Characterization of immunosensor for early detection of cucumber mosaic virus (CMV) detection in chili

By

Shahrul Aizzam Ariffin

(1331710878)

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Author's full name : SHAHRUL AIZZAM BIN ARIFFIN

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LIST OF ABBREVIATIONS

BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
C_4H_5N	Pyrrole
CMV	Cucumber Mosaic Virus
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EDTA	Ethylenediaminetetraacetic Acid
$EDTA.Na_2.2H_2O$	Disodium Ethylenediaminetetraacetic Dehydrate
Fab	Fragment Antigen-Binding
Fac	Fragment Crystallizable
GNP-Ab	Gold Nanoparticle-Antibody
H_3BO_3	Boric Acid
HRP	Horseradish Peroxidase
IgG	Immunoglobulin G
IgG-HRP	Immunoglobulin G-Horseradish Peroxide
KCl	Potassium Chloride
LOD	Limit of Detection
NaCl	Sodium Chloride
$Na_2HPO_4.2H_2O$	Disodium Hydrogen Phosphate Dehydrate
$NaH_2PO_4.H_2O$	Sodium Dihydrogen Phosphate monohydrate
NaOH	Sodium Hydroxide
OD	Optical Density
PBS	Phosphate Buffer Saline

PCR	Polymerase Chain Reaction
PPy	Polypryyrole
SAM	Self-assemble monolayer
SEM	Scanning Electron Microscopy
SPR	Surface Plasmon Resonance
SPCE	Screen Printed Carbon Electrode
TEM	Transmission Electron Microscopy
TMB	3,3',5,5'-Tetramethylbenzidine
Tris-HCL	2-Amino-2-(hydroxymethyl)-1,3-Propaneldiol Hydrochloride
Anti-CMV	Antibody-Cucumber Mosaic Virus

LIST OF SYMBOLS

%	Percentages
nm	Nano meter
µL	Micro liter
mL	Milli liter
L	Liter
g	Gram
mg	Milligram
s	Second
min	Minutes
h	Hours
M	Molar
mM	Milli Molar
Mol	Molar mass
I	Current
V	Volt
rpm	Revolutions per minute
G	Gravitational force
°C	Degree Celsius
t	Thickness

Pencirian Immunosensor untuk Pengesanan Awal Virus Mosaik Ketimun (CMV) pada Cili

ABSTRAK

Virus Timun Mozek (CMV) adalah seperti salah satu kekangan utama terhadap tanaman-tanaman cucurbit seperti timun, timun Jepun, labu, betik dan tembikai, sementara untuk tanaman-tanaman bukan cucurbit adalah seperti cili, tomato, bayam, kobis, saderi, kekacang, tembakau dan rumpai di dalam Selatan Asia dan Asia Tenggara. Berpandukan kajian yang lepas hampir 5-10 % kerugian tahunan penghasilan cili yang dicatatkan di dalam Asia yang berpunca dari CMV, di mana hampir RM 6.05 bilion kerugian tahunan yang dicatatkan di dalam penghasilan tanaman cili bagi seluruh dunia. Kerugian ini telah memberi impak yang besar terutamanya pada petani-petani jika tiada tidakkan serius diambil. Kebanyakan petani menggunakan pendekatan pemerhatian secara visual untuk mengesan CMV pada tanaman-tanaman mereka walaupun pemerhatian secara visual ini susah untuk mengenalpasti gejala yang berpuca dari CMV memandangkan visual gejala bergantung kepada kepekatan virus itu sendiri dan kerana sebab itu, kaedah ini adalah tidak boleh dipercayai untuk membasmi virus ini dari awal. Oleh itu, sebuah imunosensor elektrokimia berdasarkan pengesan virus timun mozek seperti elektrod karbon skrin tercetak dibangunkan dan ia boleh digunakan sama ada di dalam makmal dan bidang yang penting. Di samping itu, kajian mengenai penyakit CMV dan aktiviti antibodi ditunjukkan untuk membuat sensor secara aktif mengenali hanya molekul-molekul CMV dengan menggunakan antibodi yang ditentukan. Penulenan CMV digunakan untuk menghilangkan kekotoran dan kemudian, pengoptimuman CMV yang ditulenkan dilakukan menggunakan format immunoassay sandwich. Penulenan antibodi ditunjukkan untuk menghilangkan garam dan protein lain dan antibodi yang telah ditulenkan dioptimalkan menggunakan format imunoassay sandwic dan format imunoassay secara langsung. Keputusan awal untuk kedua-dua bahan yang ditulenkan mempunyai kekuatan mengikat yang tinggi. Selepas itu, antibodi yang ditulenkan telah konjugat dengan nanopartikel emas dan rumusan konjugat digunakan untuk pengubahsuaian permukaan immunosensor untuk mengubah sifat-sifat permukaan imunosensor. Isyarat elektrik yang dihasilkan dari proses pengesahan sensor diukur menggunakan teknik kronoamperometrik (CM). Dengan menggunakan teknik yang sama, set voltan potensi dijumpai pada 0.2 V dan LOD immunosensor berada pada 0.1 mg mL^{-1}. Selepas itu, imunosensor telah diuji dengan patogen lain untuk mengesahkan selektif imunosensor. Kajian awal menunjukkan imunosensor mengenal pasti sepenuhnya CMV yang telah ditulenkan dan tidak bertindak balas terhadap patogen lain di mana memberikan reaktiviti silang yang terendah. Dalam penyaringan CMV, kaedah silang-reaktif yang sama telah dilaksanakan dan daun cili mentah yang diambil di ladang-ladang MARDI digunakan untuk mengesan CMV di dalam pokok-pokok cili. Keputusan penyarigan CMV menunjukkan kehadiran CMV di dalam beberapa sampel. Oleh itu, kajian ini menyediakan alat pengesan yang sensitif dan selektif kepada para petani yang membenarkan pengesanan awal ladang cili mereka.

Characterization of Immunosensor for Early Detection of Cucumber Mosaic Virus (CMV) in Chili

ABSTRACT

Cucumber Mosaic Virus (CMV) is one of the major constraints towards cucurbit crops such as cucumbers, zucchinis, pumpkins, papayas and watermelons production, while for non-cucurbit crops such as chilies, tomatoes, spinach, lettuces, celeries, beans, tobaccos and weeds production in South and Southeast Asia. Based on previous studies approximately 5-10 % annual losses of chili yield in Asia was caused by CMV, which accounts for nearly RM 6.05 billion annual loss in chili production worldwide. This loss has given a huge impact especially to the farmers if there are no serious actions taken. Most farmers are using common approach which is visual observation to detect the CMV on their crops despite the visual observation is difficult to identify a symptom caused by the CMV since the visual of symptom depends on the concentration of virus itself and because of that reason, this method is not reliable to eradicate this virus from scratch. Therefore, a portable electrochemical immunosensor based cucumber mosaic virus detection like screen-printed carbon electrode was developed and it can be employed whether in laboratory and field that is essential. In addition, study on the CMV disease and antibody activity was demonstrated to making sensor actively recognize only the CMV molecules by using specify antibody. The CMV purification is used to eliminate the impurities and then, optimization of the purified CMV was performed using sandwich immunoassay format. The purification of antibody was demonstrated to eliminate salt and other proteins and the purified antibody was optimized using sandwich immunoassay format and direct immunoassay format. The initial results showed the both purified substance possess high binding strength. Subsequently, a purified antibody was conjugated with gold nanoparticles and the conjugated solution was used for the immunosensor surface modification to change the immunosensor surface properties. The electrical signal produced from the sensor validation process was measured using chronoamperometric (CM) technique. By using the same technique, the set voltage potential was spotted at 0.2 V and the LOD of immunosensor was at 0.1 mg mL^{-1}. After that, the immunosensor was tested with other pathogens to verify the immunosensor selectivity. The initial study shows the immunosensor fully identifies the purified CMV and did not react to other purified pathogens whereby gives the lowest cross-reactivity. In the CMV screening the same cross-reactivity method was executed and the crude chili leaves that taken around MARDI plantation were used to detect CMV in the chili trees. The results of the CMV screening show the presence of CMV in some samples. Thus, this research provides a sensitive and selective detection tool to the farmers that allow an early detection on their chili plantations.

CHAPTER 1

INTRODUCTION

1.1 Background

The agricultural sector is considered a major contributor to economic development in several countries such as Japan, Netherland, America, Taiwan, Thailand, including Malaysia. However, major challenges still plaguing the agricultural sector in those countries such as the growing demand for safe food supply as well as the high quality, the threat of climate change, increasing the risk of plant disease and others need to be addressed more seriously by all those involved. In chili production, this agriculture has an economic impact in local as well as export markets in Asia especially in South East Asia (SEA) and other parts of the world as well. More than one billion people consume chili in one or another form such as spices based on their daily basis consumption. The cucumber mosaic virus (*Bromoviridae* family, *Cucumovirus* genus) has a bigger host plants in the agriculture world because it can infect more than thousand plants species in 85 families. The outbreak of CMV in Malaysia have been reported infecting crops such as chilies, tomatoes, cucumbers, tobaccos, and weeds. Due to its worldwide distribution and polyphagous vectors, CMV is one of the five most important viruses infecting chili species worldwide (Rahman et al, 2013; Tan, 2012) and farmer may suffer crop losses from 10% to 15% and sometimes, may reach up to 60% if the plants are infected at early stage (Rahman et al., 2013). The vectors of infection may vary such as from aphids, agricultural machinery, equipment or

contaminated seeds. Due to all these factors, many researchers especially biotechnology research team from Malaysian Agriculture Research Development Institute (MARDI) starting to develop sensors to solve this kind of problem. There are many suitable sensors and one of them is an immunosensor when comes to biological, chemical pathogen, and contaminates. Immunosensors are compact analytical devices in which the event of binding of antigen-antibody complexes is detected and converted to an electrical signal, which can be recorded, processed, and displayed. Therefore, immunosensor is a good option to detect and sense a plant disease. Through this research, it can be justified that it has also contributed a great potential in the agriculture sectors (Gabor, 1966).

1.2 Problem Statement

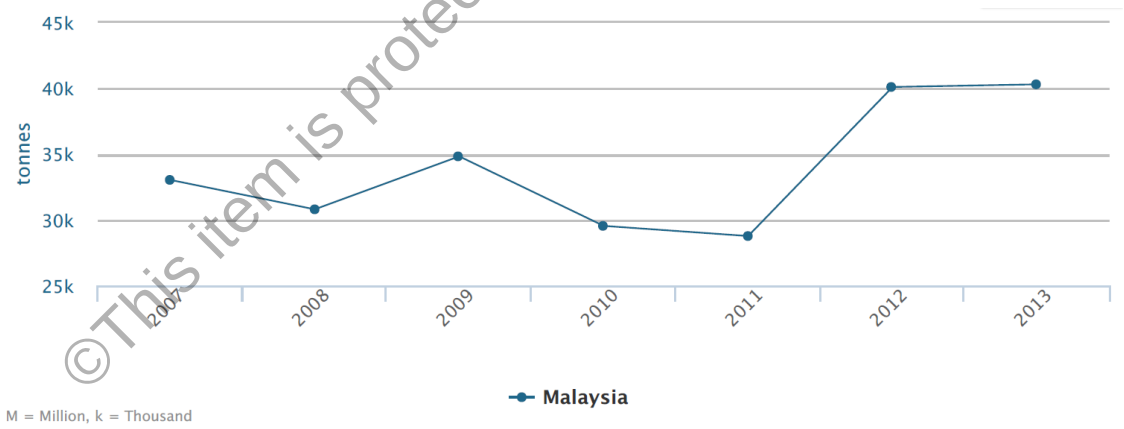

Figure 1.1: Malaysia's chili production and demand from 2007 until 2013.

Chilies are one of the major ingredients used in daily cuisine in Malaysia and also well-known among local people in the Asia region. The increment of chili's demand and consumption had risen as shown in Figure 1.1 and the addition of chili farms to meet the demand are in full swing by the authorities. Therefore, for this reason,

the spread of infectious plant diseases such as cucumber mosaic virus outbreak becomes easy to damage the plants. The cucumber mosaic virus is a well-known common disease in plants since it infects almost every variety of crops (Hidaka, 1965; Price, 1934; Zitikaitė et al, 2011) and it has an enormous destruction of chili plantations. The chili crops undergo malnutrition, become stunted and most infected crops may suffer from a low quality of production and in worst case scenarios, they are even in the verge of becoming dying crops. Meanwhile, the farmers are suffering from the low yield of chili production, low profit and this phenomenon has affected Asia as well.

The first cucumber mosaic virus symptom was discovered in 1934 (Price). One of major problems faced by farmers is late detection of virus infection because of limitation of technology. The control of the cucumber mosaic virus is still a challenge at this moment although many studies have been conducted by researchers or other authorities to prevent and eradicate cucumber mosaic virus outbreak in chili plantation. At that moment, only visual identification can be done. Hence, a monitoring system is essential to provide an early detection and to prevent a serious outbreak.

Before biosensor was invented, conventional methods such as ELISA, PCR and SPR remained as the most reliable technique for the detection of cucumber mosaic virus. However, there are several weaknesses of the conventional methods such as time-consuming process and lack of trained personnel handle the equipments. The conventional method particularly in the agriculture sectors are not very convenient because those weaknesses occurring during the virus screening generally require maximal technical expertise.

Recently, a plant disease-based biosensor promises a rapid detection and indirectly giving fast time response and result over the conventional method. The immunosensor development for the cucumber mosaic virus disease detection is very

useful as a diagnostic and analytical tool for highly sensitive detection in the chilies physiology. Thus, the immunosensor has become a great potential in agriculture sectors in order to sustain the chili production. The immunosensor optimization is operating under several specific conditions that serves only for a special purpose and problem. Most works are focused on immunosensor sensitivity, responsivity, efficiency and simplicity of the assay procedure. Additionally, the immunosensor has an ability to generate a direct electrical signal which gives a potential to monitor real-time analytes and this immunosensor is a suitable tool that can do continuous monitoring in the infected area.

In this thesis, the development of an immunosensor and designing the immunosensor which is screen-printed carbon electrode (SPCE) with a three electrodes system (working, reference and counter) will be described. Nowadays, the SPCE is widely used and additionally they are very economical (easy to fabricate in bulk and disposable), very easy to handle, miniaturized portable system, high sensitivity and selectivity on detection. Thus, this immunosensor have a huge potential to be used for the detection of cucumber mosaic virus in chili plantation.

1.3 Research Objectives

1.3.1 General Research Objective

The aim of this research is to develop high sensitivity and selective immunosensor as a transducer for immobilization and binding of plant disease detection. These objectives are accomplished with the following specific objectives.

1.3.2 Specific Research Objective

The specific objectives of this research are:

- To study the effect of a binding process for sensitivity measurement of the reaction between screen-printed carbon electrode (SPCE) and biomolecule samples.
- To assess the performance of surface modification of inorganic material (carbon) for better attachment of gold nanoparticles conjugate on the inorganic surface for immobilization and binding process.
- To explore the performance and the proposed of screen printed carbon electrode sensor to function as biosensor for detection of biomolecule samples for sensitivity and selectivity detection using cyclic amperometric measurement.
- To study gold nanoparticles performance and functionality in detection, reliability and efficiency toward biomolecule field samples for immobilization and binding process.
- To study cross-reactivity at various biomolecule samples to verify the sensor does not react to other diseases using cyclic amperometric measurement.

1.4 Research Scopes

First of all, at the early stages of the study, the literature review, and the theoretical understanding on the Cucumber Mosaic Virus (CMV) isolation and purification, Immunoglobulin G purification, SPCE as a sensor platform and biomolecule detection as a critical point or parameter for the understanding of the design and immunosensor detection.

The next scope is to extract and purify cucumber mosaic virus (CMV) from infected cucumber leaves for antibody production in rabbit, enzyme fabrication, and immunosensor antigen detection study.

Afterward, the purified cucumber mosaic virus (CMV) are optimized and characterized morphologically and serologically by using Transmission Electron Microscopy (TEM) and a microplate reader. It is very important to yield a high quality of antigen to ensure the efficiency of biosensor detection.

Furthermore, for antibody production, the rabbit is immunized with the purified CMV in order to stimulate the immune system to produce antibody against the CMV and the antibody is extracted from serum that gained after bled the rabbit for antibody purification.

The purification of antibody and chromatography process is proceed on the host's (rabbit) serum by using AKTAprime purifier to gain the specific antibody (IgG) for surface modification process which is used to binding the purified cucumber mosaic virus (CMV).

The Immunoglobulin G (IgG) is characterized in morphologically and serologically by using UV-Spectrometer and microplate reader. It is very important to do confirmation on an Immunoglobulin G (IgG) so it can binding perfectly with purified CMV.

Afterward, the activated peroxidase conjugation was demonstrated by conjugating between polyclonal antibody and Sodium Periodate using the establish method. This conjugation of activated Peroxidase to the polyclonal antibody (IgG-HRP) is a crucial part in electrical measurement using SPCE.

The conjugation of gold nanoparticles is prepared by conjugating between gold nanoparticles and polyclonal antibody and the gold nanoparticles conjugate used in immobilization process on the screen printed carbon electrode whereby the gold nanoparticles main purpose is to change the properties of the electrode surface.