ลักษณะสมบัติของเปอร์ออกซิเดสจากใบมันสำปะหลัง *Manihot esculenta* Crantz พันธุ์ KU50 และการประยุกต์ในการสลายพอลิคลอริเนตไบฟีนิลและการตรวจวัดไทออลโดยโวลแทมเมทรี

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CHARACTERISTICS OF PEROXIDASE FROM CASSAVA LEAVES *Manihot esculenta* Crantz cv. KU50 AND ITS APPLICATION IN POLYCHLORINATED BIPHENYL DEGRADATION AND THIOL DETECTION BY VOLTAMMETRY

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Cassava peroxidase (CSP) isolated from cassava leaves (*Manihot esculenta* Crantz of cv. KU50) was purified by column chromatography. It was a haem-containing cationic glycoprotein with the molecular weight of 38 and 44 kDa determined by MALDI-TOF-MS. Its characteristics and kinetic catalysis were investigated and compared to those of horseradish peroxidase (HRP). It was found that CPS showed higher stability at 70°C in 0.1 M phosphate buffer pH 6 and more tolerant in the buffer containing 50% of acetonitrile than those of HRP. In preliminary study, the inhibitory effect of thiourea on peroxidases was investigated and then the possible use of peroxidases for the detection of thiol compounds such as thiourea and thiram by voltammetry was studied. It was based on enzyme inhibition assay and square-wave voltammetric measurement of the peak height at the potential of -0.24 V versus Ag/AgCl electrode was performed in 0.04 M Britton-Robinson buffer, pH 6 containing urea hydrogen peroxide and 3, 3?-diaminobenzidine. It was found that the peak height was inversely proportional to the concentration of thiol and the relationship could be used for the detection of thiourea and thiramin in the range of 5-100 µM. In addition, the catalytic potency of CSP for the enhancement of polychlorinated biphenyl (PCB) such as 2, 4, 4?-trichlorobiphenyl (PCB-28) degradation was carried out. PCB-28 concentration of 2.2 µM was clearly decreased to 50% of its initial concentration with the higher rate than HRP catalysis in 0.04 M Britton-Robinson buffer, pH 6 during 6 hours at room temperature. From these results, it can be concluded that CSP is a novel peroxidase with thermal and acetonitrile tolerant properties which can potentially be applied as a valuable tool in bioremediation and other biotechnological applications.

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Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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ABBREVIATIONS

A	absorbance
CSP	cassava peroxidase
°C	degree Celsius
DAB	3, 3′-diaminobenzidine
g	gram
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
IC_{50}	inhibition concentration at 50%
^{app}Ki	apparent inhibition constant
^{app}Km	apparent Michaelis-Menten constant
μA	microampere
μg	microgram
μL	microliter
μM	micromolar
mL	millilitre
mM	millimolar
min	minute
M	molar
nA	nanoampare
nm	nanometer
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PCB	polychlorinated biphenyl
PCB-28	2, 4, 4′- trichlorobiphenyl
PMSF	phenylmethyl sulfonyl fluoride
PVPP	polyvinyl polypyrrolidone
RZ	Reinheitszahl
SDS	sodium dodecyl sulfate
SPE	screen-printed electrode
SBP	soy bean peroxidase
SWV	square-wave voltammetry
THU	Thiourea
TIR	Thiram

TMB	3, 3′5, 5′-tetramethylbenzidine
UHP	urea hydrogen peroxide
V	volt
V_{max}	apparent maximum velocity

CHAPTER I
INTRODUCTION

1.1 Background and rationale

Peroxidases (PODs) are classified as oxidoreductase (EC 1.11.1.7) catalyzing the oxidation of various organic and inorganic substances by hydrogen peroxide or related compounds. Peroxidase, especially horseradish peroxidase (HRP) has long been used in the area of biosciences and biotechnology, ranging from diagnostics and bioremediation. Due to the fact that HRP is readily available as a commercial enzyme with high purity, low cost and it is found to be reasonably stable, it has been applied to be used as an enzyme electrode and biosensor for the determination of hydrogen peroxide, organic peroxides and HRP inhibitors. However, there are many studies focusing on the use of peroxidases from various sources such as soybean and palm tree in order to improve the sensitivity and working range of the detection method. Cassava has been reported to be a good source of peroxidase. Its peroxidase activity can be found in many parts such as leaf, petiole, stem and root. Moreover, it was found that the extracted peroxidase from cassava leaf showed the highest specific activity and was stable in a wide range of pH as well as at high temperature. In addition, cassava is the third most important crop in Thailand and cassava leaves can be easily found and considered as agricultural waste after the cassava root harvest. Therefore, peroxidase obtained from cassava leaves is found to be interesting as a novel alternative peroxidase used for analytical applications and bioremediations. In this study, it was separated into 3 main parts. The first part was focused on the characterization of peroxidase isolated from

cassava leaves. The second part was intensively considered for the potential use of peroxidase for the detection of thiourea and thiram using voltammetric techniques and the third part was mainly studied on the potential use of peroxidase as biocatalyst for PCB degradation by peroxidation.

1.2 Studies of the thesis

1.2.1 Literature review

1.2.2 Preparation of peroxidase isolated from cassava leaves

1.2.3 Characterization of biochemical and kinetic properties of cassava peroxidase

1.2.4 Investigation of the effect of thiourea and thiram on peroxidase activity

1.2.5 Potential use of peroxidase and screen-printed electrode for the detection of thiourea and thiram by voltammetric technique

1.2.6 Potential use of peroxidase for the enhancement of PCB degradation by peroxidative reaction

1.2.7 Data analysis and dissertation writing

1.3 Objectives

1.3.1 To study biochemical properties of peroxidase isolated from cassava leaves

1.3.2 To assess the potential use of peroxidase and screen-printed electrode for the detection of thiourea and thiram by voltammetric technique

1.3.3 To investigate the potential use of peroxidase for the enhancement of PCB degradation by peroxidase reaction

CHAPTER II
LITERATURE REVIEW

2.1 Cassava and its utilizations

Cassava (*Manihot esculenta* Crantz) is a dicotyledomous plant in the botanical family of Euphorbiaceae. It is also known as manioc, manihot, yacca, mandioca, sweet potato tree and tapioca plant. Cassava is a shrubby perennial that grows to a height of 6-8 feet. It has smooth erect stems contain soft white pith and have nodes from which new plants are obtained. Cassava leaf is a large dark green compound leaf divided into about seven leaflets as shown in **Figure 2.1**. The roots, which are the most valuable portions of the plant, grow in clusters of 4-8 at the stem base. Roots are from 1-4 inches in diameter and from 8-15 inches long. The pure white interior has a very high starch content. The roots are covered with a thin fibrous bark that is reported to contain toxic hydrocyanic acid, which must be removed by washing, scraping and heating (Stephens 1994). Cassava roots are an ambiguous source of carbohydrate. So it has been used for human consumption, animal feed, along with raw materials in food industries. Moreover, Cassava starch in both native and modified can be used for the production of alcohol, glues, sweetener, pharmaceuticals and biodegradable products (Adejumo et al. 2011). Cassava leaves have also been consumed in some parts of sub-Saharan Africa and some Asian countries as a green vegetable, which provides proteins and vitamins A and B (Castellanos et al. 1994). Thailand has been the world's leading cassava exporting country for the past fifty years. Almost three fourth of the product yield was exported to China, ASEAN, Europe, and America, while the rest was sold

domestically (Poramacom et al. 2013). Many varieties have been developed and promoted for planting in our country such as Huaybong 60 (HB60), Kasetsart 50 (KU50) and Rayong 5 (R5). KU50 is the most important cassava cultivar with high starch content and also grown in extensively in Indonesia and Vietnam under the name of KM94 (Prammanee and Kamprerasart 2010). According to the national policy established to push the research trend for the utility of all part of many crops to increase the economic returns to the farmers, potential uses of cassava leaf have been initiated for animal feed preparation (Adeyemi et al. 2012, Nguyen et al. 2012) and tannin extraction (Noikondee 2008). Moreover, various enzymes such as cyanogenic beta-glucosidase (linamarase) (Haque and Branbury 1999) and peroxidase (Pereira et al. 2003) which are responsible for a defense mechanism and environmental stresses were also found in cassava fresh leaves and many parts of cassava such as petiole, stem and root. It was found that the extracted peroxidase from cassava leaf showed the highest specific activity (Suriyaprom 2000). The peroxidase was stable in a wide range of pH (4-11) as well as stable at high temperature (60°C) condition within 24 h of incubation. These are interesting properties of cassava peroxidase (CSP) and there have not yet been any reports for the study of cassava peroxidase in the field of analytical applications.

Figure 2. 1 **Cassava (*Manihot esculenta* Crantz) cultivar KU50.**

a. Cassava tree b. Leaf and Petiole c. Tuberous roots.

2.2 Plant peroxidases

Peroxidases (PODs) classified as oxidoreductase (EC 1.11.1.7) belong to a large family of enzymes that catalyzed the oxidation of a wide variety of organic and inorganic substances by hydrogen peroxide or related compounds. Generally, peroxidases play an important role in maintenance of reduced state molecules in organisms (Dunford and Stillman 1976). So, there are many isoenzyms can be isolated and purified from animals, mold, microorganisms and especially from plants. Higher plants have several peroxidase isoenzymes. These isoenzymes are differentially expressed in various tissues and organs and respond to environment conditions. According to their function and substrate preference, plant peroxidases

can be divided into two types as ascorbate peroxidases and classical peroxidases (Welinder 1992).

2.2.1 Ascorbate peroxidases (APX, EC 1.11.1.11)

Ascorbate peroxidases (APX) play an important function to prevent the damage of plant cells from excess hydrogen peroxide condition. Reactive oxygen species (ROS), including superoxide ($^{\bullet}O_2^-$), H_2O_2, hydroxyl radical ($^{\bullet}OH$) are the inevitable by-products of cell metabolism. Under stressful conditions, the formation of ROS may be excessive. These ROS attack lipids and proteins causing lipid peroxidation and protein denaturation (Bowler et al. 1992). To prevent the damage, plant cells equipped with an antioxidative system consisting of low-molecular weight anti-oxidants such as ascorbate, and protective enzyme, ascorbate peroxidase (Yu and Rengel 1999). APX has a high preference for ascorbate as electron donor. The reaction catalyzed by APX is shown below (Mittler and Zilinskas 1991):

$$2 \text{ ascorbate} + H_2O_2 \longrightarrow 2 \text{ monodehydroascobate} + 2\ H_2O$$

2.2.2 Classical peroxidases

Classical plant peroxidases can be distinguished from APX by their nonspecific use of various phenolic derivertives as electron donors and they are glycoproteins containing the prosthetic group hemin or feriprotoporphyrin IX as shown in **Figure 2.2**. Classical peroxidases play many functions in biosynthesis processes such as lignin polymerization (Nozu 1967), the

deposition and cross-linking of extension in the plant cell wall leading to a more resistant barrier against pathogen inflection (Cooper and Varner 1984). The well-known classical peroxidase is horseradish peroxidase (HRP). It was found mainly in the roots of horseradish. Nowadays, many classical plant peroxidases with their unique characteristics have been purified from various sources. The properties of some plant peroxidases obtained from various sources are summarized in **Table 2.1**.

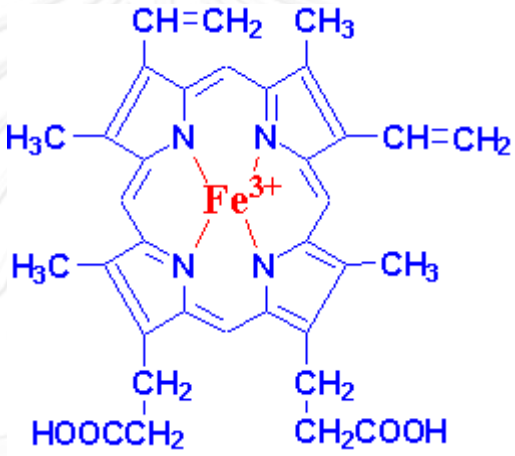


Figure 2. 2 **Ferriprotoporphyrin IX or protoferriheme.**

Table 2. 1 Biochemical characteristics of some plant peroxidases

Peroxidase	Biochemical characteristics		
	MW (kDa)	p*I*	Glycosylation sites
Horseradish peroxidase (HRP)[a]	44	8.9	8
Peanut peroxidase (PNP)[a]	40	7.9	3
Sweet potato peroxidase (SPP)[a]	38	3.5	4-7
Tobacco peroxidase (TOP)[a]	36	3.5	4
Soybean peroxidase (SBP)[b]	41	4.1	4

[a] :Lindgren et al., 2000. [b] :Ryan et al., 2006.

2.3 Application uses of peroxidases

Peroxidases have been used in the area of biosciences and biotechnology, ranging from diagnostics, bioremediation and biosensors. Here are some peroxidase applications in detail.

2.3.1 Peroxidase-lebelled antibody

Enzyme-labelled antibody is an important component in immunoassay and ELISA. Peroxidase especially horseradish peroxidase, has been found well suited for the preparation of enzyme conjugated antibodies, due to its stability and ability to yield chromogenic products. Peroxidase labelled immunoglobulins have long been used successfully as immunohistological probes for the demonstration of tissue antigens (Nakane and Pierce 1967), and

in enzyme immunoassay for the determination of chemical residues (Oplatowska et al. 2011, Vdovenko et al. 2014, Yadav et al. 2013) and also virus detection (Anderson et al. 2014).

2.3.2 Peroxidase based bioremediation

Due to the fact that peroxidases are heme proteins catalyzing a variety of oxidative transformations on organic and inorganic substrates in the presence of hydrogen peroxide or alkayl peroxides, peroxidases such as lignin peroxidase and manganese peroxidase from bacteria and fungi are useful enzymes for waste water treatment. Some phenolic compounds could be removed or detoxified from waste water by precipitation or transformation (Cheng et al. 2006, Durán and Exposito 2000). Many reports also focused on the utilization of peroxidases for removing or eliminating of industrial waste organic dyes (Rauf and Ashraf 2012), phenolic pollutants (Khan et al. 2007) and polychlorinated biphenyls (PCBs) (Köller et al. 2000).

2.3.3 Peroxidase based biosensors

One of the most common enzymes used in biosensors is HRP. It is usually immobilized on electrode as biological sensing material. HRP biosensor research has continued to develop through many forms, from the traditional voltammetric- and amperometric-based methods of detection to nano-sized devices (Ryan et al. 2006). Biosensors exploiting peroxidases have been reported which include glucose sensor (Lomillo et al. 2005), ethanol biosensors (Azevedo et al. 2003) and biosensors for the determination of hydrogen

peroxide (Kafi et al. 2008, Yu et al. 2003) and peroxidase inhibitors (Liu et al. 2008, Yu et al. 2007).

Due to the fact that the enzyme stability has been a key problem in biotechnology, peroxidases with specific properties to be able to work under a wide range of pH and temperature have been a target in many researches. The improvements of peroxidase properties by using techniques of modification (Hassani 2012), (Khosravi et al. 2012), (Feng et al. 2008)) or mutation (Lehmann and Wyss 2001) of HRP for achieving an adequate thermostability have been continuously reported. Moreover, purifications and characterization of new peroxidases from various plant sources have also been vastly explored.

2.4 Classical peroxidase reaction

The enzymatic reaction of peroxidase takes part in three steps. Firstly, the enzyme reduces H_2O_2 under the formation of oxidized enzyme (compound-I), reaction 1. Secondly, the oxidized form of the enzyme then gets reduced by a suitable electron or ireduced again to the resting state as the native form. The overall catalytic mechanism of peroxidase consists of reactions as shown (Lindgren et al. 1997):

$$POD + H_2O_2 \longrightarrow \text{Compound-I} + H_2O \quad (1)$$

$$\text{Compound-I} + AH_2 \longrightarrow \text{Compound-II} + AH\cdot \quad (2)$$

$$\text{Compound-II} + AH_2 \longrightarrow POD + AH\cdot + H_2O \quad (3)$$

Therefore, an electron or hydrogen donor for peroxidase intermediate plays an important role for peroxidase catalytic activity. Many organic substances acted as chromogens are commonly used as secondary substrates. They turn to colored product which can be detected by spectrophotometric method after the catalytic reaction occurred. Moreover, there are other substrates including phenols, amines, hetrocyclics, ferrocences and ferrocyyanide which can be detected based on fluorescence, chemiluminasecence and amperometric methods.

2.5 Spectrophotometric measurement

All biochemicals absorb energy from at least one region of spectrum of electromanatic radiation. The energy at which absorption occurs is dependent on the available electronic, vibrational and rotational energy levels of the molecule. Spectrophotometric techniques involve the measurement of these transitions in precise and are analytical procedure used for the characterization and quantification of (biological) molecules (Harri and Bashford 1988).

2.5.1 Spectrophotometric measurement of enzyme activity

An assay where the color is changed and the absorption of product differs from that of substrate, the enzyme activity can be measured directly by the following the change in absorption in time. The color change of enzyme catalyzed product can adopt from one of the following approaches for spectrophotometric measurement; firstly, i) stop the reaction with acid, alkali or organic solvent and add a reagent (chromogen), which produces a color on the reaction with one product. This is termed a discontinuous assay. Secondly,

ii) couple the reaction under study with an indicator reaction, which produces a color ‘instantaneously’. This is called coupled assay. Finally, iii) use a synthetic chromogenic substrate instead of natural substrate where the true substrate is complex and little different chemically from the product (Harri and Bashford 1988).

2.5.2 Chromogen reaction for monitoring peroxidase activity

In colorimetric or spectrophotometric method, many chromogens such as 2, 2′-azino-di (3-ethyl-benzthiazoiine-sulphonate) (ABTS), o-dianisidine (ODD), o-phenylenediamine (OPD), and 3, 3′, 5, 5′-tetramethylbenzidine (TMB) are commonly used for monitoring peroxidase activity. They are considered as secondary substrates which turn to oxidized form with specific color after catalyzing by peroxidase in the presence of peroxide. Here, their properties are briefly summarized in **Table 2.2**. Recently, TMB are mostly used as a chromogen for the determination of peroxidase activity. That is because TMB has the advantage of being a non-mutagenic chromogen with a very high molar absorption coefficient (67,300 $M^{-1}cm^{-1}$). As a result, the use of TMB for peroxidase activity monitoring is not only led to improve the sensitivity of the assay but also protect the user and environment from toxic substance exposure. Moreover, there are some reports showed that TMB could be used as a mediator for the determination of HRP activity by electrochemical technique (Crew et al. 2007).

Table 2. 2 **Spectrochemical property and toxicity of some chromogens.**

Chromogen	Spectrochemical property		Toxicity
	Maximum adsorption (λ_{max})	Extriction coefficient (ε)	
2, 2´-Azino-di (3-ethyl-benzthiazoiine-sulphonate) (ABTS)	414 nm (Sakharov et al. 2001)	31,000 $M^{-1}cm^{-1}$	Mutagen (Voogd et al. 1980)
o-Dianisidine (ODD)	420 nm (Sakharov et al. 2001)	30,000 $M^{-1}cm^{-1}$	Carcinogen (Casero et. al., 1997)
o-Phenylenediamine (OPD)	445 nm (Sakharov et al. 2001)	11,100 $M^{-1}cm^{-1}$	Mutagen (Voogd et al. 1980)
3, 3´, 5, 5´-Tetramethylbenzidine (TMB)	450 nm	67,300 $M^{-1}cm^{-1}$	Non-mutagen (Garner et al. 1975)
	655 nm (Loung et al. 1995)	39,000 $M^{-1}cm^{-1}$	

2.6 Electrochemical measurement

Electrochemistry is the branch of chemistry concerned with the interrelation of electrical and chemical effects. This field deals with the study of chemical change caused by the passage of an electric current and the production electrical energy by chemical reactions. In electrochemical system, the process and factors that affect the transport of charge across between an electrode and electrolyte are concerned. An overall electrode reaction (O + *ne* ⇆ R) composed of a series of steps that cause the conversion of the dissolved oxidized species (O) to a reduced form (R) in solution as shown in **Figure 2.3**. In general, the current (or electrode reaction rate) is governed by the rates of processes such as mass transfer of O from the bulk solution to the electrode surface. At the electrode surface, the electron transfer is followed after the chemical reaction proceeding. These might be the protonation or catalytic decomposition occurred on the electrode surface and other surface reactions, such as adsorption, desorption, or crystallization (Bard and Faulkner 2001).

2.6.1 Mediator reaction for monitoring peroxidase activity

Mediators are low molecular weight molecules that can transfer electrons between redox centre of the enzymes and working electrodes, thus facilitate electrical communication between them as shown in **Figure 2.4**. The use of mediators made it possible to decrease the applied potential and greatly reduce the influent signals caused by electrochemically easily oxidizable interfering compounds present in real samples. Some mediators

used for monitoring enzyme activity by voltammetric measurement are presented in **Table 2.3**.

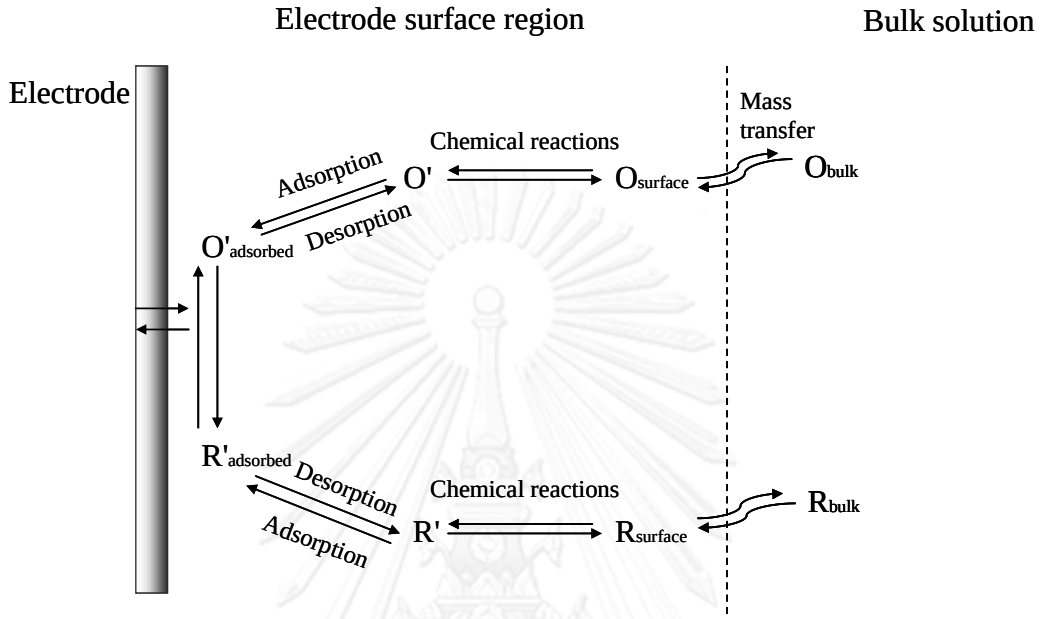


Figure 2. 3 **Pathway of a general electrode reaction.**

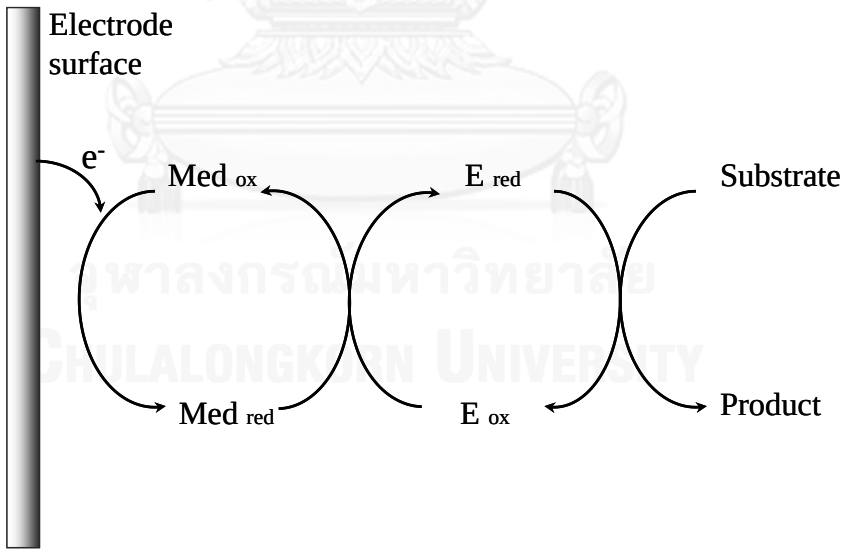


Figure 2. 4 **Reaction scheme depicting the role of a mediator for an enzyme-catalyzed reaction.**

E is the enzyme and Med is the mediator.

Table 2. 3 **Electrochemical properties and application use of some mediators.**

Mediator	Electrochemical property		Application use
	Redox potential/ Electrode	Product form	
2, 2´-Azino-di (3-ethyl-benzthiazoline-sulphonate) (ABTS)	472 mV (E^{o} vs. Ag/AgCl)	$ABTS/ABTS^{+}$	Electrochemical analysis of lignin oxidation by laccase and mediator (Bourbonnais et al. 1998)
	885 mV (E^{o} vs. Ag/AgCl)	$ABTS^{+}/ABTS^{2+}$	
3, 3´-Diaminobenzidine (DAB)	-0.62 V (vs. SCE)	DAB/DAB^{2+}	$DAB-H_2O_2$-HRP voltammetric enzyme-linked immunoassay (Zhang et al. 2008)
3, 3´, 5, 5´-Tetramethylbenzidine (TMB)	0.60 V (vs. Ag/AgCl)	TMB/TMB^{+}	Peroxidase for sensitive voltammetric detection of H_2O_2 with 3, 3´, 5, 5´-tetramethylbenzidine (Sun et al. 2013)
	0.95 V (vs. Ag/AgCl)	TMB^{+}/TMB^{2+}	

2.6.2 Voltammetric techniques

Voltammetry is a branch of electrochemistry techniques based on the application of a potential (*E*) to an electrode and the monitoring of the resulted current (*i*) flowing through the electrochemical cell over a period of time (*t*). They are considered active techniques because the applied potential forces a change in the concentration of an electroactive species at electrode surface by electrochemically reducing or oxidizing it. The analytical advantages of voltammetric techniques include excellent sensitivity with a very large useful linear concentration range for both inorganic and organic species (10^{-12} to 10^{-1} M). A large number of useful solvents and electrolytes and a wide range of temperatures can be employed with rapid analysis times in seconds and this can be used to determine several analysts, simultaneously. In addition, the ability to determine kinetic parameters, a well-developed theory and thus the ability to reasonably estimate the values of unknown parameters can be achieved. Furthermore, there are different potential waveforms that can be generated for measuring small currents (Marken et al. 2001).

2.6.2.1 Cyclic voltammetry (CV)

Cyclic voltammetry (CV) has become an important and widely used as electroanalytical technique in many area of chemistry. Normally, it is used for qualitative determination such as the study of redox processes, for understanding reaction intermediates, and obtaining stability of reaction products. This technique is based on the applied potential to a working

electrode in both forward and reverse direction while monitoring the current. For example, the initial scan could be in the negative direction to the switching potential. At that point the scan would be reverse and run in the positive direction. The cycle of analysis could be performed as one full cycle, a partial cycle, or a series of cycles. The response obtained from a CV called cyclic voltammogram as shown in **Figure 2.5** for the reversible redox system.

2.6.2.2 Pulse Voltammetry

In order to increase speed and sensitivity of voltammetric techniques, many forms of potential modulation have been tried over the years and three pulse techniques are widely used. The potential wave forms and their respective current response were shown in **Figure 2.6.**

2.6.2.2.1 Normal Pulse Voltammetry (NPV)

This technique uses a series of potential pulse of increasing amplitude. The current measurement is made near the end of each pulse, which allows time for charging. It is usually current to decay carried out in an unstirred solution. The resulting voltammogram displays the sampled current on the vertical axis and the potential pulse is stepped on the horizontal axis.

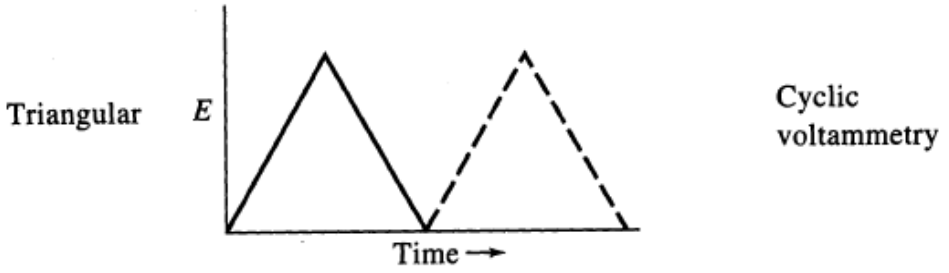
2.6.2.2.2 Differential Pulse Voltammetry (DPV)

This technique is comparable to normal pulse voltammetry in that the potential is also scanned with a series of pulses. However, it differs from NPV because each potential pulse is fixed and superimposed on a slowly changing base potential. Current is measured at two points for each pulse, the first point just before the application of the pulse and the second at the end of the pulse. These sampling points are selected to allow for the decay of the nonfaradaic current. The difference between current measurements at these points for each pulse is determined and plotted against the base potential.

2.6.2.2.3 Square wave voltammetry (SWV)

The excitation signal in SWV consisted of a symmetrical square-wave pulse of amplitude (E_{sw}) superimposed on a staircase waveform of step height (ΔE), where the forward pulse of the square wave coincides with the staircase step. The net current (i_{net}) is obtained by taking the difference between the forward and reverse currents (i_{for} - i_{rev}) and is centered on the redox potential. The peak height is directly proportional to the concentration of electroactive species and direct detection limits as low as 10^{-8} M is possible. Square-wave voltammetry has several advantages. Among these techniques, it shows excellent sensitivity with lowest background currents and the speed of analysis is very fast.

a.)



b.)

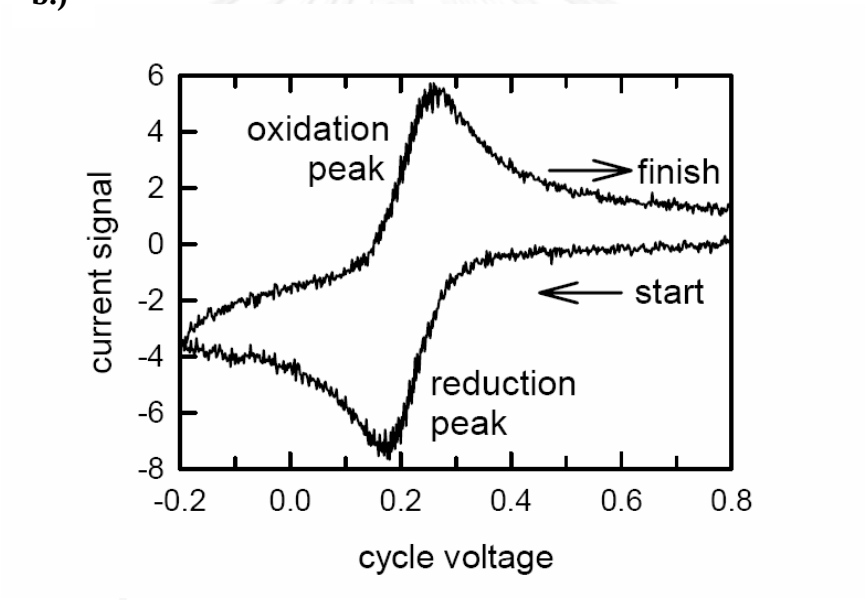


Figure 2. 5 **A typical cycle voltammogram showing reduction and oxidation peak.**
a.) Wave form of potential and b.) Current peak.

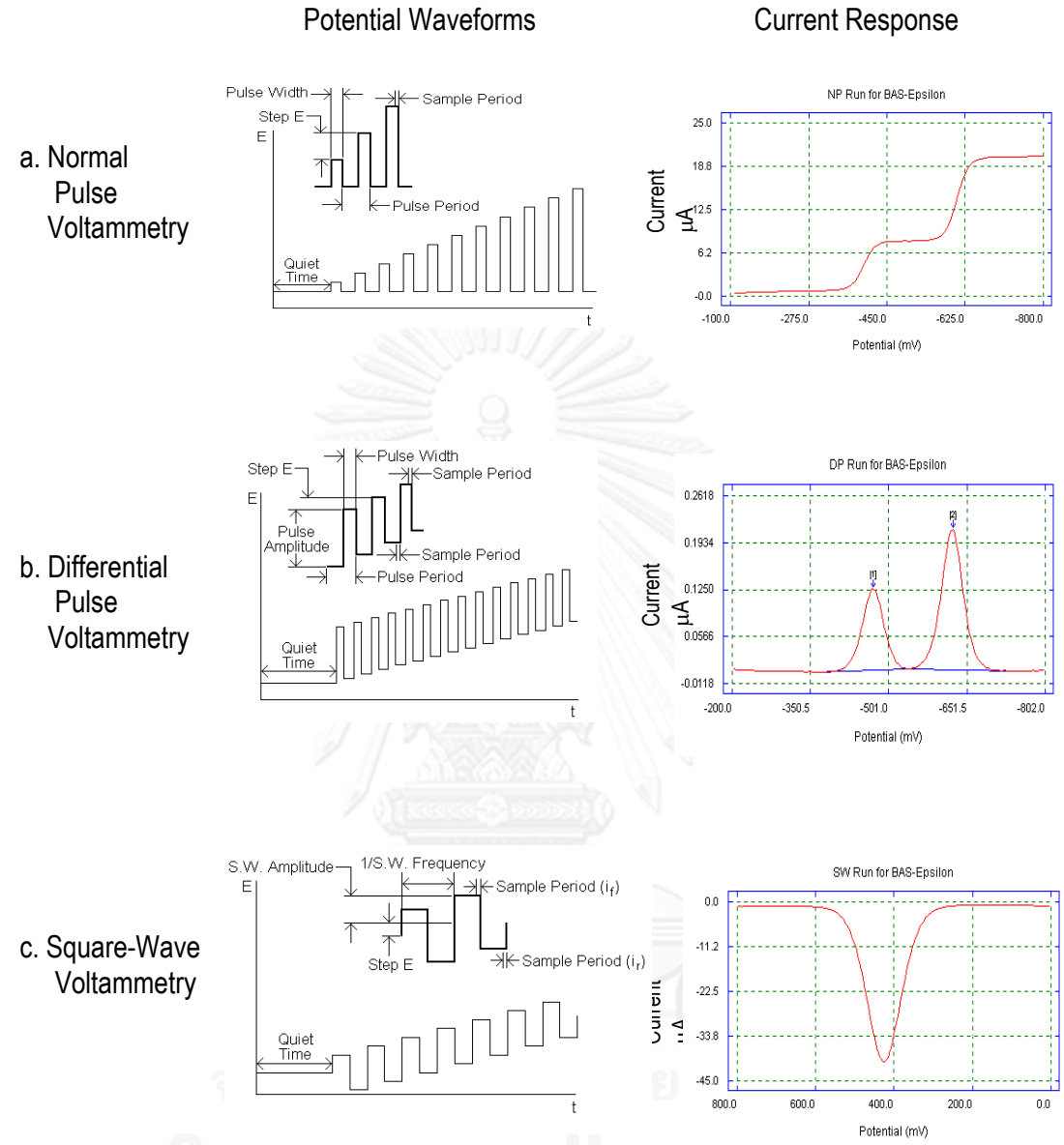


Figure 2. 6 **Potential wave forms and the respective current responses of pulse voltammetry.**

a.) Normal Pulse Voltammetry b.) Differential Pulse Voltammetry c.) Square-Wave Voltammetry

2.6.3 Screen-printed electrodes (SPEs)

Screen-printed electrode technologies have been impressively improved the advances in SPE fabrication and applications in environmental monitoring. SPE in particular can combine ease of use and apply electrochemical techniques for environmental analysis outside the laboratory. As the mass production makes SPEs with consistent chemical performance and the moderate cost, it also allows SPEs to become disposable. It may be the most appropriate electrochemical sensors for in situ analysis because of their linear output, low power requirement, quick response. In addition, the surface of SPEs can be easily modified for multiple purposes related to a wide range of different pollutants and a variety of techniques for improvements. Many kinds of SPEs modifiers including noble metals, inorganic nanomaterials, enzymes, and DNA sequences have been developed over the past several years (Li et al., 2012).

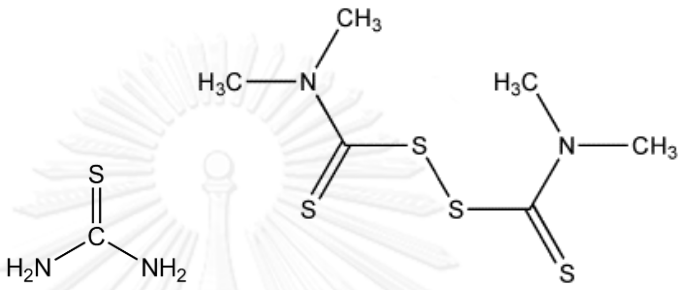
In biosensor applications, the screen-printed electrodes have advantages of miniaturization, versatility and cheapness. They particularly lends themselves to be widely used for the determination of analytes by inhibition assay and used as disposable enzyme sensor and immunosensors for biomedical, environmental and industrial analysis (Hart et al. 2004). Biosensors based on the principle of enzyme inhibition have been applied for a wide range of significant analytes such as organophosphorus pesticides, organochlorine pesticides, derivatives of insecticides, heavy metals and glycoalkaloids.

2.7 Thiol compounds and thiram

In general, thiol compounds are considered as low molecular weight, they associated enzymatic recycling systems which are responsible for the protection of cells against oxidative stress and maintenance of the optimal intracellular redox state. The major thiol compounds found in plasma are cysteine, cysteineglycine, homocysteine and glutathione. Moreover, the interactions of other thiol compounds such as thiourea, thiouracil drug or mercaptoacetic acid with peroxidase were studied in many researches in order to know the mechanism of catalysis (Aspuru and Zatón 1997, Sariri et al. 2006, Zatón and Aspuru 1995). In this study, thiourea which is commonly known as a strong inhibitor of peroxidases was used for the inhibition study.

Thiram is a dithiocarbamate fungicide that has been widely used for the protection of fruits, vegetables and crops from fungal diseases and also used as accelerator for rubber vulcanization. The Integrated Risk Information System has set up the toxicological constant of thiram at 0.005 mg/kg/day. For routine analysis, HPLC has been used for the determination of thiram in crop matrices as well as in soil and water in environmental compartments (Sharma et al. 2003). Thiram was determined based on its maximum absorption and retention time of HPLC system which was required large volume of mobile phase and time-consuming. Although its chemical structure consists of thiol groups like thiourea, an effective peroxidase inhibitor, there are no reports on the interaction between thiram and peroxidases. Therefore, this work is intended to investigate and characterize the biochemical as well as catalytic properties of peroxidase isolated from cassava leaves in comparison to HRP and also study the interaction of some thiol compounds such as thiourea and

thiram towards cassava and horseradish peroxidase activity. In order to improve the sensitivity and specificity for thiram detection, the voltammetric technique will be chosen for the investigation of peroxidase reaction in the presence of thiram. Chemical structures of thiourea and thiram are shown in **Figure 2.7**.



Thiourea Thiram

Figure 2. 7 **Chemical structures of thiourea and thiram.**

2.8 Polychlorinated biphenyls (PCBs)

Polychlorinated biphenyls (PCBs) are made up of a biphenyl nucleus with 1–10 chlorine atoms having a chemical formula of $C_{12}H_{10-n}Cl_n$. The basic formula structure of PCBs is shown in **Figure 2.8**. They are a complex mixture of multiple isomers with different degrees of chlorination yielding up to 209 products called congeners (Borja et al. 2005). PCB congeners with the same number of chlorine atoms are known as homologs, and the homologs with different chlorine positions are called isomers. For several decades PCBs were extensively used in a wide range of industrial applications such as: oil in transformers, dielectrics in capacitors, hydraulic fluids in hydraulic tools and equipment and heat exchange liquids. PCBs were also used as plasticizers surface coatings, adhesives, pesticides, carbonless copy paper, inks, dyes, and waxes (Borja et al. 2005). Although their production was

prohibited a long time ago, they still persist in the environment. One of the few available techniques to decontaminate PCB-polluted matrices consists in thermal physico-chemical methods that represent technically and financially demanding approaches. Alternatively, bioremediation has attracted greater public interest as an effective and economical strategy for the removal of pollutants. Many reports have been dealing with bacterial and fungal (Čvančarova et al. 2012) degradation of PCB and also other have been investigated for PCBs degradation by using enzymes (Cheriyan and Abraham 2010, Magee et al. 2008, Takagi et al. 2007). Peroxidases and laccase are a group of oxidoreductases catalyzing the reduction of peroxides, have been used to transform various xenobiotics, polycyclic aromatic hydrocarbons, and other pollutants found in industrial waste and contaminated water (Cheng et al. 2006).

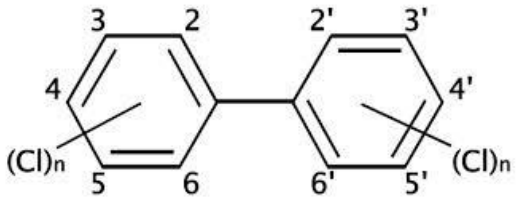


Figure 2. 8 **Chemical structures of polychlorinated biphenyls PCBs.**

In this study, cassava leaf was attempted to be used as an alternative material for exploring a novel peroxidase with specific characteristics that might be overcome some drawbacks of HRP. Therefore, characteristics of peroxidase isolated from cassava leaves of cultivar KU50 such as its catalytic activity, ability and stability in high temperature and high organic solvents content condition were studied and compared to those of horseradish peroxidase (HRP). Besides this, the potential use of

CSP for the detection of thiourea and thiram as well as its capability for catalyzing the degradation of some polychlorinated biphenyls (PCBs) were also carried out.

CHAPTER III

MATERIALS AND METHODS

3.1 Equipments

Analytical balance:-Sartorius MC 210S, Scientific Promotion Co., Ltd., Germany

-Sartorius LC 6200S, Scientific Promotion Co., Ltd., Germany

Autopipette: Pipetman, Gilson, France and Finnpipette digital multichannel pipette, Labsystem Inc., Findland

Centrifuge: Hettich zentrifugen EBA 21, Germany

Electrophoresis apparatus: Hoefer Mighty Small, Phamacia Biotech, U.S.A.

ELISA microtiter plate: Corning, U.S.A.

ELISA microtiter plate reader: Titertek multiskan plus plate reader: Labsystems Ins., Findland

Fraction collector: Redi Frac, Pharmacia LKB, Sweden

HPLC: Agilent 1200 series with UV detector, Agilent technologies, U.S.A. with ZORBAX SB-C18, Agilent Technologies and UV detector: Agilent, Agilent technologies, U.S.A.

Mass spectrometer: Flight-Mass Spectrometry (MALDI-TOF-MS).

pH meter: PHM 83 Autocal pH meter, Radiometer, Denmark

Peristaltic pump: P-1, Phamacia Biotech, Sweden

Potentiometric instrument: PalmSens + PSTrace software and assessories, Netherland

Rotary vacuum evaporator: Rotavapor-RE, Buchi, Switzerland.

Spectrophotometer: Beckman 800 UV/VIS, U.K.

Vortex mixture: Geine model K-550-GE, Sciencetific Industries, U.S.A.

3.2 Chemicals

Acetic acid, Sigma-Aldrich, U.S.A.

Acetone, J.T. Baker, U.S.A.

Acetonitrile, J.T. Baker, U.S.A.

Acrylamide, Sigma-Aldrich, U.S.A.

Ammonium sulfate, Fluka, Switzerland

Boric acid, Sigma-Aldrich, U.S.A.

Bovine serum albuminutes (BSA), Sigma-Aldrich, U.S.A.

2, 4, 4′-Chlorinated biphenyl (PCB-28), Dr. Ehrenstorfer GmbH, Germany

Concanavalin-A Sepharose 4B, Sigma-Aldrich, U.S.A.

Coomasie brilliant blue R-250, Sigma-Aldrich, U.S.A.

3, 3′-Diaminutesobenzidine (DAB), Sigma-Aldrich, U.S.A.

Dichloromethane, HPLC grade, J&T Barker, U.S.A.

Ethylacetate, J&T Barker, U.S.A.

Extrelute NT 20, Merck, Germany

Hexane, HPLC grade, J&T Barker, U.S.A.

Methanol, HPLC grade, Berdick&Jackson, Korea

α-D-Methylglucopyranoside, Sigma-Aldrich, U.S.A.

Phenol, Sigma-Aldrich, U.S.A.

Phenylmethylsulfonyl fluoride (PMSF), Sigma-Aldrich, U.S.A.

Phosphoric acid (85% wt in water), Sigma-Aldrich, U.S.A.

Polyvinyl polypyrrolidone (PVPP), Sigma-Aldrich, U.S.A.

Standard molecular weight marker protein, Sigma-Aldrich, U.S.A.

Sephacryl S-200, Phamacia, Sweden

3, 3′, 5, 5′ Tetramethylbenzidine, Sigma-Aldrich, U.S.A.

Thiourea, Sigma-Aldrich, U.S.A.

Thiram, Dr. Ehrenstorfer GmbH, Germany

Urea hydrogen peroxide (carbamide peroxide), Sigma-Aldrich, U.S.A.

Other common chemicals were obtained from Merck, Fluka or Sigma-Aldrich.

3.3 Buffers and reagents

Buffers for enzyme preparation:

-0.1 M acetate-citric acid buffer, pH6

-20 mM, 50 mM and 100 mM sodium phosphate buffer pH 6.5

-0.1 M sodium phosphate buffer pH 6.5 containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 2% w/v of polyvinyl polypyrrolidone (PVPP)

-50 mM sodium phosphate buffer pH 6.5 containing 0.2 M NaCl

Buffers for characterization and spectrophotometric method:

-0.1 M acetatate-citric acid (pH 3.0-6.0)

-0.1 M sodium phosphate (pH 5.0-8.0)

-0.1 M Tris-HCl (pH 8.0-9.0)

-0.1 M Tris-glycine (pH 8.0-10.0)

-0.1 M Britton-Robinson buffer (pH 6.0)

Buffers for electrochemical method:

-0.1 M Britton-Robinson buffer (pH 5.0-6.0)

Ultra-pure water was obtained from reverse osmosis (RO) MilII-Q system (R ≥18 MΩ) was used for HPLC analysis and sterilized reverse osmosis water was used for buffers and reagents preparation for testing PCB-28 degradation.

3.4 Enzymes

Horseradish peroxidase (II), Sigma-Aldrich, U.S.A.

Cassava peroxidase purified from cassava leaves

3.5 Preparation of peroxidase from cassava leaves

Fresh cassava leaves (KU50) 500g were washed in distilled water, chopped and homogenized in a blender with cold solution of 0.1 M sodium phosphate buffer pH 6.5 containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 2% (w/v) of polyvinyl polypyrrolidone (PVPP) with the ratio of 1:1 (w/v). The homogenate was filtered through a cloth filter and kept on ice before centrifuged at 12,000g for 30 minutes at 4°C. The supernatant was collected as crude extract and then it was purified by sequential steps of purification. The procedure for preparation and purification was performed as described in (Jongmevasna et al. 2013).

3.6 Purification of peroxidase from cassava leaves

3.6.1 Ammonium sulfate precipitation

Crude extract was precipitated by the addition of 40% ammonium sulfate in a continuous stirring condition at 4°C. The precipitation was allowed to form for 4 hours and collected by centrifugation at 12,000g for 30 minutes at 4°C. The supernatant ionic strength was raised by the addition of

ammonium sulfate concentration to 80% saturation. After 4 hours, the second precipitate was collected with the same procedure. Each precipitate was redissolved in a small volume of 50 mM sodium phosphate buffer pH 6.5 and dialyzed overnight against a large volume of the same buffer. Any precipitate formed after dialysis, it was removed by centrifugation at 12,000g for 20 minutes at 4°C.

3.6.2 DEAE-Cellulose column chromatography

The supernatant dialyzed solution was concentrated and loaded onto DEAE column (∅ 3.4 cm x height 7.7 cm) that was equilibrated with 50 mM sodium phosphate buffer pH 6.5 at least 4 times of column volume. The 2-mL fractions were collected from the column eluted with 300 mL of the same buffer at a flow rate of 60 mL/hour as unbound protein solution. The bound solution was collected after the column was eluted by the same buffer containing 0.5 M NaCl. Each fraction was tested for protein and heme content as well as peroxidase activity in order to plot the purification profile. All fractions containing peroxidase activity were pooled for further purification.

3.6.3 Concanavalin A-Sepharose 4B column Chormatography

Concanavalin A -Sepharose 4B gel (Con-A) needs a prewash step with 4 column volume of distilled water before use in order to remove ethanol used for gel preservation. After the gel was settled, it was suspended in 2 column volume of 50 mM sodium phosphate buffer pH 6.5 and packed into plastic column (1.5 x 8.0 cm) using peristaltic pump at a flow rate of 15 mL/hour. The

packed column was pre-equilibrated by passing 10 column volume of 50 mM sodium phosphate buffer, pH 6.5 containing 0.2 M NaCl at a flow rate of 15 mL/hour.

After the unbound DEAE portion was dialyzed in a large volume of 50 mM sodium phosphate buffer, pH 6.5 containing 0.2 M NaCl overnight at 4 $^{\circ}$C, it was centrifuged at 12,000g for 20 minutes at 4 $^{\circ}$C. The supernatant was concentrated to 3 mL before loading onto the column. The Con-A unbound protein was collected in 2 mL fractions eluted by the addition of 200 mL of 50 mM sodium phosphate buffer, pH 6.5 containing 0.2 M NaCl at a flow rate of 15 mL/hour. After that, the column was washed by the addition of 0.5 M α-D-methyl glucopyranoside over 10 column volume for Con-A bound protein collection. Each fraction was tested for protein, heme content and peroxidase activity in order to plot the purification profile.

3.7 Protein content determination

Protein concentration of extract and purified portion was determined by the method of Bradford (Bradford 1976) with bovine serum albumin used as a standard. Sample, 100 μl, was mixed with Coomassie blue reagent and left for 10 minutes before the absorbance at 595 nm was read.

3.8 Peroxidase activity assay

Activity of peroxidase was determined by using spectrophotometric method based on (Bos et al. 1981) and (Jongmevasna et al. 2013). Each peroxidase in storage buffer; 50 mM sodium phosphate buffer, pH 6.0 containing 0.2 M NaCl was diluted

with 0.1 M phosphate buffer, pH 6.0 to prepare 1.5 units/mL working solution and it was used in all experiments. The substrate solution was prepared as following procedure; 1 mg TMB was firstly dissolved in 200 μL of dimethylsulfoxide (DMSO), then it was gently diluted in 0.1 M acetate-citric acid, pH 6.0. After 10 minutes, 1 mg UHP in 500 μL of 0.1 M acetate-citric acid, pH 6.0 was added and the final volume of 10 mL was adjusted. The reaction solution was consisted of enzyme solution, 10 μL, pre-incubated with 100 μl of 0.1 M acetate-citric acid buffer, pH 6.0 in microtiter well at 25°C for 3 minutes. The reaction was then initiated by the addition of 200 μL substrate solution. After 3 minutes of incubation, it was stopped by the addition of 40 μL of 2 M sulfuric acid. The absorbance at 450 nm was then measured by Titertek Multiscan plus plate reader (Labsystems Ins., Finland). One unit of enzyme is the amount of enzyme, which oxidizes 1 μmole of TMB per minute. The molar extinction coefficient was 67,300 $M^{-1}cm^{-1}$ (Misono et al. 1997).

3.9 Characterization of cassava peroxidase

3.9.1 Absorption spectrum

The Con-A bound portion with peroxidase activity referred to as cassava peroxidase (CSP) was concentrated in 50 mM phosphate buffer containing 0.2 M NaCl, pH 6.0 and its absorption spectrum in the range of 250-800 nm was investigated by using a single-beam UV/VIS spectrophotometer. Moreover, the absorption spectra of HRP and SBP were also carried out and compared to that of cassava peroxidase under the same condition.

3.9.2 Molecular weight

The molecular weight of Con-A bound protein was estimated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a minutesi Protean III Electrophoresis Cell (Bio-Rad, Laboratories, Hercules, MA, U.S.A.), with 12% resolving gel and 3% stacking gel using buffer system according to method reported by LaemmLi (LaemmLi 1970). The molecular weight markers used were β-galactosidase (116 kDa), bovine serum albuminutes (66.2 kDa), ovalbuminutese (45 kDa), lactate dehydrogenase (35 kDa), β-lactoglobulin (18.4 kDa) and lysozyme (14.4 kDa). Both markers and enzyme were mixed with 5x sample buffer (0.3 mM Tris-HCl, 50% glycerol, 20% SDS, 5% 2-mercaptoethanol and 0.05% bromophenol blue) and boiled for 5 minutes. After electrophoresis, proteins were stained with Coomasie brilliant blue. Moreover, its molecular weight was also determined by Matrix Assisted Laser Desorption Ionization-Time of Flight-Mass Spectrometry (MALDI-TOF-MS). One microliter of sample was mixed with 10 μL of saturated sinapinic acid in 0.1% TFA: ACN (70:30). Then 1 μL of the mixture was spotted on a stainless steel target and allowed to dry at room temperature. The dried mixture was measured on an AutoflexTOF/TOF (Bruker Daltonik, Bremem, Germany). The instrument was equipped with a N_2-laser and all measurements were done in linear mode.

3.9.3 Iso-Electric Focusing Point (IEF)

The p*I* values of purified CSP and HRP were examined using a Bio-Rad mini gel IEF. The method and applied potential condition based on the

instruction manual of mini IEF cell model 111 was carried out (100 V for 20 minutes, 200 V for 20 minutes and 450 V for 90 minutes). After electrophoresis, the standard p*I* lane was cut and immersed in the fixative solution for 30 minutes prior to protein staining. For activity staining, the peroxidase sample lane was cut and stained with the solution containing 0.1 mg/mL of DAB and 0.1 mg/mL of UHP as substrates in acetate-citric acid buffer, pH 6.0 (Fernandes et al. 2006) and isoelectric points were determined by comparing with IEF markers (IEF mix 3.6–9.3, Sigma, St. Louis, USA).

3.9.4 Effect of pH on peroxidase activity

The optimal pH of CSP for the catalysis of substrates containing UHP and TMB in the pH range of 3-10 was investigated. The assay was carried out at 25°C and the buffers used were 0.1 M acetatate-citric acid (pH 3.0-6.0), 0.1 M phosphate (5.0-8.0), Tris-HCl (8.0-9.0) and Tris-glycine (8.0-10.0). The enzyme activity was assayed as described in 3.8. Moreover, HRP were also performed in the same condition in order to compare with that of CSP.

3.9.5 Effect of temperature on peroxidase activity

The activity of the purified CSP and HRP for the catalysis of substrates containing UHP and TMB at different temperatures; 5°C, 25°C, 35°C, 55°C and 70°C were investigated. Moreover, three buffer systems; 0.1 M acetate-citric acid (pH 6.0), 0.1 M Britton-Robinson buffer (pH 6.0) and 0.1 M phosphate (pH 6.0) were also used to investigate their effect on peroxidase activities.

In order to study the temperature working range of peroxidases, the

reaction solution containing either CPS or HRP was incubated in chosen buffer system at various temperature ranging from 5 to 70°C. at The residual activity was measured at pre designated temperature and buffer systems under standard condition as described in 3.8. To investigate heat inactivation of the enzymes, both peroxidases were incubated in chosen buffer system and incubated at temperatures ranging from 5 to 70°C for 0-6 hours. Samples were withdrawn periodically and the residual enzyme activity was measured at 25°C under standard condition.

3.9.6 Effect of organic solvents on peroxidase activity

Peroxidase activities in solvent-aqueous mixed solution containing acetonitrile or methanol in the range of 5-60% (v/v) were carried out. Peroxidases were incubated in 0.1 M phosphate buffer (pH 6.0) containing substrate solution and various concentrations of solvents for 3 minutes at 25°C and their activities were tested as described in 3.8. The relative activities of CSP and HRP were compared to their activities in the reaction solution without any solvents. Working ranges of both peroxidases in the presence of acetonitrile or methanol in the phosphate buffer systems were then determined. In addition, stabilities of both peroxidases in reaction buffers containing various concentrations of both solvents during 12 hours were also observed by taking aliquots for activity testing as shown in 3.8.

3.10 Kinetic parameters study of peroxidases

3.10.1 Kinetic parameters of peroxidase activity

Peroxidases were kept in storage buffer; 50 mM sodium phosphate buffer, pH 6.0 containing 0.2 M NaCl and it was diluted with 0.1 M phosphate buffer, pH 6.0 to prepare 1.5 units/mL working solution for peroxidase assay. The catalytic activity of CSP 0.1 M acetate-citric acid buffer, pH 6.0 at 25°C was studied and compared to that of HRP. Kinetic parameters such as Michaelis-Menten constant (K_M) and maximum velocity (V_{max}) were determined by Lineweaver-Burk and secondary plots. The K_M for UHP was determined by using various concentration of UHP in the range of 28-228 μM under 4 fixed concentrations of TMB in the range of 32-260 μM in the reaction. CSP activity in reaction buffer were carried out and compared to those of HRP under the same condition. Two microliters of 1.5 units/mL peroxidase solution in 0.1 M phosphate buffer, pH 6.0 was added into microtiter well and followed by the addition of 100 μl of 0.1 M acetate-citric acid buffer, pH 6.0 and 100 μl of TMB solution. The reaction was initiated by the addition of 50 μL UHP solution. The change in absorption at 650 nm (ΔA/minutes) due to the oxidation of TMB was recorded during 3 minutes with 30 seconds interval using the microtiter plate reader. The initial rate was measured and calculated using the molar absorption coefficient of its product, 39,000 $M^{-1}cm^{-1}$ (Misono et al. 1997). One unit of enzyme is the amount of enzyme, which oxidizes 1 μmole of TMB per minute.

3.10.2 Kinetic parameters of peroxidase activity in the presence of thiourea

The concentration of UHP and TMB were kept constant at their K_M and the inhibitor concentrations were varied from 1 to 500 μM. Thiourea were prepared in methanol as stock solution and diluted to have certain concentration with 0.1 M acetate-citric acid buffer, pH 6 containing 10% of methanol. The assay was carried out as the determination of kinetic parameters except that 100 μL of inhibitor solution was used instead of 100 μL of 0.1 M acetate-citric acid buffer. The decrease in activity (%) was then plotted against inhibitor concentration in μM. Their dissociation constants (K_i) and inhibition mechanisms were graphically determined by secondary plot of ${}^{app}K_M$.

3.11 Spectrophotometric method for peroxidase inhibition study

In the preliminary study, the colorimetric or spectrophotometric method for the determination of thiourea, a strong inhibitor of peroxidase, was developed. Peroxidase activities of CSP and HRP were assessed by using UHP and TMB as peroxidase substrates. The inhibitory effect of thiourea and its possible detection rage for thiourea determination based on peroxidase inhibition assay were performed as follow.

3.11.1 Inhibitory effect of thiourea on peroxidases activity

In order to study the effect of thiourea on CSP activities, the concentration of UHP and TMB were kept constant at its K_M. TMB solution, 1

mg TMB was firstly dissolved in 200 μL of dimethylsulfoxide (DMSO), then it was gently diluted in 0.1 M acetate-citric acid, pH 6.0 and the final volume of 15 mL was adjusted. For UHP solution, 1 mg of UHP was dissolved in 20 mL of 0.1 M acetate-citric acid, pH 6.0. The stock solution of 10 mM thiourea was prepared in methanol and diluted to a certain concentration in 0.1 M acetate-citric acid buffer, pH 6.0 containing 5% of methanol as a working solution. Thiourea concentrations were varied from 1 to 500 μM. The assay was carried out as the determination of kinetic parameters as described in 3.10 except that the 100 μL of thiourea working solution was used instead of 100 μL of 0.1 M acetate-citric acid buffer.

3.11.2 Dose response curve of thiourea on peroxidases activity

The half-maximal inhibitory concentration (IC_{50}) values were also determined by dose response curve (Copeland 2000). The decrease in activity (%) was calculated and then graphically plotted against thiourea concentration in μM. The relative activity (% RA) was calculated as the equation below.

$$\% \text{ RA} = \frac{\text{Optical density of solution in the presence of thiourea}}{\text{Optical density of solution in the absence of thiourea}} \times 100$$

3.12 Electrochemical method for peroxidase inhibition study

The electrochemical method for the assessment of peroxidase activity of CSP and HRP and their inhibition in the presence of thiourea and thiram was developed. The electrochemical measurement was performed with a disposable screen-printed

electrode consisting of a 2 x 3 mm^2 carbon working electrode and an Ag/AgCl reference electrode. Peroxidase activities were examined by using UHP and 3, 3′diaminutesobenzidine (DAB) as peroxidase substrates. Voltammetric measurement based on the redox reaction of DAB was modified from (Zhang et al. 2008).

3.12.1 Fabrication of screen-printed electrodes (SPEs)

Electrodes were screen-printed onto a polyvinylchloride (PVC) substrate. Screen-printing was performed semi automatically by the use of DEK 248 CERD and polyester screen mesh screens. Initially, a carbon conductive layer (screen mesh size 90Tand MCK carbon ink) was applied with curing temperature of 55°C for 2 hours, followed by a silver/silver chloride ink (screen mesh size 90T and silver/silver chloride ink) layer with curing at temperature of 55°C for 2 hours. Finally, a non-conducting insulation tap was covered over non-working surface and the working area of an electrode was defined as 2x3 mm^2. The electrode preparation was performed at sensor laboratory, pilot plant development and training institute, King Mongkut's University Technology, Thonburi.

3.12.2 Electrocmemical measurement for peroxidase activity

Reaction solutions containing peroxidase and its substrate in the presence and absence of thiourea and thiram in 0.04 M Britton-Robinson buffer, pH 6 were performed in a well of 96 microtiter plate. Electrochemical

measurements in both cyclic voltammetry and square wave voltammetry were carried out by using SPEs and PalmSens potentiometric instrument. The

3.12.2.1 Cyclic voltammetry (CV)

CV was used for investigation of the electrochemical characteristic of DAB at SPE surface. It was performed by starting an initial potential of 0.0 V and a switching potential in the range of -0.6 to +0.8 V with a scan rate of 20 m Vs^{-1} using a PalmSens potentiostate with the PalmSens PC software operating system.

3.12.2.2 Square-wave voltammetry (SWV)

SWV was used for the determination of oxidized DAB concentration corresponding to peroxidase activity. In the preliminary study, HRP was used for the condition optimization. Initially, the relationship between DAB concentration and the current response of DAB product catalyzed by peroxidase was investigated. The stock solution of 5,000 μM DAB was prepared by dissolving 1 mg DAB in 920 μL of RO water and the two-fold serial dilutions were performed to get the concentration of 2,500, 1250, 625 and 312 μM. UHP stock solution, 4 mg of UHP was dissolved in 4.2 mL of RO water for a concentration of 10,000 μM and it was diluted to 500 μM as a concentration of working solution. Peroxidase was kept in storage buffer; 50 mM sodium phosphate buffer, pH 6.0 containing 0.2 M NaCl and it was diluted to 0.3 units/mL in 0.1 M phosphate buffer, pH 6.0 as working solution for peroxidase assay. Ten microliters of peroxidase working solution

was added into microtiter well and followed by the addition of 100 μL of 0.04 M Britton-Robinson buffer, pH 6 containing 5% methanol and 50 μL of DAB solution. It was thoroughly shaken by hand and incubated for 3 minutes, and then the reaction was initiated by the addition of 50 μL UHP solution. After incubated at 25°C for 3 minutes, the enzyme reaction was stopped and mixed by the addition of 40 μL, 0.2 M sulfuric acid.

In order to achieve the accuracy of SWV measurement by the used of disposable SPEs, the repeatability of SPEs should be examined before using. Five electrodes were selected by random sampling and they were used for the determination of oxidized DAB concentration corresponding to peroxidase activity. Five reactions and SWV measurements with duplicate reading were performed by using fixed concentrations of 125 μM DAB and 100 μM UHP in the reaction as the procedure in 3.12.2.2. Moreover, before the electrochemical measurement was done, the SPE was rinsed with RO water and allowed to dry at room temperature. SWV current response of DAB product was measured by setting the potential from 1.0 to -0.8 V with a step potential of 10 mV, pulse 75 mV and frequency 50 Hz at the scan rate of 50 mVs^{-1}.

3.12.3 Detection of thiol compounds based on inhibition assay by using electrochemical measurement

The effect of thiourea and thiram on peroxidase activity was tested by peroxidase inhibition assay. The stock solution of thiourea and thiram was prepared in methanol at a concentration of 10 mM and diluted to a certain concentration in 0.04 M Britton-Robinson buffer, pH 6 containing 5% methanol

as working solutions. The concentrations were prepared in range of 1 - 100 μM. The peroxidase inhibition assay was performed as described in 3.12.2 except that 100 μL of thiourea or thiram working solution was used instead of 100 μL of 0.04 M Britton-Robinson buffer. The change of current responses in the presence and absence of target compounds were measured and calculated for the percentage (%) of relative activity. They were graphically plotted as a function of thiourea and thiram concentrations. The percentage of relative activity (% RA) was calculated as shown in 3.11.1. The detection range of thiourea or thiram could be observed from the inhibition curve.

3.13 Degradation of thiram based on peroxide reaction

3.13.1 Reaction solutions for thiram degradation

Thiram stock solution was prepared in methanol at the concentration of 10 mM and diluted to 100 μM in 0.04 M Britton-Robinson buffer, pH 6 containing 20% methanol as working solutions. DAB solution (6,000 μM in sterilized RO water) was diluted to 2,400 μM and UHP solution (12,000 μM in sterilized RO water) was diluted to 2,400 μM for peroxidase assay. The first experiment, the thiram reaction solution was incubated with UHP at various temperatures of 25°C, 35°C and 55°C in order to investigate the degradation rate of thiram within 90 minutes. The reaction solution was consisted of 1,000 μL of thiram in 0.04 M Britton-Robinson buffer, pH 6 containing 20% methanol and 250 μL of UHP. The final volumn was adjusted to 1,550 μL by the addition of BR buffer, pH 6 containing 20% methanol. The second experiment,

the effect of peroxide and peroxidase on thiram degradation was studied. Therefore, the reaction solution was consisted of 50 μL of 0.5 units/mL of CSP, 1,000 μL of thiram in 0.04 M Britton-Robinson buffer, pH 6 containing 20% methanol and 250 μL of DAB and UHP. The reaction final volume was kept at 1,550 μL and it was incubated at 25°C for 24 hours. In addition, the reactions in the absence of peroxidase and/or UHP as control solutions were also performed and incubated at 25°C for 24 hours.

3.13.2 HPLC determination of thiram

Each reaction solution was subsequently taken for the determination of residual thiram in the solution using HPLC. Aliquots of reaction solutions were taken into a vial and 20 μL was injected to HPLC with dual wavelength UV detector; 275 nm and 290 nm. The analytical column used was Zorbax SB-C18 15 X 44.5 mm with column temperature of 30°C. The mobile phase was an acetonitrile-water gradient started at the ratio of 30:70 and run for 1 minute, and then the ratio of 90:10 was further run for 5-10 minutes with a flow rate of 1.0 mL/minutes. HPLC condition was modified from (Ekroth et al. 1998).

3.14 Degradation of PCB-28 based on peroxidation catalyzed by peroxidase

3.14.1 Reaction solutions for PCB-28 degradation

In order to investigate the potential of CSP and HRP on the degradation of PCB-28, two experiments of peroxide reactions catalyzed by either CSP or HRP in the presence and absence of phenol were carried out. PCB-28 stock solution at the concentration of 110 μM (28 μg/mL) was prepared in

acetonitrile. UHP as peroxidase substrate solution at a concentration of 7.5 mM was prepared in sterilized RO water. In the first experiment, the reaction solution was composed of 50 μL of 15 units/mL peroxidase (CSP or HRP), 1,200 μL of 0.1 M phosphate buffer, pH 6.0 containing 5% acetonitrile, 30 μL of PCB-28 and 50 μL UHP. The reaction final volume was adjusted to 1,500 μL by the addition of sterilized distilled water. In the second experiment, phenol at a concentration of 1.0 mM (150 μL) was added and the final volume of 1,500 μL was adjusted with sterilized distilled water. In addition, the reactions in the absence of peroxidase and/or PCB-28 were also performed as control experiments. All reactions were incubated at 25°C for 24 hours.

3.14.2 HPLC determination of PCB-28

The residual concentration in each reaction solution was subsequently taken and monitored by HPLC for 0-24 hours. Aliquots of reaction solutions containing PCB-28 were taken into a vial and 60 μL were injected into a reverse phase column (ZORBAX SB-C18, 15X44.5 mm), heated at 30°C, with a flow rate of 1 mL/min for 15 minutes. PCB-28 was analyzed at 240 and 250 nm under isocratic conditions with 60% acetonitrile in water as mobile phase and standard calibration curve in the range of 0.14-1.68 μg/mL were prepared.

CHAPTER IV
RESULTS

In this study, the characteristic of a novel peroxidase isolated from cassava leaves were investigated. Its advantages which were overcome the limitations of horseradish peroxidase (HRP) and its potential use for the detection of thiourea as a representative of thiol compounds based on peroxidase inhibition were studied. For inhibition assay, the applicable uses of both spectrophotometric and electrochemical techniques for the measurement of peroxidase activity in the presence of thiourea or thiram were carried out. In addition, the capability of cassava peroxidase (CSP) as biocatalyst for PCB degradation was examined and compared to that of HRP. These findings from all experiments were described in this chapter.

4.1 Preparation and purification of cassava peroxidase

4.1.1 Crude enzyme preparation

According to the extraction procedures described in section 3.5, crude enzyme containing 170 mg protein was extracted from 500 g of fresh cassava leaves. The specific activity of the extract determined by the use of urea hydrogen peroxide (UHP) and 3, 3′, 5, 5′-tetramethylbenzidine (TMB) as substrates was 550 units/mg of protein. Results of each purification steps were summarized in **Table 4.1**. (See calibration curve for protein determination by Bradford method in Appendix C)

4.1.2 Ammonium sulfate precipitation

Crude extract was further purified by ammonium sulfate precipitation as mentioned in section 3.6.1. After the fraction 40-80% ammonium sulfate saturation was redissolved and dialyzed in 20 mM phosphate buffer pH 6.5, it was found that the fraction contained 70% yield of crude enzyme. In this step, the enzyme was purified 1.2 fold with the specific activity of 690 units/mg protein.

4.1.3 DEAE-Cellulose column chromatography

The enzyme solution obtained from 4.1.2 was loaded onto DEAE-cellulose column chromatography as the procedure in 3.6.2. The chromatographic profile in **Figure 4.1** showed that most of proteins did not bind to the DEAE-cellulose column. They were rapidly eluted from DEAE column within 20 fractions as unbound portion with a high specific activity of 1,200 units/mg protein. In this step, the unbound DEAE was purified 2.2 with the recovery yield of 60% compared with crude enzyme.

4.1.4 Concanavalin A column chromatography

The unbound DEAE portion obtained from above was concentrated and then loaded onto Concanavalin A-Sepharose 4B by using 50 mM sodium phosphate buffer pH 6.5 containing 0.2 M NaCl as running buffer. In this step, the unbound DEAE portion could be separated into bound and unbound protein portions with peroxidase activity as shown in **Figure 4.2**. The bound proteins of Concanavalin A showed high peroxidase activity of 8,200 units/mg

protein and 15 fold of purification. The characteristics and catalytic properties of Concanavalin A bound portion was considered as cassava peroxidase (CSP) and used for further studies.

Table 4. 1 Partial purification of peroxidase from cassava leaves.

Sample	Total activity (10^3 x Units[a])	Total proteins (mg)	Specific activity (Unit[a]/mg)	Yield (%)	Purification fold
Crude	94	170	550	100	1.0
40-80% $(NH_4)_2SO_4$	66	96	690	70	1.2
Unbound-DEAE	58	57	1,000	60	2.2
Bound-Concanavalin A	3.3	0.40	8,200	4	15

[a] One unit of enzyme activity was defined as the amount of enzyme, which oxidizes 1 μmole of TMB per minute at room temperature

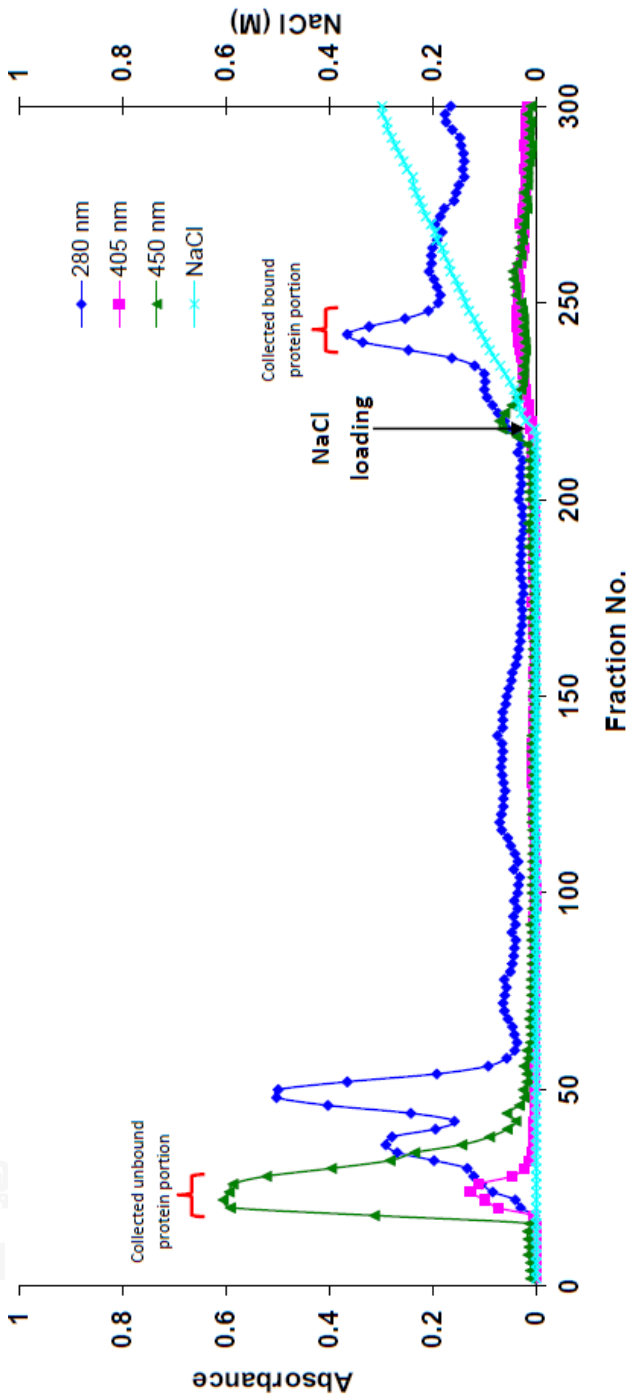


Figure 4. 1 **Chromatographic profile of 40-80% saturated ammonium sulfate fractions loaded onto DEAE-cellulose column**

(∅ 3.4 cm x height 7.7 cm). The unbound protein solution was eluted with 50Mm sodium phosphate buffer, pH 6.5 and the bound protein was eluted after the addition of the phosphate buffer, pH 6.5 containing 0.5 M NaCl.

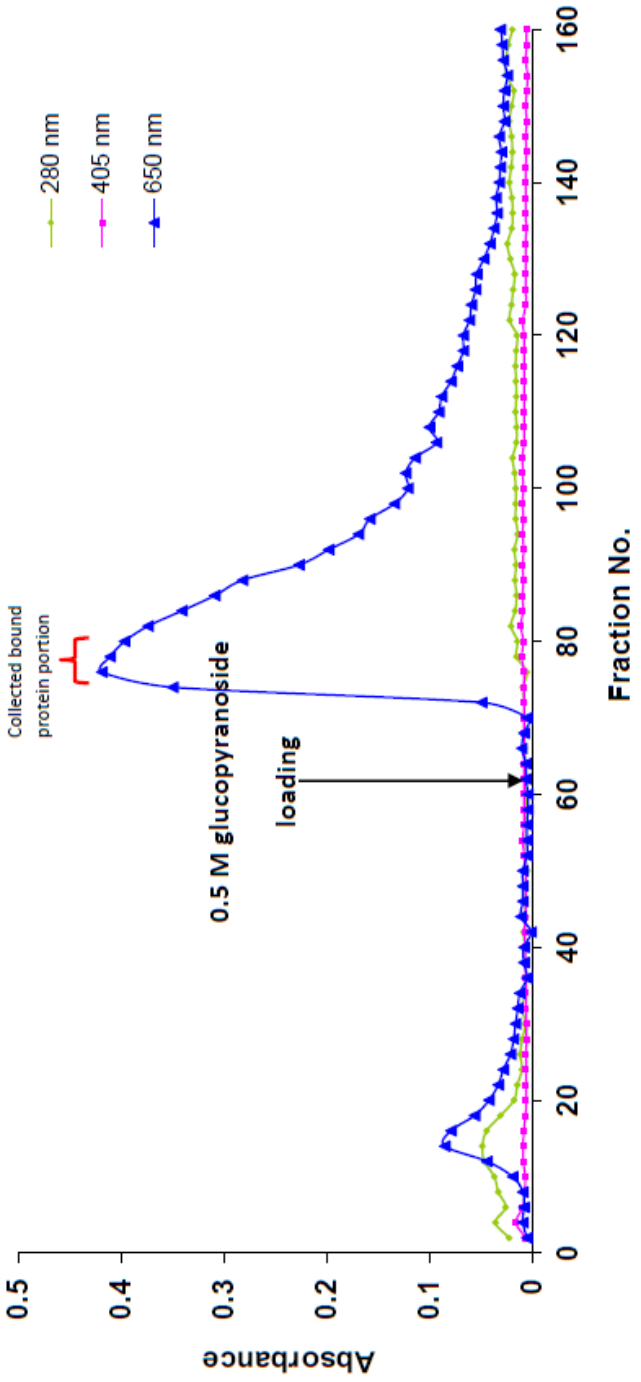


Figure 4. 2 **Chromatographic profile of unbound DEAE-cellulose fractions loaded onto Concanavalin-A column**

(∅ 1.5 cm x height 8.0 cm). The unbound protein solution was eluted with 50Mm sodium phosphate buffer saline, pH 6.5 containing 0.2 M NaCl and the bound protein was eluted after the addition of the phosphate buffer saline, pH 6.5 containing 0.5 M α-D-methyl glucopyranoside.

4.2 Characterization of cassava peroxidase

The biochemical properties including absorption spectra, the molecular weight and the catalytic activity of cassava peroxidase (CSP) were determined and compared to those of horseradish peroxidase (HRP) in order to investigate some unique characteristics of prepared cassava peroxidase. All characteristics were described and summarized in **Table 4.2.**

4.2.1 The Absorption spectrum of cassava peroxidase

The absorption spectrum of native cassava peroxidase was examined in the range of 200 – 800 nm and compared to that of the native HRP as shown in **Figure 4.3.** It was found that the absorption spectrum of cassava peroxidase was similar to HRP, but CSP showed the different Soret maximum at 400 nm whereas HRP showed at 403 nm, respectively.

4.2.2 The molecular weight of cassava peroxidase

The purified CSP showed two protein bands on SDS-PAGE by Coomassie brilliant blue staining (**Figure 4.4**.) and the estimated molecular weight of them were found to be 41 and 45 kDa which were corresponded to the MALDI-TOF-MS results as shown in **Figure 4.5.** The total ion chromatogram of CSP showed 2 forms of molecular masses of 38 kDa and 44 kDa. The MW of purified CSP obtained was closely similar to those of HRP (43 kDa).

4.2.3 Iso-electric focusing point of cassava peroxidase

The p*I* value of purified CSP was examined by polyacrylamide gel isoelectrofocusing (IEF) as described in section 3.9.3. The activity staining pattern of the IEF gel in Figure 4.6 showed that 4 isoforms of cassava peroxidase were observed with different p*I* values. The major isoform of CSP was determined at p*I* = 6.4 and three minor isoenzymes were observed at p*I* value of 6.0, 6.8 and 7.2 HRP was found to have more than 5 isoforms with various p*I* in the range of 5.2-8.2. (See calibration curve for p*I* determination in Appendix D).

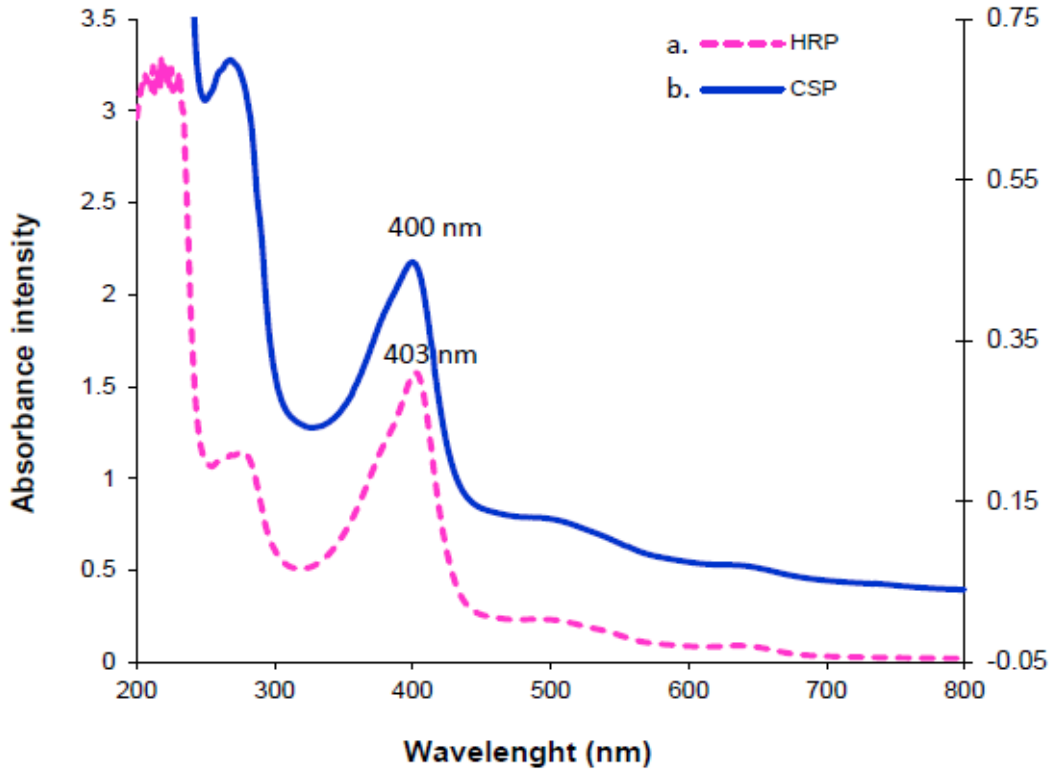


Figure 4. 3 Absorption spectra of plant peroxidases in 20 mM phosphate buffer. pH 6.0.

a) Cassava peroxidase (CSP) and b) Horseradisd peroxidase (HRP).

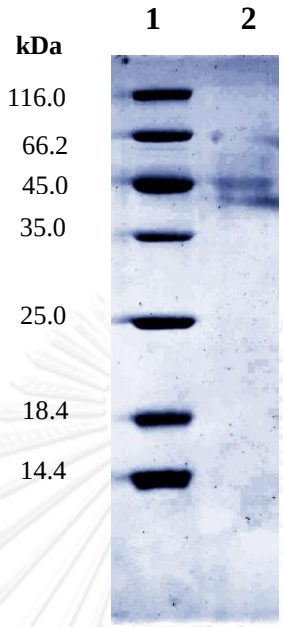


Figure 4. 4 SDS-PAGE of purified cassava peroxidase (CSP) from cassava leaves. Samples were run on 12% acrylamine gel and stained with Coomassie brilliant blue. Lane1) Mass molecular mrkers and lane 2) Purified CSP

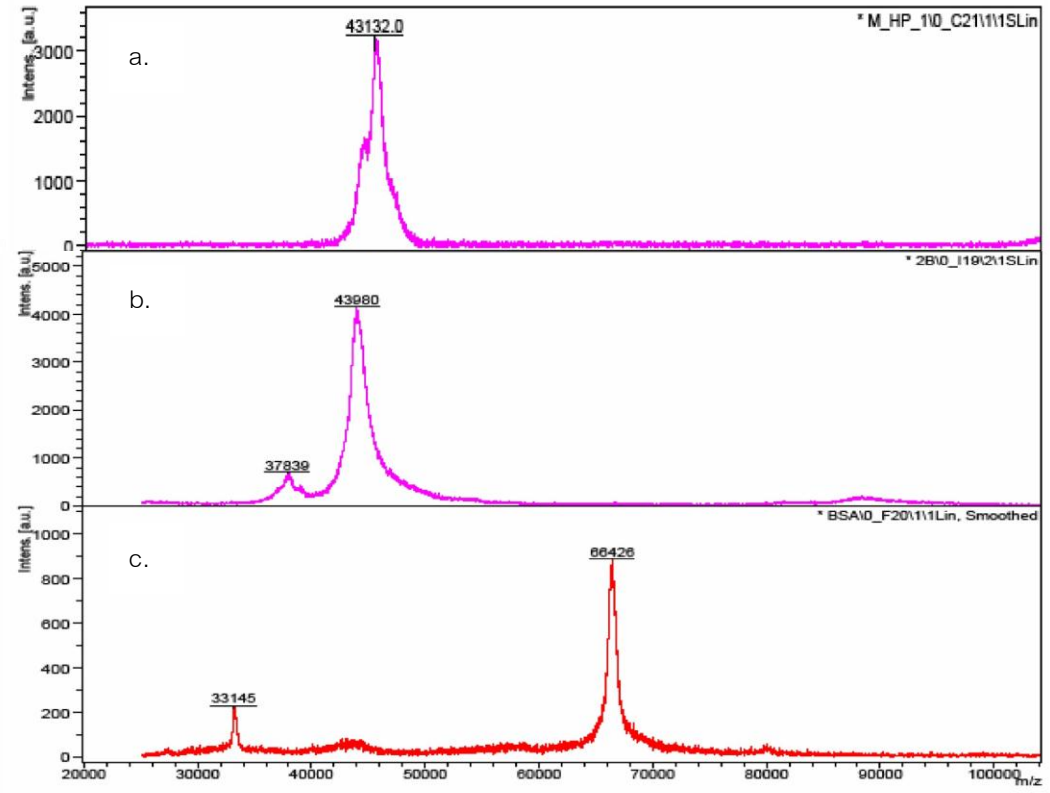


Figure 4. 5 Molecular weight of peroxidases by LC-MALDI-TOF.

Experiment: The dried sample mixture is measured on an AutoflexTOF/TOF (Bruker Daltonik, Bremem, Germany). The instrument is equipped with a N2-laser, and measurement is done in linear mode. a.) Horseradish peroxidase (43 kDa), b.) Cassava peroxidase (38 kDa and 44 kDa) and c.) Bovine serum albumin (66 kDa).

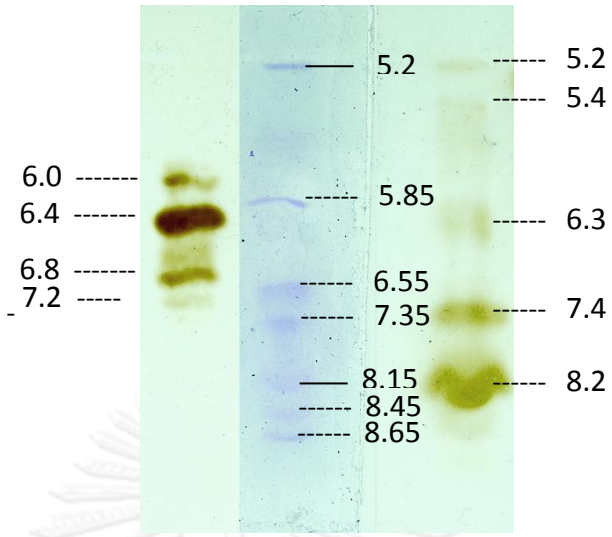


Figure 4. 6 Isoelectric focusing gel with ampholyte solution (pH 3.0-10.0) and peroxidases.

The p*I* values of cassava peroxidase (CSP2) compared with standard p*I* markers: β- lactoglobulin (5.2), Bovine carbomic anhydrase (5.85), Myogloblin-base (7.35), Lentil lectin-acidic (8.15), Lenil lectin-middle (8.45) and Lenil lectin-basic (8.65).

4.2.4 Effect of pH on cassava peroxidase activity

The peroxidase activity of purified enzyme obtained from bound Concanavalin-A portion in the buffer with various pH from 3 to 10 was investigated. CSP showed its optimal pH for the catalysis of UHP and TMB oxidation at pH 6 and it was able to work with the activity over 80% in the range of pH 5-8. On the contrary, HRP could catalyze both substrates in a range of pH 3-9 which was a wider range than that of CSP. The optimal pH profiles of peroxidases are shown in **Figure 4.7**.

4.2.5 Effect of temperature on peroxidase activity

4.2.5.1 Optimal temperature of peroxidase

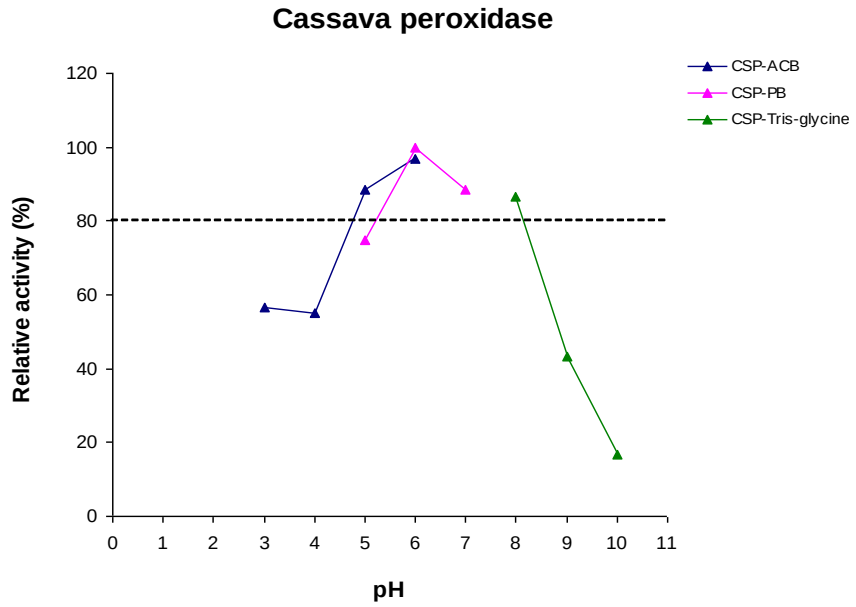
CSP showed its optimal temperature for the catalysis of UHP and TMB oxidation in the range of 5-70°C. It is remarkably able to work in a wide range of temperature and still retained its activity more than 75% when it was incubated in 0.1 M acetate-citric and 0.1 M Britton-Robinson buffer. For HRP, the optimal temperature and its relative activity were found to be in the range of 25-60°C and 75-100%, respectively. The optimal temperature profiles of peroxidases are shown in **Figure 4.8**.

4.2.5.2 Thermal stability of peroxidases

For the thermal stability test, it was found that CSP was obviously more stable than HRP when it was incubated in 0.1 M phosphate buffer. After 2 hours of incubation at 5-70°C, only CSP in phosphate buffer still retained more than 75% of its initial activity. In phosphate buffer, HRP

relative activity seemed to be more stable at 55°C than that in other buffers. However, activities of both peroxidases in 0.1 M acetate-citric acid and 0.1 M Britton- Robinson buffers were dramatically decreased when the temperature was raised higher than 40°C and incubated for 4 hours. Nevertheless, HRP could not retain as high activity as that of CSP when the temperature was raised higher than 55°C and its activity was found to be almost disappeared after incubated in 70°C for 2 hours as shown in **Figure 4.9**.

a.



b.

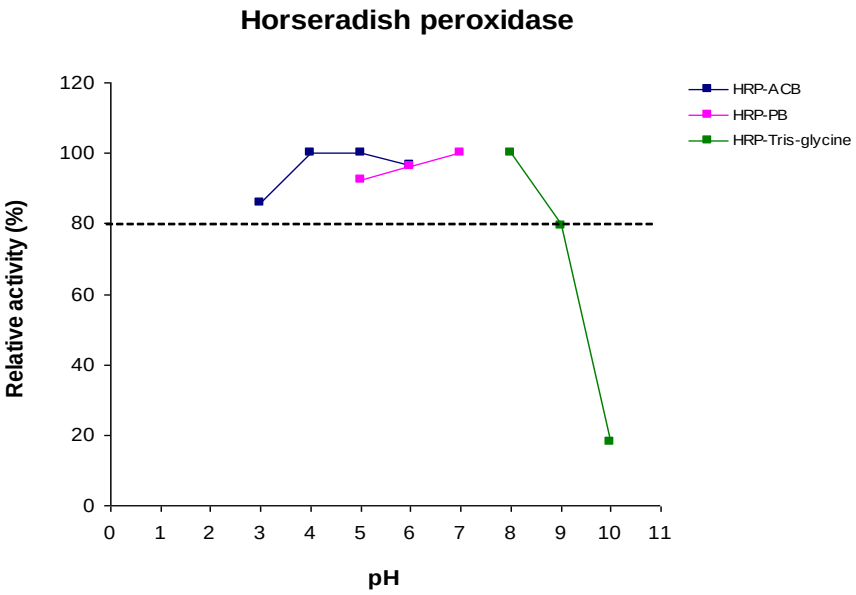
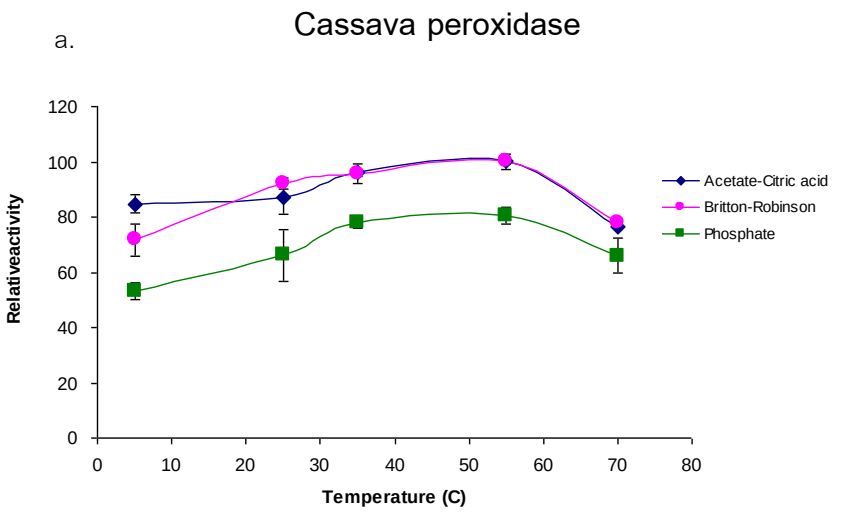


Figure 4. 7 The optimal pH profile of peroxidase activity.

a.) Cassava peroxidase (CSP) b.) Horseradish peroxidase (HRP).

a.



b.

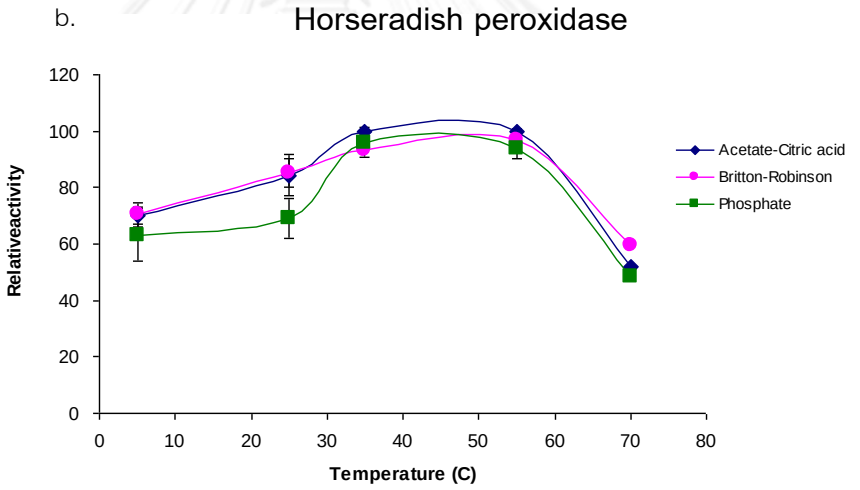


Figure 4. 8 The optimal temperature profile of peroxidase activity.

a.) Cassava peroxidase (CSP) b.) Horseradish peroxidase (HRP)

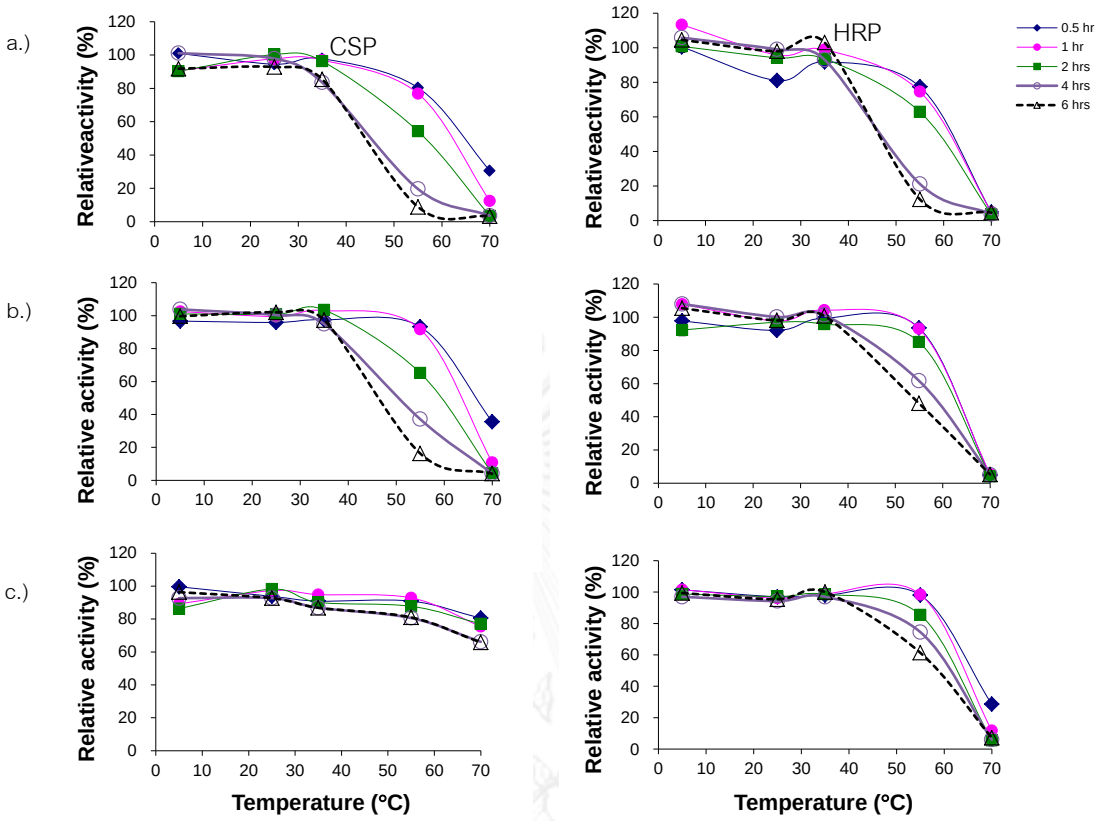


Figure 4. 9 Thermal stability of CSP and HRP for 0-6 hours in various types of buffer, pH 6.0

a.) 0.1 M acetate-citric, b.) 0.1 M Britton-Robinson and c.) 0.1 M phosphate buffer (Peroxidase activity was determined in triplicate.).

Table 4. 2 Characteristics of cassava peroxidase (CSP) versus horseradish peroxidase (HRP)

Peroxidases	Characteristics					
	RZ ratio	Molecular weight (kDa)	pI	Isoforms	pH working range[1]	Temperature working range[1]
Cassava peroxidase (CSP)	0.6	38, 44	6.0, 6.4, 7.0, 7.2	4	5-8	5-70°C
Horseradish peroxidase (HRP)	1.5	43	5.2, 5.4, 6.3, 7.4, 8.2	>5	3-9	25-60°C

Peroxidase activity was performed in 0.1M phosphate buffer, pH 6 at 25°C using 3, 3′, 5, 5′-tetramethylbenzidine and urea hydrogen peroxide as peroxidase substrates

4.2.6 Effect of organic solvent on peroxidase activity

4.2.6.1 Ability to work in solvent-aqueous solution

The effect of acetonitrile and methanol concentration on CPS and HRP activities were investigated. Experiments using buffer solution containing acetonitrile or methanol in the concentration of 5-60% were explored. After CSP were incubated in the solvent-aqueous system for 3 minutes, it was found that CSP was able to work in a solution containing 25% acetonitrile and methanol with its relative activities approximately 65% (**Figure 4.10 a**.). On the other hand, HRP relative activity in the solution containing 25% methanol was found to be approximately 80% which was higher than its activity in 25% acetonitrile solution which retained its activity to only 50% (**Figure 4.10 b.**).

4.2.6.2 Tolerance of peroxidase in solvent-aqueous solution

Peroxidase stabilities in both solvent-aqueous systems were elucidated by plotting peroxidase relative activities against solvent concentrations and time of incubation as shown in the **Figure 4.11.** Result of stability test showed that CSP presented its stability in a wide concentration range in both organic solvents when it was incubated in acetonitrile and methanol concentration ranging from 10 to 60% for 6 hours at 25^{o}C and its relative activity retained higher than 75% of its initial activity. On the contrary, HRP was sensitive to acetonitrile content; its activity almost disappeared after 6 hours but it was stable in the solution

containing a narrow range of 20-35% methanol which stilled retained its activity to 70-85%.

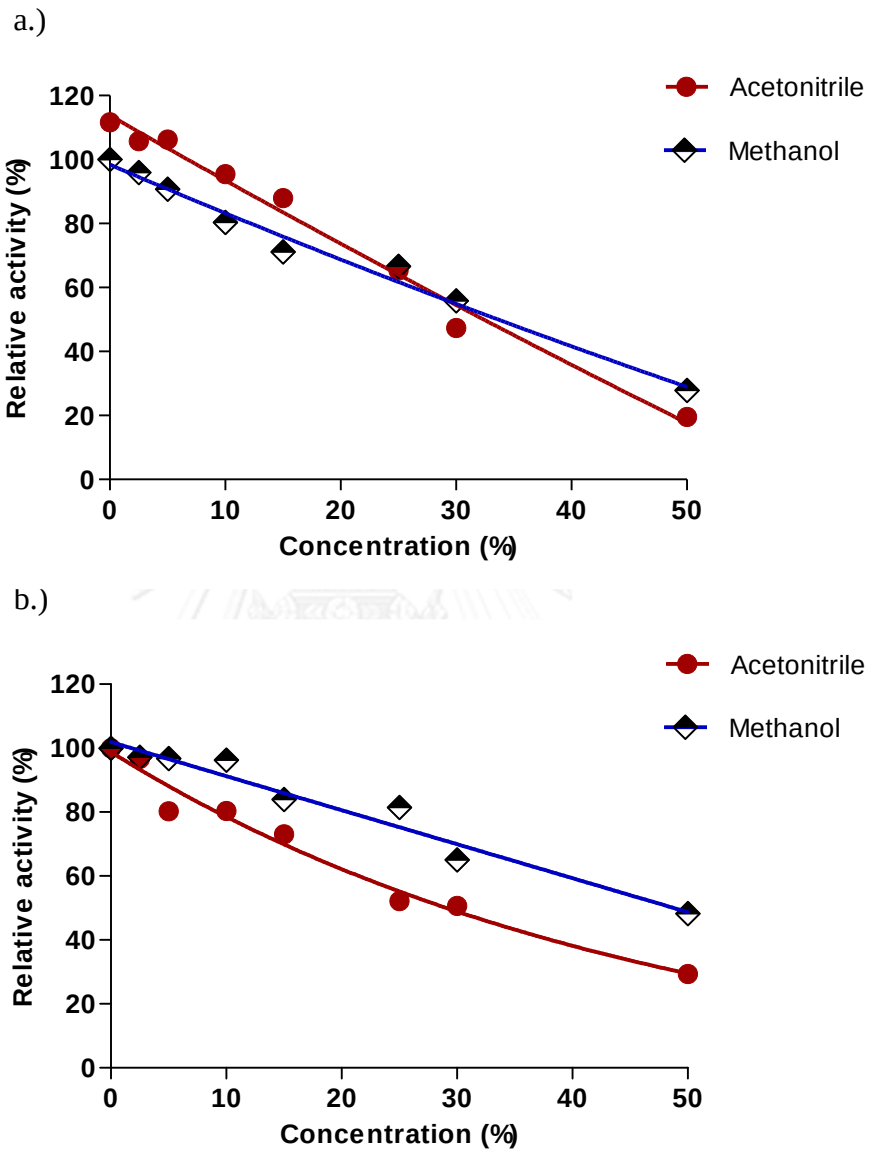


Figure 4. 10 Solvent tolerance of a.) CSP and b.) HRP in 0.1 M phosphate buffer, pH 6.0 containing 0-50 % of acetonitrile or methanol.

Both enzymes were incubated in organic solvent-aqueous mixed solution for 3 minutes (Peroxidase activity was determined in triplicate.).

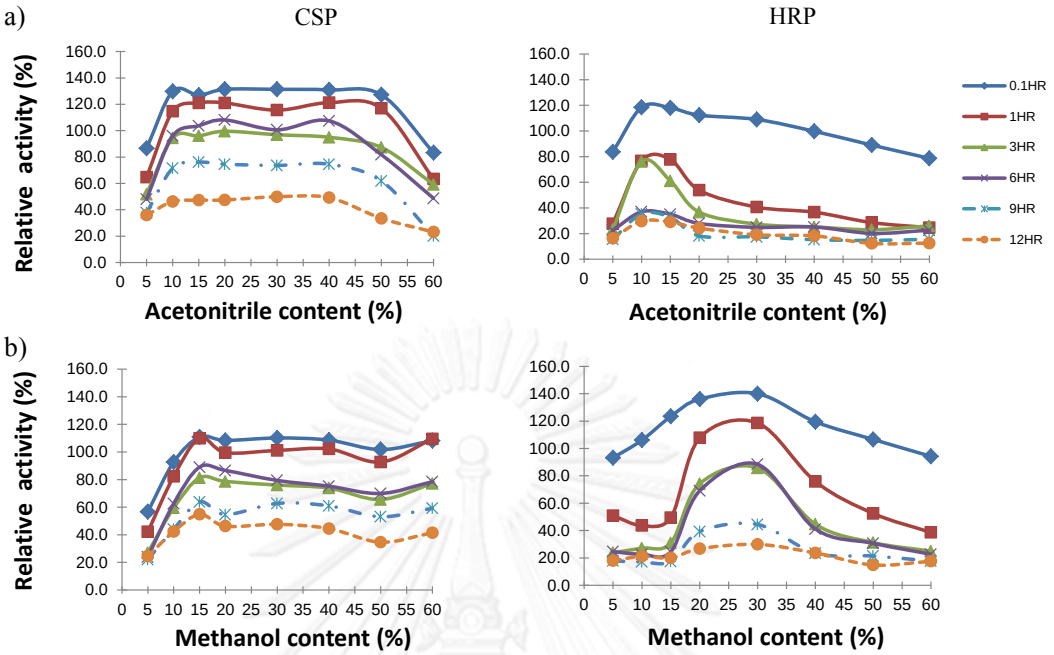


Figure 4. 11 Stability of CSP and HRP in 0.1 M phosphate buffer, pH 6.0 containing a.) acetonitrile and b.) methanol.

The solvent tolerance was investigated from 0-12 hours. (Peroxidase activity was determined in triplicate.).

4.3 Kinetic catalysis of peroxidases

After the catalytic activities of peroxidases in 0.1 M acetate-citric acid buffer, pH 6 at 25°C were performed, kinetic parameters such as Michaelis-Menten constant (K_M) and maximum velocity (V_{max}) were determined by Lineweaver-Burk (primary plots) and secondary plots. It was found that the primary plots of CSP and HRP for UHP and TMB catalysis demonstrated as a parallel line pattern as shown **in Figure 4.12-4.13** respectively. The effect of substrate concentrations on CSP initial rate was carried out and Michaelis-Menten constant ($^{app}K_M$) and maximum velocity (V_{max}) were calculated and shown in **Table 4.3**. $^{app}K_M$ values for UHP of CSP and HRP were found to be 100±10 and 420±30 μM. This indicated that CSP had higher affinity towards UHP as a primary substrate than HRP. Additionally, CSP also showed higher affinity towards TMB which was a secondary substrate with the lower $^{app}K_M$ values.

4.4 Spectrophotometric method for peroxidase inhibition assay

In the preliminary study, the spectrophotometric method was developed in order to investigate the potential use of peroxidase inhibition assay for the detection of target inhibitors. In this experiment, we attempted to use UHP as a source of hydrogen peroxide and TMB as a chromogenic substrate. As TMB can generate the catalyzed product with a high extinction coefficient, it is currently considered as a sensitive chromogen for monitoring peroxidase activity by colorimetric or spectrophotometric method. Moreover, thiourea which is a strong inhibitor of peroxidase was used as a model for peroxidase inhibition study. Therefore, the effects of thiourea on CSP and HRP were investigated and detection ranges of the proposed inhibition assay by spectrophotometric measurement were carried out.

4.4.1 Inhibition mechanism of thiourea towards peroxidase activity

Inhibition patterns of peroxidases in the presence of thiourea at various concentrations were studied using Lineweaver-Burk plot. It was found that the degree of inhibition depended on the increment of thiourea concentration. The effects of thiourea on CSP and HRP were elucidated in **Figure 4.14**. The dissociation constants (K_i) and the inhibition type of thiourea were defined based on the patterns of Lineweaver-Burk plot. It can be seen that the increment of thiourea concentrations could affect ${}^{app}K_M$ values but could not affect V_{max} values, thiourea was thus, considered as a competitive inhibitor of both CSP and HRP.

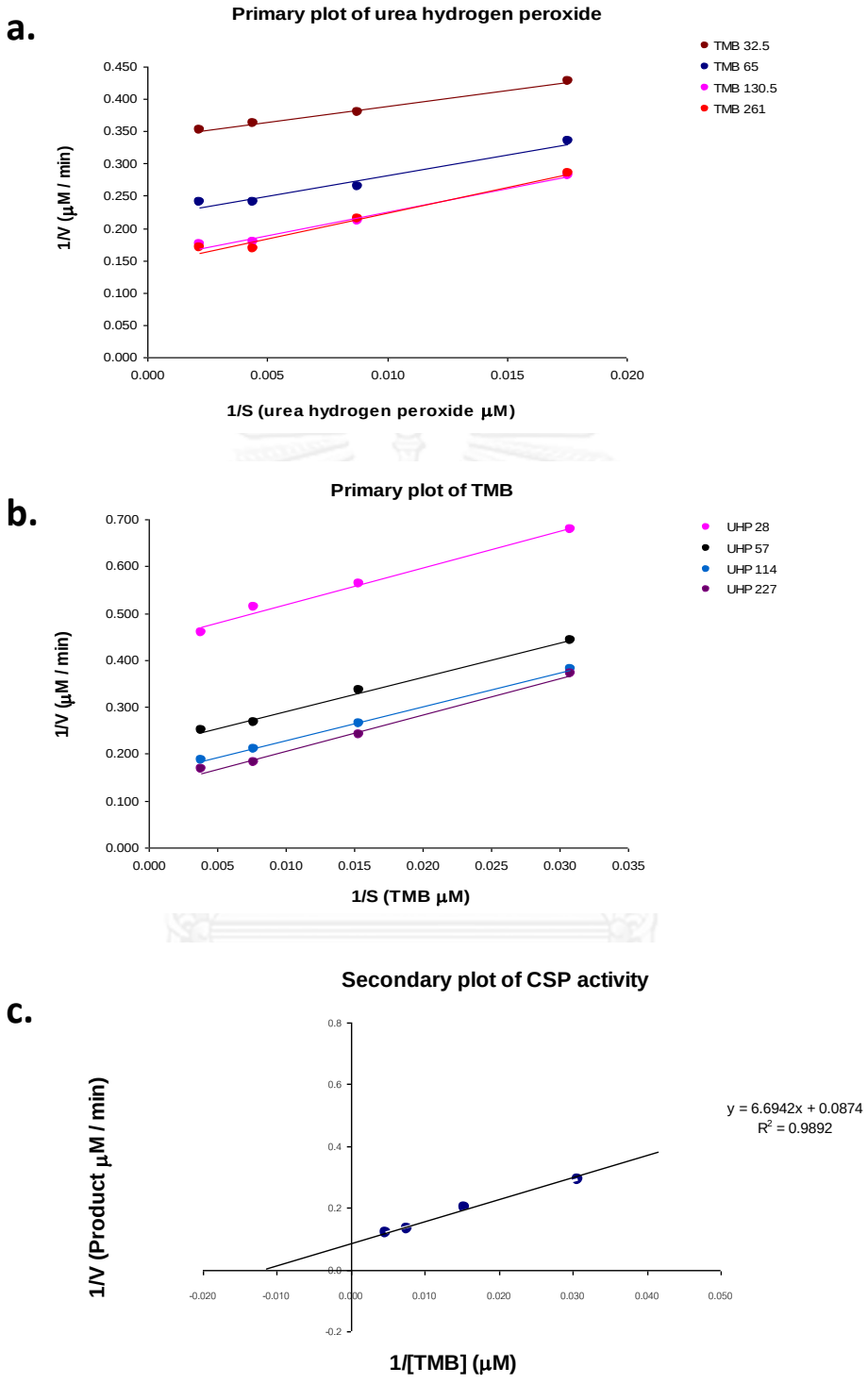
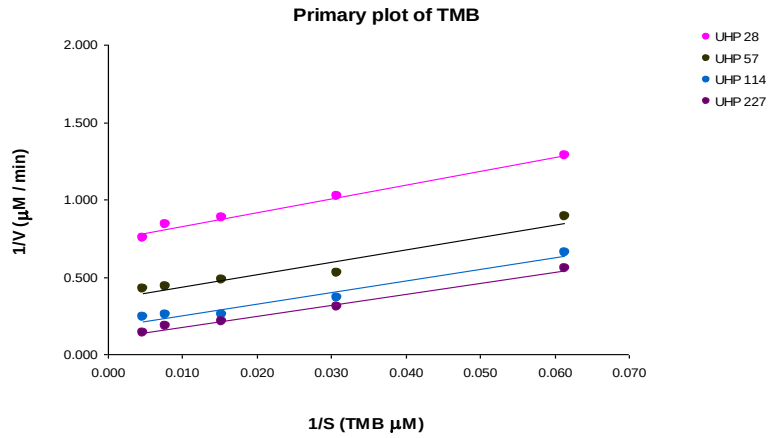
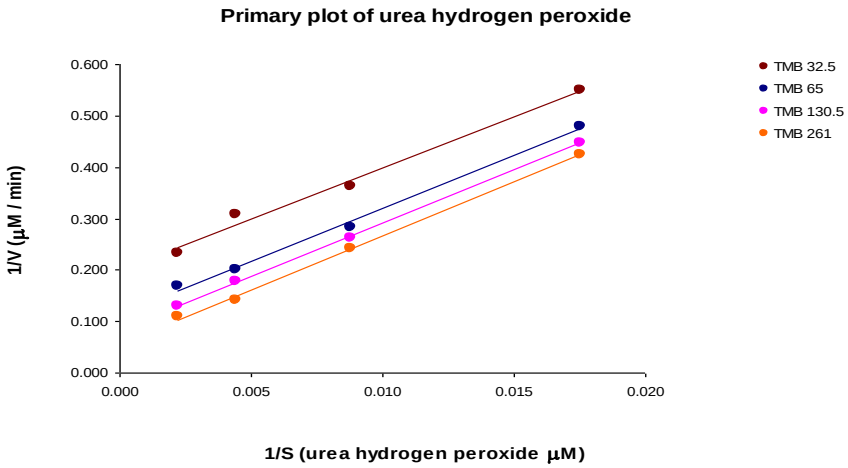


Figure 4. 12 Primary and secondary plots of cassava peroxidase (CSP) for the catalysis of urea hydrogen peroxide (UHP) and 3, 3´, 5, 5´-tetramethylbenzidine (TMB).

a.



b.



c.

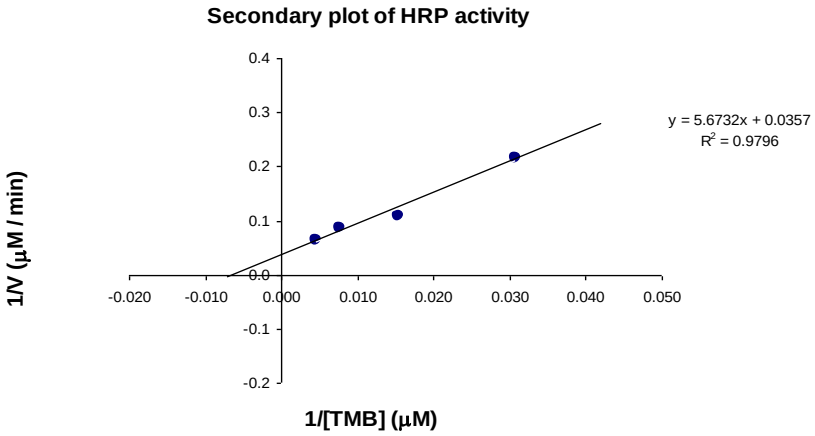


Figure 4. 13 Primary and secondary plots of horseradish peroxidase (HRP) for the catalysis of urea hydrogen peroxide (UHP) and 3, 3´, 5, 5´-tetramethylbenzidine (TMB).

Table 4. 3 Kinetic parameters of cassava and horseradish peroxidases.

(Catalysis of urea hydrogen peroxide and 3, 3, 5, 5′-tetramethyl benzidine as substrates)

Peroxidases	$^{app}V_{max}$ (μM min^{-1})		$^{app}K_M$ (μM)	
		UHP		TMB
Cassava peroxidase (CSP)	12±3	100±10		80±12
Horseradish peroxidase (HRP)	28±5	420±30		160±20

The initial rate was the mean of triplicate determination and calculated using the molar absorption coefficient of its product, 39,000 $M^{-1}cm^{-1}$.

Figure 4. 14 Inhibition effects of thiourea concentrations on the velocity of peroxidase for tetramethylbenzidine catalysis in the presence of urea hydrogen peroxide.

a.) Horseradish peroxidase (HRP) and b.) Cassava peroxidase (CSP).

4.4.2 Dose response curve of thiourea towards peroxidase activity

The half maximal inhibitory concentration (IC_{50}) value of thiourea was determined by dose response curve. The IC_{50} value is a practical readout of the relative effects on enzyme activity of different substances under a specific set of solutions. As the result of IC_{50} value and The ${}^{app}K_i$ value of thiourea as shown in **Table 4.4**., it was found that THU was an effective inhibitor of HRP rather than CSP. Moreover, the applicable range for thiourea detection by enzymatic inhibition was defined as shown in **Table 4.4** indicated that the detection range of thiourea based on CSP and HRP inhibition was found to be in the range of 25-500 μM. In addition, the concentration and inhibition-dependent plots of thiourea elucidated linear relationship between them as shown in **Figure 4.16**. Therefore, these preliminary results indicated a good perspective for the practical use of CSP for the detection of thiourea and other thiol compounds.

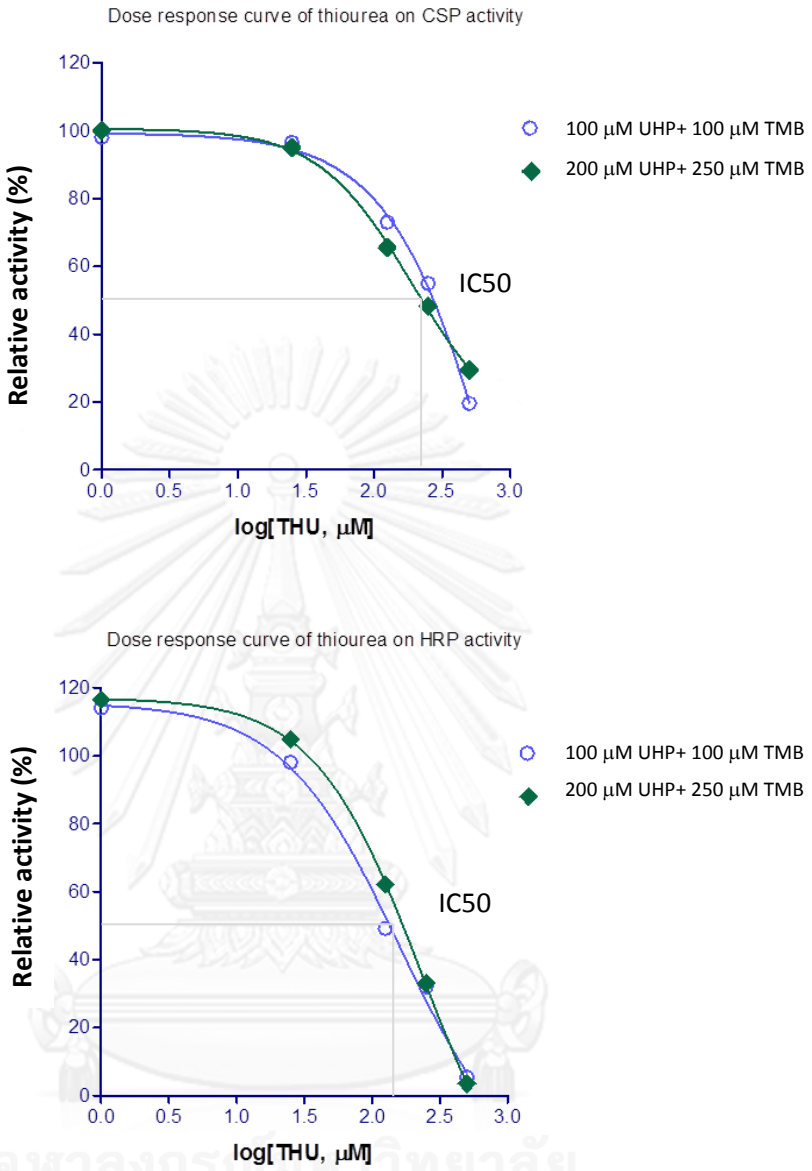


Figure 4. 15 Dose response curves of thiourea concentrations on peroxidase activity for UHP and TMB catalysis.

a.) Horseradish peroxidase (HRP) and b.) Cassava peroxidase (CSP)

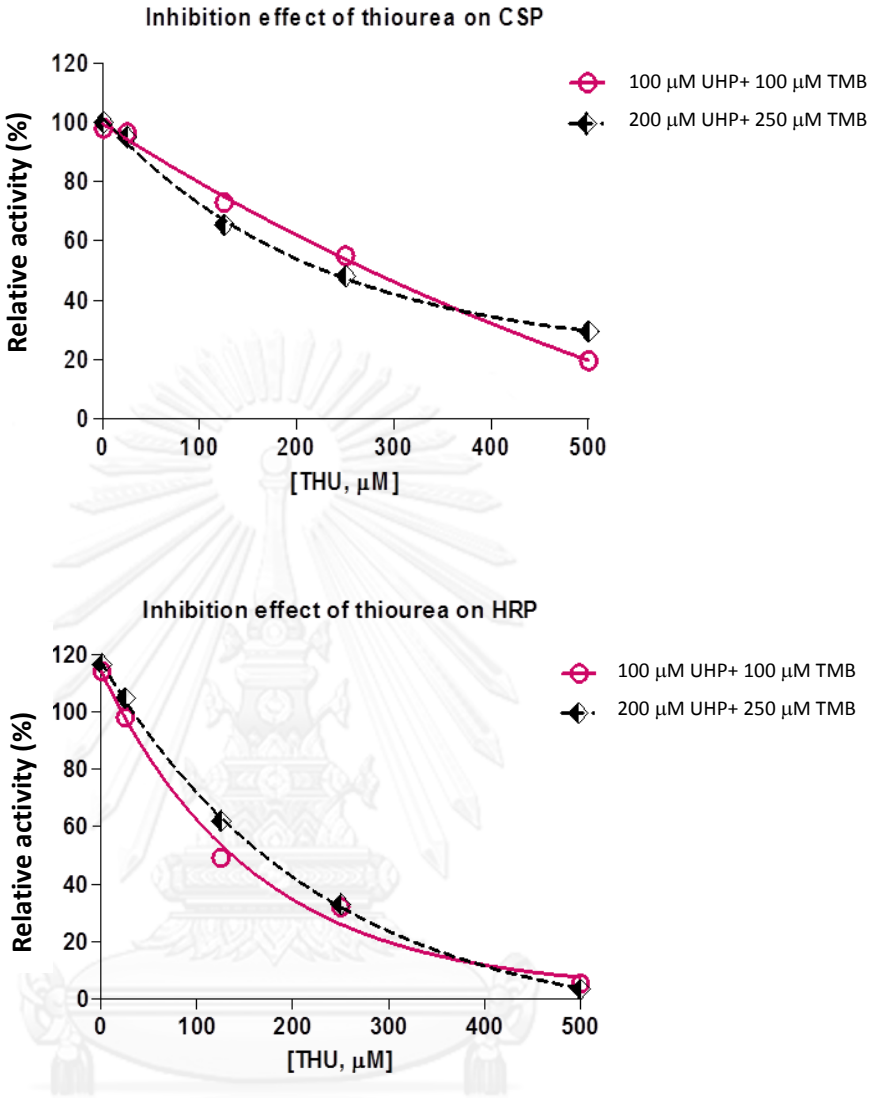


Figure 4. 16 The relationship between thiourea concentrations and peroxidase inhibition for UHP and TMB catalysis.

a.) Horseradish peroxidase (HRP) and b.) Cassava peroxidase (CSP).

Table 4. 4 Inhibitory effects of thiourea on peroxidases and its detectable range based on spectrophotometric method.

4.5 Electrochemical method for peroxidase inhibition assay

From the result of spectrophotometric method, it indicated the potential usage of peroxidase for the detection of thiourea based on the inhibition assay. Therefore, we applied the inhibition assay for the detection of thiram based on electrochemical measurement. In the preliminary test, the inhibition assay was performed by the use of thiourea and HRP as a model for the optimal condition trial.

4.5.1 Fabrication of screen-printed electrodes (SPEs)

The electrode used in this study was fabricated semi automatically by screen printer. The appearance of designed SPE is shown in **Figure 4.17**. After SPE was already prepared, it was used for the characterization of DAB and measurement of its oxidized product in the reaction solution. The repeatability of SWV measurement by the use of prepared SPEs was investigated, the percentage of relative standard deviation (%RSD) of the peak position at potential of -0.24 V and the current peak height approximately 400 nA obtained from 5 electrodes were found to be less than 5% and 15%, respectively.

4.5.2 Electrochemical measurement for peroxidase activity

4.5.2.1 Cyclic voltammograms of DAB

The cyclic voltammogram of DAB was carried out by the use of SPE applied voltage in the range of -0.6 to +1.0 V and the reaction of DAB solutions in 0.04 Britton-Robinson (BR) buffer containing peroxidase and UHP with and without the addition of sulfuric acid (H_2SO_4) as stop solution was

investigated. It was found that there was an oxidation peak of DAB at 0.36 V (peak height 1.3 μA) in the solution without the addition of H_2SO_4 (**Figure 4.18. a**) but two peaks of DAB oxidation at -0.19 V (peak height 3.1 μA) and 0.45 V (peak height 2.7 μA) were found in the solution when the H_2SO_4 was added as elucidated in **Figure 4.18.b**.

4.5.2.2 Square - wave voltammogram (SWV) of DAB

Square wave voltammetric measurements in the potential range from 0.0 to -0.6 V with a step potential of 10 mV, pulse 75 mV and frequency 50 Hz at scan rate of 50 mVs^{-1} were performed for the determination of current response of DAB in 0.04 BR buffer containing HRP and UHP with and without the stop solution. As a result from **Figure 4.19**, the square-wave voltammogram of DAB response obtained from the solution containing HRP, UHP and H_2SO_4 a votammetric peak at the potential of -0.24 V with the peak height of 2.2 μA was clearly observed. It was the highest peak whereas the votammetric peak obtained from other solution such as BR buffer with H_2SO_4, BR buffer containing DAB and H_2SO_4 and BR buffer containing DAB, HRP and H_2SO_4 could not be detected, except for BR buffer containing DAB, HRP, UHP and H_2SO_4. However, low signal of current peak height could be observed from solution containing only DAB without peroxidase and UHP.

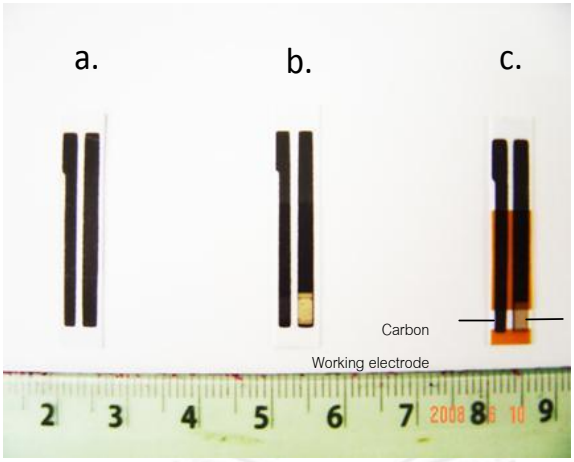


Figure 4. 17 A Screen-printed electrode consisting of a 2x3 mm^2 carbon working electrode and an Ag/AgCl reference electrode

a.) PVC applied with a carbon conductive layer, b.) PVC applied with carbon and Ag/AgCl ink layer and c.) a screen-printed electrode with reference and working electrodes.

Table 4. 5 Repeatability of SWV measurement obtained from screen-printed electrode.

	Peak position (V)			Peak height (nA)		
Electrode No.	Reading 1	Reading 2	Average	Reading 1	Reading 2	Average
1	-2.40	-2.40	-2.40	308	302	305.00
2	-2.28	-2.40	-2.34	329	344	336.50
3	-2.29	-2.29	-2.29	405	411	408.00
4	-2.40	-2.40	-2.40	334	373	353.50
5	-2.49	-2.49	-2.49	396	396	396.00
		Average	-2.38		*Average*	359.80
		SD	0.08		*SD*	42.48
		%RSD	3.36		*%RSD*	11.81

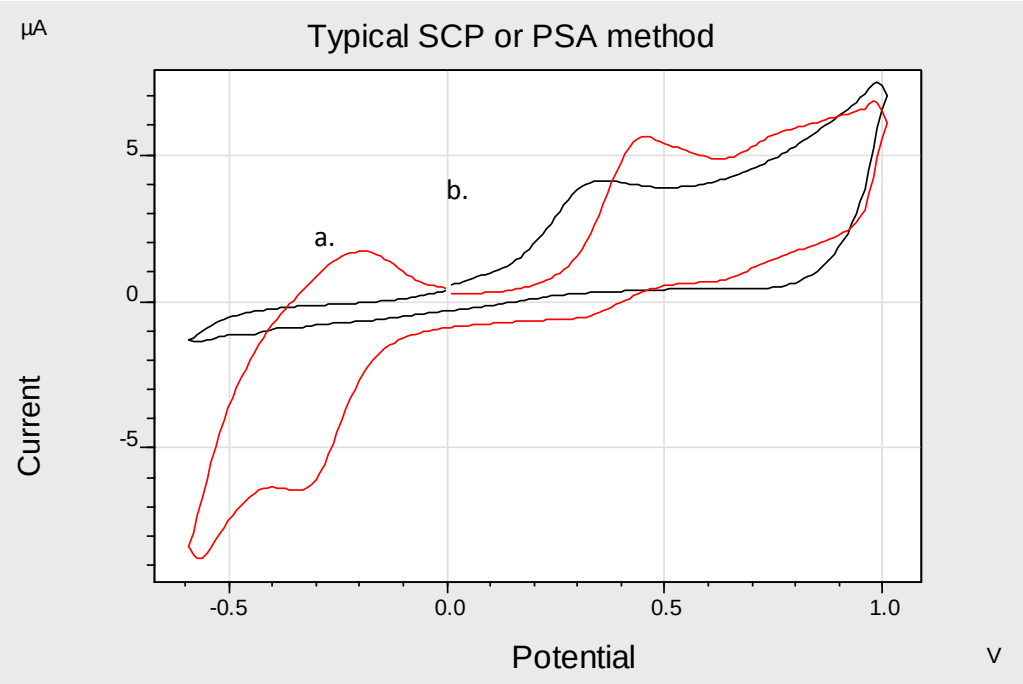


Figure 4. 18 Cyclic voltammograms of DAB in 0.04 M Britton-Robinson buffer pH 5 containing HRP and UHP.

(a.) The oxidative peak was at potential of 0.36 V in the solution without the addition of sulfuric acid and b.) Two oxidative peaks were at potential of -0.19 V and 0.45 V in the solution with the addition of sulfuric acid)

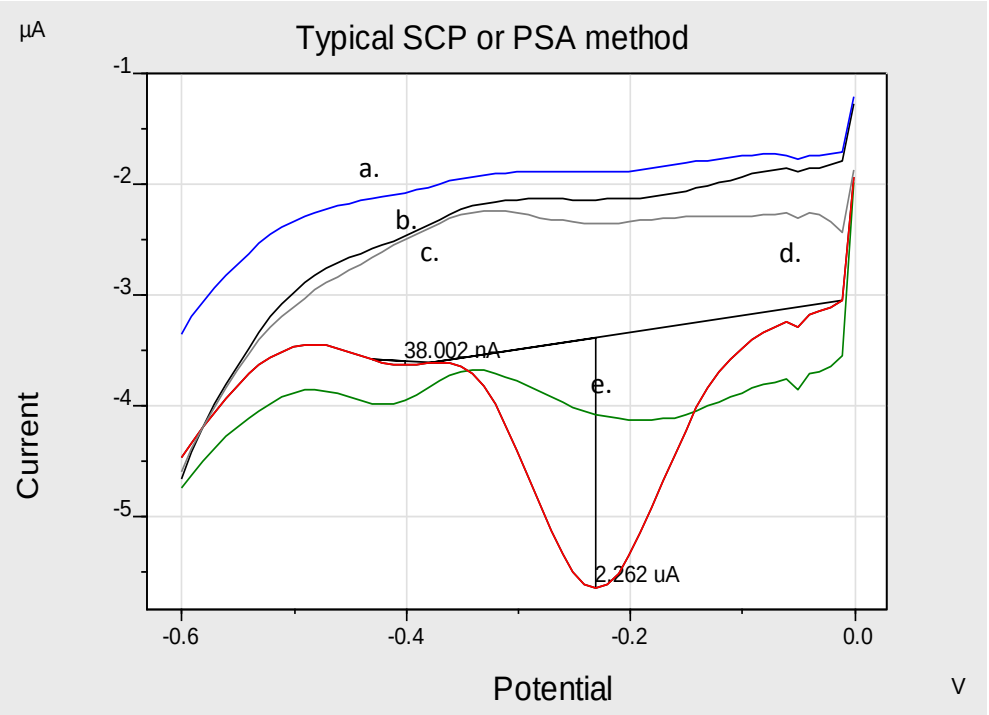
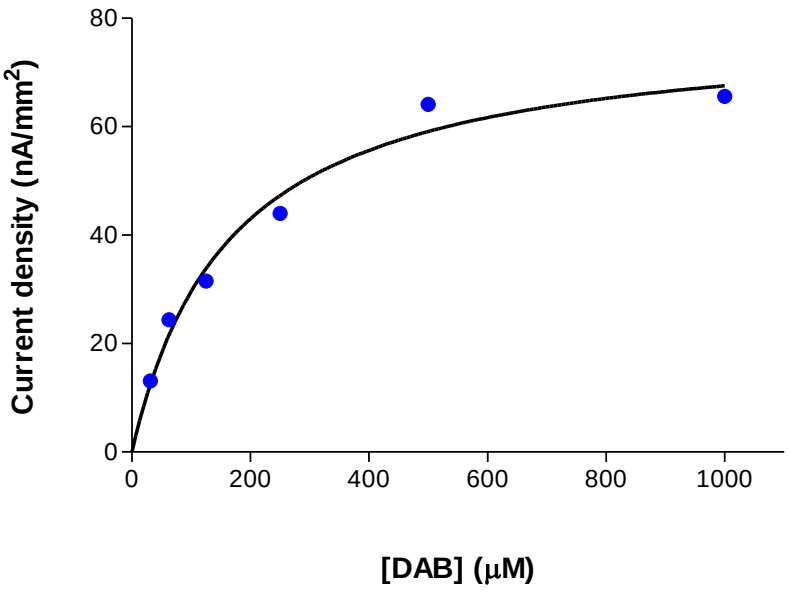


Figure 4. 19 Square-wave voltammograms of DAB in 0.04 M Britton-Robinson buffer pH 6.

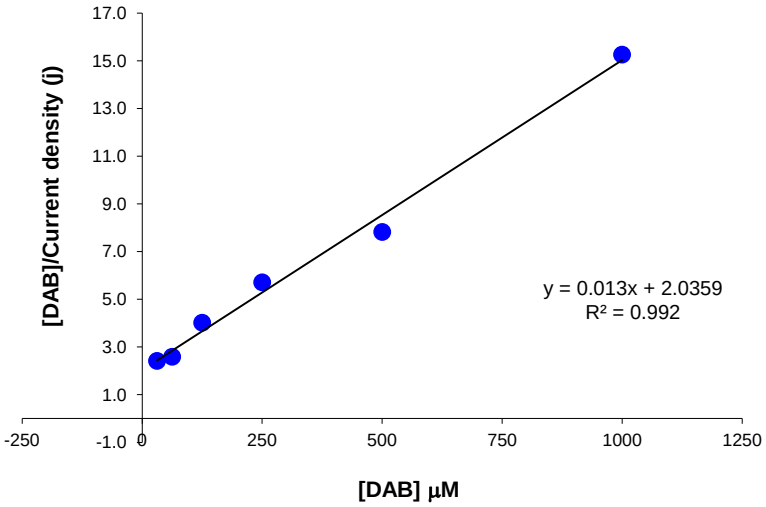
(The highest reductive current was obtained from DAB product in the presence of HRP, DAB, UHP with the addition of H_2SO_4, a.) BR + H_2SO_4, b.) BR + DAB + H_2SO_4, c.) HRP+ DAB + H_2SO_4, d.) BR+ DAB + UHP+ H_2SO_4, e.) HRP+ DAB + UHP+ H_2SO_4)

According to our procedure for SWV experiment, HRP reaction solution containing a varied concentration of DAB in the range of 31-1,000 μM with the constant concentration of UHP at 100 μM was incubated at 25°C for 3 minutes. The reaction was then stopped by the addition of 2 M sulfuric acid and the SWV measurement was performed by using SPE with the working area of 6 mm^2 (2 mm x 3 mm). It was found that the relationship between current response and DAB concentration illustrated as a non-linear graphical plot as shown in **Figure 4.20**. The non-linear fit curve was analyzed by the GraphPad Prism 5 program and the maximum current density was predicted at 78.8 nA/mm^2. In addition, the linear relationship with the coefficient of determination (R^2) of 0.992 could be found when the ratio of DAB concentration and current density was plotted as a function of DAB concentration as shown in **Figure 4.21**.



[DAB] μM	Average current peak height at E -0.24 V i (nA)	Average current density j (nA/mm^2)	SD (n=3)	RSD%
31.2	236	13.1	2.0	15.6
62.5	439	24.4	2.9	12.0
125	566	31.5	4.0	12.8
250	792	44.0	4.0	9.0
500	1154	64.1	3.7	5.8
1000	1180	65.6	1.9	2.9

Figure 4. 20 Non-linear relationship between current density and DAB concentration. (The reaction solution was 0.04 M Britton-Robinson buffer, pH 5, containing 10 μL HRP with a constant concentration of UHP at 100 μM and varied concentrations of DAB in the range of 31.2-1,000 μM. It was incubated for 3 minutes and stopped by the addition of 2 M sulfuric acid and the current peak height at E -0.24 Volts was determined by SWV measurement obtained from screen-printed electrode cooperated with PalmSens.)



[DAB] μM	Average current density at E -0.24 V j (nA/mm^2)	Average [S]/j	SD (n=3)	RSD%
31.2	13.1	2.4	0.35	14.5
62.5	24.4	2.6	0.32	12.4
125	31.5	4.0	0.48	12.0
250	44.0	5.7	0.52	9.1
500	64.1	7.8	0.45	5.7
1000	65.6	15.3	0.44	2.9

Figure 4. 21 The linear relationship between the ratio of DAB concentration and current density and DAB concentration.

(The reaction solution was 0.04 M Britton-Robinson buffer, pH 5, containing 10 μL HRP with a constant concentration of UHP at 100 μM and varied concentrations of DAB in the range of 31.2-1,000 μM. It was incubated for 3 minutes and stopped by the addition of 2 M sulfuric acid and the current peak height at E -0.24 Volts was determined by SWV measurement obtained from screen-printed electrode cooperated with PalmSens.)

4.5.3 Inhibitory effect of thiols on peroxidase activity

The experiments were carried out as in 4.5.2. CSP and HRP reaction solutions containing varied amount of DAB (125, 250 and 500 μM) with the constant concentration of UHP at 100 μM in the absence of neither thiourea nor thiram were prepared and these considered as a control reactions. For thiol inhibition study, the test solutions contained both DAB and UHP as previously described for a control reaction but thiourea or thiram was added instead of 0.04 M BR buffer at a final concentration of 100 μM in the reaction. It was found that the SWV current peak height of DAB at the potential of E -0.24 V obtained from each test reaction was lower than that of control reaction. From this result, it indicated that the activity of peroxidase was decreased and corresponded to the concentration of both thiols. According to the ratio of DAB concentration and current density, it was proportional to DAB concentration. The linear regression lines of three solutions containing different DAB concentrations and in the presence of either 20 μM thiourea or 20 μM thiram as well as the solution in the absence of both thiols are illustrated as shown in **Figure 4.22**. The result showed that thiram elucidated its strong inhibitory effect higher than thiourea on both CSP and HRP activity with different manner. In the presence of thiram for CSP inhibition, the linear regression line was found to cross over with the line that contained no thiol (control line). On the contrary, the linear regression line of thiram for HRP inhibition and the control line were found to be parallel.

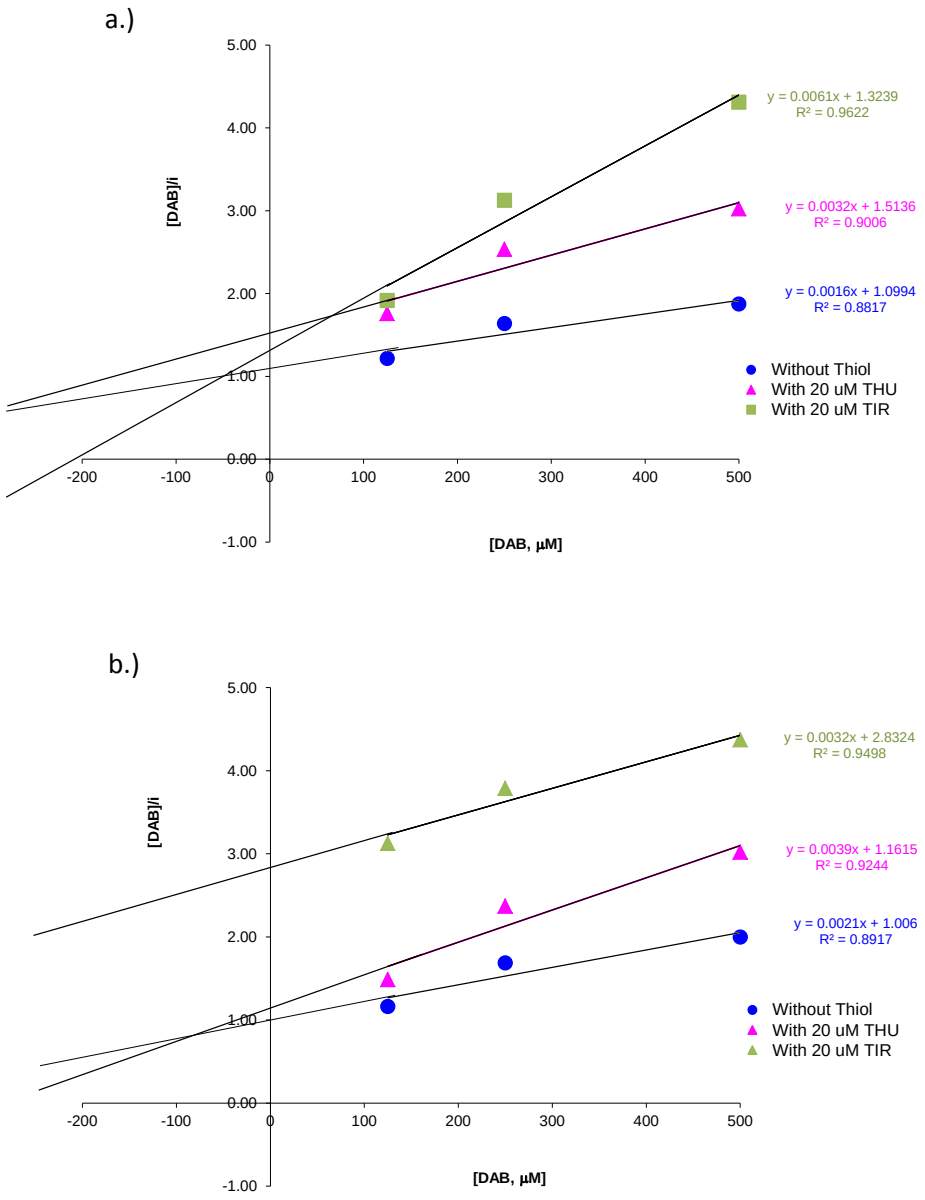


Figure 4. 22 Linear regression line of the response obtained from solution.

a.) CSP and b.) HRP solution in 0.04 M Britton-Robinson buffer, pH 6 containing a constant concentration of UHP at 100 µM and varied concentrations of DAB in the range of 62-250 µM. the current peak height at E -0.24 Volts was determined by SWV measurement obtained from screen-printed electrode cooperated with PalmSens.)

4.5.4 Detection of thiols based on peroxidase inhibition assay and SWV measurement

In order to develop a method for the detection of thiols based on peroxidase inhibition, the relationship between current response of CSP activity and thol concentration was primarily investigated. The peroxidase solution was prepared in 0.04 M Britton-Robinson buffer, pH 6, containing 5% methanol with a constant concentration of DAB (62 or 125 or 250 μM) and 100 μM of UHP in the presence of a varied concentration of either thiourea or thiram in the range of 1 -100 μM. The reaction was incubated for 3 minutes and the current peak height at the potential of -0.24 V was determined by SWV measurement.

4.5.4.1 Detection of thiols based on CSP inhibition assay

The relationship between current response of CSP activity and thiol concentrations was investigated and illustrated in different types of graphical plots. As the current peak height was plotted as a function of thiol concentration shown in **Figure 4.23**, it was found that the linear inverse relationship between current response and thiourea concentration with the coefficient of determination (R^2) 0.970 could be observed. The detectable range of thiourea inhibition curve was found in the range of 10-100 μM. Although the relationship between current peak height and thiram concentration elucidated as a nonlinear line, the semi-log plot of signal current and thiram concentration elucidated the linear inverse relationship. It was clearly observed that the regression line of thiram inhibition at 125 μM DAB showed the coefficient of determination (R^2) 0.999 and it could be

used for the detection of thiram in range of 5-100 μM as shown in **Figure 4.24**.

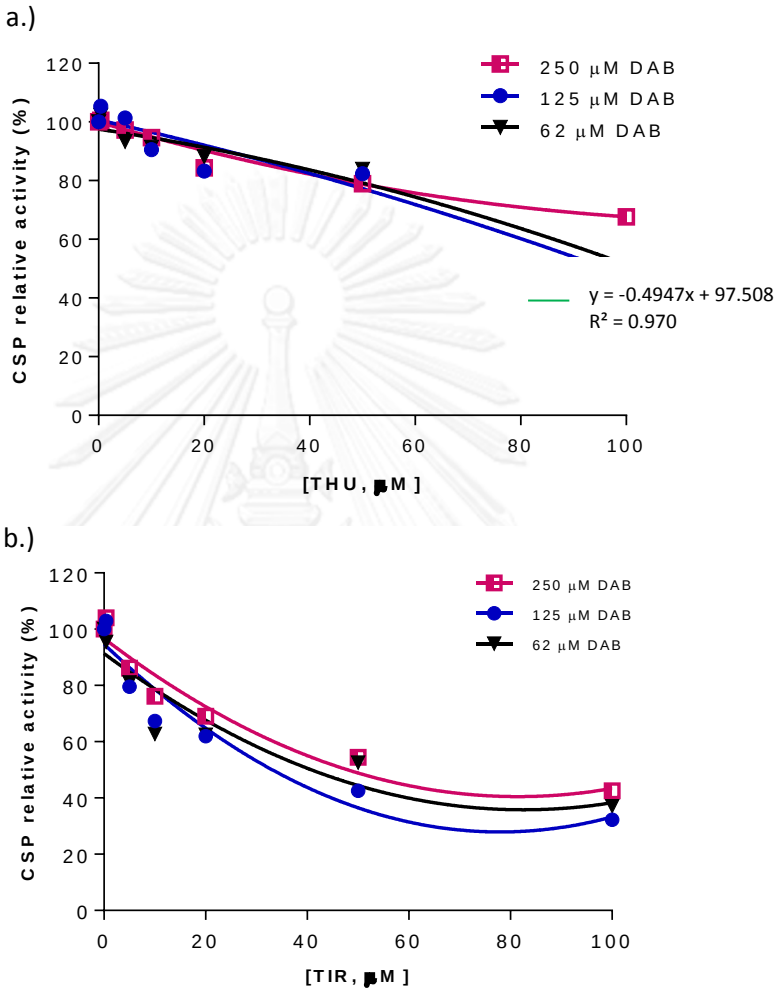
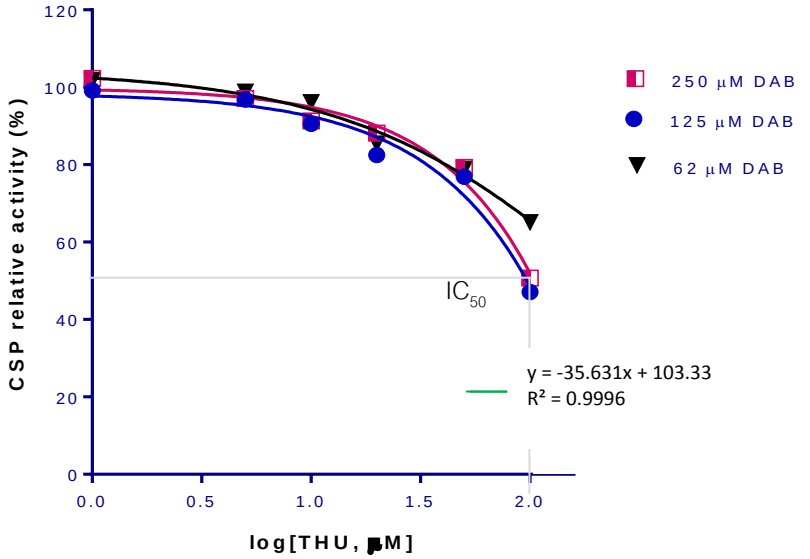


Figure 4. 23 Inhibition curve of a.) thiourea and b.) thiram concentrations on CSP activity for UHP and DAB catalysis.

(The reaction solution was 0.04 M Britton-Robinson buffer, pH 6, containing 10 μL CSP with a constant concentration of 62, 125 and 250 μM of DAB and 100 μM of UHP in the presence of varied concentration of 1-100 μM thiol. It was incubated for 3 minutes and stopped by the addition of 2 M sulfuric acid and the current peak height at E -0.24 Volts was determined by SWV measurement obtained from screen-printed electrode cooperated with PalmSens.)

a.)



b.)

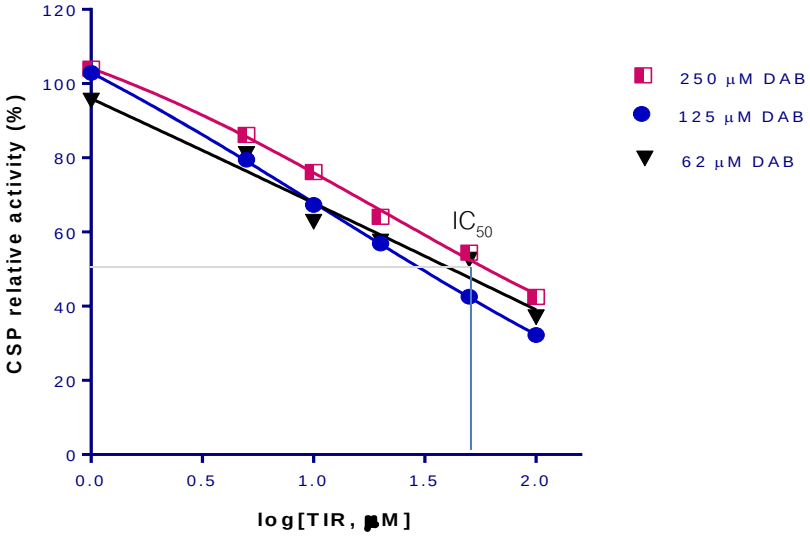


Figure 4. 24 Semi-log inhibition curve of a.) thiourea and b.) thiram concentrations on CSP activity for UHP and DAB catalysis.

(The reaction solution was 0.04 M Britton-Robinson buffer, pH 6, containing 10 µL CSP with at certain concentration of DAB (62, 125 and 250 µM) and 100 µM of UHP in the presence of varied concentration of 1-100 µM thiol. After 3 minutes incubation, it was stopped by 2 M sulfuric acid and the current peak height at E -0.24 Volts was determined by SWV measurement obtained from screen-printed electrode cooperated with PalmSens.)

4.5.4.2 Detection of thiols based on HRP inhibition assay

The relationship between current response of HRP activity and thiol concentrations was also investigated. It was found that the non-linear inverse relationship between current response and thiol concentration was observed in both types of graphical plots such as the related function between current peak height and thiol concentration as shown in **Figure 4.25** and the semi-log corresponded function between current peak height and log concentration of thiol as shown in **Figure 4.26**. It indicated that the detectable range of both thiourea and thiram was found to be in the range of 10-100 μM and 5-100 μM, respectively.

As a result, it could be suggested that the developed method has the potential use for the detection of thiol compounds such as thiol and thiram based on peroxidase inhibition and SWV measurement and the method details was summarized as in **Table 4.**6.

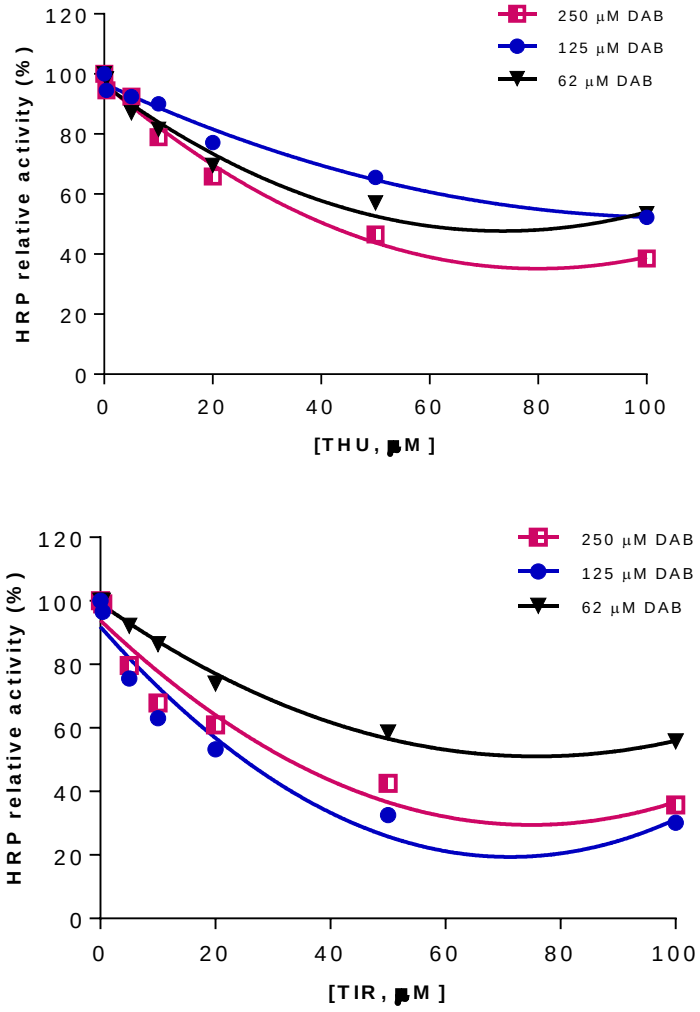
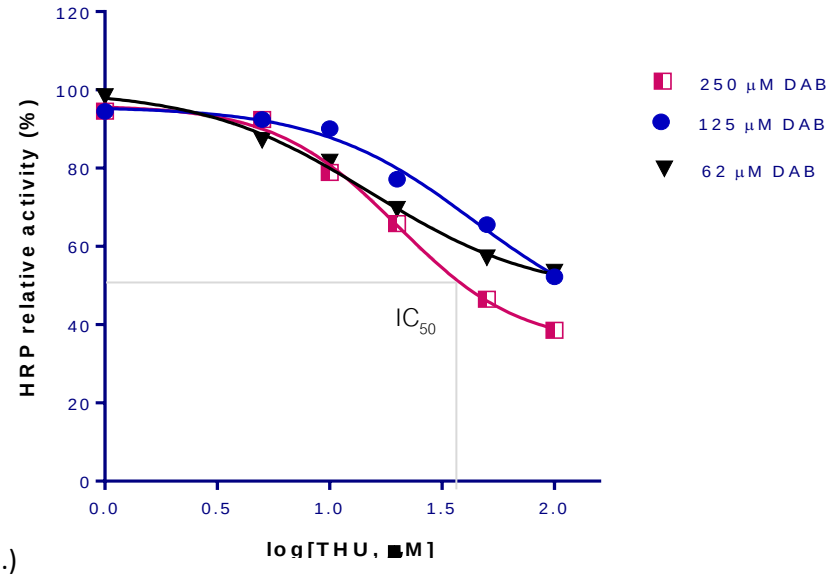


Figure 4. 25 Inhibition curve of a.) thiourea and b.) thiram concentrations on HRP activity for UHP and DAB catalysis.

(The reaction solution was 0.04 M Britton-Robinson buffer, pH 6, containing 10 µL HRP with a constant concentration of 62, 125 and 250 µM of DAB and 100 µM of UHP in the presence of varied concentration of 1-100 µM thiol. It was incubated for 3 minutes and stopped by the addition of 2 M sulfuric acid and the current peak height at E -0.24 Volts was determined by SWV measurement obtained from screen-printed electrode cooperated with PalmSens.)

a.)



b.)

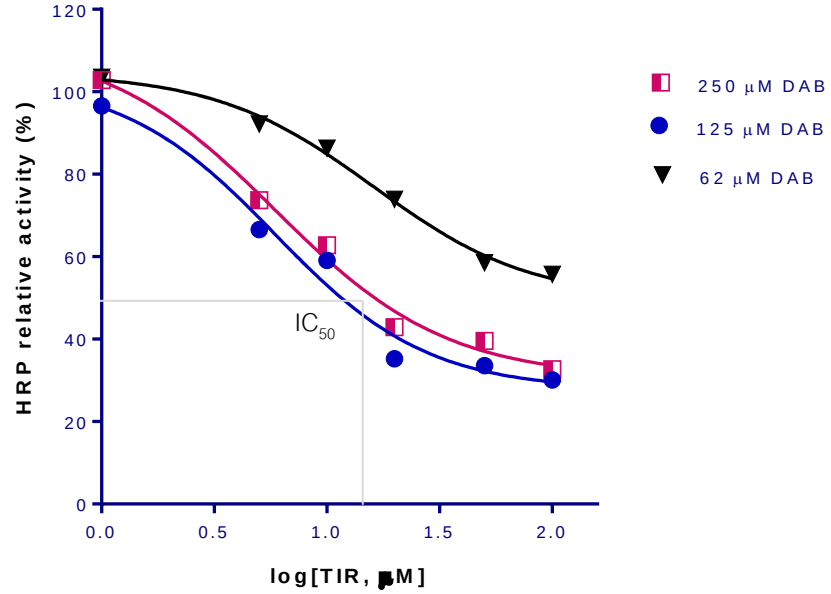


Figure 4. 26 Semi-log ihibition curve of a.) thiourea and b.) thiram concentrations on HRP activity for UHP and DAB catalysis.

(The reaction solution was 0.04 M Britton-Robinson buffer, pH 6, containing 10 μL HRP with a constant concentration of 62, 125 and 250 μM of DAB and 100 μM of UHP in the presence of varied concentration of 1-100 μM thiol. It was incubated for 3 minutes and stopped by the addition of 2 M sulfuric acid and the current peak height at E -0.24 Volts was determined by SWV measurement obtained from screen-printed electrode cooperated with PalmSens.)

Table 4. 6 Summary of electrochemical measurement for the detection of thiols.

Peroxidase	Thiourea		Thiram	
	Graphical plot: detection range (μM)	IC_{50} (μM)	Graphical plot: detection range (μM)	IC_{50} (μM)
Cassava peroxihdase (CSP)	Linear plot: 10-100	100 ± 16	Semi-log plot: 5-100	50 ± 8
Horseradish peroxihdase (HRP)	Semi-log plot:10-100	40 ± 8	Semi-log plot: 5-100	16 ± 4

IC_{50} : Inhibition concentration at 50% of thiol was estimated from the semi-log inhibition curve at 250 μM of DAB

4.6 Degradation of thiram based on peroxide reaction

For the application of peroxidase in the area of bioremediation, we tried to investigate the degraded rate of thiram treated with peroxide under high temperature and peroxidase catalyzed reaction.

4.6.1 Chromatographic separation of thiram by reverse phase-HPLC

Thiram could be practically determined by reverse phase-HPLC with UV detector at the wavelength of 275 and 290 nm and it was eluted from the analytical column with the retention time of 5.96 minutes as shown in **Figure 4.27**.

4.6.2 Effect of peroxide and temperature on thiram degradation

Thiram at the concentration of 64 μM (15 μg/mL) in 0.04 M Britton-Robinson buffer, pH 6 containing 20% methanol was treated with 400 μM of UHP and incubated at various temperatures (25°C, 35°C and 55°C). It was clearly be seen that thiram incubated in solution containing UHP at 55°C was dramatically decreased and nearly disappeared within 90 minutes whereas the thiram incubated in solution containing UHP at 35°C was slightly decreased. Unfortunately, the peak of the degraded product could not be observed by the HPLC system. Moreover, thiram was found to be stable in a control solution when UHP was not added and in the solution containing UHP which was incubated at 25°C. The profile of thiram degradation was elucidated and shown in **Figure 4.28.**

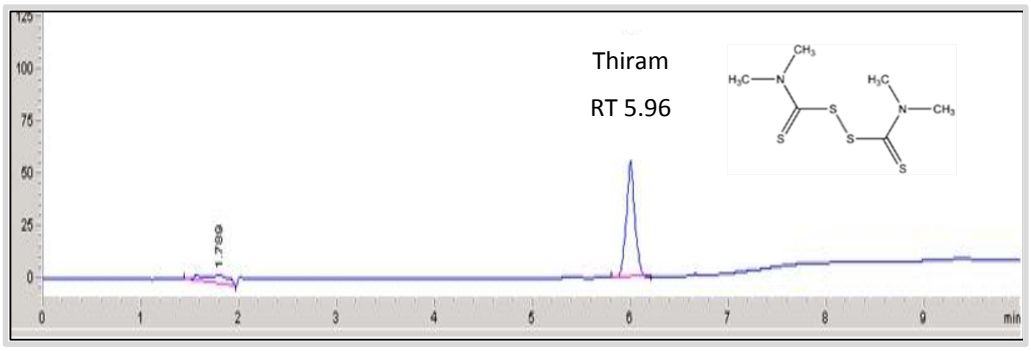


Figure 4. 27 Chromatogram of thiram separated by HPLC.

(HPLC with reverse phase column: ZORBAX SB-C18 incubated at 30°C and mobile phase; acetonitrile-water gradient, 30:70 for 1 minute and 90:10 for 10 minutes with flow rate of 1.0 mL/minute)

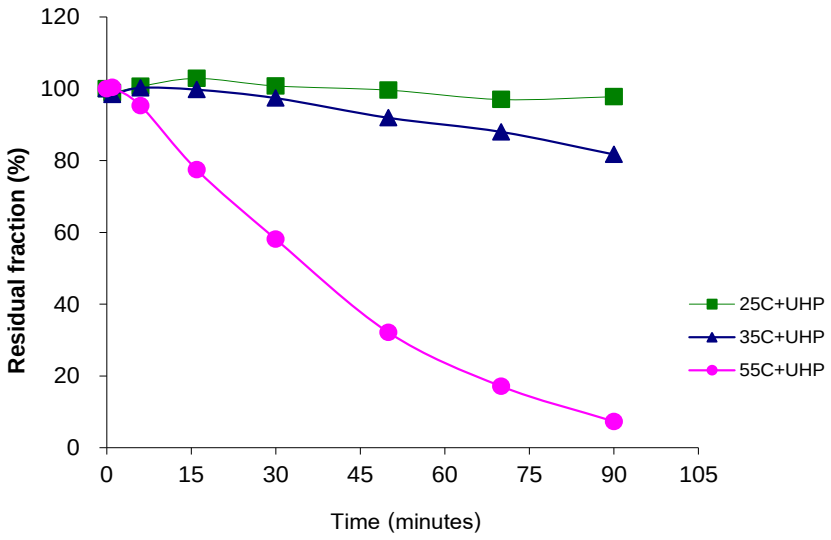


Figure 4. 28 Effect of peroxide and temperature on thiram degradation

(Thiram at a concentration of 64 μM was treated with 400 μM of UHP and incubated at 25°C, 35°C and 55°C for 90 minutes).

4.6.3 Effect of peroxide and peroxidase on thiram degradation

This experiment, 64 μM of thiram in the same buffer as above was treated with CSP in the presence of 400 μM of UHP and incubated at 25°C for 90 minutes. It was found that thiram in both solutions containing UHP in the presence and absence of CSP were decreased and the residual fraction retained approximately 20% of the initial concentration within 24 hour reaction time. The thiram in a control solution when neither CSP nor UHP was added was slightly decreased but its residual fraction retained 80%. Moreover, the remarkable peak of degraded product could not be observed as previous experiment. The effect of peroxide and peroxidase on thiram degradation was illustrated in **Figure 4.29.**

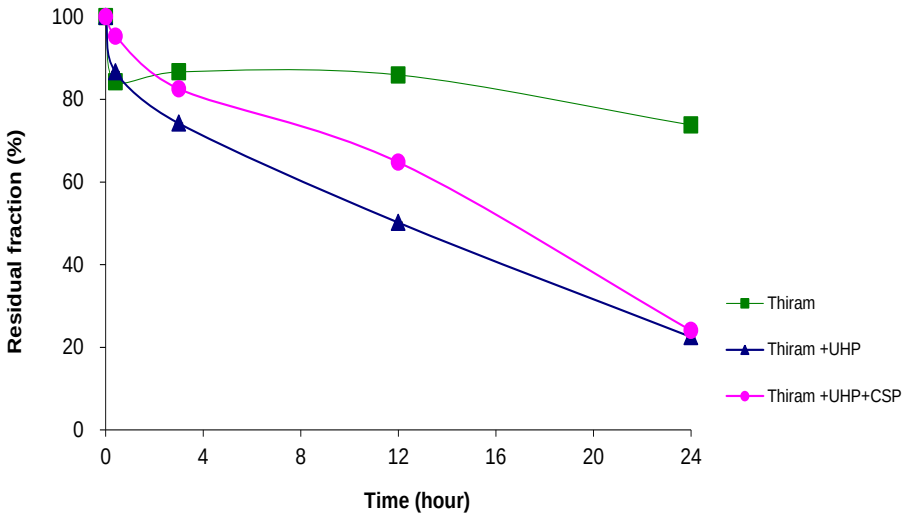


Figure 4. 29 Effect of peroxide and peroxidase on thiram degradation

(Thiram at a concentration of 64 μM was treated with CSP, 400 μM of UHP and incubated at 25°C for 24 hours).

4.7 Degradation of PCB-28 based on peroxide reaction

In the section, the capability of cassava peroxidase (CSP) as biocatalyst for PCB degradation was examined and compared to that of HRP. The degradation of PCB-28 (2, 4, 4′-Chlorinated biphenyl) which was selected as a target pollutant of trichlorobiphenyls under the peroxide reaction was studied.

4.7.1 Chromatographic separation of PCB-28 by HPLC

PCB-28 was effectively determined by reverse phase-HPLC with UV detector at the wavelength of 250 nm and it was eluted from the analytical column with the retention time of 7.61 minutes as shown in **Figure 4.30**.

4.7.2 Effect of peroxide and peroxidase on PCB-28 degradation

During 14 hours of incubation time, the residual PCB-28 obtained from peroxidase catalyzed reactions in 0.1 M phosphate buffer, pH 6 containing 5% acetonitrile in the absence of phenol was determined by HPLC. For CSP catalysis, PCB-28 was readily decreased to approximately 50 % of its initial concentration (560 ng/mL, 2.2 μM) within 6 hours in the reaction containing hydrogen peroxide (from UHP) (**Figure 4.31 a.1**). The degradation rate of PCB-28 in solution containing UHP catalyzed by HRP was slowly decreased, the residual PCB-28 was found to be 80% after 6 hours of incubation whereas 90% of residual PCB-28 in a control solution containing only UHP was still remained in the solution (**Figure 4.31 b.1**). Moreover, the peroxidase activities were also determined during the incubation time. CSP activity was dramatically decreased in a solution containing UHP with or without PCB-28. However, its

activity was continuously retained as its initial activity in a solution containing only CSP or in the presence of phenol (**Figure 4.31 a.2**). On the contrary, HRP activity in all reaction solutions was decreased during the time of incubation. In addition, its activity in a control solution containing only HRP was also decreased with a slower rate. Graphical plots of PCB-28 residual and peroxidase relative activities during 14 hours of incubation are shown in **Figure 4.31.**

4.7.3 Effect of phenol and peroxidase on PCB-28 degradation

In the presence of phenol, the degradation rate of PCB-28 in the solution containing UHP catalyzed by CSP was found to be 70% of the initial concentration which was slower than that solution in the absence of phenol after 6 hours of incubation (**Figure 4.31 a.1**). On the contrary, a solution catalyzed by HRP was found to be 60% but the degradation rate of PCB-28 in this solution was faster than the solution in the absence of phenol after 6 hours of incubation (**Figure 4.31 b.1**). Moreover, it can obviously be seen that HRP activity in the reaction solution containing PCB-28, UHP in the presence of phenol catalyzed by HRP was dramatically decreased within 0.1 hours of incubation as shown in **Figure 4.31 b.2**.

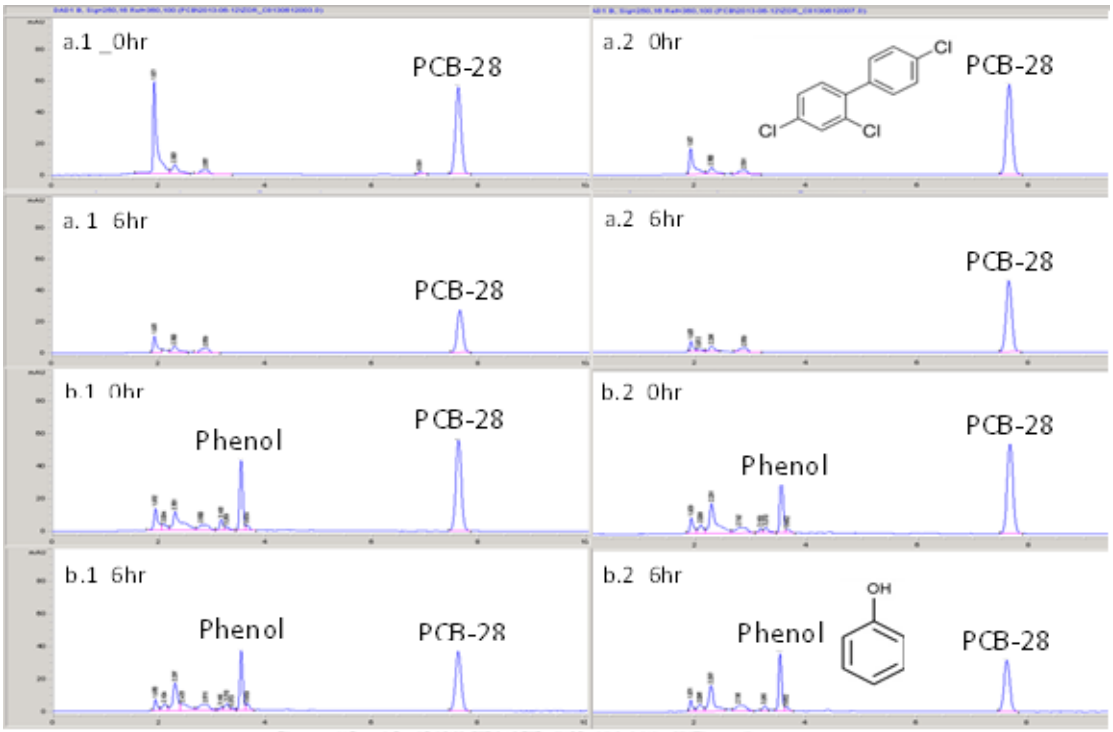


Figure 4. 30 Chromatogram of PCB-28 separation.

(HPLC with reverse phase column (ZORBAX SB-C18) under isocratic condition 60% acetonitrile, residual PCB-28 in the reaction solution containing 2.2 M PCB-28, 250 μM UHP and peroxidase, a.1) CSP solution without phenol addition, a.2) CSP solution with phenol addition, b.1) HRP solution without phenol addition, b.2) HRP solution with phenol addition, all reaction solutions were incubated for 0-6 hours).

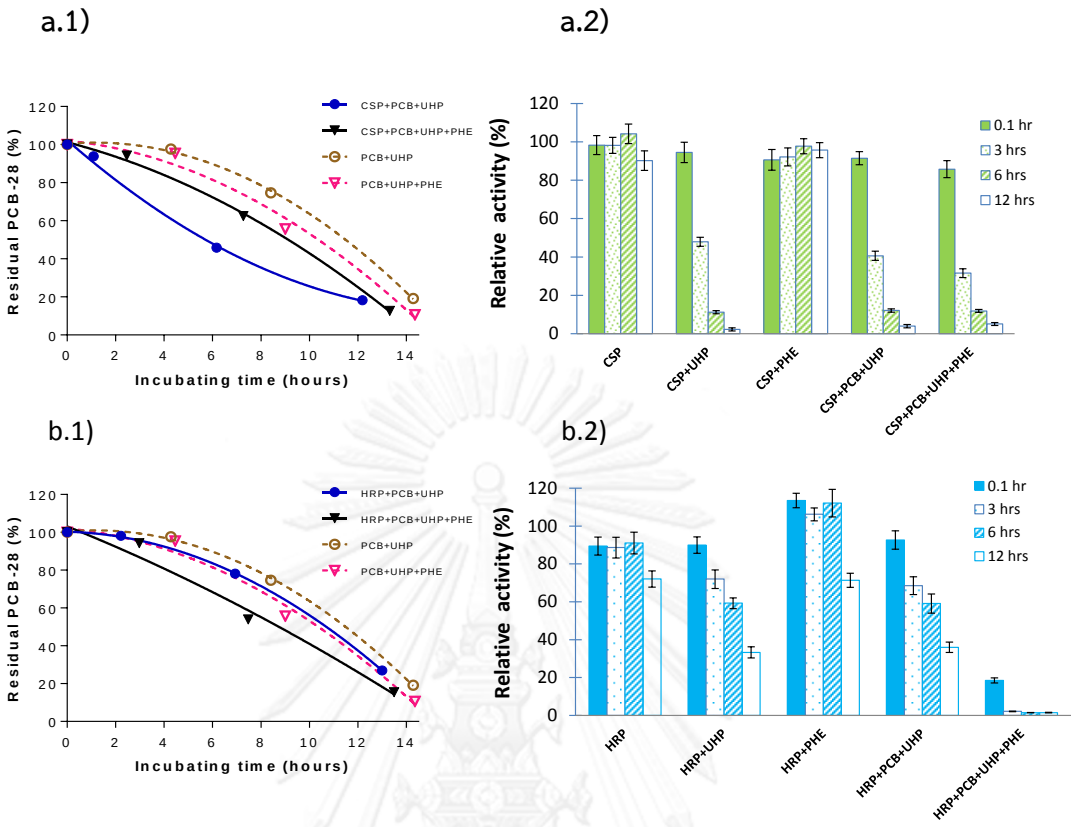


Figure 4. 31 Degradation of PCB-28 in 0.1 M phosphate buffer, pH 6 containing 5% acetonitrile at 25°C.

Residual PCB-28 in the reaction solution containing 2.2 M PCB-28, 250 μM UHP and peroxidase, a.1) CSP solution in the presence and absence of phenol, a.2) CSP activity in solutions during 24 hours, b.1) HRP solution in the presence and absence of phenol, b.2) CSP activity in solutions during 24 hours.

CHAPTER V
DISCUSSIONS

According to the objectives of this study, the characteristic of a novel peroxidase isolated from cassava leaves were investigated and its potential use for the detection of thiourea and thiram based on peroxidase inhibition assay was studied. For inhibition assay, the applicable uses of both spectrophotometric and electrochemical techniques for the measurement of peroxidase activity were carried out. In addition, the capability of cassava peroxidase (CSP) as a biocatalyst for thiram and PCB degradation was examined. In this chapter, these results were interpreted and discussed to providing the research findings.

5.1 Preparation of cassava peroxidase

Peroxidase was successfully extracted from cassava leaves as crude with approximately 190 units/g of leaf. After ammonium sulfate fractionation and DEAE-cellulose column chromatography, the unbound portion with peroxidase activity was observed as a major protein and it was further purified by Concanavalin-A affinity column which specifically binds molecules containing α-D-mannopyranosyl and α-D-glucopyranosyl residues (Miranda et al. 2002). The bound portion was desorbed with buffer containing α-D-glucopyranoside. After sequential purifications, CSP was purified to 15 folds with the overall protein yield of 4% and the specific activity was found to be 8,200 units/mg protein. Additionally, the prepared peroxidase was found to be a cationic glycoprotein.

5.2 Characteristics of cassava peroxidase

Comparative study of biochemical characteristics of both peroxidases (CSP and HRP) was investigated. Their RZ (Reinheitszahl) values which were the absorbance ratio of A_{403}/A_{275} were determined in deionized water. It is a measurement of heme and total protein content which is a conventional indication of peroxidase purity (Das et al. 2011). The Soret spectrum of CSP which are the maximum absorbances were found to be at 280 and 400 nm. They were the characteristics of heme protein and found to be the same as that of HRP (Welinder 1979, Welinder and Larsen 2004). However, the Soret maximum was slightly different between them and the RZ value of CSP was found to be approximately 0.6 whereas that of HRP 1.5, respectively. The purity and molecular weight (MW) of CSP obtained from bound Concanavalin-A column was determined by SDS-PAGE and MALDI-TOF. The purified CSP showed two protein bands on SDS-PAGE by Coomassie brilliant blue staining and the estimated molecular weight were found to be 38 and 44 kDa which were corresponded to the MALDI-TOF-MS results. The total ion chromatogram obtained from MALDI-TOF-MS showed that there were 2 forms of CSP with molecular masses of 37.84 kDa and 43.98 kDa. The MW of purified CSP obtained were closely similar to that of HRP (43 kDa). Moreover, it was almost similar to that of secretary peroxidase PX3 which was specially found in only cassava root during post-harvest deterioration and reported as 39 kDa (Reilly et al. 2007). The variation in isoenzyme composition of peroxidases was carried out by isoelectrofocusing technique with activity staining. CSP showed 4 bands at p*I* of 6.0, 6.4, 7.0 and 7.2 with peroxidase activity while on SDS gel electropholysis the enzyme showed only two bands. This result suggested that CSP had multiforms. For HRP, 5 isoforms with p*I* in the range of

5.2-8.2 were observed. The existence of isoform among peroxidases isolated from plants was commonly occurred and correlated with peroxidase glycosylation (Gobaldon et al. 2007).

5.2.1 Optimal pH and temperature

Due to the fact that the enzyme stability has been a key problem in biotechnology, peroxidases with specific properties to be able to work under a wide range of pH and temperature have been a target in many researches. The improvements of peroxidase properties by using techniques of modification (Feng et al. 2008, Hassani 2012, Khosravi et al. 2012) or mutation (Lehmann and Wyss 2001) of HRP for achieving an adequate thermostability have been continuously reported. Moreover, purifications and characterization of new peroxidases from various plant sources have also been vastly explored. Soybean peroxidase (SBP) is one of the attractive peroxidase alternatives. SBP was isolated form by-product of soybean seed hull and considered as inexpensive peroxidase catalyst for application such as in wastewater treatment (Bo´dalo et al. 2006, Genga et al. 2001, Steevensz et al. 2014). Artichoke peroxidase C (AKPC) is a basic enzyme purified form artichoke flowers which is widely grown in Mediterranean countries. AKPC was considerably more resistant to inactivation by pH and temperature (Sidrach et al. 2006). Other studies tried to investigate new peroxidases from leaves of economic plants such as *Jatropha cucas* which is a drought-resistant plant and its seed contain 40% viscous oil which is an important source of bio-diesel. Recently, purification of peroxidase form *Jatropha cucas* leaves (JcGP1) was reported. Its biochemical

characteristics were found to be stable under high temperature, extreme pH environment and organic solvent content (Feng et al. 2012). In this study, we also investigated peroxidase from leaves of cassava which is an economic plant in Thailand. Its leaves are considered as non-valuable parts of cassava. CSP obtained from cassava leaves was a heme-containing cationic glycoprotein with the Soret maximum at 400 nm (Jongmevasna et al. 2013). CSP showed its ability to work in a narrow pH range of 5-8 compared to other peroxidases. However, CSP is able to remarkably work in a wide range of temperature ranging from 5 to 70°C and still retained its activity more than 76%. The optimal temperature was in the range of 30-60°C which was comparable to that of AKPC. The most active peroxidase was JcGP1. It was found to be able to work in almost full range of pH from 3 to 12 and it can retain its activity more than 90% at temperature ranging from 55-75°C. Characteristics of some peroxidases from difference sources are summarized in **Table5.1**.

Table 5. 1 Characteristics of some peroxidases from difference sources.

5.2.2 Thermal stability of peroxidase

Several industrial applications of peroxidase such as wastewater treatment and phenolic synthesis need thermostable peroxidases in order to increase yields and cost-effective capability of their processes under high temperature was also required. Therefore, the thermal stability of CSP in the range of temperature from 5°C to 70°C was studied. The results showed that activities of both peroxidases in acetate-citric acid and Britton- Robinson buffers were dramatically decreased when the temperature was raised higher than 40°C and incubated for 4 hours. On the contrary, CSP still retained more than 75% of its activity in phosphate buffer for 2 hours at temperature ranging from 5°C to 70°C. In phosphate buffer, HRP relative activity seemed to be more stable at 55°C than that in other buffers. Nevertheless, HRP could not retain as high activity as that of CSP when the temperature was raised higher than 55°C. From these results, it can clearly be seen that phosphate buffer should be used as a suitable buffer system for peroxidase storage and for usage of enzyme at high temperature. This finding was in agreement with previous report that studied the effect of phosphate buffer on HRP thermal stability (Asad et al. 2011). It was found that the stability of HRP increased when the enzyme was incubated in 10 mM potassium phosphate buffer, pH 7 at 80°C. HRP half-life time in phosphate buffer was nearly 1.5 times of that in Tris-HCl buffer. However, there was another report that studied the effect of sodium phosphate buffer concentration on thermal stability of HRP. The result showed that the denaturation temperature decreased as sodium phosphate buffer concentration increased (Haifeng et al. 2008).

Due to the fact that a phosphate ion can be an exogenous ligand of heme protein, the proximal ligand is assumed to play an important role in controlling the chemistry and function of them. The majority of peroxidases utilize histidine as their proximal ligand (Sun et al. 2013) and (Gajhede et al. 1997). (Pond et al. 2002) reported that phosphate ion from the buffer could bind to ferric H175G cytochrome c peroxidase cavity mutant. Recent attention has focused on replacing the proximal ligand with a small residue such as glycine or alanine, thus forming an artificial cavity on the proximal side of the heme. Termed cavity mutants, these proteins have the unique ability to bind exogenous ligands in the cavity in order to reconstitute their wild-type activity. This approach was firstly applied for sperm whale myoglobin (Barrick 1994) and has since been accomplished for cytochrome c peroxidase (CCP) (McRee et al. 1994), heme oxygenase (Sun et al. 2013) and horseradish peroxidase (HRP) (Newmyer et al. 1996). Moreover, the effect of phosphate buffer on the kinetic catalysis of jack bean urease was studied (Krajewska and Zaborska 1999). It was found that at pH 5.80–7.49 the buffer was a competitive inhibitor and the true competitive inhibitor of urease is $H_2PO^-_4$ ion.

5.2.3 Working range and tolerance of peroxidase in solvent-aqueous solution

There are many advantages of using enzyme in organic solvent or aqueous solution containing organic solvents compared to traditional aqueous reaction systems. For industrial advantages, organic solvent reaction systems provide the increment of non-polar substrate solubility, suppression of water-

dependent side reactions, alteration of substrate specificity or enantiomer selectivity and elimination of microbial contamination (Iyer and Ananthanarayan 2008). In order to find out the potential ability and stability of CSP in aqueous-solvent mixed media, acetonitrile and methanol which are commonly used in a wide range of applications in chemical analysis were selected. Experiments using buffer solution containing acetonitrile or methanol in the concentration of 5-60% were investigated. It was found that CSP was able to work in a solution containing 25% acetonitrile and methanol with its relative activities approximately 65%. On the other hand, HRP relative activity in the solution containing 25% methanol found to retain approximately 80% which was more effective than its activity in 25% acetonitrile which retained only 50%. For stability testing, CSP presented its stability in a wide concentration range in both organic solvents when CSP was incubated in acetonitrile and methanol concentration ranging from 10 to 60% for 6 hours at 25°C, its relative activity was found to be higher than 75%. On the contrary, HRP was found to be stable in only methanol mixed aqueous solution in a narrow concentration range of 20-35%. This result suggested that the conformation of CSP protein structure was more resistant to the denaturation causing by hydrophilic solvent such as acetonitrile and methanol than that of HRP. The effect of solvents on enzyme deactivation is mostly caused by interruption of conformation flexibility and loss of crucial water on its surface. In general, the hydrophilic solvent possesses more ability to strip the essential water off enzyme molecules than other solvents with hydrophobic property (Doukyu and Ogino 2010). However, the effects of some organic solvents on

the activity/stability of various peroxidases have been reported. SBP exhibited its activity approximately 70% and 60% in the presence of acetone and methanol, respectively at the concentration of 33% in 10 mM sodium phosphate buffer, pH 6.0 (Genga et al. 2001). Moreover, JcGP1 was found to be stable against many organic solvents at the concentration up to 25%. After 54 hours of incubation, more than 95% relative activity was retained in the solution containing methanol, acetone, isopropanol, n-hexane, iso-octane, ethanol, acetonitrile, acetoacetate, glycerol, 1-butanol and n-heptane. Furthermore, incubation of the enzyme with acetoacetate and 1-butanol for 2 hours significantly activated JcGP1 activity with its relative activities in the range of 130-140% (Feng et al. 2012). For CSP, we found that its activity was increased up to 110-130% in a solution containing 10-50% of acetonitrile content within 3 hours of incubation. This enhancement may be described by the fact that acetonitrile could increase the solubility of a chromogenic substrate, TMB and/or interact with hydrophobic residue of CSP, resulting in a suitable conformation for its catalysis. In addition, acetonitrile tolerance of CSP might be related to natural cyanide content in cassava leaves. KU50 is a widely grown cultivar with high cyanogen content; fresh leaves containing approximately HCN 500 mg/kg dry mass (Hular-Bograd et al. 2011, Phuc et al. 2000). Therefore, peroxidase isolated from cassava leaf of cv. KU50 displayed unique characteristic for acetonitrile tolerance which might be caused by natural adaptation.

5.3 Kinetic catalysis of peroxidases

In this study, UHP and TMB were particularly useful substrates due to their advantage properties. As a certain mass of UHP can be obtained, it can provide a reproducible amount of hydrogen peroxide after dissolving in aqueous solution and TMB is an effective chromogen with high absorption coefficients and lacks mutagenicity and carcinogenicity (Bos et al. 1981). Peroxidases can catalyze the oxidation of TMB into two colored products. In excess of H_2O_2 or acidic condition, the blue colored product will be further oxidized to a yellow species. Due to the particular characteristic of TMB, it is practically useful for the investigation of peroxidase activity in both non-stopped and stopped reaction with acid addition. The kinetic characterization of CSP for the oxidation of UHP and TMB showed the pattern of Lineweaver-Burk plots which followed the ping-pong mechanism (Copeland 2000) as HRP catalysis (Banci 1997). As the effect of substrate concentrations on CSP initial rate was carried out, Michaelis-Menten constant ($^{app}K_M$) and maximum velocity (V_{max}) were calculated and showed that $^{app}K_M$ values for UHP of CSP were found to be 100±10 while that of HRP was 420±30 μM. This indicated that CSP had higher affinity towards UHP as a primary substrate than HRP. Additionally, they also showed higher affinity towards TMB which was a secondary substrate with the lower $^{app}K_M$ values of 80 for CSP. Therefore, CSP with its beneficial characteristic for catalysis could be an interesting choice as a new alternative peroxidase.

5.4 Spectrophotometric method for peroxidase inhibition assay

In preliminary test, the spectrophotometric method was developed in order to investigate the potential use of peroxidase inhibition assay for the detection of

target inhibitors. In experiment, we attempted to use UHP as a source of hydrogen peroxide and TMB as chromogenic substrate. As TMB can generate the catalyzed product with high extinction coefficient, it is currently considered as a sensitive chromogen for monitoring peroxidase activity by colorimetric or spectrophotometric method. Moreover, thiourea which is a strong inhibitor of peroxidase was used as a model for peroxidase inhibition study. Therefore, the effects of thiourea on CSP and HRP were investigated and detection ranges of the purposed inhibition assay by spectrophotometric measurement were carried out. The procedure was performed in microtiter plates and the intensity of enzyme catalyzed product was measured by the microtiter plate reader. This method can provide accurate results of data sets because the reaction can be performed under the same condition and same reaction time.

5.4.1 Inhibition mechanism of thiourea towards peroxidase activity

The inhibition types of inhibitor towards the enzyme activity can be defined based on the patterns of Lineweaver-Burk plot (Copeland 2000). According to the increment of thiourea concentrations, it could affect ${}^{app}K_M$ values but could not affect the V_{max}, thiourea was thus considered as a competitive inhibitor for the oxidation of TMB catalyzed by both of CSP and HRP. Moreover, thiourea was shown to be a noncompetitive inhibitor of SBP and the inhibitory effect of some thiol compounds were reported in (Jongmevasna et al. 2013). This work suggested that thiourea (THU) and other thiol pesticides such as thiosemicarbazide (TSC), thiophanate-methyl (TPM) and ethylenethiourea (ETU) elucidated their inhibitory effects on CSP, HRP and SBP

activities. The inhibitory effect of these thiols on peroxidase activity could occur by the influence of amino ($-NH_2$) group and sulfide anion (S^{2-}) with appropriate position to access into an enzyme molecule. Amino groups were effective electron donors of TMB oxidation catalyzed by HRP which was described by (Misono et al. 1997). Therefore, amino group of TSC showed higher ability than THU to compete with that of TMB for the reduction of compound I and II containing an oxyferryl center and a porphyrin radical. In addition, the sulfide anion (S^{2-}) of thiols was an effective residue which reacted with heme porphyrin in the active site (Adeyoju et al. 1995).

5.4.2 Dose response curve of thiourea towards peroxidase activity

In many biological assays, a plot of the signal response as a function of the concentration of exogenous substance that describes the change in signal with changing concentration of substance is known as a dose response curve. The plot can be used to follow the inhibitor effect on the initial velocity of an enzymatic reaction at a fixed concentration of substrate. The concentration of inhibitor required to achieve a half-maximal degree of inhibition is referred to the IC_{50} value (Copeland 2000). In the case of THU, it was found to be an effective inhibitor of HRP rather than CSP. Therefore, these preliminary results indicated a good perspective for the practical use of CSP for the detection of some thiols and other related compounds.

5.5 Electrochemical method for peroxidase inhibition assay

The applicable uses of electrochemical measurement for enzyme inhibition

assays have been reported in many literatures. They focused on the development of inhibitor-based sensors as a tool for the determination of contaminants and pollutants in food and environmental samples. For examples, the detection of malathion and 2, 4-dichlorophenoxyacetic acid (2, 4-D) based on alkaline phosphatase (ALP) inhibition with the amperometric measurement and the limit of detection (LOD) 0.5-6 μg/L was defined (Mazzei et al. 2004). Also the biomonitoring of methomyl pesticide by laccase inhibition and square-wave voltammetry with the LOD of 0.235 μM was reported (Zapp et al. 2011). In addition, the screen printed electrode immobilized with peroxidase was developed for amperometric sulfide detection by using inhibition based enzyme detector.

5.5.1 Screen-printed electrodes (SPEs) used for electrochemical measurement

In this study, a batch of SPEs with approximately 400 pieces was homemade fabricated by semi automatically screen printer. The repeatability of electrochemical measurements obtained from prepared SPEs was found to be acceptable.

5.5.2 Electrochemical characteristic of 3, 3′-diaminobenzidine (DAB)

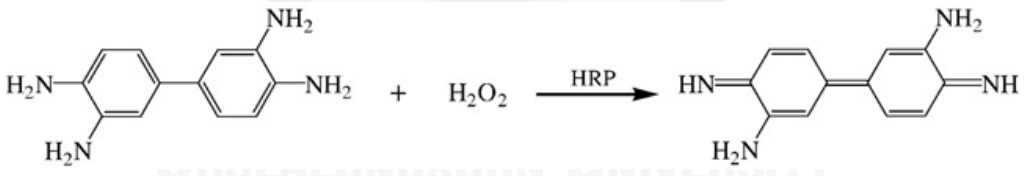
DAB was considered as a peroxidase secondary substrate with electrochemical property, it was used as a mediator for monitoring peroxidase activity by electrochemical measurements. Basically, DAB was used as a peroxidase substrate in both monomeric chromogen or mediator and also electroactive polymer modified on electrode surface. There were

some reports proposed that the oxidative polymerization of 3, 3′-diaminobenzidine (DAB) on gold electrode could form a stable complex selectively with SE(IV) in test solutions (Nateghi et al. 2005). The determination was based on the selective accumulation of Se (IV) by anodic stripping voltammetry. Other report suggested that phenol can be bound onto an electrode surface thus a polymerization process takes place on the modified electrode surface. The binding of phenol on the electrode surface enables us to determine phenol at very low concentrations. Phenol determination is very difficult in natural samples due to its dissolution mechanism in water. Voltammetric techniques require a small amount of sample volume and selective for the determine phenol and its derivatives. This method could be applied for the detection of phenol and chlorophenol content in tap water samples (Mülazımoğlu et al. 2011).

5.5.2.1 Cyclic voltammetry (CV) of DAB

The electroactive product obtained from the oxidation of DAB in the presence of HRP-H_2O_2 system in pH 5.0 BR buffer solution was 4, 4′-diimino-bicyclohexylidene-2, 5, 2′, 5′-tetraene-3, 3′-diamine. (Zhang et al. 2008) proposed the method for the detection of carcinoembryonic antigen (CEA) in human serum based on a new voltammetric enzyme immunoassay system and DAB was firstly used as substrate for HRP catalysis reaction. The process of the HRP-catalyzed oxidation reaction of DAB by H_2O_2 is shown in **Figure 5.1**. They reported that DAB catalyzed product produced a sensitive voltammetric peak at a potential of -0.62 V (vs. SCE) in BR buffer solution. In

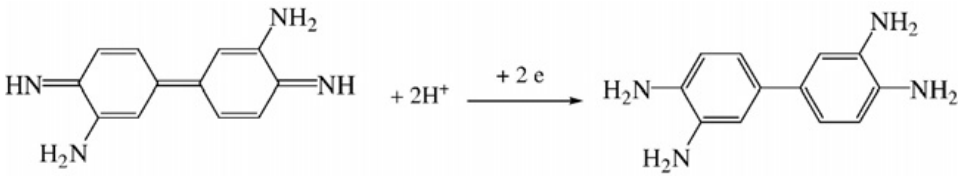
addition, DAB as an electroactive polymer has also been used for electrode surface modification such as the oxidative polymerization of 3, 3′-diaminobenzidine (DAB) on gold electrode to form a stable complex selectively with SE(IV) in test solutions (Nateghi et al. 2005). They reported that E_{pox} (DAB) was found to be at potential of +0.335 V (vs. Ag/AgCl) in $H_2O/HClO_4$ solution (pH = 3.51). The E_{pox} of DAB at potential of +0.36 V (vs. Ag/AgCl) obtained from our result was in agreement with their result which they suggested that it was the potential for the oxidation of DAB to be DAB^{+}. Moreover, they also suggested that CVs of DAB films deposited on gold electrode displayed a redox pair with oxidation and reduction potentials at negative potentials more than -0.20 V. However, our result obtained from CV showed that the other oxidation peak of DAB at the potential of -0.19 V was found in only the reaction solution containing sulfuric acid as a stop solution, the oxidation peak might be represented the further oxidation of DAB^{+} to be DAB^{+2}.



a. 3, 3′-diaminobenzidine b. DAB electroactive product

Figure 5. 1 **The process of the HRP-catalyzed oxidation reaction of DAB by H_2O_2 in BR buffer pH 3.5.**

a.) 3, 3′-diaminobenzidine and b.) 4, 4′-diimino-bicyclohexylidene-2, 5, 2′, 5′-tetraene-3, 3′-diamine (DAB electroactive product) (Zhang et al. 2008).



a. DAB electroactive product b. DAB

Figure 5. 2 **The reduction of the HRP-catalyzed oxidation reaction of DAB with a two-electron transfer process in BR buffer solution.**

a.) 3, 3-diaminobenzidine and b.) 4, 4′-diimino-bicyclohexylidene-2, 5, 2′, 5′-tetraene-3, 3′-diamine (DAB electroactive product) (Zhang et al. 2008).

5.5.2.2 Square - wave voltammetry (SWV) of DAB

According to our procedure for SWV experiment, DAB was successfully used as a mediator for monitoring peroxidase activity by electrochemical measurement obtained from SPE. The ratio of DAB concentration and current density illustrated the linear relationship between them with R^2 = 0.992 in the range of DAB concentration (31-1,000 μM). At the concentration of peroxidase substrates (500 μM of DAB and 100 μM of UHP), the reductive current after 3 minutes of the reaction catalyzed by CSP could be generated at a current density of 1.92 μA/cm^2. Although the current density of DAB obtained from SWV under our condition was lower than the current density 7.14 μA/Cm^2 of phenol and 59.5 μA/cm^2 of o-phenilendiamine catalyzed by HRP - H_2O_2 reaction detected with graphite-epoxy composite (GCE) ((Kergaravat et al. 2012). The working area of electrode used in the method was smaller. it was only 0.21 cm^2 and the reaction was measured after 3 minutes of incubation. However,

the current density of our system can be improved by the increment of H_2O_2 in the system in order to reach the optimal ratio of DAB and H_2O_2 for CSP catalysis.

5.5.3 Detection of thiourea by SWV measurement and peroxidase inhibition

From the result of the double-reciprocal plot of substrate concentration versus current density, the difference of both lines in the presence and absence of 100 μM thiourea was clearly observed via their slopes. The higher slope found in the presence of thiourea, the lower current density obtained from the reaction. It indicated that the electrochemical measurement under our condition could be used to study the inhibitory effect of thiourea on peroxidase for the catalysis of DAB oxidation in the presence of UHP. Unfortunately, we did not design the electrochemical measurement to monitor the initial velocity of peroxidase-catalyzed reaction. Basically, the SWV could be used for the determination of kinetic parameters as previously described by (Kergaravat et al. 2012). The evaluation of electrochemical characteristics of seven cosubstrates for HRP catalysis was performed using SWV technique. If the SWV was performed as an interval of 10s to monitor the inhibitory effect of thiourea on the initial velocity of the DAB oxidation, then the type of inhibition could be classified and compared to the results from spectrophotometric measurement.

For dose response curves of thiourea inhibition toward CSP and HRP activity for the catalysis of DAB oxidation, SWV measurement could detect the inhibitory effect when the concentration of thiourea was higher than 10 μM. From the semi-log plot, thiourea showed it ability to be a strong inhibitor for HRP than CSP. HRP activity was dramatically decreased in the presence of thiourea with the IC_{50} of 40±8 μM whereas IC_{50} of CSP was found to be 100±8 μM. The inhibition curve could be used for the detection of thiourea in the range of 10-100 μM. Although three inhibition curves were constructed with 3 different concentrations of DAB in the range of 62-250 μM, the tendency of thiourea inhibitory effect on peroxidase seem to be almost the same. It indicated that the degree of thiourea inhibitory effect on both peroxidases did not depend on concentration of DAB in that range. Moreover, its detectable concentration range of thiourea obtained from electrochemical measurement was found to be lower than thiourea detection range (25-500 μM) based on the inhibition of TMB oxidation with HRP and UHP catalysis using spectrophotometric measurement. However, there was another report suggested that amperometric measurement of HRP electrode using 1,1- dimethylferrocence (DMFc) as electron transfer mediator could detect thiourea in 1.5 % (v/v) methanol solution in the range of 0.05-1.0 mM (Adeyoju et al. 1995). In principle, the degree of inhibition of peroxidase activity depends on types and chemical structure of cosubustrate and inhibitors (Metelitza et al. 2004).

5.5.4 Detection of thiram by SWV measurement and peroxidase inhibition

For electrochemical measurement, it indicated that the proposed method has a potency to be used for the detection of thiol compounds based on peroxidase inhibition assay and SWV measurement using screen-printed electrode and PalmSens. According to the linear inverse relationship between the percentage of relative CSP activity and log concentration of thiram in the reaction, thiram could be detected in the concentration range of 5-100 μM. The linear of inhibition lines obtained from the reaction containing different concentrations of DAB were not significantly different among them. This might indicate the inhibitory effect of thiram CSP did not depend on concentration of DAB in that range. On the contrary, the inhibition of thiram on HRP activity was observed as a higher degree than its effect on CSP activity, the thiram IC_{50} for HRP (16±4 μM) was less than that of CSP (50±8 μM). Moreover, the inhibitory effect of thiram on HRP activity seems to be dependent on DAB concentration and it demonstrated the relationship as a non-linear curve which could be used for the detection of thiram in the concentration range of 5-100 μM.

5.6 Degradation of thiram based on peroxide reaction

This section, we tried to investigate the degraded products of thiram treated with peroxide under high temperature and peroxidase catalysis. The result showed that the amount of thiram was decreased when it was incubated at 55°C in the presence of urea hydrogen peroxide and nearly disappeared within 90 minutes. It indicated that thiram was sensitive to temperature. Moreover, it was easily and

directly degraded in the presence of only hydrogen peroxide without the addition of peroxidase. Therefore, the function of peroxidase as biocatalyst for thiram degradation was slightly observed. From the literature review of thiram degradation, applications and analytical methods (Sharma et al. 2003), thiram degrades more rapidly in acidic soils and the major metabolites are copper dimethyl - dithiocartbarmate, dimethylamine and carbon disulfide. In water, it is rapidly broken down by hydrolysis and photodegradation, especially under acidic condition. In addition, (Horsfall 1989) was the first to propose that the fungicide effect of thiram was connected with their ability to form complexes with heavy metal ions; Fe, Zn, Cu, Mn and Mo.

5.7 Degradation of PCB-28 based on peroxide reaction

Polychlorinated biphenyl (PCB) is a large group of stable organic chemicals containing up to 209 compounds called congeners with different degrees of chlorination. Due to the fact that it is in the priority list of persistent organic pollutants (POPs) for eventual elimination by 2025, technologies associated with the utilizations of plants, bacteria, white rot fungi and enzymes such as aromatic dehydrogenase, cytochrome 450, peroxygenases, peroxidases including lignin and horseradish peroxidase have been conducted on PCBs bioremediation (Aken et al. 2010, Borja et al. 2005, Seeger et al. 2010). In this study, the potency of cassava peroxidase for the enhancement of PCB-28 (2, 4, 4′-trichlorobiphenyl) degradation in peroxidase catalyzed reactions with and without phenol was performed and compared to that of HRP.

5.7.1 Effect of peroxide, phenol and peroxidase on PCB-28 degradation

During 6 hours of incubation, it was clearly observed that CSP displayed catalysis activity for the enhancement of PCB-28 degradation with a higher rate than HRP catalysis the reaction in the absence of phenol. On the contrary, the degradation rate of PCB-28 catalyzed by CSP seems to be inhibited in the presence of phenol. In case of HRP catalysis, the degradation rate of PCB-28 in the presence of phenol was higher than that in the absence of phenol. These findings indicated that there were some differences between CSP and HRP catalysis. In addition, it was found that PCB-28 can also be degraded without the involvement of any peroxidases and PCB-28 degradation rate in the control solution was found to be higher than that in the absence of phenol. The result indicated that PCB-28 could directly be degraded by hydrogen peroxide and phenol as non-enzymatic reaction. Phenol and substituted phenols can undergo extensive oxidative coupling and eventual polymerization in natural systems via reactions catalyzed by naturally occurring extracellular enzymes and mineral oxides (Bollag 1992, Stone and Morgan 1984). Phenoxy radicals that remain soluble can continue to serve as substrates for further oxidative coupling, eventually forming larger polymers that precipitate from solution (Nicell 1994, Wu et al. 1999). Certain nonphenolic contaminants such as PCBs and PAHs may be incorporated into the polymeric products by adsorption and/or covalent bonding (Walter et al. 2003).

5.7.2 The degraded product of PCB-28 degradation

Moreover, we expected to investigate the degraded products from reactions such as dechlorinated and hydroxylated products in the sample solution containing buffer and 5% acetonitrile by HPLC. According to previous study (Köller et al. 2000), they investigated the mechanism of PCB-9 (2, 5-dichlorobiphenyl) and PCB-52 (2, 2′, 5, 5′-tetrachlorobipenyl) degradation catalyzed by HRP/H_2O_2 mixture, they suggested that the main steps in PCB degradation by peroxidase is dechlorination and hydroxylation. After 220 minutes of reaction time, the residual PCB-9 was found to be 10% of initial amount (5 μM). For PCB-52, due to PCB-52 consists of higher chlorine content than PCB-9, the degradation rate was found to be slower and PCB-52 was decreased to 45% of initial concentration (0.15 μM). Moreover, the degradation products were identified by using GC-MS. The degradation of PCB-9 and PCB-52 generated the less chlorinated PCB and unchlorinated 1, 1′-biphenyl as well as hydroxylated PCBs, hydroxylatebiphenyls and aromatic acid such as dichlorobenzoic acids and benzoic acid. However, the degraded products of PCB-28 did not seem to be detected under our experimental condition. The HPLC with C-18 reversed-phase system and isocratic 60% acetonitrile mobile phase was used for determination of residual PCB-28 and its degraded products in buffer solutions. Basically, PCBs can be separated by reversed-phase HPLC system based on the difference in theirs hydrophobic properties. The sequential separation of PCBs mixed solution containing trichloro-, tetrachloro-, pentachloro- and hexachlorobiphenyl was reported that PCBs with lower chlorine content can be eluted easier than those

containing higher chlorine content (Olšovská et al. 2010). In addition, the HPLC system was applied for the determination of hydroxylated forms of PCB-3 and PCB-15 as hydroquinones and quinones (Amaro et al. 1996, Qun et al. 2013). The HPLC spectra showed that peaks of hydroquinones which are metabolite of PCB-3 and PCB-15 and quinones, oxidized forms of hydroquinones were typically detected in front of their PCB parent's peaks. As the result of chromatograms, there were no remarkable peaks found before the peak of PCB-28 (RT 7.6 minutes) in both CSP and HRP catalyzed reaction which contained UHP with and without the addition of phenol for 6 hours. This could be implied that the degraded products as pollutant forms; dechlorinated and hydroxylated PCB and quinones in our experimental solution, were not detected. Beside this, it might the cause of the degraded products were successively degraded or oxidized to other forms which could not be detected by UV at the wavelength of 240-250 nm.

CHAPTER VI
CONCLUSIONS

1. Peroxidase was successfully extracted from cassava leaves as crude with approximately 190 units/g of leaf. After purification by DEAE cellulose and Concanavalin A column chromatography, it was concluded that it was a cationic glycoprotein cassava peroxidase (CSP). The overall protein yield was 4% with 15 folds of purification and the specific activity was found to be 8,200 units/mg protein.

2. CSP from cassava leaves showed its unique characteristics which were overcome those of HRP. It was considered as thermo tolerant peroxidase which was stable at 70°C for 2 hours in phosphate buffer, pH 6 and able to work in a wide range of temperature from 5-70°C. Moreover, it was an organic solvent- tolerant enzyme. It was stable in acetonitrile or methanol mixed aqueous solution in the range of 10-60% for 6 hours.

3. Peroxidase-catalyzed reaction under experimentally designed condition had a potential for the detection of thiourea and thiram based on enzyme inhibition assay by using both spectrophotometric and electrochemical measurements. Furthermore, the CSP could be an alternative peroxidase with the potency in the chemical analysis applications.

4. Homemade screen-printed electrode cooperated with PalmSens was considered as a small set of portable electrochemical device which was effectively used for monitoring peroxidase activity. The square wave voltammetric (SWV) technique was a sensitive measurement for providing reductive current obtained from the reduction of 3, 3′-diaminobenzidine (DAB) product catalyzed by peroxidase in a well of microtiter plate. As the method consisted of a set of portable electrochemical devices, an adequate sensitivity of electrochemical measurement under experimentally designed condition and small amount of sample needed, the proposed method had a potency to be a useful tool in chemical analysis which could be available outside laboratory.

5. The potential use of peroxidase and screen-printed electrode for the detection of thiol in solution by voltammetric technique based on peroxidase inhibition assay could be achieved. The proposed method could be used for the detection of thiourea and thiram in the concentration range of 10-100 μM.

6. Capability of CSP as a biocatalyst for the enhancement of thiram and PCB-28 degradation by peroxide reaction was found to be satisfactory. For thiram, it was easily and directly degraded in the presence of only hydrogen peroxide. Therefore, the function of peroxidase as a biocatalyst for thiram degradation was slightly observed. On the contrary, PCB-28 degradation in the reaction catalyzed by CSP, the concentration of 560 ng/mL (2.2 μM) was readily decreased to approximately 50 % within 6 hours in the presence of hydrogen peroxide (from UHP). Therefore, the reaction could be applied for the enhancement of some

related toxic substances or pollutants degradation in the aspect of bioremediation.

7. CSP isolated from cassava leaves shows its attractive characteristics and it will be interesting if further study focuses on the development of the proposed method for the determination of target pollutants in the samples and much attention should be paid in the direct electron transfer of a novel peroxidase which might be useful in the area of electrochemical and biosensor applications.

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APPENDIX

APPENDIX A

Preparation for polyacrylamide gel electrophoresis

30% Acrylamide, 0.8% bis-acrylamide, 100 mL

Acrylamide	29.2 g
N, N´–methylene-bis-acrylamide	0.8 g
Adjusted volume to 100 mL with distilled water	

1 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethame	6.06 g

Adjusted pH to 6.8 with 1M HCl and adjusted volume to 100 mL with distilled water

Solution B (SDS PAGE)

2 M Tris-HCl pH 8.8	75 mL
10% SDS	4 mL
Distilled water	21 mL

Solution C (SDS PAGE)

1 M Tris-HCl pH 6.8	50 mL
10% SDS	4 mL
Distilled water	46 mL

Coomassie Gel Stain, 1 litre

Coomassie Blue R-250	1.0 g
Methanol	450 mL
Distilled water	450 mL
Glacial acetic acid	100 Ml

Coomassie Gel Destain, 1 litre

Methanol	100 mL
Glacial acetic acid	100 mL
Distilled water	800 mL

SDS-PAGE

12.5% separating gel

30% acrylamide solution	4.2 mL
Solution B	2.5 mL
Distilled water	3.3 mL
10% $(NH_4)_2S_2O_8$	50 μL
TEMED	10 μL

5.0% stacking gel

30% acrylamide solution	0.67 mL
Solution C	1.0 mL
Distilled water	2.3 mL
10% $(NH_4)_2S_2O_8$	30 μL
TEMED	5.0 μL

Sample buffer

1 M Tris-HCl pH 6.8	0.6 mL
50% glycerol	5.0 mL
10% SDS	2.0 mL
2-mercaptoethanol	0.5 mL
1% bromophenol blue	1.0 mL
Distilled water	0.9 mL

One part of sample buffer was added to four parts of sample. The mixture was heated 5 minutes in boiling water before loading to the gel.

Electrophoresis buffer, 1 litre

Tris (hydroxymethyl)-aminomethane	3.0 g
Glycine	14.4 g
SDS	1.0 g

Adjusted volume to 1 litre with distilled water
(pH should be approximately 8.3).

APPENDIX B

Preparation of buffers

M Sodium acetate/citric acid buffer pH 5 (4x)

Citric acid	210.14 g
Sodium acetate	82.0 g
Add distilled water to make total volume	500 mL

50 mM PBS pH 6.5, 0.2 M NaCl

NaCl	2.34 g
Na_2HPO_4	0.87 g
NaH_2PO_4	0.88 g
Add distilled water to make total volume	250 mL

50 mM PBS pH 6.5, 0.5 M NaCl

Nacl	5.84 g
Na_2HPO_4	0.70 g
NaH_2PO_4	0.70 g
Add distilled water to make total volume	200 mL

0.1 M Sodium acetate/citric acid buffer pH 5 (4x)

Citric acid	210.14 g
Sodium acetate	82.0 g
Add distilled water to make total volume	500 mL

0.1 M Britton-Robinson buffer pH 6 (4x)

0.4 M Boric acid	2.47 g
0.4 M Phophoric acid , 85% wt in water	2.40 mL
0.4 M Acetic acid	2.40 g
Add 0.2 M sodium hydroxide for pH adjustment	
Add distilled water to make total volume	250 mL

Substrate solution

3, 3;, 5, 5; Tetramethylbenzidine (TMB)	1.0 mg
Dimethylsulfoxide (DMSO)	100 μL
Urea hydrogen peroxide	1.0 mg
0.1 M Sodium acetate/citric acid buffer pH 5	10 mL

Note: 1.0 mg was first dissolved in DMSO, and then diluted with 0.1 M Sodium acetate/citric acid buffer pH 5. It has to mix gently and thoroughly and wait for 10 minutes before adding to urea hydrogen. Moreover, the substrate solution must be fresh preparation before using.

Stop solution:

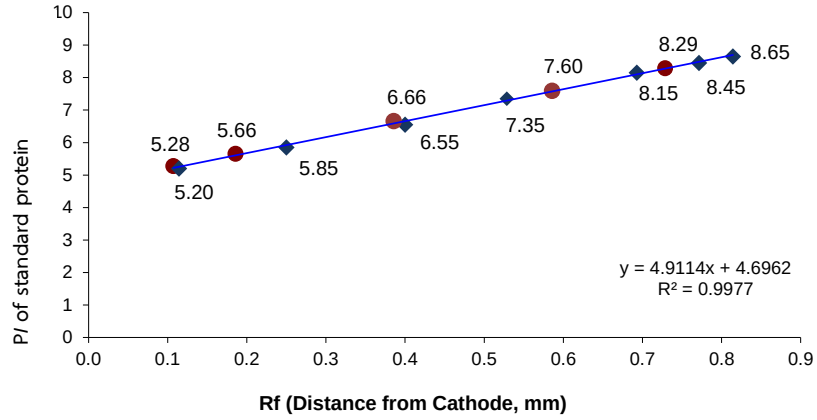
2 M Sulfuric acid

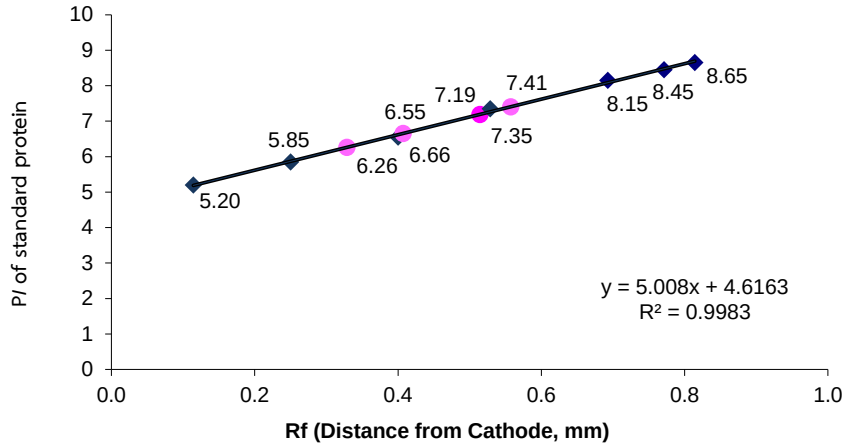
Distilled water	22.3 mL
Conc. Sulfuric acid	2.7 mL
Total volume	25.0 mL

(Note: Add sulfuric acid into distilled water)

APPENDIX C

Calibration curve for p*I* determination of peroxidases





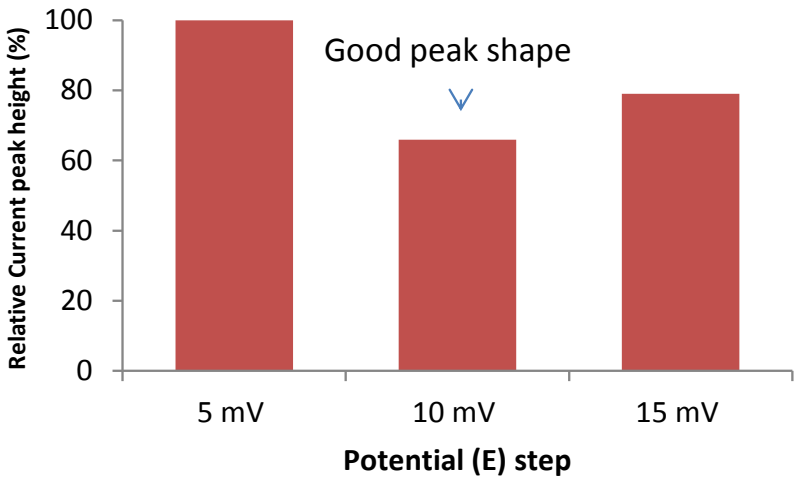
Standard p*I*	Distance from cathode (mm)	Rf
5.2	8.0	0.114
5.85	17.5	0.250
6.55	28.0	0.400
7.35	37.0	0.529
8.15	48.5	0.693
8.45	54.0	0.771
8.65	57.0	0.814

APPENDIX D

Optimization of square wave voltammetry (SWV)

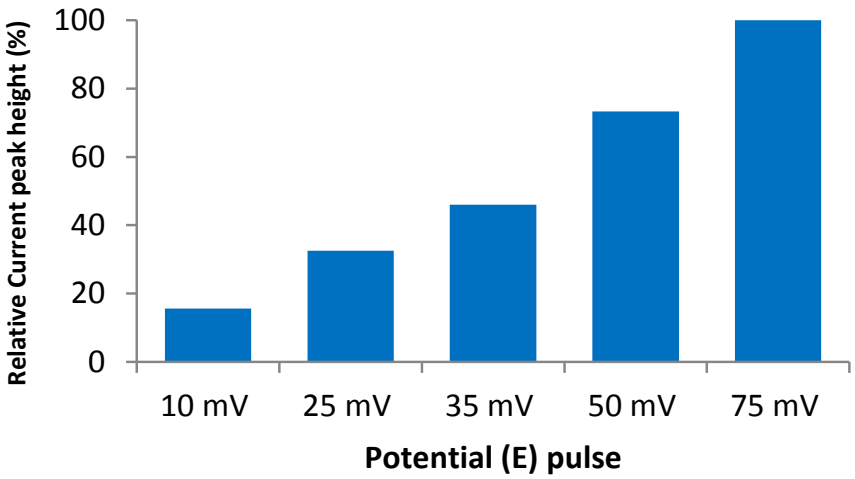
Reaction: HRP solution in 0.04 Britton-Robinson buffer containing 250 μM DAB and 250 μM UHP, the reaction was stopped after 3 minutes of incubation by the addition of 2 M sulfuric acid as a stop solution.

1. Effect of potential (E) step



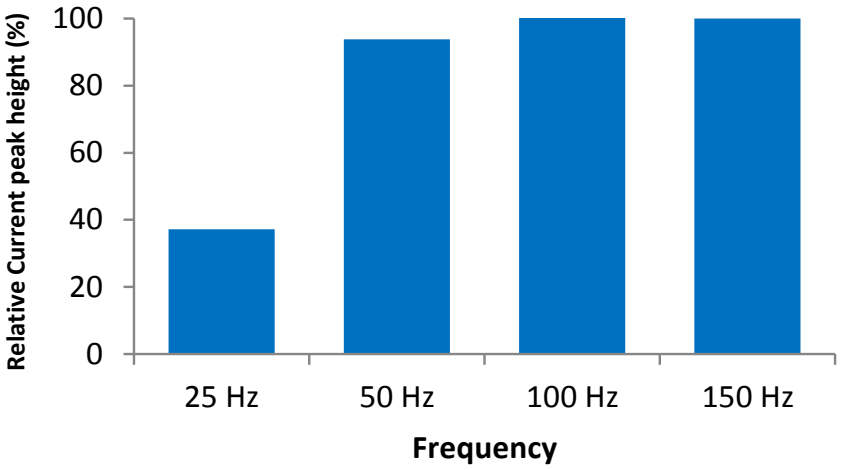
E step	Relative current peak height (%)	Current peak height (nA)
5 mV	100.0	253
10 mV	66.0	167
15 mV	79.0	200

2. Effect of potential (E) pulse at the E step: 10 mV



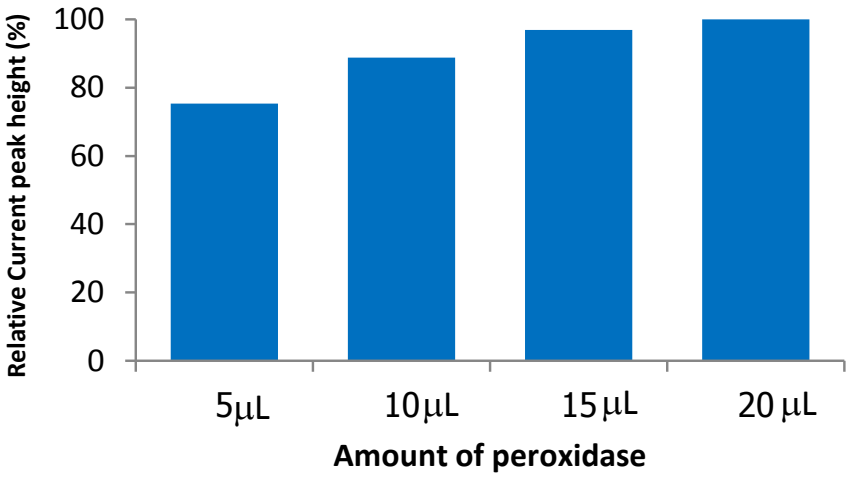
E pulse	Relative current peak height (%)	Current peak height (nA)
10 mV	15.6	155
25 mV	32.6	324
35 mV	46.0	458
50 mV	73.3	729
75 mV	100.0	995

3. Effect of frequency at the E step: 10 mV and the E pulse: 75 mV



Frequency	Relative current peak height (%)	Current peak height (nA)
25 Hz	37.2	162
50 Hz	93.8	408
100 Hz	129.9	565
150 Hz	100.0	435

4. Effect of amount of peroxidase at the E step: 10 mV, the E pulse: 75 mV and frequency 50Hz



Amount of POD µL	Relative current peak height (%)	Current peak height (nA)
5	75.3	956
10	88.8	1127
15	96.9	1230
20	100.0	1270

VITA

Mrs. Wischada Jongmevasna was born on December 10, 1967, at Tak Province. In 1989, she got the Bachelor degree of Science (biology) from Chiang Mai University. After that she had two-year experience in fruit canning manufactory. Since1991, she has been a medical scientist at Division of Food, Department of Medical Sciences. After she has experience in the field of pesticide residues analysis for 7 years, she continued to study the master degree in Biotechnology Program at Chulalongkorn University and graduated in 1999. Nowadays, she works for the Bureau of Quality and Safety of Food, Department of Medical Sciences. As her interesting area related in new technologies and method developments for the determination of contaminants in food, the progress in electrochemical technologies and biosensors become popular, she decided to study the doctoral degree in Biotechnology Program at Chulalongkorn University and work in this area since 2009.