard deviation
SDS-PAGE	Sodium-dodecyl sulfate-polyacrylamide gel electrophoresis
TBS	Tris buffer saline
TEMED	*N*,*N*,*N*,*N*-tetra-methyl-ethylenediamine
TSE	Transmissible spongiform encephalopathy
TTBS	Tris buffer saline with Tween-20
V_H	Variable domain (heavy chain)
V_L	Variable domain (light chain)
v/v	Volume per volume
w/w	Weight per weight
µm	Micrometer
µg	Microgram

CHAPTER 1

INTRODUCTION

Animal blood has been utilized in human food, animal feed and in the field of the laboratory, medical, industrial and fertilizer since several years ago. Currently, advance technology in animal blood collection and processing techniques have led to a production of numerous blood protein ingredients that could give benefits to the environment, nutrition and economic derived from the maximal utilization of animal blood. The blood can be separated into two parts, a liquid and cellular portion. Plasma, the liquid portion of blood is mostly utilized in the food industry because it is tasteless and contains no dark color associated with the red blood cells. Meanwhile, the cellular portion of blood is not extensively used as compared to the plasma due to its heme component, which possesses an undesirable color, odor and gives a metallic taste to the end-product.

The production of blood products involved 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 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 inhibitor in the manufacturing of surimi. In addition, the porcine plasma can be used as an egg replacer in bakery products as foaming, jellification and solubility agent. The plasma and albumin are used as solubility and jellification agent in yogurt (Álvarez et al., 2012). Besides, fibrinogen and plasma from porcine blood can be added as an emulsifier in cakes and pastries. Stabilized haemoglobin is commonly used in meat products as binding and colouring agents. Decoloured globin obtained from haemoglobin can be used as solubility agent in biscuits while globulins can be used as emulsifier, solubility and jellification agent in meat products (Álvarez et al., 2012). Thus, the increasing demand of porcine plasma will occur as much as the current requirement of bovine plasma in the food industry.

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

The prion proteins or infectious proteins are extremely resistant to heat, ultraviolet light, ionizing radiation, normal sterilization 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 various consumptions 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 no evidences of natural case of transmissible spongiform encephalopathy (TSE) in non-ruminants including porcine (Greenlee et al., 2016), development of scientific method to detect porcine blood derivative in food is imperative because although the religious law to ban non-Halal or non-Kosher blood has been stated, the addition of porcine blood in food still occurred. This is due to the lacking in existing methods to help to enforce the laws and the manufacturer may adulterate the expensive meats with a high amount of blood proteins to fraud the protein content in their food products.

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

The general objective of this study is to produce hybridoma that secretes monoclonal antibody (mAb) against porcine blood; where the mAb(s) will react to the heated protein of porcine blood for qualitative detection of porcine plasma in fish-based products especially surimi. The use of mAb will offer an extra advantage since the mAb itself is species-specific and could differentiate specific animal blood from other blood species, other tissues and common food proteins. The specific objectives of this study comprised of:

a) to produce hybridoma through the fusion of immunized splenocytes and myeloma cells (P3X63Ag8.653, ATCC® CRL-1580™)
b) to characterize the mAb(s) that are specific to porcine blood especially porcine plasma
c) to develop indirect non-competitive ELISA for detection of porcine plasma in a laboratory model of fish ball and commercial fish surimi

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