UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT AND EVALUATION OF ENZYME-LINKED IMMUNOSORBENT ASSAYS FOR THE DETECTION OF GELATIN SOURCES

NUR AZIRA BINTI TUKIRAN

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By

NUR AZIRA BINTI TUKIRAN

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DEVELOPMENT AND EVALUATION OF ENZYME-LINKED IMMUNOSORBENT ASSAYS FOR THE DETECTION OF GELATIN SOURCES

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NUR AZIRA TUKIRAN

June 2016

Chairman : Professor Amin Ismail, PhD
Institute : Halal Products Research Institute

Gelatin is derived from partial hydrolysis of type I collagen (connective tissues such as skin, bone, tendon and ligament), which is commonly obtained from mammalian sources (bovine and porcine). However, the use of gelatin derived from mammalian sources in confectionery and pharmaceutical products have become a controversial issue regarding to religious and health concern. Additionally, it was reported that porcine gelatin has been surreptitiously added to edible bird's nests (EBNs) to increase their net weight prior to sale. Thus, a reliable technique for the determination and detection of gelatin sources is necessary in order to protect and reassure consumers against food fraud. This study concerns two issues; (i) determination of gelatin sources in confectionery and pharmaceutical products and (ii) determination and detection of porcine gelatin adulterant in EBN. There are some reports for gelatin sources differentiation by analysis of their amino acids. Those specific peptides can be used as specific biomarkers for gelatin sources differentiation and detection. Thus the aims of this study were to develop and evaluate the potential biomarker of porcine marker peptides using enzyme-linked immunosorbent assay (ELISA) for the detection and determination of porcine gelatin in processed products.

Collagen α2 (I) chain protein of 125 kDa molecular weight showed resistance against heat and detectable in the studied commercial processed products when analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Three porcine species-specific amino acid sequences of GFPGSPGNVGPAGK (Peptide 1) and GIPGEFGLPGPAGPR (Peptide 2) of collagen α2 (I) chain and SGDRGETGPAGPAGPVGPVGAR (Peptide 3) of collagen α1 (I) chain were selected for raising polyclonal antibodies (pAbs). Three competitive indirect ELISAs were developed using pAbs against the aforementioned porcine species-specific amino acid sequences of collagen α2 (I) chain to obtain pAb1 and pAb2, and α1 (I) chain to obtain pAb3. The limit of detection (IC_{15}) of the three competitive indirect ELISAs was 0.033, 0.082 and 0.052, µg/mL respectively. The median inhibitory

concentration (IC_{50}) of pAb1, pAb2 and pAb3 was 0.265, 0.394 and 0.228 µg/mL, respectively. All pAbs were able to recognize mammalian gelatin, while pAb2 and pAb3 exhibited moderate cross-reactivity (≤15 – 1%) toward fish and chicken gelatin. The specificity of the developed ELISAs was not influenced by the process type (type A or type B) and the origin of the collagen (skin or bone). Fourier transform infrared spectroscopy (FTIR) results showed that slight difference of the infrared spectra between Amide II and III regions of gelatin may influence the sensitivity of this immunoassay. Seventy commercially processed products (i.e. jellies, gummies, premix powders and hard shell capsules) were examined, 7% of samples showed false-positive results when analyzed using competitive indirect ELISA based on pAb2.

Competitive and non-competitive ELISAs were developed as to provide quantitative and qualitative measurements of porcine gelatin in EBN respectively. Based on developed competitive ELISAs, pAb1 and pAb2 have showed moderate cross-reactivity with cave nest and blood cave nest, respectively. Both pAbs exhibited moderate cross-reactivity with egg white. No cross-reactivity (<1%) was observed with EBNs and egg white for pAb3. pAb3 has showed best sensitivity, specificity, accuracy and repeatability compared to pAb2 and pAb1. While for non-competitive indirect ELISAs, all pAbs were able to detect at least 0.05% porcine gelatin in spiked samples of EBNs. However, pAb1 exhibited slight cross-reactivity toward orange, cave and house nests. In conclusion, the study indicated that the developed ELISAs utilizing the anti-peptide pAbs would be a good strategy for the detection and determination of gelatin sources in processed products.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

PEMBANGUNAN DAN PENILAIAN *ENZYME-LINKED IMMUNOSORBENT ASSAYS* UNTUK PENENTUAN SUMBER GELATIN

Oleh

NUR AZIRA BINTI TUKIRAN

Jun 2016

Pengerusi : Profesor Amin bin Ismail, PhD
Institut : Institut Penyelidikan Produk Halal

Gelatin terhasil dari proses separa hidrolisis terhadap kolagen jenis I (tisu penghubung seperti kulit, tulang, tendon dan ligamen), yang kebiasaannya diperolehi daripada sumber mamalia (*bovine* dan *porcine*). Namun begitu, penggunaan gelatin yang diperolehi daripada sumber mamalia terhadap pembuatan produk makanan (manisan) dan farmaseutikal menjadi isu kontroversi apabila mengambil kira tuntutan agama dan kesihatan. Tambahan pula, terdapat laporan yang mana gelatin *porcine* telah ditambah secara tidak sah.ke dalam sarang burung walit bagi menambahkan berat sarang burung walit sebelum ia dijual. Oleh itu, teknik yang boleh dipercayai untuk menentukan dan mengesan sumber gelatin adalah perlu untuk melindungi dan memberi jaminan kepada para pengguna terhadap penipuan makanan. Kajian ini adalah mengenai dua isu; (I) menentukan sumber gelatin dalam makanan (manisan) dan produk farmaseutikal dan (ii) penentuan dan mengesan pencemaran gelatin *porcine* dalam sarang burung walit. Terdapat beberapa laporan berkenaan pembezaan sumber gelatin oleh analisis asid amino. Peptida tertentu boleh digunakan sebagai penanda biologi untuk pembezaan dan pengesanan sumber gelatin. Oleh sebab itu, tujuan kajian ini dijalankan adalah untuk menbangunkan dan menilai potensi petanda biologi terhadap petanda peptida pada *porcine* dengan menggunakan *enzyme-linked immunosorbent assay* (ELISA) bagi menentukan sumber gelatin yang digunakan di dalam produk yang telah diproses.

Apabila ujikaji natrium dodesil sulfat-elektroforesis gel poliakrilamida (SDS-PAGE) dijalankan pada gelatin yang telah melalui perlakuan haba, dan beberapa produk komersial, rantaian protein kolagen α2 (I) yang mempunyai berat molekul 125 kDa telah dikesan. Sebanyak tiga jujukan asid amino yang mempunyai ciri khusus pada *porcine* yang mana terdiri dari GFPGSPGNVGPAGK (Peptida 1) dan GIPGEFGLPGPAGPR (Peptida 2) daripada rantaian kolagen α2 (I) serta SGDRGETGPAGPAGPVGPVGAR (Peptida 3) daripada rantaian kolagen α1 (I) telah dipilih untuk pembangunan ELISA. Sebanyak tiga ELISA kompetitif tidak langsung telah dibangunkan dengan menggunakan antibodi poliklonal (pAb) daripada arnab terhadap jujukan asid amino yang telah dinyatakan di atas, yang mana

jujukan asid amino daripada rantaian kolagen α2 (I) untuk menghasilkan pAb1 dan pAb2 serta rantaian kolagen α1 (I) untuk menghasilkan pAb3. Ketiga-tiga ELISA yang dibangunkan mempunyai had pengesanan (IC_{15}) masing-masing sebanyak 0.033, 0.082 dan 0.052 µg/mL. Manakala kepekatan perencatan median (IC_{50}) masing-masing mencatat sebanyak 0.265, 0.394 dan 0.228 µg/mL. Kesemua pAb mampu mengenalpasti gelatin daripada sumber mamalia. PAb2 dan pAb3 menunjukan tindak balas silang yang sedarhana (≤15 – 1%) terhadap gelatin daripada sumber ikan dan ayam. Di dalam konteks kekhususan, ELISA yang dibangunkan ini menunjukkan yang ia tidak dipengaruhi oleh jenis proses (jenis A atau jenis B) dan tempat sumber kolagen tersebut diperolehi (kulit atau tulang). Keputusan daripada ujikaji Fourier transform inframerah (FTIR) telah mendapati perbezaan diantara jalur inframerah gelatin di kawasan Amide II dan III, yang mana berkemungkinan mempengaruhi kepekaan ELISA. Hasil kajian dengan menggunakan ELISA kompetitif tidak langsung berdasarkan pAb2, 7% daripada 70 produk komersial yang diuji (seperti jeli, *gummy*, serbuk pracampur dan kapsul berkulit keras), didapati menunjukkan keputusan positif palsu.

ELISA kompetitif dan ELISA tidak kompetitif tidak langsung turut dibangunkan untuk menyediakan ukuran kuantitatif dan kualitatif gelatin *porcine* dalam sarang burung walit masing-masing. Berdasarkan ELISA kompetitif tidak langsung yang telah dibangunkan, pAb1 dan pAb2 telah menunjukkan tindak balas silang yang sedarhana terhadap sarang burung walit jenis gua dan sarang burung walit jenis gua bewarna merah masing-masing. Kedua-dua pAb juga turut menunjukkan tindak balas yang tindak balas silang yang sedarhana terhadap putih telur. Tiada tindak balas silang (<1%) dipamirkan terhadap kesemua sampel sarang burung walit dan putih telur apabila diuji dengan pAb3. pAb3 telah menunjukkan keputusan terbaik dari segi kepekaan, kekhususan, ketepatan dan kebolehulangan berbanding pAb2 dan pAb1. Hasil kajian dari pembangunan ELISA tidak kompetitif tidak langsung telah berjaya mengesan sekurang-kurangnya sebanyak 0.05% gelatin *porcine* di dalam sampel. Walau bagaimanapun, pAb1 mempamerkan sedikit tindak balas silang ke arah sarang burung oren, gua dan rumah. Kesimpulannya, kajian ini menunjukkan bahawa ELISA yang dibangunkan dengan menggunakan anti-peptida pAb berpotensi untuk menjadi satu strategi yang baik untuk mengesan dan menentukan sumber gelatin dalam produk diproses.

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Amin bin Ismail, PhD
Professor
Halal Products Research Institute
Universiti Putra Malaysia
(Chairman)

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

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

BUJANG BIN KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

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Signature: _____
Name of Chairman of Supervisory Committee: Professor Dr. Amin bin Ismail

Signature: _____
Name of Member of Supervisory Committee: Professor Dr. Shuhaimi bin Mustafa

Signature: _____
Name of Member of Supervisory Committee: Associate Professor Dr. Muhajir bin Hamid

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
AOAC	Association of Analytical Communities
ATR	Attenuated total reflectance
B	Bovine gelatin
BCN	Blood cave nest
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
C	Chicken gelatin
CBB	Coomassie Brilliant Blue
CFA	Complete Freund's Adjuvant
CN	Cave nest
CR	Cross-reactivity
CV	Coefficient of variation
Da	Dalton
DTT	Dithiothreitol
EBN	Edible bird nest
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
F	Fish gelatin
FTIR	Fourier-transform infrared spectrometer
H	House nest
HMW	High molecular weight
HRP	Horseradish peroxidase
IC	Inhibitory concentration
IFA	Incomplete Freund's Adjuvant
KDa	Kilo dalton
KLH	Keyhole limpet hemocyanin
LC-MS	Liquid chromatography–mass spectrometry
LMW	Low molecular weight

LOD	Limit of detection
Log	Logarithm
LOQ	Limit of quantification
M	Molarity
MAb	Monoclonal antibody
mM	micromolar
MS/MS	Tandem mass spectrometry
MW	Molecular weight
m/z	Mass-to-charge ratio
N/A	Not available
NCBI	National Center for Biotechnology Information
nd	Not detected
OD	Optical density
ON	Orange nest
P	Porcine gelatin
PAb	Polyclonal antibody
PBS	Phosphate-buffered saline
PMF	Peptide mass fingerprinting
PVDF	Polyvinylidene fluoride
Q-TOF	Quadrupole time-of-flight
R^2	Correlation
Rf	Relative electrophoretic mobility
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SN	Small strip nest
TBS	Tris buffered saline
TEMED	Tetramethylethylenediamine
TMB	3,3',5,5'-Tetramethylbenzidine
Tris-HCI	Tris in hydrochloric acid
v/v	Volume per volume

w/v	Weight per volume
WHN	White nest
α	Alpha
β	Beta
γ	Gamma

CHAPTER 1

GENERAL INTRODUCTION

Annually, tons of gelatin are used in food and pharmaceutical products due to its favourable price, availability and ease of use. Gelatin is derived from a partial hydrolysis of type I collagen (connective tissues, such as skin, bone, tendon and ligament), which is commonly obtained from bovine and porcine sources (Shakila *et al.*, 2012). Gelatin that is obtained from bovine or porcine can provide a high stability of gels, excellent physicochemical properties and a high melting point compared to fish gelatin (Cho *et al.*, 2005). This circumstance explains high production of porcine and bovine gelatin compared to fish gelatin.

Gelatin source is particularly of concern because of the extensive use of gelatin ingredients in wide variety of processed products (Grand View Research, 2014). Commonly, in the food industry, gelatin has been used as a thickener, a food stabilizer and a food texture enhancer. While, in the pharmaceutical industry, gelatin has been utilized as suspending agent, an encapsulating agent, a tablet binder, and a coating agent. Mammalian source of gelatin is controversial in terms of religious beliefs and food safety. For example, Muslim and Jewish are strictly prohibited to consume any pig-related products, as well as unslaughtered animals which do not comply with their corresponding religious requirement (Regenstein *et al.*, 2003). Similarly, Hindu customs are restricted to consume any cattle-derived products. Moreover, the outbreak of Bovine Spongiform Encephalopathy (BSE) crisis in 1986 has raised the public concern about the bovine gelatin safety. In addition, it was reported that gelatins are capable to trigger allergic reaction (Wang *et al.*, 2005; Sakaguchi *et al.*, 1996). Thus, proper product labeling plays an important role. Nevertheless, the predicament of fraudulent and mislabeling making avoidance is the only approach.

In addition, it was reported that porcine gelatin has been used as an adulterant. It was clandestinely added to edible bird's nests (EBNs) to increase their net weight prior to sale (Lin *et al.*, 2006; Goh *et al.*, 2001). Edible bird's nest is one of the famous Chinese traditional products that are reported to have high nutritional and medicinal values (Vimala *et al.*, 2012; Guo *et al.*, 2006).

In light of these concerns, it is of great significance to develop a reliable analytical method for gelatin sources differentiation or gelatin detection in processed products. Recently, a number of methods such as polyacrylamide gel electrophoresis (Nur Azira *et al.*, 2014; Aina *et al.*, 2013), liquid chromatography (Azilawati *et al.*, 2015; Yilmaz *et al.*, 2013; Zhang *et al.*, 2009) and Fourier transform infrared (FTIR) spectroscopy (Hashim *et al.*, 2010) have been established for the detection of gelatin sources. These methods demonstrate the successful development for gelatin sources differentiation. However, limited studies have been published on the development of immunological-based method (Waber *et al.*, 2010; Doi *et al.*, 2009; Venien and

Levieux, 2005a; Venien and Levieux, 2005b). Owing to its high sensitivity and throughput making immunoassay an expedient alternative. Moreover, this method is able to offer simplicity in sample preparation which does not need high sample purity particularly when dealing with commercial processed products. In addition, protein-based method such as the enzyme-linked immunosorbent assay (ELISA) is preferable to the polymerase chain reaction (PCR) based method as DNA could be degraded during the gelatin manufacturing process thus making the determination process troublesome (Cai *et al.*, 2012).

A prerequisite for the development of ELISA is the choice of antigen. However, the production of specific antibodies to particular gelatin source is challenging due to poor immunogenicity. Previously, Venien and Levieux (2005a), have demonstrated that the polyclonal antibodies (pAbs) obtained from tyrosine enrichment were very sensitive to gelatin type (type A or B). With this limitation, Venien and Levieux (2005b) extended their works by using bovine species-specific amino acid sequences as immunogen for pAbs production. This approach demonstrated good reactivity towards bovine gelatin and was able to detect bovine gelatin in porcine gelatin down to 1.5 – 2%. As bovine and porcine gelatins has different amino acid compositions, those specific peptides can be used as specific biomarkers for gelatin sources differentiation (Zhang *et al.*, 2009). Therefore, the aims of this study were to develop and evaluate the potential biomarker of porcine marker peptides using ELISA for the detection and determination of porcine gelatin in processed products.

In deciding the protein that will be used as the antigen, gelatin was analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and peptide mass fingerprinting (PMF). Polyclonal rabbit antibodies (pAbs) were raised against the porcine species-specific amino acid sequences. The performance of ELISAs was assessed based on specificity, accuracy, sensitivity, and repeatability. Commercial pharmaceutical and confectionery products from several countries were tested. The spiked samples of porcine gelatin in EBN were also evaluated. Currently, this is the first report of anti-peptide pAbs capable to detect and determine the gelatin sources in commercial confectionery and pharmaceutical products, as well as in the EBNs. The developed ELISAs would be paramount beneficial to quality control laboratories and enforcement authorities for ingredients verification and quality control EBNs products.

1.1 Problem statements

The utilization of gelatin ingredient in a wide variety of products has led to a huge concern of various group of consumers especially those who restricted to religion-based dietary. Therefore, there is a necessity to develop reliable methods for gelatin sources determination in food products. Immunoassays offer an analytical alternatives to instrumental methods. However, one of the restrictions is the fact that gelatin is a non-antigenic protein. Previous reports showed that gelatin sources might be identified based on detection of marker peptides by high-performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS). Taking advantage of

this information, the development of ELISA based on species-specific marker peptides would offer a great alternative to overcome the non-antigenic problem. The approach consisted of raising pAbs against species-specific amino acid sequences and using them in ELISA. This study would give valuable information about the reproducibility of anti-peptide pAbs in determining porcine gelatin in processed products.

1.2 Significance of the study

This study was aimed to detect and determine the gelatin sources in commercially processed products. The approach relates to the development of ELISA by utilizing peptide immunogens of collagen α1 (I) and α2 (I) chain proteins. The study further relates to the evaluation of developed ELISAs to different matrices particularly in confectionery and pharmaceutical capsules as well as in EBNs. The proposed ELISAs would be beneficial to the enforcement authorities for ingredient verification in processed products, as well as offers new technique for quality control in EBN industry.

1.3 Hypotheses

The utilization of anti-peptide polyclonal antibodies based on ELISA will produced good specificity in term of gelatin sources determination either in raw or processed products.

1.4 Objectives

The objectives of the study were as follows:

1.4.1 General objective

The general objective of this study was to develop and evaluate the potential biomarker of porcine marker peptides using ELISA for the detection and determination of porcine gelatin in the processed products.

1.4.2 Specific objectives

i. To identify the potential marker peptides of porcine gelatin by combination of SDS PAGE and PMF.

ii. To develop and evaluate the efficiency of pAbs (pAb 1, 2 and 3) for the determination of gelatin sources in raw materials, pharmaceutical and confectionery products by competitive indirect ELISA.

iii. To develop and evaluate the efficiency of pAbs (pAb 1, 2 and 3) for the determination of porcine gelatin in EBN by competitive indirect ELISA.

iv. To develop and evaluate the efficiency of pAbs (pAb 1, 2 and 3) for the detection of porcine gelatin in EBN by non-competitive indirect ELISA.

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BIODATA OF STUDENT

Nur Azira Tukiran was born in Batu Pahat, Johor on 25th October 1986. She was attended primary school at Sekolah Kebangsaan Long Gafar, Kelantan and had her secondary education at Sekolah Menengah Kebangsaan (A) Tok Bachok, Kelantan. She was completed her matriculation at Kolej Matrikulasi Kedah (KMK), Kedah in 2005. She was graduated from Universiti Malaysia Terengganu (UMT), Terengganu in 2008 and earned a Bachelor of Science in Agrotechnology. In 2010, she pursued her study at Halal Products Research Institute (HPRI), Universiti Putra Malaysia (UPM), and was awarded Master of Science in Halal Food Analysis in 2012. In 2013, she earned a scholarship from International Islamic University Malaysia (IIUM) and Ministry of Higher Education Malaysia for continuing her study at PhD level. Currently, she is doing her PhD program in the same institute.

LIST OF PUBLICATIONS

The following publications are resulted from the dissertation:

Journals:

Nur Azira, T., Amin, I., Shuhaimi, M. and Muhajir, H. (2015). Enzyme immunoassay for the detection of porcine gelatine in edible bird's nest. *Food Additives and Contaminants – Part A*. 32: 1023 – 1028.

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Nur Azira, T., Nur Illiyin, M. R. and Amin, I. SDS-PAGE and enzyme immunoassay methods for detection of porcine gelatin in edible bird's nest. In Proceeding of Malaysia International Halal Research and Education Conference 2014. Putrajaya, Malaysia, December 2-4, 2014. Universiti Putra Malaysia; pp 34 - 39.

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