IN-VITRO AND *IN-SILICO* SELECTION OF DNA-BASED APTAMER TOWARDS PORK DETECTION USING SELEX

By

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Meat authentication is a growing global concern in the food industry, necessitating accurate and efficient methods for source identification. Established techniques such as PCR and mass spectrometry are sensitive and specific, but require high-quality DNA or protein for testing, and are time-consuming. In contrast, aptamer-based detection, a newer approach in food authentication shows promise as a more accessible alternative. Hence, this study attempted to address the need for porcine-specific aptamer capable of binding to porcine proteins. The study aimed to develop and characterize aptamers that bound to any pork protein through SELEX process, combined with Next Generation Sequencing (NGS) analysis, Liquid Chromatography Mass Spectrometry (LC-MS) analysis, and molecular docking simulation. Fourteen rounds of selection using the centrifugal-ultrafiltration separation technique against four negative controls (chicken, duck, beef and lamb) led to the identification of potential pork-binding aptamers. The final pool library was subjected to Sanger sequencing and NGS. Sequence analysis yielded 67 sequences, with the most frequently occurring aptamer, APT#A1, exhibiting the highest binding affinity (27.61 ±1.92 nM) as determined by

ELONA. Clustering analysis, combined with motif and network analysis also resulted in five aptamers (APT#A1, APT#A2, APT#A4, APT#A8, APT#A17), to be used for further analysis. However, protein blotting revealed cross-reactivity with multiple proteins from negative samples, necessitating further specificity enhancement. LC-MS analysis consistently identified troponin subunits (TnI and TnT) as potential target markers that bound to the aptamers. Aptamer truncation analysis showed that the removal of the flanking region could affect the stability and binding efficacy of APT#A1. On the other hand, the forward primer binding site was identified as crucial to aptamer binding, as its retention in the truncated sequences of APT#A2 and APT#A4 resulted in improved binding affinity. Molecular docking simulations demonstrated more stable and stronger interactions between aptamers and troponin, notably with specific chains of troponin (TnT and TnI), as compared to that of myosin complex structure. Amino acids that were responsible for the interactions were glutamine (GLN), valine (VAL), glycine (GLY), serine (SER), isoleucine (ILE), methionine (MET), and glutamic acid (GLU) with interactions occurring at a distance of < 3.0 Å by hydrogen bonding. As a conclusion, through combinations of approaches, this study has successfully developed and characterized a high affinity porcine-binding aptamer. These aptamers are a promising element that can use for porcine detection and thus will improve the authentication limit.

Keywords: meat authentication, molecular docking, porcine aptamer, rapid identification, SELEX

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

PEMILIHAN DNA APTAMER BABI SECARA *IN-VITRO* DAN *IN-SILICO* UNTUK PENGENALPASTIAN DAGING BABI MELALUI KAEDAH SELEX

Oleh

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Dalam industri makanan, pengesahan keaslian daging telah menjadi tumpuan penggunanya secara global dan memerlukan kaedah pengesahan yang untuk mengenalpasti sumbernya. Kaedah yang sedia ada seperti tindak balas berantai polimeras (PCR) dan spektrometri jisim adalah cukup sensitif dan spesifik, tetapi kaedah ini memerlukan ekstrak DNA atau protein yang berkualiti tinggi serta memakan masa yang lama. Sebaliknya, alat pengesanan berasaskan aptamer merupakan pendekatan terbaharu yang menjadi kaedah alternatif yang lebih mudah diakses oleh penggunanya. Oleh itu, kajian ini dijalankan untuk membangunkan dan mencirikan aptamer yang terikat kepada mana-mana protein dari daging babi melalui kombinasi proses SELEX dengan penjujukan generasi seterusnya (NGS), analisis kromatografi cecair-spektrometri jisim (LC-MS) dan simulasi dok molekul. Aptamer berpotensi yang terikat dengan protein daging babi telah dipilih melalui empat belas pusingan pemilihan menggunakan teknik pemisahan emparan-ultrapenapisan berbanding lima sampel kawalan negatif (daging ayam, ikan, lembu dan biri-biri). Koleksi DNA yang terakhir telah disasarkan kepada penjujukan Sanger dan NGS. Analisis penjujukan

menghasilkan 67 jujukan. Kajian ELONA menunjukkan aptamer APT#A1 dengan frekuensi yang paling tinggi mempunyai kekuatan perlekatan yang tertinggi iaitu 27.61 ±1.92 nM. Walaubagaimanapun, *protein blotting* menunjukkan bahawa aptamer yang dipilih tidak spesifik kepada babi sahaja, malah ianya turut terlekat kepada protein daripada sampel-sampel negatif. Analisis LC-MS menunjukkan aptamer yang dipilih melekat secara konsisten kepada subunit protein troponin iaitu TnI dan TnT. Analisis pemangkasan aptamer menunjukkan bahawa penyingkiran bahagian primer pada aptamer boleh mempengaruhi kestabilan dan keberkesanan perlekatan bagi APT#A1. Sebaliknya, bagi APT#A2 dan APT#A4, penyingkiran bahagian primer hadapan telah meningkatkan tahap perlekatan aptamer dengan protein. Simulasi dok molekul juga membuktikan bahawa aptamer-aptamer ini berinteraksi dengan lebih kuat dan stabil terhadap troponin kompleks dan subunit tertentu troponin (TnT dan TnI), berbanding miosin kompleks. Asid amino yang terlibat dengan interaksi antara aptamer dan protein adalah glutamin, valina, glisina, serin, isoleusina, metionina dan asid glutamik, dengan jarak interaksi kurang daripada 3.0 Å melalui ikatan hidrogen. Kesimpulannya, aptamer yang menyasarkan protein daripada daging babi dengan kekuatan perlekatan yang tinggi telah berjaya dipilih menggunakan gabungan beberapa kaedah. Aptamer ini mempunyai potensi digunakan sebagai elemen untuk mengidentifikasi spesis dan membaikpulih kaedah pengesahan spesis di masa akan datang.

Kata kunci: pengesahan daging, dok molekul, aptamer babi, kaedah pengesanan pantas, SELEX

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LIST OF ABBREVIATIONS

ΔG	Gibbs free energy
µg	microgram
µl	microliter
3D	three dimensional
Å	Angstrom
APS	ammonium persulfate
ATR-FTIR	attenuated total reflectance-Fourier Transform Infrared
B_{max}	maximal binding capacity
bp	base pair
BSA	bovine serum albumin
CE	capillary electrophoresis
CV	coefficient of variation
DART-HRMS	Direct analysis in real time-High Resolution Mass Spectrometry
dd-PCR	droplet digital polymerase chain reaction
DNA	deoxyribonucleic Acid
dsDNA	double-stranded DNA
e.g.	Exempli Gratia (For Example)
ELAA	enzyme-linked aptamer assay
ELISA	enzyme-linked immunosorbent assay
ELONA	enzyme-linked oligonucleotide assay
EMA	economically motivated adulteration
EMSA	electrophoretic mobility shift assay

GC-MS	Gas Chromatography-Mass Spectrometry
GHP	Good Hygiene Practice
GMP	Good Manufacturing Practice
HACCP	Hazard Analysis and Critical Control Point
HADDOCK	High Ambiguity Driven Docking
HPLC-HRMS	High Performance Liquid Chromatography-High Resolution Mass Spectrometry
HPLC-UV-FLD	High Performance Liquid Chromatography-UV Photolysis-Fluorescence Detection
hr	hour
HTS	high throughput sequencing
IEF	isoelectric focusing
IR-MS	isotope ratio-mass spectrometry
JAKIM	Department of Islamic Development Malaysia
K_D	dissociation constant
kDa	kilo Dalton
LB	lysogeny broth
LC-MS	Liquid Chromatography-Mass Spectrometry
LFA	lateral flow assay
MEME	Multiple EM for Motif Elicitation
min	minute
mM	milimolar
MS	mass-spectrometry
ng	nanogram
NGS	Next Generation Sequencing

NIR	Near-infrared spectroscopy
nM	nanomolar
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
PCR	polymerase chain reaction
QMEAN	Qualitative Model Energy Analysis value
QMQE	Global Model Quality Estimate
QSQE	Quaternary Structure Quality Estimate
RFLP	Restriction Fragment Length Polymorphism
RMSD	root-mean-square deviation
RNA	ribonucleic acid
rpm	revolutions per minute
rt-PCR	reverse transcription polymerase chain reaction
SA-HRP	streptavidin-conjugated Horseradish peroxidase
SD	standard deviation
SDS	sodium dodecyl-sulphate
SDS-PAGE	sodium dodecyl-sulphate polyacrylamide gel electrophoresis
sec	second
SELEX	Systematic Evolution of Ligands by Exponential enrichment
SPPIDER	Solvent accessibility-based Protein-Protein Interface iDEntification and Recognition
ssDNA	single-stranded deoxyribonucleic acid
STML	SWISS-MODEL Template Library

TMB	tetramethylbenzene
TnC	troponin C
TnI	troponin I
TnT	troponin T
TPP	Trans Protein Pipeline
UPLC	Ultra-Performance Liquid Chromatography
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

1.1 General introduction

Meat adulteration has become a major global concern due to its impact on food quality and safety. Meat adulteration can be either intentional or unintentional (Banti, 2020), with intentional substitution being the most fraudulent practice throughout the supply chain, such as the substitution of meat products with other meat species (Fengou et al., 2021). For instance, the incidence of the horsemeat scandal in 2013 triggered worldwide concern regarding the sources of meat and the products' authenticity (Zhang et al., 2023). Since then, various research has been conducted to authenticate the truth of meat sources. As a result, many government bodies all over the world have been tasked to conduct the products' authenticity checking. In Malaysia, the responsibility for these checks falls under the Department of Islamic Development Malaysia (JAKIM) (Abdullah & Alias, 2019).

Recently, researchers have found several meat products adulterated with pork (Qin et al., 2022; Fengou et al., 2021; Li et al., 2019). The occurrence of meat cartel scandal in Malaysia has alarming the consumer regarding the issue of meat authenticity (Mohd Riza et al., 2022). The incident of unintentional meat contamination during packaging and processing is less reported (Li et al., 2020). However, it can cause serious public health, such as exposure to toxins, pathogens,

or allergens (Li et al., 2020). Hence, consistent inspections and effective traceability system are critical in monitoring the food crime.

In food authentication and analysis, the availability of reproducible and stable detection methods is a significant aspect for consistent detection and identification of food sources. Various methods of authentication such as polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA), imaging approaches and liquid chromatography-mass spectrometry (LC-MS) have been developed. However, meat adulteration is difficult to detect without the aid of the laborious detection techniques (He et al., 2021). In addition, the existing authentication methods facing several limitation, including undetectability, extraction difficulty of complex matrix sample and require proper sample handling and storage (Ungerer et al., 2020; Sajali et al., 2018). These limitations lead to long process to be completed. Hence, the advance of an aptamer-based detection method is one of the alternatives that can overcome these limitations and provide a rapid and on-site detection of meat adulteration.

Aptamers, which are also known as synthetic ligands, are single-stranded oligonucleotide molecules developed by *in-vitro* and *in-vivo* selection from random libraries of synthetic oligonucleotides and selected by Systematic Evolution of Ligands by Exponential enrichment (SELEX). To date, aptamers for different targets including protein, whole cells, metal ions, drugs, amino acids, co-factors, antibiotics, and nucleic acids have been developed (McKeague & Derosa, 2012). Uniquely, these aptamers have shown an ability to mimic the function of antibodies

by folding into a tertiary structure, allowing for the recognition and binding specifically to targets with high affinity.

Besides, DNA aptamers also have various advantages as compared to antibodies, including more stable, smaller in size and low cost of synthesis (Schmitz et al., 2020; Röthlisberger & Hollenstein, 2018; Xuan et al., 2018). Aptamer can adopt numerous folding topographies, which are composed of the stem, hairpin, loop and quadruplex structures. These structures either bind to the sequence's complementary nucleotide or serve as a binding spot for the target molecules (Zhao et al., 2015). In addition, the aptamer structure is reversible and can be restored to its natural state. Due to its stability, aptamer can tolerate a broad range of conditions, including temperature, pH or ion concentrations. This makes aptamer more robust and flexible for modification, processing and storage (Walter et al., 2012).

The use of *in-silico* molecular docking for aptamer-target interaction is quite popular nowadays. This method involved algorithm-based simulations to predict the interaction of aptamer and its target (Navien et al., 2021). Several studies performedthe *in-silico* study to determine the binding strength of the aptamer sequences towards the cognate target (Liang et al., 2024; Ma et al., 2022; He et al., 2021). Moreover, this method can also be used to determine the specific binding site at the atomic level. Thus, due to these advantages, aptamer-protein interaction was employed in this study to demonstrate the aptamer binding.

The aptamer has been widely applied in various fields of applications including clinical, toxicology, pharmaceutical, and food analysis. As for food authentication, the number of aptamer-based detection methods is still growing. In this study, the porcine protein was targeted for aptamer selection through SELEX. Although there are no reports on aptamer generated for porcine thus far, the potential to utilize this technology for such use is highly promising. Therefore, this study aims to determine a porcine-specific aptamer, which can be used as a probe for porcine detection in meat and its derivatives.

1.2 Problem statement

Determining food quality and safety is the responsibility of many sectors, including the government, food industry and researchers. Various methods for traceability and authentication have been developed including mass-spectrometry (MS), PCR, nuclear magnetic resonance (NMR) and more. However, the existing detection methods suffer several challenges and limitations; including the requirement of good quality of extracted DNA and protein before detection, time-consuming and costly. The requirement of high specific and efficient primers from gene for PCR amplification, lengthy preparation of sufficient samples or templates and necessity of performing an appropriate DNA extraction method are among other challenges. Besides, there is scarce information on aptamer development for porcine detection or any animal speciation. Moreover, DNA aptamers is known to have inherent advantages as compared to RNA aptamers, which are more stable and flexible in structure and properties. Therefore, the aptamer-based detection method is sought to provide a faster and on-site tool for authentication purposes. This study is conducted to develop a porcine DNA aptamer that could be useful for the detection of porcine and its derivatives in the future.

1.3 Research hypotheses

1. Porcine-binding ssDNA aptamer(s) can be screened and identified through the combination of the SELEX process, sequencing analysis and clustering analysis.
2. The selected aptamers will display several characteristics such as strong binding affinity and selectivity. Each aptamer may have a unique secondary structure and motifs, which will enable the specific binding. Post-SELEX truncation analysis also can improve the aptamer binding capability and affinity.
3. The interaction between the aptamer and targeted protein can be demonstrated by evaluating the Z-score and HADDOCK score through docking simulation analysis.

1.4 Research objectives

This study aims to develop and characterize a novel porcine-bound DNA aptamer for future porcine detections in meat products. The specific objectives of this study are:

1. To identify potential porcine aptamer(s) by the combination of SELEX, sequencing and clustering analysis.
2. To characterize the selected aptamer through prediction of its secondary structure, motif analysis, assessment of binding affinity and truncation analysis.
3. To analyze the binding between aptamer and the target porcine protein through molecular docking simulation.

1.5 Significance of the study

Significantly, aptamer can recognize its target molecules with extraordinary selectivity and affinity. The developed and characterized aptamer is useful for the detection and authentication of porcine in meat products. In addition, aptamer allows direct detection of target samples without the need for pure DNA or protein extraction. The future-developing aptamer-based detection tools can be an alternative method to replace the costly and time-consuming method. The developed biosensory tools are also applicable for porcine detection, where their application is user-friendly and non-laborious application.

1.6 Overview of the study

This thesis is separated into three major sections. Figure 1.1 illustrates the general overview of the studies. The first working chapter (Chapter 3) described the screening and selection process of the porcine-bound aptamer, combining SELEX with sequencing through Sanger sequencing and Next Generation Sequencing as well as clustering analysis. The second working chapter (Chapter 4) designated the characterization of the candidate aptamer, by prediction of secondary structure, identification of sequence and structural motifs, determination of binding affinity, truncation analysis and identification of protein-bound aptamer by LC-MS. The selected aptamer was therefore subjected to molecular docking, which is described in Chapter 5. This chapter focuses on the simulation of aptamer-protein interaction using HADDOCK platform. In order to perform the simulation, three-dimensional model of aptamer and protein was built and evaluated. The specific molecule that involved in the interaction was identified. The conclusion and future recommendations are summarized in the last chapter.

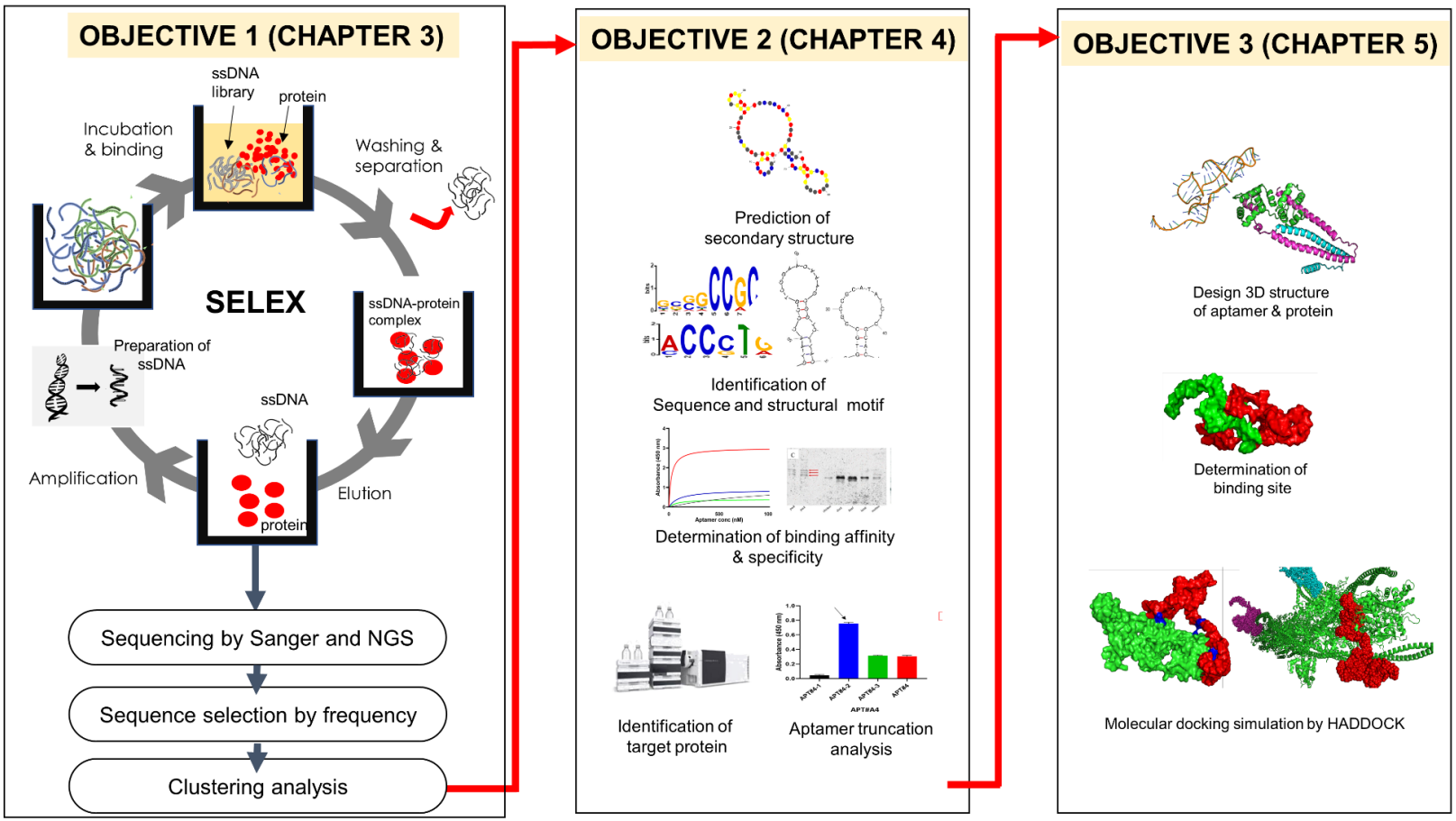

Figure 1.1: Flow chart of the study

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