CHARACTERIZATION OF PEROXIDASE FROM CASSAVA LEAVES

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT

OF THE REQUIREMENTS FOR

THE DEGREE OF MASTER OF SCIENCE (BIOCHEMISTRY)

FACULTY OF GRADUATE STUDIES

MAHIDOL UNIVERSITY

2000

ISBN 974-664-247-2

Thesis

entitled

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CHARACTERIZATION OF PEROXIDASE FROM CASSAVA LEAVES

was submitted to the Faculty of Graduate Studies, Mahidol University

for the degree of Master of Science (Biochemistry)

on

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ACKNOWLEDGEMENT

I wish to express my deepest appreciation and sincere gratitude to my advisers, for his understanding, helpfulness, kindness and encouragement throughout this study. I am most thankful for the time he spared me during this thesis. I am also most grateful to Prof. Dr. M.R. Jisnuson Svasti and Dr. Nuanchawee Wetprasit for their valuable suggestions and comments.

I am very grateful to Ms. Panpaka Chalermisrachai and Mr. Thakorn Sornwatana for teaching me the experimental techniques and for their help throughout this work.

I would like to thank the Chulaporn Research Institute for the analysis amino acid composition of the enzyme.

My special thanks to Mrs. Thitika Vajrodaya for her excellent typing and her kind understanding.

I am very grateful to all members in the Department of Biochemistry, especially Ms. Suwatchara Leelamaneepong and the member in room B. 303, for their wonderful friendships and their help during my study.

My success would have been impossible without my parents and members in my family for their unlimited love, understanding, support and encouragement which will never be forgotten.

Kanjana Suriyaprom

4136783 SCBC/M : MAJOR : BIOCHEMISTRY ; M. Sc (BIOCHEMISTRY)
KEY WORD : PEROXIDASE, CASSAVA
KANJANA SURIYAPROM : CHARACTERIZATION OF PEROXIDASE FROM CASSAVA LEAVES. THESIS ADVISOR : MONTRI CHULAVATNATOL, Ph. D., M.R. JISNUSON SVASTI, Ph. D., NUANCHAWEE WETPRASIT, Ph.D. 94 p. ISBN 974-664-247-2

Peroxidase (EC 1.11.1.7) is an ubiquitous plant enyzyme that catalyzes the oxidation of cellular components by H_2O_2. A peroxidase was purified from the leaves of cassava (*Manihot esculenta* Crantz.) 74 folds to a specific activity of 386 U/mg. The purification procedure consisted of 60-80% ammonium sulfate precipitation, followed by affinity chromatography using a concanavalin A Sepharose 4B column and gel filtration chromatography using a Sephadex G-200 column. The native molecular weight for the enzyme was found to be 112 kD by gel filtration and the subunit molecular weight was estimated to be 56 kD by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. So the enzyme was a homodimer. The isoelectric point of the purified enzyme was estimated by polyacrylamide isoelectrofocusing. It existed in two forms with pI values of 6.4 and 6.25. The enzyme contained a higher amount of the amino acids (GLX +ASX) than the basic amino acids. It was shown to be heme proteins with a Soret band at 404 nm. The enzyme was stable in a broad pH range of 4-11 and had a slightly acidic optimum pH of 6. An optimul temperature of the enzyme activity was 60°C. The enzyme retained about 70% of its activity during incubation at temperature upto 65°C for 24 hr. The cassava leaf peroxidase catalyzed the oxidation of the following substrates: coniferyl alcohol (K_m = 0.003 mM), o-dianisidine (K_m = 0.037 mM), quercetin (K_m = 0.054 mM), syringaldazine (K_m = 0.077 mM), 3,3'-diaminobenzidine (K_m = 0.022 mM), pyrogallol (K_m = 0.89 mM) and guaiacol (K_m = 5.52 mM). KCN, NaN_3 and thiourea were inhibitory to the enzyme.

4136783 SCBC/M: สาขาวิชา : ชีวเคมี; วท. ม. (ชีวเคมี)

กาญจนา สุริยะพรหม : การศึกษาคุณสมบัติของเปอร์ออกสิเดสในใบมันสำปะหลัง (CHARACTERIZATION OF PEROXIDASE FROM CASSAVA LEAVES) คณะกรรมการควบคุมวิทยานิพนธ์ : มนตรี จุฬาวัฒนกล, Ph. D., ม.ร.ว. ชิษณุสรร สวัสดิวัตน์, Ph. D., นวลฉวี เวชประสิทธิ์, Ph. D. 94 หน้า. ISBN974-664-247-2

เปอร์ออกซิเดสเป็นเอ็นไซม์ที่พบได้ทั่วไปในพืช ซึ่งจะเร่งปฏิกิริยาออกซิเดชั่นของส่วนประกอบต่างๆในเซลล์ โดยใช้ไฮโดรเจนเปอร์ออกไซด์ร่วมด้วย ในการศึกษาได้ทำการสกัดเอ็นไซม์เปอร์ออกซิเดสจากส่วนใบของต้นมันสำปะหลังและผ่านกระบวนการทำให้บริสุทธิ์ขึ้น 74 เท่า และมี specific activity เท่ากับ 386 U/mg ซึ่งประกอบด้วยการตกตะกอนด้วยเกลือแอมโมเนียมซัลเฟตที่ 60-80% อิ่มตัวตามด้วย affinity คอลัมน์โดยใช้ concanavalin A และ เจล ฟิลเตรชั่น (gel filtration) บน คอลัมน์เซฟฟาเด็กซ์ จี-200 (Sephaedx G-200). จากผลการวิเคราะห์โดย SDS-PAGE พบว่า ได้แถบของโปรตีนในตำแหน่ง น้ำหนักโมเลกุลประมาณ 56 กิโลดาลตัน และ จากคอลัมน์เซฟฟาเด็กซ์ พบว่า เอ็นไซม์นี้มีน้ำหนักโมเลกุล ประมาณ 112 กิโลดาลตัน ดังนั้น จึงสรุปได้ว่าโมเลกุลของเอ็นไซม์นี้ประกอบด้วย 2 หน่วยย่อย จากการวิเคราะห์โดย polyacrylamide isoelectrophoresis พบว่าเอ็นไซม์นี้มี 2 รูปแบบซึ่งมีค่า pI เท่ากับ 6.4 และ 6.25 เอ็นไซม์เปอร์ออกซิเดสนี้ประกอบด้วยปริมาณของกรดอะมิโน GLX+ASX มากกว่าปริมาณของกรดอะมิโนที่เป็นด่าง ในการศึกษาหมู่โปรสเธติก พบว่า เอ็นไซม์นี้มี ฮีมเป็นส่วนประกอบ เนื่องจาก พบ Soret band ในสเปคตรัมของเอ็นไซม์ที่ 404 นาโนเมตร เอ็นไซม์นี้มีความทนทานต่อ pH ในช่วงที่กว้าง คือ 4 ถึง 11 และสามารถทนอุณหภูมิได้สูงถึง 65 องศาเซลเซียส ซึ่งที่อุณหภูมินี้เอ็นไซม์จะมีแอกติวิตีเหลืออยู่ประมาณ 70% เอนไซม์มี pH และอุณหภูมิที่เหมาะสมต่อการทำงานของเอ็นไซม์คือ pH เท่ากับ 6 และที่อุณหภูมิ 60 องศาเซลเซียส เอ็นไซม์เปอร์ออกซิเดสจากใบมันสำปะหลังสามารถเร่งปฏิกิริยาออกซิเดชั่นได้ดังต่อไปนี้ coniferyl alcohol (K_m = 0.003 mM), o-dianisidine (K_m = 0.037 mM), quercetin (K_m = 0.054 mM), syringaldazine (K_m = 0.077 mM), 3,3'-diaminobenzidine (K_m = 0.022 mM), pyrogallol (K_m = 0.89 mM) และ guaiacol (K_m = 5.52 mM) KCN, NaN_3 และ thiourea เป็นตัวยับยั้งแอกติวิตีของเอ็นไซม์เปอร์ออกซิเดสได้

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LIST OF ABBREVIATIONS

%	percentage
°C	degree celsius
µg	microgram
µl	microlitre
A	Absorbance
A. araucana	*Araucaria araucana*
APX	ascorbate peroxidase
BLP1	Barley leaf peroxidase
BSA	bovine serum albumin
cm	centimeter
Con A	Concanavalin A Sepharose 4B
CRP	Carrot root peroxidase
DAB	3,3'-diaminobenzidine
DMSO	Dimethylsulfoxide
DNP-lysine	dinitrophenyl-lysine
g	gravitational acceleration
gm	gram
hr	hour
HRP	horseradish peroxidase
JRP	Japanese-radish peroxidase
Kav	partition coefficient
kD	kilodalton
K_m	Michaelis-Menten constant

MeOH	methanol
mg	milligram
min	minute
ml	milliliter
mM	millimolar
Mr	relative molecular weight
nd	not determined
ND-PAGE	Non-denaturing polyacrylamide
nm	nanometer
OD	optical density
pI	Isoelectric point
PITC	phenylisothiocyanate
PMSF	phenyl methyl sulfonyl fluoride
POD	peroxidase
PP	Potato peroxidase
PVPP	polyvinyl polypyrrolidone
PXP_3	Poplar xylem peroxidase
Rf	relative mobility
SDS	sodium dodecyl sulfate
TEA	triethylamine
TEMED	N, N, N',N'-tetramethyl ethylenediamine
TP	Turnip peroxidase
Tris	Tris(hydroxymethyl)aminomethane
V	voltage

v/v	volume by volume
V_{max}	maximum velocity
w/v	weight by volume

CHAPTER I

INTRODUCTION

1.1 Cassava

Cassava (*Manihot esculenta* Crantz.) (Fig. 1) is a member in family Euphobiaceae. It is a perennial shrub growing up to about 4 m tall, and forms storage roots or tubers. The storage roots vary in shape, size, and number, usually 5-10 roots per plant. Stems and branches are predominantly brownish or greyish. Leaves are thick and 5-10 leaves form a finger-like arrangement (1).

Cassava is an economic crop in Thailand. It was brought into Thailand from Malaysia. So it was first planted in the South of Thailand. It is called in Thai "Mun Mai", "Mun Samrong" and "Mun Sampalang". Cassava is called yaca in Spanish, mandioca in Protuguese and manioc in French. This shrubby plant is thought to have originated in tropical America, from where it spread to other parts of the world (2). In Thailand presently, the major cassava-planting areas are in the east and northeast.

Cassava seems like an ideal crop for tropical region because it has a number of good characteristics. Firstly, it can be grown through out the year because it has no critical period for propagation and harvesting. Secondly, it can be cultivated using seeds, tubers or stem cuttings. Thirdly, it grows well in both arid and wet climate conditions, producing storage roots even in poor soil. Fourthly, it is relatively resistant to insects and fungi and gives a high yield per unit area. It requires minimal attention during growth and so it is relatively inexpensive to cultivate. Planting consists simply of clearing the ground and inserting into it 30 cm long pieces of the stems with several

Fig. 1 Cassava (*Manihot esculenta* Crantz.)

auxillary buds. Each plant produces several storage roots which can be harvested in about 18 months or left in the ground until needed (3). Cassava roots have about 34% dry matter of which 74 to 85% is starch. Its amylose content is of 13 to 28% and its protein content is low (4).

Worldwide, 65% of cassava is used for human consumption, 20% for animal feed and the remaining 15% for starch and industrial uses such as production of alcohol, sorbitol, sweetening agent, monosodium glutamate, powder, pelletized medicine, paper, textiles. For human consumption, the storage roots are peeled and chopped and then boiled, steamed, fried or roasted. Production of fresh storage roots from cassava in Asia is about 50 million tons per year which represents about on third of world production. A main producer is Thailand which produces about 21 million tons per year. Thailand exported cassava to Europe starting in the 1960s. The exported cassava pellets serves as a component of animal feed. Because of population growth, cassava production for human consumption, especially in Africa, is steadily increasing. In Asia, however, production remains rather stable during the last decade. It appears that when average income in society increases, the relative importance of cassava for human consumption decreases. Cassava may become more important as a feedstuff when the use of storage roots in local animal feed receives more attention. Production of cassava in Thailand and Indonesia for the feedstuff market of western Europe is expected to remain quite important (1).

1.2 Peroxidases

Peroxidases (donor: H_2O_2 oxidoreductase; EC.1.11.1.7) belong to a large family of enzymes capable of oxidizing the oxidation of several different substrates in the presence of H_2O_2. Higher plants have several peroxidase isozymes. These isozymes are differentially expressed in various tissues and organs and respond to environmental conditions. According to current classification, the plant peroxidases are divided into two types ascorbate peroxidases and classical peroxidases (5).

1.2.1 Ascorbate peroxidase (APX) (EC 1.11.1.11)

Plants have APX to rid themselves of excess hydrogen peroxide under normal and stress conditions such as extreme salinity and sandy soil. Reactive oxygen species (ROS), including superoxide ($^{\circ}O^{-}$), H_2O_2, hydroxyl radical ($^{\circ}OH$) which 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 denaturing (6). To prevent the damage, plant cells are equipped with an antioxidative system consisting of low-molecular-weight antioxidants such as ascobate, glutathione and protective enzymes, ascorbate peroxidase (7). APX has a high preference for ascorbate as an electron donor. The enzyme catalyzes the reaction:

$$2 \text{ ascorbate} + H_2O_2 \longrightarrow 2 \text{ monodehydroascorbate} + 2H_2O \quad (8)$$

Cellular regeneration of ascorbate is accomplished through the direct reduction of monodehydroascorbate (MDA) to ascorbate by MDA reductase (MDAR) using NADH as the electron donor (Fig. 2) (9-10).

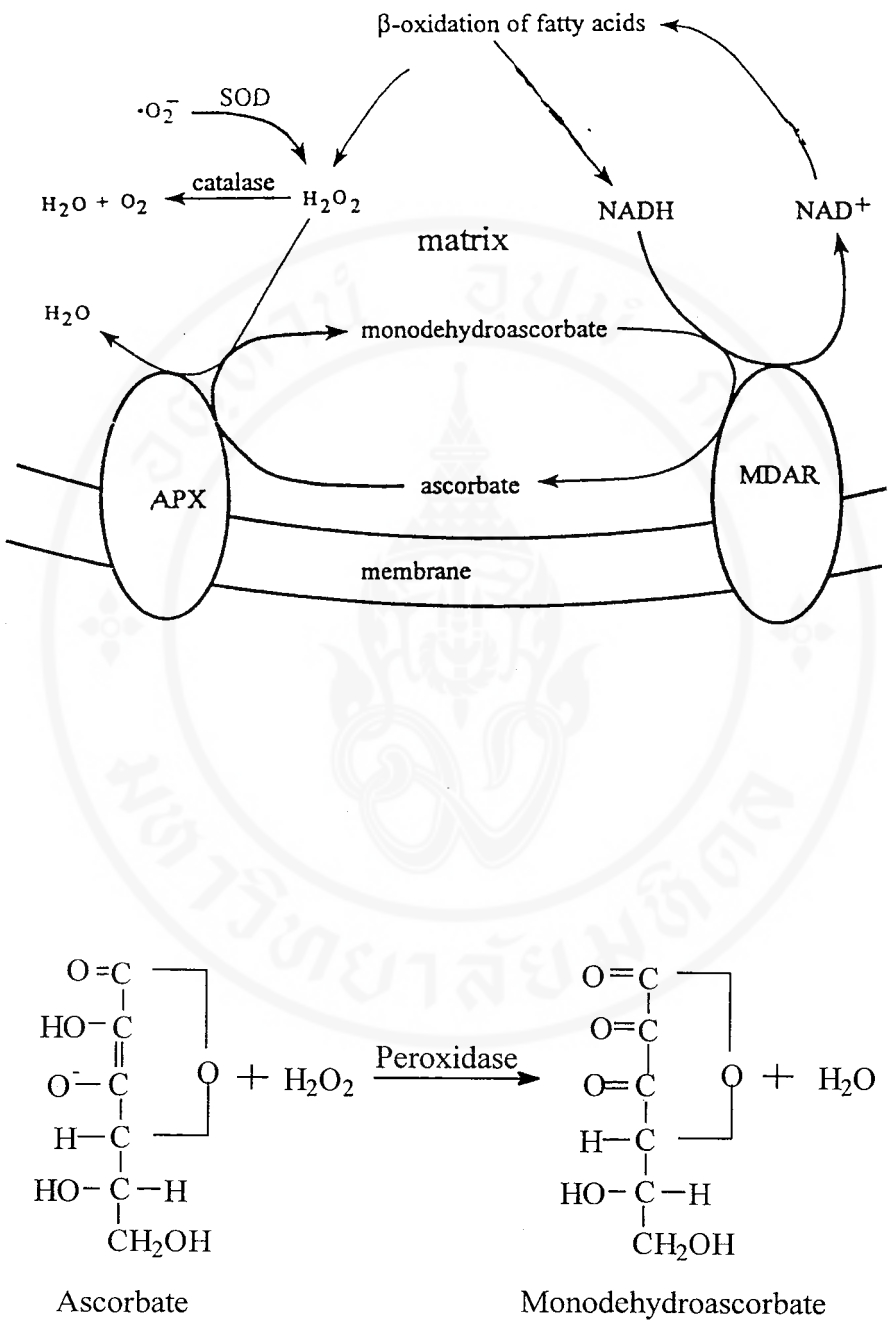


Fig. 2 Proposed model illustrating H_2O_2 scavenging under excess H_2O_2 or stress condition (10)

There are two types of APX in plants (11), one of which is associated with the chloroplasts while the other is localized in non-photosynthetic tissues (cytosolic isozyme). However, the chloroplastic and cytosolic isozymes of ascorbate peroxidase differ from each other as follows (12). a) The chloroplastic isozyme has a very short life time in an ascorbate-depleted medium; b) the sensitivities of the chloroplastic isozyme to inhibitors such as thiol reagents ,p-chloromercurisulfonic acid, p-hydroxymercuribenzoate are higher than the cytosolic isozyme; c) the chloroplastic isozyme is more specific to ascorbate as the electron donor than the cytosolic isozyme. For example, the cytosolic isozyme can oxidize pyrogallol at an appreciable rate; and d) the chloroplastic isozymes has a narrow pH optimum.

APX is distinct from guaiacol peroxidase with respect to the properties shown in Table 1 (13).

Examples of ascorbate peroxidases are:

1. Pea cytosolic ascorbate peroxidase is homodimer with molecular weight of 57,500, composed of two subunits with molecular weight of 29,500. The enzyme have a high stability, a broader pH optimum for activity and the capacity to utilize alternate electron donor such as pyrogallol , guaiacol (8).

2. Ascorbate peroxidase in roots of Japanese radish is monomeric with molecular mass of 28 kD and was stabilized by ascorbate. The enzyme has a narrow optimum around pH 6. It cannot use guaiacol, 3,3'-diaminobenzidine, pyrocatechol. The enzyme is labile in absence of ascorbate (14).

Table 1. Comparison between ascorbate peroxidases and classical peroxidases

Properties	Ascorbate peroxidases	Classical peroxidases
Glycoprotein	No	Yes
Heme	Yes/No	Yes
Ca^{2+} binding	No	Yes
Substrate	Ascorbate	Guaiacol or phenolic compounds: DAB, o-dianisidine, pyrogallol, coniferyl alcohol
Inhibition by thiol reagents	Yes (12)	No (12)
Physiological roles	To scavenge excess H_2O_2 formed in plant cell under normal and stress conditions	Biosynthetic process (18): - lignin polymerization (17) - pathogen defense - wound-healing - suberization - involved in the biosynthesis and polymerization of extensin - phenol oxidation - the oxidative deterioration of vegetables

Summarized from (13) and other sources as indicated.

3. Ascorbate peroxidase in potato tubers is labile in the absence of ascorbate. It has a molecular weight of 30 kD, a pH optimum of ~7. Ascorbate is specifically required as the electron donor. The enzyme is inhibited by thiol reagents (15).

1.2.2 Classical peroxidase (guaiacol peroxidase)

Classical plant peroxidases can be distinguished by their nonspecific use of phenolic derivatives and their involvement in polymerizing reactions (16). Guaiacol peroxidase is distinct from APX as shown in Table 1.

Examples of classical peroxidase are:

1. Horseradish peroxidase (HRP) EC (1.11.1.7) is the well-known classical peroxidase . HRP consists of more than 30 multiforms (19) which can be usually classified with 3 groups: acidic, neutral and basic isozymes by their isoelectric points. HRP is found mainly in the roots of horseradish. It is a hemoprotein catalyzing the oxidation by H_2O_2 of a number of substrates. It has a molecular weight of 40 kD and reversibly inhibited by 5-10 M cyanide and sulfide (20).

HRP is glycoprotein. The glycan of the peroxidase is important for the enzyme activity and protein stability (21). As a lyophilized dry powder, it may be stored for several years in a refrigerated condition. In an aqueous solution, it can maintain its activity for over a year at 4°C (22). Because of its good stability, ease of assay and low molecular weight, HRP has been widely used as a marker in enzyme immunoassays and histochemical studies.

2. Peroxidase in vacuoles of pea (*Vicia faba*) leaves is a basic peroxidase with molecular weight of 49 kD. It is hemoprotein and has a pH optimum at pH 5. Cyanide and azide inhibit its activity. The phenolics such as quercetin, kaempferol

and rutin have been found in the vacuoles of *Vicia faba* leaves and they can be oxidized by the peroxidase in vacuoles in the presence of H_2O_2 (23).

3. Three different classes of primary cell wall structural proteins (24) in plants are hydroxyproline-rich glycoproteins (extensins), proline-rich glycoproteins and glycine-rich proteins. Extensins are synthesised as soluble precursors but are immobilized in the cell wall by a hydrogen peroxide/peroxidase-mediated process (25). Enhanced deposition and cross-linking of extensins in the plant cell wall lead to a more resistant barrier against pathogen infection. Induction or inhibition of extensins deposition can result in increased or decreased pathogen resistance respectively (22).

4. Lignin peroxidases

Lignin is a polymer of phenylpropane units (monolignol precursors), such as p-coumaryl, coniferyl, and sinapyl alcohols (26). The lignin synthesis, a phenolic hydrogen atom is removed from coniferyl alcohol enzymatically (Fig. 3). The phenoxy radical thus formed is in resonating state among several structures, and a radical reacts with another radical to form lignin.

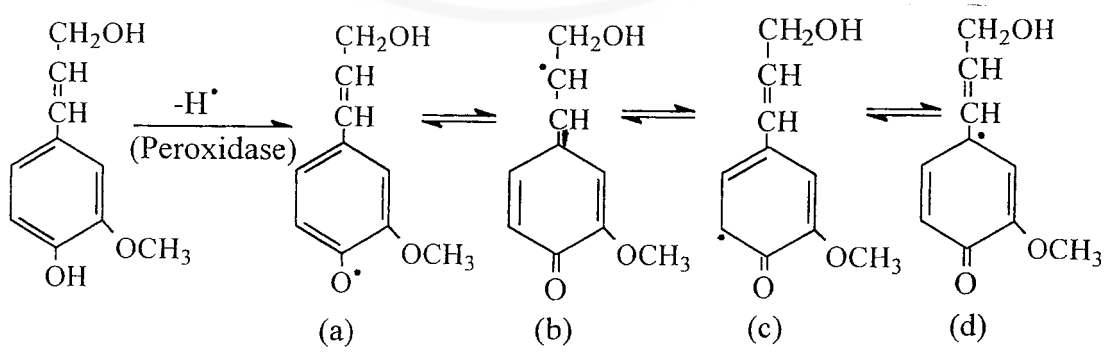


Fig. 3. Scheme of dehydrogenative polymerization products of coniferyl alcohol (27).

(a) - (d) are the resonant forms.

Peroxidase participates in the biogenesis of lignin. The enzyme can convert coniferyl alcohol into a lignin-like material which is called the dehydrogenative polymerization product (DHP) (26).

- In bamboo shoot, peroxidase participaties in the formation of DHP from coniferyl alcohol. The peroxidatic reaction can be inhibited by KCN while the polymerization step to form lignin is not. These result suggests that H_2O_2 and peroxidase are essential for dehydrogenation of coniferyl alcohol (26). The bamboo peroxidase has a heme group as its prosthetic group.

- Cell wall-associated peroxidase from needles of Norway Spruce (*Picea abies* L.) plays a role in lignification. It catalyzes the reaction between coniferyl alcohol and H_2O_2. The wall of lignifying cells contains a higher peroxidase activity than the wall of non-lignifying or dormant tissues (28-29). The net oxidation of confieryl alcohol by cell wall-bound peroxidase from spruce needles is partially inhibited in the presence of reduced ascorbate (30). The work by Takahama and Oniki has provided evidence that ascorbate inhibits the peroxidase-mediated oxidation of coniferyl alcohol by reducing the radical products of the reaction catalyzed by peroxidase (31) (Fig. 4).

- In Poplar xylem (PXP), peroxidase isozymes (PXP 3-4 and PXP 5) can oxidize a lignin monomer analog syringaldazine (SYR). The pI values of PXP 3-4 are 3.43, 3.53 and that of PXP 5 is about 3.73. Their molecular weights are between 46-48.5 kD and all isozymes are glycosylated (32).

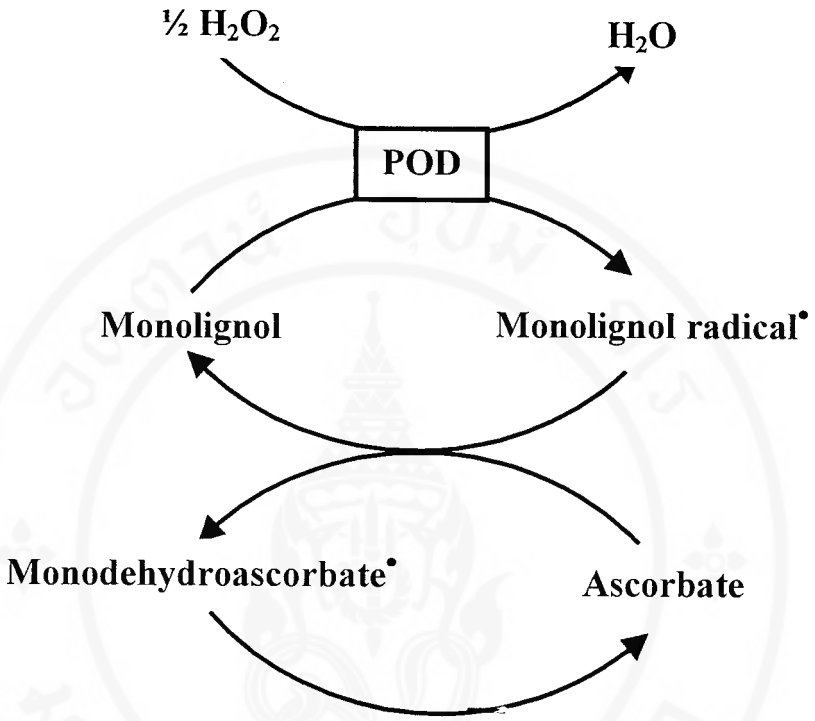


Fig. 4 The monolignol-coupled oxidation of ascorbate by peroxidase (30)

- An anionic peroxidase is associated with the suberization response in potato tubers during wound healing. It is a 45 kD peroxidase that is localized to suberizing tissues. The poly(aromatic) of suberized cell walls is polymerized via a peroxidase/H_2O_2-mediated process. Ascorbate is a very poor substrate (33).

5. In post-harvest deterioration, the activity of peroxidase isozyme A from the parenchyma of cassava (*Mahihot esculenta* Crantz.) increases. It has a pI value of 6.8 and its molecular weight is about 105 kD. It is a dimer, consisting of 54 kD subunits. It is a hemoprotein, sodium azide and potassium cyanide can inhibit its activity (34).

6. In Hevea bark, peroxidase activity is induced by wounding from tapping injury. The enzyme can convert wound-induced latex phenols into phenolic polymers. Thus the Hevea bark peroxidase may have an opposing effect on the latex flow (35).

1.3 Peroxidase reactions

Peroxidase catalyzes the oxidation of a electron donor (AH) using hydrogen peroxide as the hydrogen accepter. The stable oxidation product (A) is produced by the reaction. The overall reaction mechanism is shown below (36).

$$\text{Native Enzyme} + H_2O_2 \longrightarrow \text{Compound I}$$

$$\text{Compound I} + AH \longrightarrow \text{Compound II} + A$$

$$\text{Compound II} + AH \longrightarrow \text{Native Enzyme} + A + 2H_2O$$

$$\text{Sum:} \quad H_2O_2 + 2\,AH \longrightarrow 2A + 2H_2O$$

Oxidation of the substrate (AH) involves the activation of ferric peroxidase by H_2O_2. In the process, two electrons are removed from the native enzyme, producing ferryl (IV) porphyrin cation radical (or protein radical) of the enzyme known as compound I. Compound I is an active form of enzyme. It is unstable and has a characteristic green color. It can oxidize organic compounds (AH) and itself is reduced by an electron to compound II. Compound II is also an active form. It is quite stable and has a characteristic red color. A subsequent one-electron oxidation returns the peroxidase to its ferric state. The excess of H_2O_2 converts compound II into compound III. Compound III is known as oxyperoxidase which is an inactive form of the enzyme as shown the following equation.

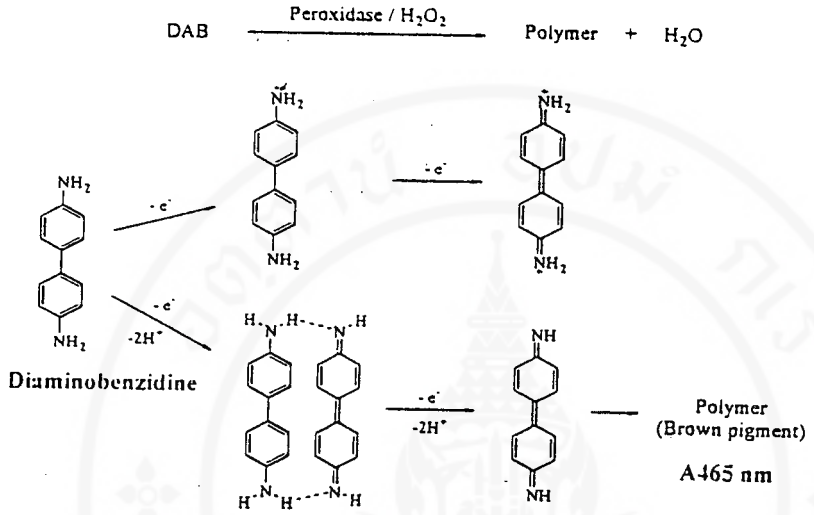
$$\text{Compound II} + H_2O_2 \longrightarrow \text{Compound III, inactive}$$

The reaction are generally considered as irreversible change. The rate-determining step in peroxidation is the return of compound II back to the native enzyme (38).

A number of substrates can be used as hydrogen donors in peroxidase reaction, eg. phenols 3',3-diaminobenzidine (DAB), coniferyl alcohol, guaiacol, pyrogallol and o-dianisidine. The DAB assay provides a suitable procedure for quantitation and biochemical characterization of peroxidases, immunochemical studies using peroxidase-labeled antigen-antibody reactions and histochemical staining for peroxidase.

The reactions using some selected substrates of peroxidases are shown below:

(a) Diaminobenzidine (38)



(b) Pyrogallol (39)

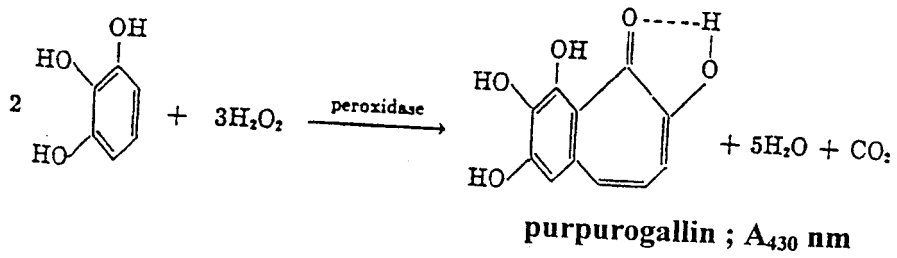
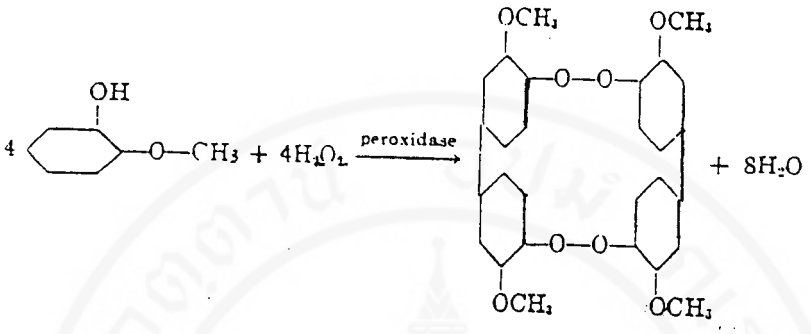


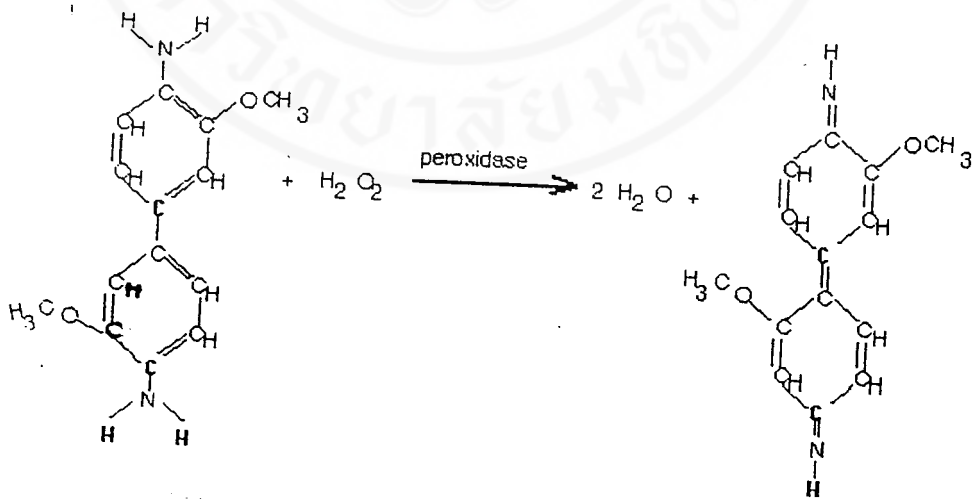
Fig. 5 The reaction of peroxidase using selected substrates

(c) Guaiacol (40)



tetraguaiacol ; A_{470} nm

(d) o-Dianisidine (41)



Oxidized O-dianisidine A_{460}

Fig. 5 (cont'd.) The reaction of peroxidase using selected substrates

1.4 Applications of peroxidases

Peroxidases have been used in many techniques in experimental bioscience. Here are selected popular applications.

1.4.1 Peroxidase-conjugated antibody for enzyme-linked immunosorbant assay (ELISA)

The use of enzyme-labelled antibody in immunochemistry and ELISA is popular because of their simplicity and sensitivity (42). This technique allows detection and quantitation of small amounts of antigen or antibody in biological fluid. Many different enzymes have been used. Among these, horseradish peroxidase is widely employed.

1.4.2 Electronmicroscopic staining

Immunoelectron microscopy is a technique characterized by the association of various electron-dense markers with antibody or other immunoreactive macromolecules. Under electron microscope, it is possible to identify the antigen-antibody complexes at the ultrastructural level.

Histochemical methods for peroxidase localization in tissues or cells was first developed in 1966 by Graham and Karnovsky. The visualization of the enzyme localizaton is based on the use of DAB. The polymer end products form the precipitate, insoluble and electron-opaque product at the site of the peroxidase reaction (43).

1.4.3 Enzyme coupled assays

Hydrogen peroxide is produced by enzymic conversion of a number of clinically significant compounds. One of the methods to follow the formation of H_2O_2 is by using a phenolic compound to form a colored product in the presence of a

peroxidase. For example, in uric acid assay, chromogen ABTS (2,2'-azinodi(3-ethylbenzthiazoline-6-sulfonate) can be used.

$$\text{Uric acid} + O_2 \xrightarrow{\text{uricase}} \text{allantonin} + H_2O_2 + CO_2$$

$$H_2O_2 + ABTS_{red} \longrightarrow ABTS_{ox} + 2H_2O \qquad (44)$$

A new chemiluminescent assay of linamarin consists of coupled reactions. The first reaction is the conversion linamarin to glucose by linamarase. The second reaction is the conversion of glucose to H_2O_2 by glucose oxidase. Ultimately, H_2O_2 is assayed by adding luminol solution containing of HRP to give 3-aminophthalate with the emission of light (45).

$$\text{Linamarin} \xrightarrow[\text{linamarase}]{H_2O,\ \text{glucose}} \text{Cyanohydrin}$$

$$\text{Glucose} + H_2O + O_2 \xrightarrow{\text{glucose oxidase}} H_2O_2 + \text{gluconic acid}$$

$$H_2O_2 + \text{luminol} \xrightarrow{\text{HRP}} \text{3-aminophthalate} + \text{light}$$

1.5 Aim of thesis

The aim of this study is to purify and characterize peroxidase from cassava leaves.

During the preliminary investigation leading up to this thesis, the peroxidases from different parts of cassava were found in the 60-80% $(NH_4)_2SO_4$ of leaf (40 U/mg), petiole (35 U/mg), stem (20 U/mg) and root (6 U/mg). 1 U is defined as a change of 1 absorbance unit at 465 nm per min. According to the result, cassava leaves had the highest specific activity. So cassava leaf was chosen for this study

CHAPTER II

MATERIALS AND METHODS

2.1 Plant Materials

Cassava leaves (*Manihot esculenta* Crantz.) were purchased from Pakkret market, Nonthaburi.

2.2 Chemicals

Name of Chemicals	Company
Ammonium sulfate	Sigma
PVPP	Sigma
Acrylamide	Sigma
Concanavalin A sepharose 4B	Sigma
N,N-methyl-bis-acrylamide	Sigma
Mmercaptoethanol	Sigma
IEF marker kit	Sigma
Standard marker proteins for SDS-PAGE	Sigma
Coomasie brilliant blue R-250	Sigma
DAB	Sigma
BSA	Sigma
Tris	Sigma
Glycine	Sigma
SDS	Sigma
Guaiacol	Sigma
Pyrogallol	Sigma
o-Dianisidine	Sigma
Coniferyl alcohol	Sigma
Syringaldazine	Sigma
Quercetin	Sigma
Sodium azide	Sigma
α-D-methylglucopyranoside	Sigma
Bromophenol blue	Sigma
Amino acid standard solution	Sigma
Pharmalyte pH 3-10	Sigma
DNP-Lysine	Sigma
Sephadex G-200	Pharmacia
H_2O_2	Merck
DMSO	Merck

Name of Chemical	Company
Methanol	Merck
Sodium carbonate	Merck
Folin-ciocalteau's phenol reagent	Merck
Glacial acetic acid	Merck
Sodium monohydrogen phosphate	Merck
Sodium dihydrogen phosphate	Merck
Ethanol	Merck
HCl	Merck
Ascorbic acid	National Biochemical Corp.
TEMED	Fluka
Thiourea	Fluka
Phenylisothiocyanate	Pierce
Copper sulfate	May and Baker
Ammonium persulfate	May and Baker
Potassium cyanide	Carlo-Erba
Sodium chloride	Carlo-Erba
Glycerol	Carlo-Erba
Sulfuric acid	Carlo-Erba
Trichloroacetic acid	Carlo-Erba

2.3 Preparation of crude cassava leaf peroxidase

Fresh cassava leaves (1,500 gm) were washed in distilled water. It was then chopped and homogenized in a blender in ice cold 0.1 M sodium phosphate buffer pH 6.5 with ratio of 1:1 (w/v), containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 2% w/v of polyvinyl polypyrrolidone (PVPP). The homogenate was filtered through a filter cloth and centrifuged at 12,000 x g for 30 min at 4°C in a Sorvall RC-5C plus refrigerated centrifuge. The supernatant was collected as a crude extract for further assay of its activity as described in section 2.6.

2.4 Purification of cassava leaf peroxidase

2.4.1 Ammonium sulfate precipitation

The crude extract was fractionated by slowly adding solid fine ammonium sulfate to 40% saturation with continuous stirring at 4°C. Precipitation was allowed to form for 4 hours at 4°C. Afterward, the precipitate was collected by centrifugation at 12,000 x g for 30 min at 4°C. The supernatant was raised to 60% saturation by further adding an appropriate amount of ammonium sulfate. After collecting the second precipitate, the saturation as raised to 80% by a further addition of ammonium sulfate and the third precipitate was similarly collected. Each precipitate was redissolved in a small volume of 20 mM sodium phosphate buffer pH 6.5 and dialyzed overnight against and a large volume of the same buffer. Any precipitate formed during dialysis was removed by centrifugation at 10,000 x g for 20 min at 4°C.

2.4.2 Concanavalin A-Sepharose 4B column chromatography

Concanavalin A-Sepharose 4B was washed 4 times in distilled water to remove the ethanol used to preserve the gel. The settled gel was then suspended in 2 volumes of 50 mM sodium phosphate buffer, pH 6.5. The gel suspension was packed into a glass column (1.8 x 8 cm) using a peristaltic pump at a flow rate of 15 ml/hr. The column was pre-equilibrated by passing 10 column volumes of 50 mM sodium phosphate buffer, pH 6.5 containing 0.2 M NaCl at a flow rate of 15 ml/hr.

The ammonium sulfate fraction at 80% saturation was dialysed against an excess volume of the 50 mM sodium phosphate buffer, pH 6.5 containing 0.2 M NaCl overnight at 4°C. The dialyzed sample was centrifuged at 10,000 x g for 20 min at 4°C. The supernatant fraction (15 ml, about 36mg protein) was loaded onto the column. The

column was washed with the starting buffer for 10 column volumes, using flow rate of 15 ml/hr. The elution of bound proteins can be achieved by using a stepwise of α-D-methyl glucopyranoside (at 0.3 M and 0.5 M) in the starting buffer over 10 column volumes, at the same flow rate. Fractions of 2 ml each were collected. The elution profile was monitored for protein by measuring the absorbance at 280 nm and the peroxidase activity was determined as described in section 2.6. The fractions containing the bound peroxidase activity were pooled for further purification.

2.4.3 Sephadex G-200 column

Sephadex G-200 was swollen in distilled water for 5 hours at 90°C. The settled gel was then suspended in 2 volumes of 20 mM sodium phosphate buffer pH 6.5. The gel suspension was degassed and packed into a glass column (52 x 2.5 cm) using a peristaltic pump at flow rate of 15 ml/hr. The column was calibrated with a molecular weight marker kit (β-amylase Mr = 200,000, alcohol dehydrogenase Mr = 150,000, bovine serum albumin Mr = 66,000, carbonic anhydrase Mr = 29,000, cytochrom C Mr = 12,400) at a flow rate of 11.5 ml/hr, 4°C. Blue dextran and DNP-lysine were used to determine the void volume and total volume of the column respectively.

The partially purified bound enzyme from the Concanavalin A Sepharose 4B column (section 2.4.2) was dialyzed against the starting buffer. An aliquot (2.5 ml, about 2.5 mg) was loaded onto the column and eluted at a flow rate of 11.5 ml/hr. Fractions of 2 ml each were collected using LKB 7000 Ultrorac fraction collector. The elution profile was monitored for protein by measuring the absorbance at 280 nm and the peroxidase activity was determined as described in section 2.6. The fraction containing peroxidase were collected and used for further characterization.

2.5 Determination of protein content

Protein concentration was determined by the method of Lowry *et al* (46) and bovine serum albumin (20-100 μg) was used as standard protein.

The assay mixture contained 100 μl of diluted protein solution and 3 ml of freshly prepared alkaline copper solution (100 ml of 2% Na_2CO_3 in 0.1 M NaOH was mixed with 1 ml of 1% potassium sodium tartrate and 1 ml of 0.5% copper sulfate). The mixture was left at room temperature for 10 min and then 0.3 ml of 1 M Folin reagent was added and mixed in thoroughly. The mixture was left for another 30 min and then the absorbance at 650 nm was measured using a Beckman DU-40 spectrophotometer.

2.6 Determination for peroxidase activity using DAB

The peroxidase activity was measured by the modified method of Herzog and Fahimin (47) by following the initial rate in the conversion of freshly prepared 3,3'-diaminobenzidine (DAB) to the product which has absorbance at 465 nm using a Shimadzu UV visible spectrophotometer. The assay mixture (3 ml) contained 0.25 mM DAB in 0.1 M sodium phosphate buffer pH 6.5, enzyme extract and the reaction was started by the addition of 1 mM H_2O_2 (blank containing DAB and enzyme extract in the absence of H_2O_2). The enzyme activity was expressed as A_{465}/min. One unit was defined as a change of one absorbance unit at 465 nm per min.

2.7 Gel electrophoresis

2.7.1 Denaturing polyacrylamide gel electrophoresis (SDS-PAGE)

The peroxidase preparation from each step of purification was analyzed by denaturing gel electrophoresis on a slab gel system according to the modified method described by Laemmli (48). The slab gel system consisted of a stacking gel (10 x 2 x 0.001 cm) of 3% acrylamide and separating gel with 10% (w/v) acrylamide (10 x 8.8 x 0.001 cm). The preparations of both separating gel and stacking gel were simplified as shown in Table 2.

Table 2 Preparation of polyacrylamide gel for SDS-PAGE

Reagent		**Stacking gel 3%**	**Separating gel 10%**
30% Acrylamide, 0.8% Bis	(ml)	1	5.3
1.5 M Tris-HCl, pH 8.8	(ml)	-	4
0.5 M Tris-HCl, pH 6.8	(ml)	2.5	-
10% SDS	(ml)	0.1	0.16
Distilled water	(ml)	6.4	6.5
10% Ammonium persulfate	(µl)	25	40
100% TEMED	(µl)	5	10
Final volume	(ml)	10	16

Samples were treated with a solubilizing buffer with a final concentration 1% (w/v) SDS, 10% glycerol, 5% (v/v) β-mercaptoethanol and small amount of bromophenol blue in 62 mM Tris-HCl, pH 6.8, and boiled for 1-2 min. Electrophoresis was performed with 0.1% SDS in 25 mM Tris-glycine pH 8.3 as electrode buffer at constant voltage of 100 V until the dye marker reached the bottom of the gel.

After electrophoresis, the gel was stained for protein with 0.2% Brilliant blue R-250 as described in section 2.7.5

Subunit molecular weight was calculated from standard molecular weight maker kit (phosphorylase b Mr = 94,000, bovine serum albumin Mr = 67,000, ovalbumin Mr = 43,000, carbonic anhydrase Mr = 30,000, soybean trypsin inhibitor Mr = 20,100, α-lactabumin Mr = 14,000).

2.7.2 Non-denaturing gel electrophoresis

Non-denaturing gel electrophoresis was used to determine the electrophoresis pattern of peroxidase isozymes. The system used was a modification of that reported by Cameo and Blaquier (49). The native gel was prepared from a stock solution of 30% (w/v) of acrylamide and 0.8% (w/v) of N,N-bis-methyl-eneacrylamide. The separating gel contained 5% (w/v) acrylamide, whereas the stacking gel contained 3% acrylamide. The gel was polymerized chemically by the addition of TEMED and ammonium persulfate. The preparation of both separating and stacking gel were simplified in the following Table 3.

Table 3 Preparation of polyacrylamide for non-denaturing gel electrophoresis

Reagent		**Stacking gel 3%**	**Separating gel 5%**
30% Acrylamide, 0.8% Bis	(ml)	1	1.3
1.5 M Tris-HCl, pH 8.4	(ml)	-	2
0.5 M Tris-HCl, pH 6.8	(ml)	2.5	-
Distilled water	(ml)	6.5	4.68
10% Ammonium persulfate	(µl)	25	20
100% TEMED	(µl)	5	5
Final Volume	(ml)	10	8

Samples were mixed with a sample buffer containing 62 mM Tris-HCl pH 6.8, 10% glycerol and small amount of bromophenol blue in the ratio 5:1. The running buffer was 25 mM Tris-glycine pH 8.3. The gel was run at a constant voltage of 100 V unit the dye marker reached the bottom of the gel.

After electrophoresis, the gel was stained enzyme activity as described in section 2.7.4

2.7.3 Isoelectric focusing on polyacrylamide gel (IEF)

Differences in charge of the purified proteins in their native state was studied by isoelectric focusing according to the method of BIO-RAD (50). IEF was performed in a BIO-RAD mini gel IEF.

First; One glass plate was attached with gel supported film on the side that contact to the gel.

Second; The acrylamide gel was prepared from a stock solution of 24.25% (w/v) of acrylamide and 0.75% (w/v) of N,N-bis-methyleneacrylamide. The acrylamide slab gels (10 ml) contained 5% acrylamide, 5% (w/v) glycerol, 5% ampholyte, pH 3-10. The gel was polymerized chemically by the addition TEMED and ammonium persulfate.

Third; The gel solution was layered on the casting tray and covered with a support film attached to a glass plate. The gel was allowed to set about 2 hr and after the gelling was complete, the casting tray was removed.

Fourth: 2 µl of sample was loaded onto the gel attached to the support film and focusing was performed at 100 V for 15 min, 200 V for 15 min and 450 V for

60 min, successively. The standard pI marker used were trypsin inhibitor (pI = 4.55), β-lactoglobulin (pI = 5.2), bovine carbonic β (pI = 5.85), human carbonic (pI = 6.55).

After electrophoresis, the protein bands in the focused gel were fixed in a solution of 30% methanol, 5% trichloroacetic acid and 3.5% sulfosalicylic acid for 15 min and then transferred into a solution of 27% ethanol, 10% acetic acid and 0.04% Coomassie brilliant blue R-250 for 2-4 hr at room temperature for protein stained. The other part of gel stained for enzyme activity band as described in section 2.7.4.

2.7.4 Staining for peroxidase activity

The gel was also submerged in phosphate buffer pH 6.5 prior to soaked in reaction mixture, which consisted of 0.5 mM DAB (freshly prepared), 1 mM H_2O_2 in 0.1 M phosphate buffer pH 6.5 until activity band appeared.

2.7.5 Protein staining

After electrophoresis the gel was stained for protein in 0.2% Brilliant blue R-250 in 50% methanol and 10% acetic acid in for 2-4 hr at room temperature with moderate shaking. Destaining was performed by immersing the gel overnight in a solution 25% ethanol and 7% acetic acid until the background of the gel was clear.

2.8 pH stability

The pH stability was determined by incubating 10 µl of purified peroxidase for 24 hr at 4°C with 90 µl of the universal buffer (Gritton & Robinson type) pH between 3-11 and then readjusted to pH 6.5 by adding 2.9 ml of 0.1 M sodium phosphate buffer pH 6.5. The peroxidase activity was assayed by using DAB as described in section 2.6. The result was expressed as the percentage of the enzyme activity remained.

2.9 pH optimal

The purified peroxidase was used to determine optimum pH for the reaction of DAB. The substrate solution containing universal buffer at various pH ranging from 3-11 was mixed with purified peroxidase at 25°C. The peroxidase activity at various pH was measured as described in section 2.6.

2.10 Temperature stability

Enzyme stability was investigated over the range of 25°C - 70°C (25°C, 40°C, 50°C, 60°C, 65°C, 70°C). The purified peroxidase was incubated at various temperature for 0-24 hr (2 hr, 4 hr, 8 hr, 16 hr, 24 hr). The enzyme solution was removed and cooled immediately to 25°C before measuring the remained activity by using DAB as substrate in section 2.6. The result was expressed as the percentage of the enzyme activity remained.

2.11 Temperature optimal

To study the effect of temperature on the activity, the purified enzyme was assayed at various temperatures (4°C, 25°C, 37°C, 50°C, 60°C, 65°C, 70°C) using DAB as substrate as described in section 2.6. The reaction mixture (in the absence of H_2O_2) was incubated in the desired temperature for about 3 min. The reaction was started by the addition of 1 mM H_2O_2. Result was expressed as the percentage of the enzyme activity.

2.12 Spectral analysis

The UV-visible spectra of native purified peroxidase and horseradish peroxidase in range 220-600 nm were recorded using a Shimazu, uv-250/PC at 25°C. A cuvette (1

cm light path) containing 150 μg enzyme solution in 20 mM sodium phosphate buffer pH 6.5 was used.

The absorption spectrum of substrate coniferyl alcohol was recorded using a Shimazu, uv-250/PC at 25°C. Its spectrum obtained after the addition of 1 mM H_2O_2 and purified enzyme was similarly recorded.

2.13 Determination of kinetic constants

The purified peroxidase from section 2.4 was used in the kinetic study to determine K_m for selected substrates. The substrates were:

a) H_2O_2

b) DAB (465 nm) in 0.1 M phosphate buffer

c) Guaiacol (470 nm) in 0.1 M phosphate buffer

d) o-dianisidine (460 nm) in 2% methanol

e) Pyrogallol (nm 430) in 0.1 M phosphate buffer

f) Coniferyl alcohol (260 nm) in 5% methanol

g) Syringaldazine (530 nm) in 7% DMSO

h) Quercetin (370 nm) in 2% methanol

i) Ascorbate (290 nm) in 0.1 M phosphate buffer

Activities with each electron donor was determined in the same assay mixture as described in section 2.6 but DAB was replaced with one of the above substrates..

To determine K_m of H_2O_2, H_2O_2 concentration was varied while DAB was kept constant at 0.25 mM. K_m for DAB was determined by varying the concentration of DAB and keeping a fixed 1 mM H_2O_2. K_m for other electron donor were determined in

the same manner as DAB. The K_m values for all substrates were estimated by Lineweaver-Burk plots using Enzfitter computer program.

2.14 Effects of inhibitors

The purified enzyme was incubated with various concentrations of inhibitors at 25°C for 30 min. Then, the enzyme was assayed for the remaining activity in reaction mixture as described in section 2.6.

2.15 Determination of amino acid compositions

Amino acid composition was determined using the Waters Pico Tag system which involves three steps (51). First, about 10 μg of sample was dried and hydrolyzed with 6M HCl containing 1 mg/ml phenol in vacuum sealed tube at 105-110°C for 22-24 hr in Picrotag work station. After hydrolysis, sample was dried and redried under vacuum by using a solution containing MeOH:TEA:H_2O (2:1:1). The hydrolyzed sample was derivatized with phenylisothiocyanate (PITC) to yield phenylthiocarbamyl (PTC) amino acids. Lastly, the amino acid derivatives were analyzed by HPLC using a Waters Pico Tag column.

CHAPTER III

RESULTS

3.1 Purification of cassava leaf peroxidase

Peroxidase from cassava leaf was purified according to the methods outlined in section 2.6. The crude enzyme (6 U/mg) was sequentially precipitated by ammonium sulfate at 0-40%, 40-60% and 60-80% saturation. The highest specific activity was found that the fraction 60-80% ammonium sulfate saturation (the average specific activity of 40 U/mg) (Fig. 6). This step gave 61% activity yield and 7.6 fold purification (Table 4).

Further purification of the enzyme was achieved by affinity chromatography on Concanavalin A Sepharose 4B column. The most protein (peak I) did not bind to the Concanavalin A column and it contained some but low specific activity of the peroxidase (Fig. 7). The bound material was eluted as single major peak (bound peak II) with 0.3M α-D-methyl glucopyranoside. When 0.5 M α-D-methyl glucopyranoside was used to elute the column, only a small activity peak appeared. When the unbound peak was reloaded to the Concanavalin A column which was rewashed with the buffer, the activity remained unbound. Elution with 0.3 M α-D-methyl glucopyranoside yielded no peroxidase activity (Fig. 8). So there were two fractions of the peroxidase activity, namely ConA-bound and ConA-unbound (Fig 7). Since the bound fraction had a higher specific activity, it was further purified.

The bound fraction from the Concanavalin A column was dialyzed and concentrated by using aqua-sorb and then loaded onto a Sephadex G-200 column. The protein profile and the activity profile are shown in Fig. 9. The column was precali-

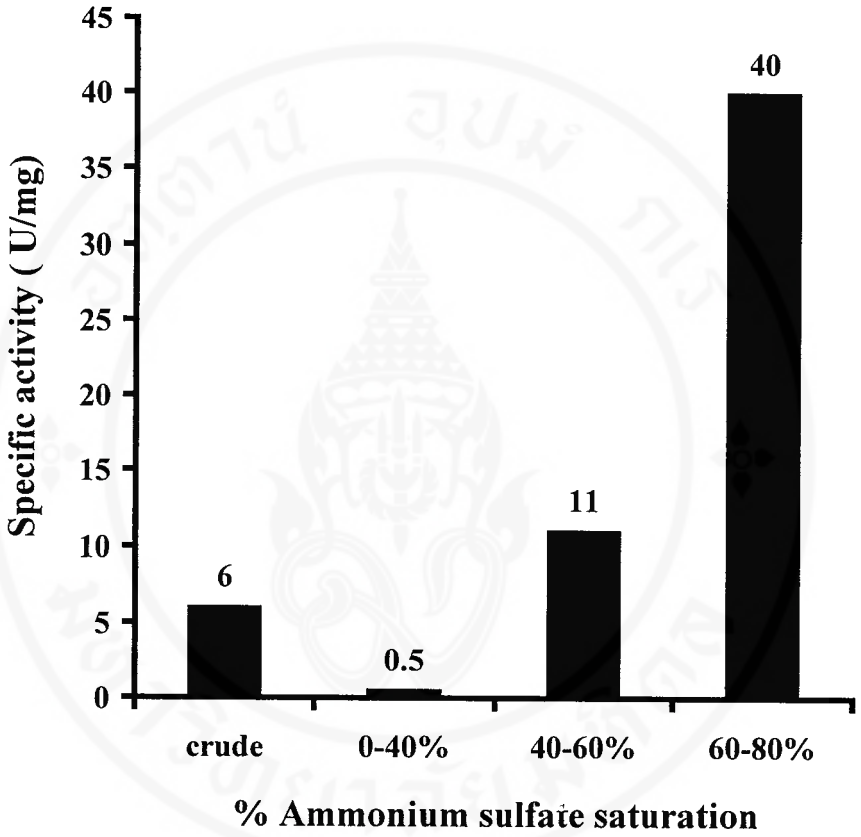


Fig. 6 The specific activity of peroxidase of the crude extract of cassava leaf and the fractions obtained by ammonium sulfate fractionation.

Table 4 Purification of peroxidase from cassava leaves

Sample	Total protein (mg)	Total activity (Unit)	Specific activity (Unit/mg)	% Yield	Fold purification
Crude	2815	14728	5.23	100	1
80%$(NH_4)_2SO_4$	224	8939	39.9	60.7	7.63
ConA	21.2	3805	179.5	25.8	34.3
G-200	4.95	1910	385.85	12.97	73.78

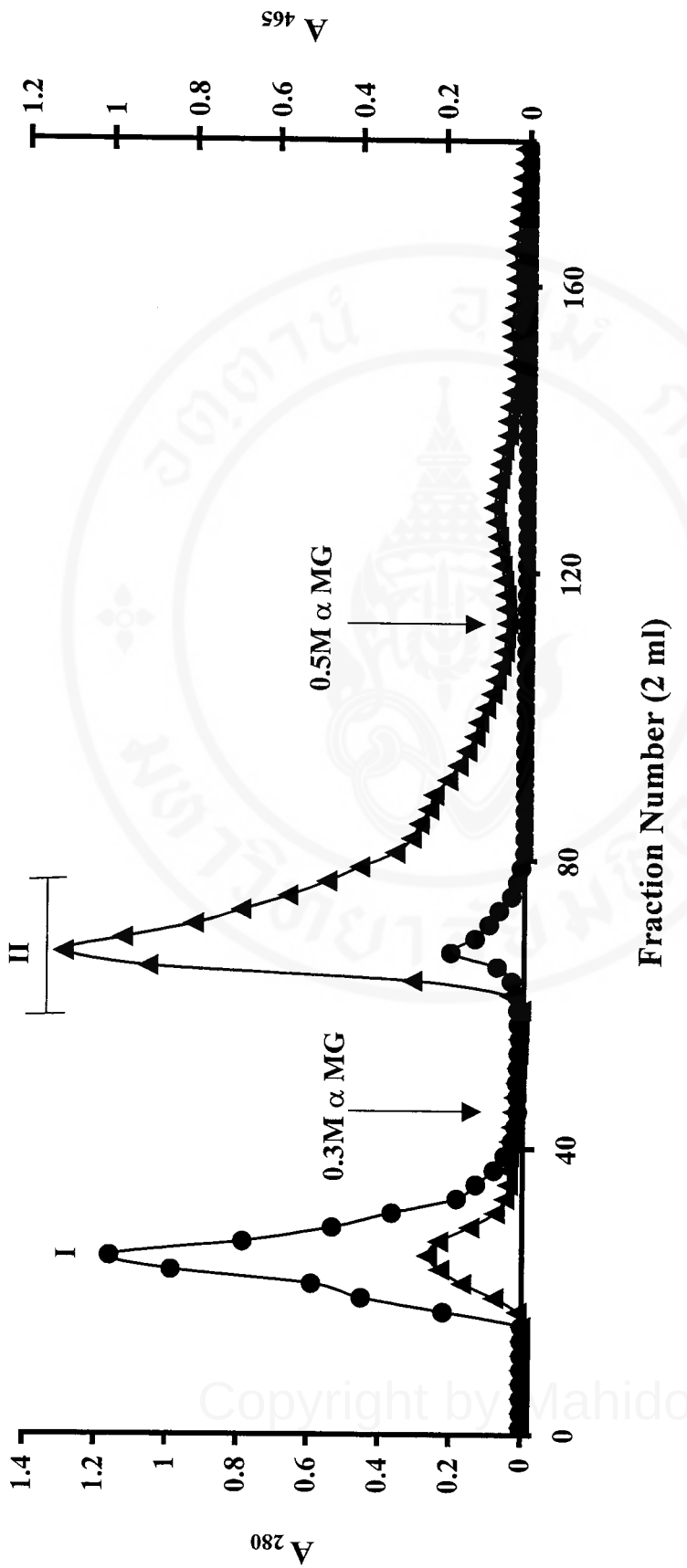


Fig. 7 Chromatography on Concanavalin A Sepharose of 60-80% ammonium sulfate precipitate of peroxidase from cassava leaf. The profile shows two peaks of protein (I and II).

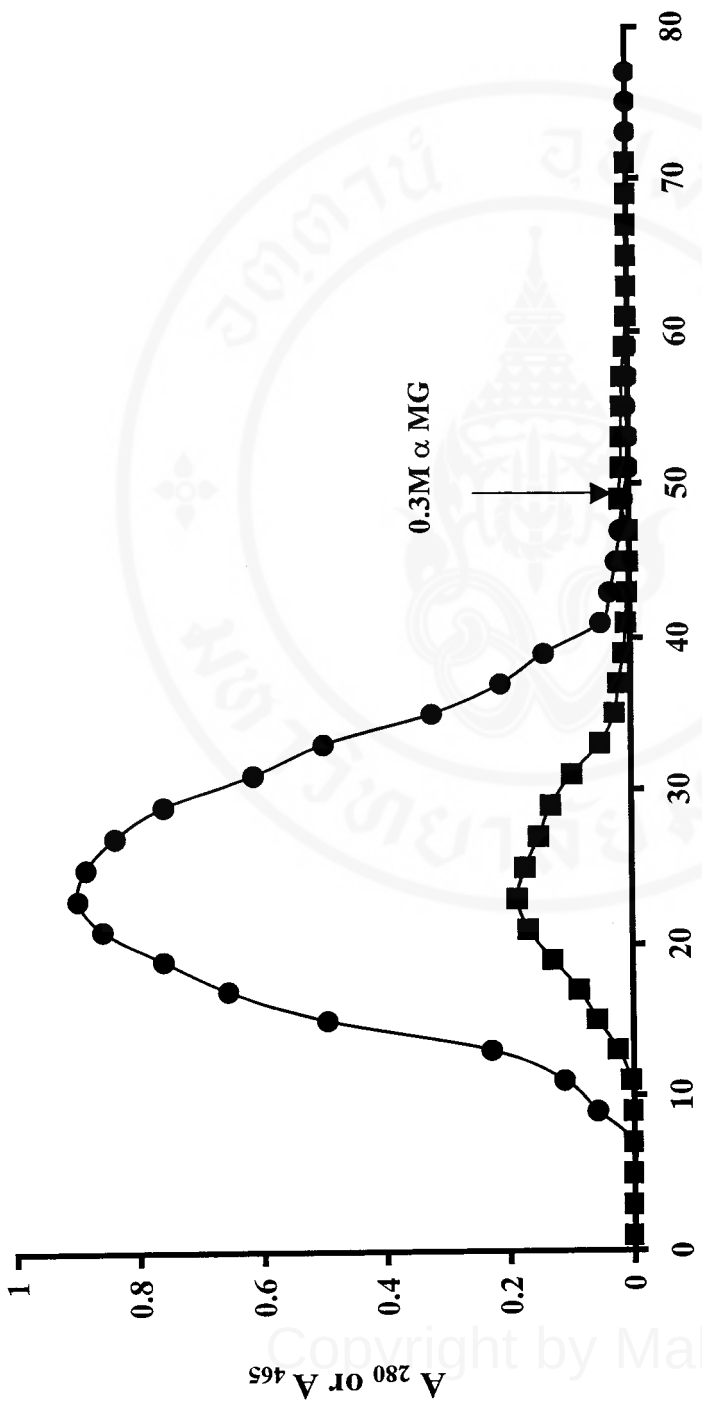


Fig. 8 Chromatography on concanavalin A Sepharose of reloaded unbound protein peak. The profile showed one peak of protein.

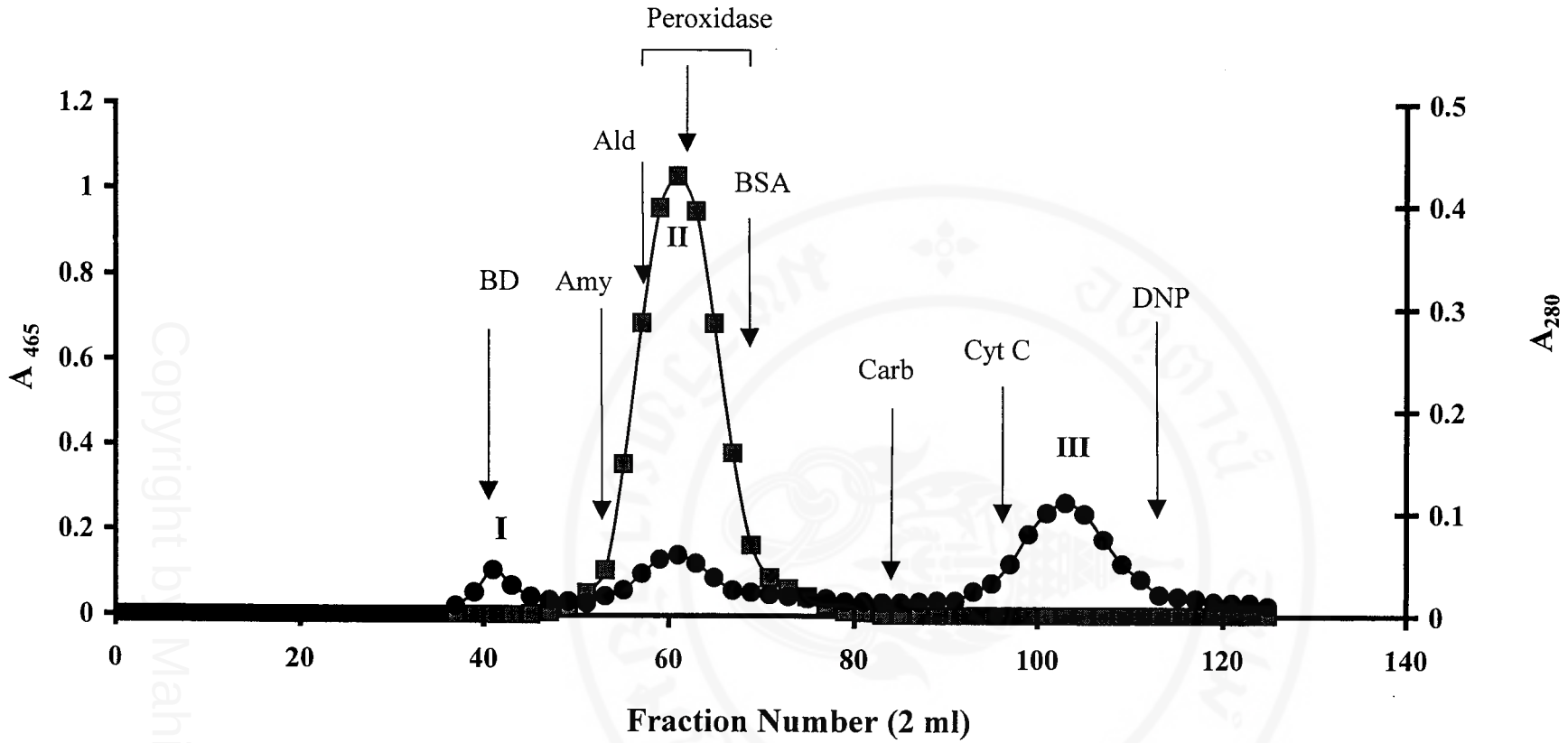


Fig. 9 **Chromatography on Sephadex G-200 column (52 x 2.5 cm) of bound protein peak from Concanavalin A. The profile shows three peaks of protein (I, II and III).**

protein A_{280} nm **peroxidase activity A_{465} nm** **the peroxidase fraction being pooled**

BD = Blue dextran, Amy = β-Amylase Mr = 200,000, Ald = Alcohol dehydrogenase Mr = 150,000

Carbo = Carbonic anhydrase Mr = 29,000, Cyt C = Cytochrom C Mr = 12,400

brated using protein markers as described in section 2.4.3. A standard curve for molecular weight determination is shown in Fig. 9. Among the 3 protein peaks (I, II, III), the peroxidase activity was found in peak II. The fractions with the peroxidase activity (peak II) were pooled. The purification of the cassava leaf peroxidase is summarized in the Table 4. The enzyme was purified 74 folds with 13% activity yield.

3.2 Characterization of cassava peroxidase

3.2.1 Physical properties

3.2.1.1 Native and subunit molecular weights

The native molecular weight of the purified peroxidase from cassava leaf was estimated to be 112 kD by chromatography on a Sephadex G-200 gel filtration column (Fig. 10). Its electrophoresis mobility on SDS-PAGE was compared with those of standard proteins under the same conditions (Fig. 11). A plot of Rf versus the logarithm of molecular weights of the standard proteins was linear (Fig. 12). Based on the Rf value, the purified cassava peroxidase had a subunit molecular weight of 56 kD. This data on the native and the subunit molecular weights indicated that the purified peroxidase from cassava leaf was a homodimer.

3.2.1.2 Isoelectric point (pI)

The purified cassava peroxidase was analyzed for its pI value by polyacrylamide gel isoelectrofocusing as described in section 2.7.3. The activity staining pattern of the IEF gel shown in Fig.13 revealed at 2 isoenzymes. From the standard curve (Fig. 14) the major isozyme with pI 6.4 and the minor isozyme with pI 6.25.

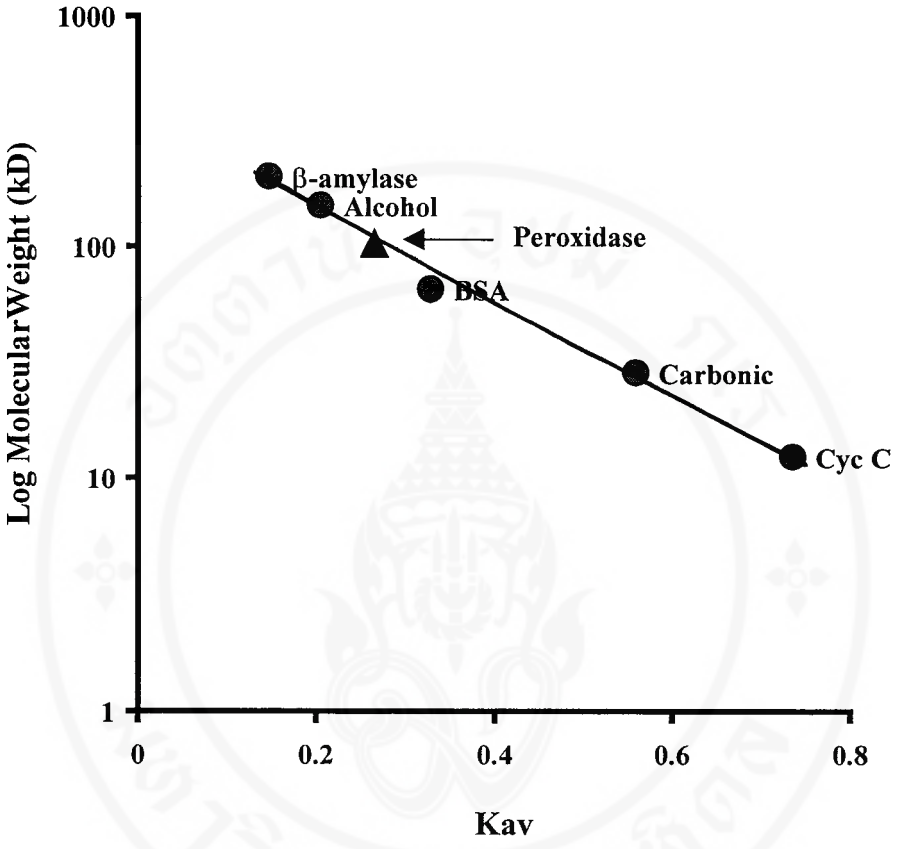


Fig. 10 **Calibration curve for native molecular weight determination by chromatography on Sephadex G-200 column (52 x 2.5 cm).**

▲ = peroxidase from cassava leaf.

β-amylase Mr= 200,000,

Alcohol = Alcohol dehydrogenase Mr = 150,000,

BSA = Albumin bovine serum Mr = 66,000,

Carbonic = Carbonic anhydrase Mr = 29,000

Cyt C = Cytochrom C Mr = 12,400

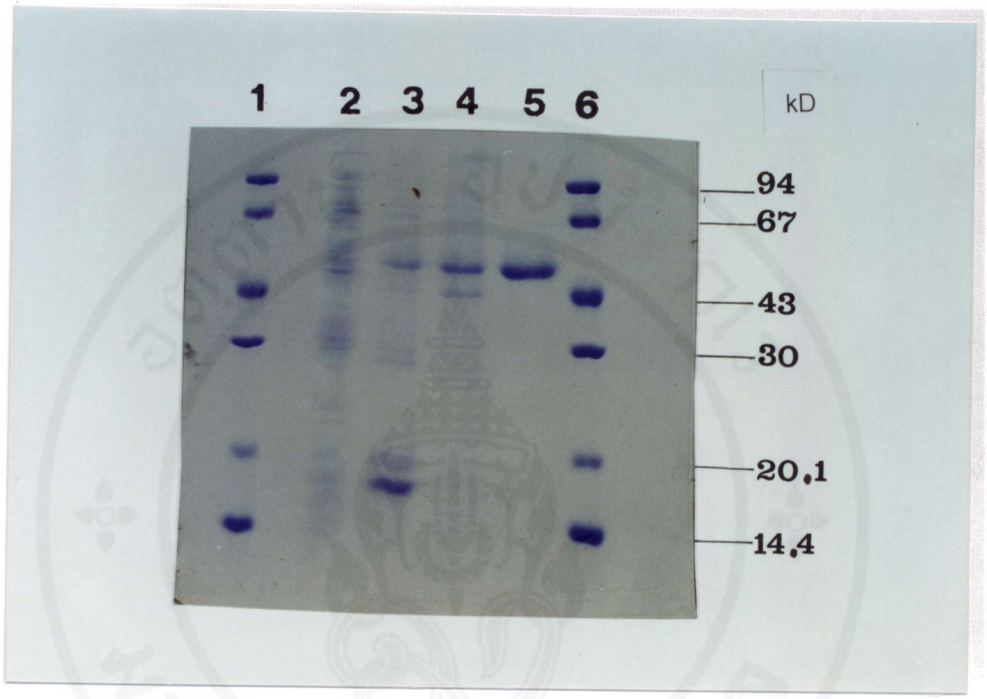


Fig. 11 SDS-PAGE of fractions from the purification of peroxidase from cassava leaf.

Lane 1, 6 = standard protein markers (12 μg)

Lane 2 = crude extracts

Lane 3 = 60-80% ammonium sulphate precipitate

Lane 4 = Con A, peak II

Lane 5 = Sephadex G-200, peak II

Amount of loaded in each lane (2-5) = 30 μg

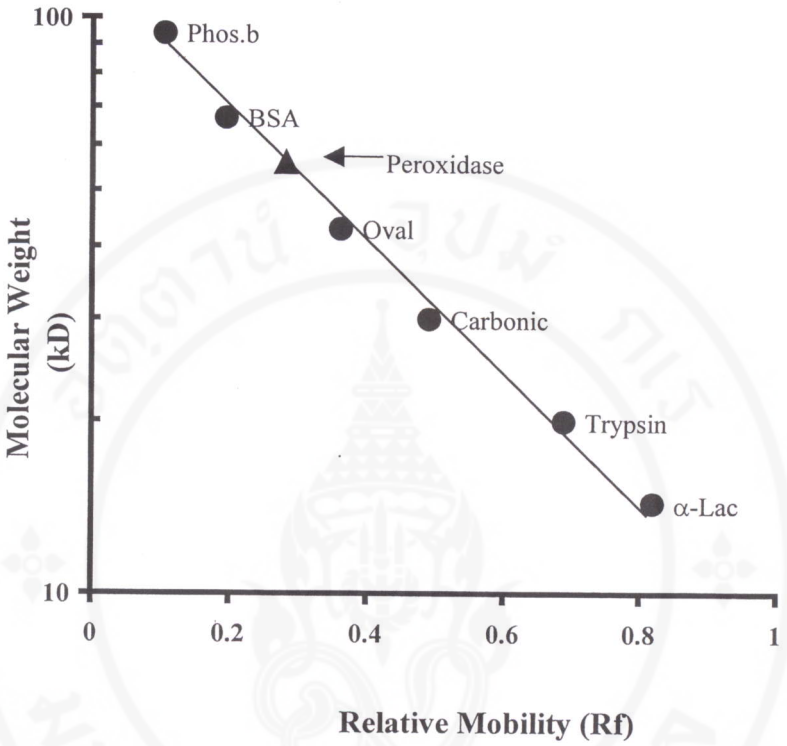


Fig. 12 Standard curve for molecular weight determination by SDS-PAGE . The peroxidase from Sephadex G-200 gave one major protein band.

▲ = peroxidase from cassava leaf

Standard protein markers: Phos. b = Phosphorylase b Mr = 94,000

BSA = Bovine serum albumin Mr 67,000

Oval = ovalbumin Mr = 43,000,

Trypsin = Soybean trypsin inhibitor Mr = 20,000

Carbonic = carbonic anhydrase Mr = 30000

α-Lac = α-Lactabumin Mr = 14,400

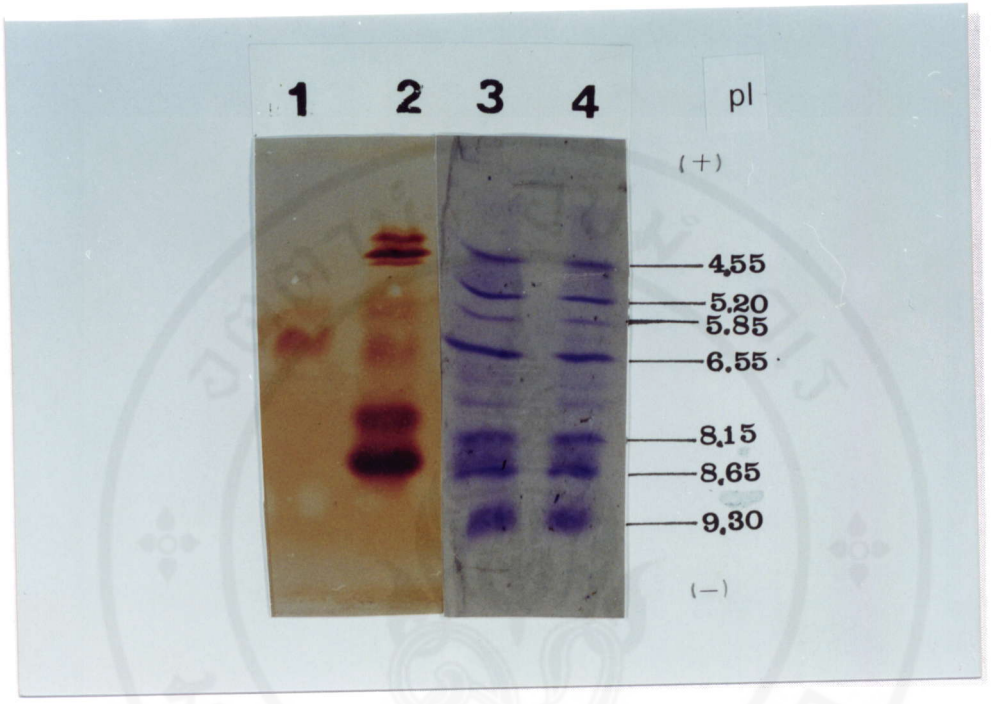


Fig. 13 Isoelectrofocusing gel eletrophoresis of purified cassava leaf peroxidase

Lane 1 = Purified peroxidase from Sephadex G-200 column (0.1 unit)

Lane 2 = Horseradish peroxidase (0.42 unit)

Lane 3,4 = Standard pI markers (14 μg)

pI 4.55, 5.2, 5.85, 6.55, 8.15, 8.65 and 9.3 from anode to cathod.

Lane 1 ,2 = peroxidase activity stain

Lane3 ,4 =protein stain

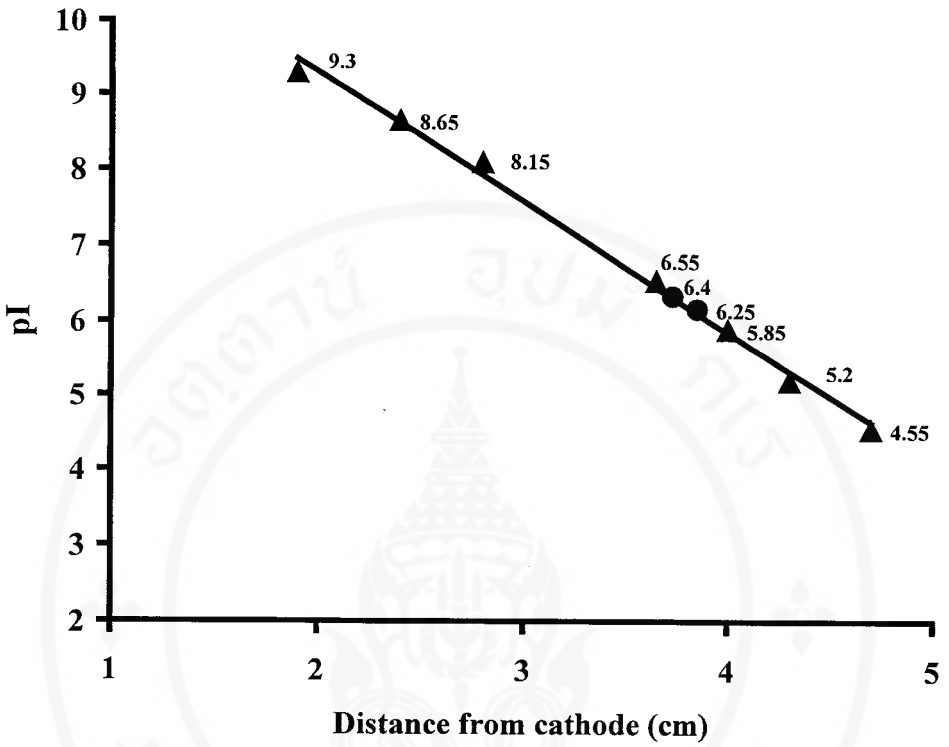


Fig. 14 **Calibration curve of standard pI markers of isoelectric focusing gel electrophoresis. Soybean trypsin inhibitor, pI = 4.55; β-lactoglobulin A, pI = 5.20; Bovine carbonic anydrase β, pI = 5.85; Human carbonic anhydrase, pI = 6.55; Lentil lectin, pI = 8.15; Lentil lectin, pI = 8.65; Trypsinogen, pI = 9.3**

● = peroxidase from cassava leaf

3.2.1.3 Non-denaturing gel electrophoresis

The Non-denaturing PAGE of the enzyme fractions from in each step of the purification was performed as described in section 2.7.2. The activity staining of the bound enzyme (Fig. 15) revealed a series of peroxidase bands. One major band (slow moving) was retained throughout the purification. The fast moving band were lost during the purification.

3.2.2 Chemical properties

3.2.2.1 UV-visible absorption spectrum

The uv-visible spectra of the purified cassava peroxidase and of horseradish peroxidase are shown in Figs. 16 and 17. Both spectra exhibited a distinct absorbance peak at 403-404 nm.

In the experiments using coniferyl alcohol as electron donor the product (DHP) formation was followed at 262 nm because the difference in the spectrum of the substrate and that of its product was observed at that wavelength (Fig. 18).

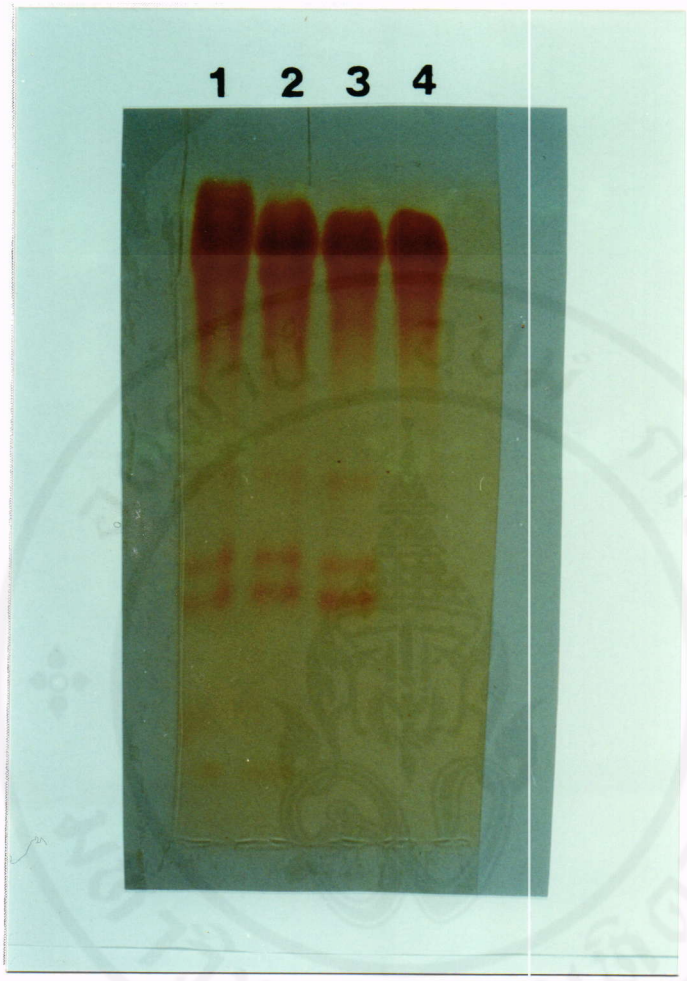


Fig. 15 Non-SDS-PAGE of cassava leaf peroxidase.

Lane 1 = crude enzyme

Lane 2 = 60-80% $(NH_4)_2SO_4$

Lane 3 = Concanavalin A

Lane 4 = Sephadex G-200

Amount loaded in each lane = 0.3 U

The gel was stained for peroxidase activity.

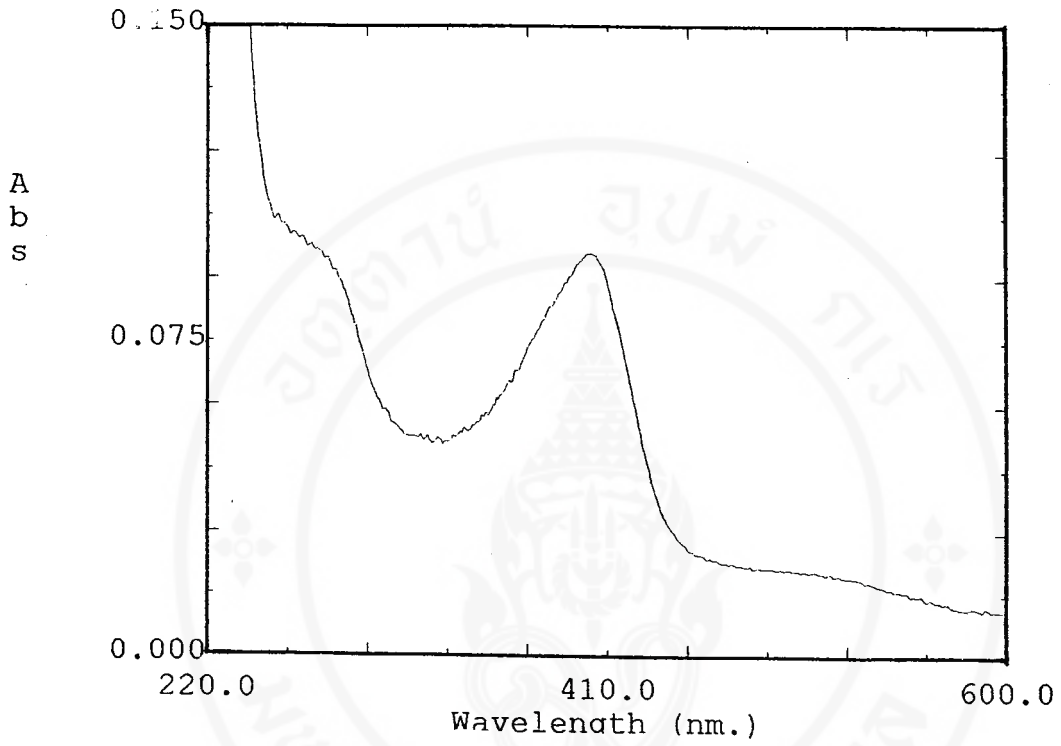


Fig. 16 UV-visible absorption spectrum of purified cassava leaf peroxidase showing a Soret band at 404 nm.

Protein = 150 μg/3 ml

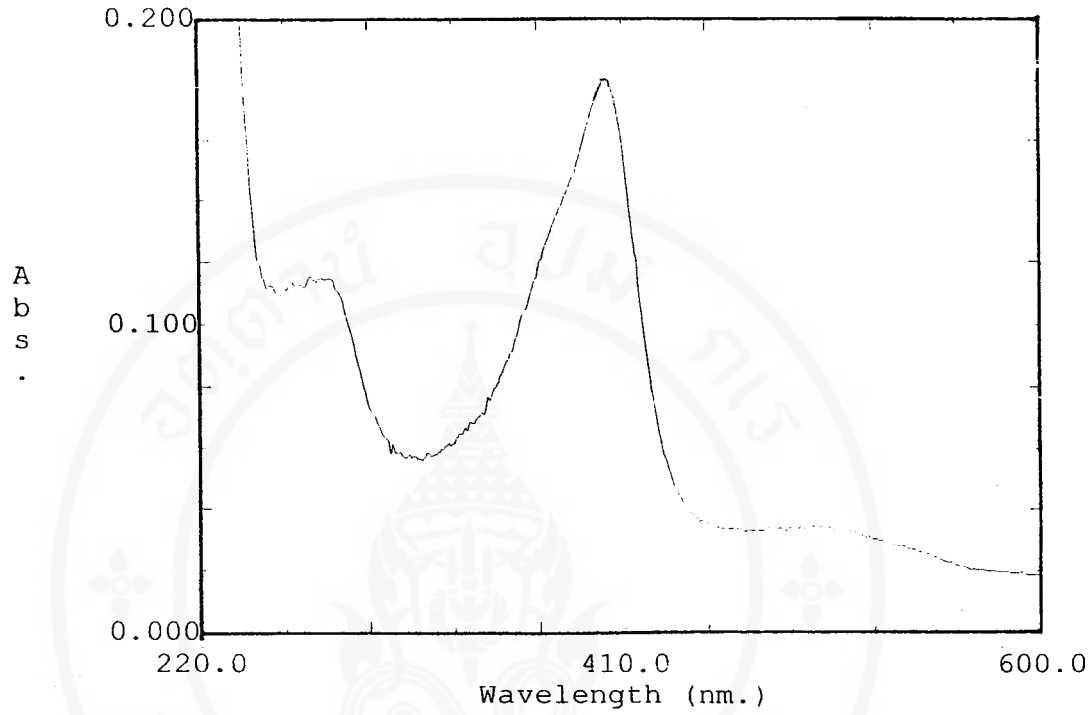


Fig. 17 UV-visible absorption spectrum of horseradish peroxidase showing a Soret band at 403 nm.

Protein = 150 μg/3 ml

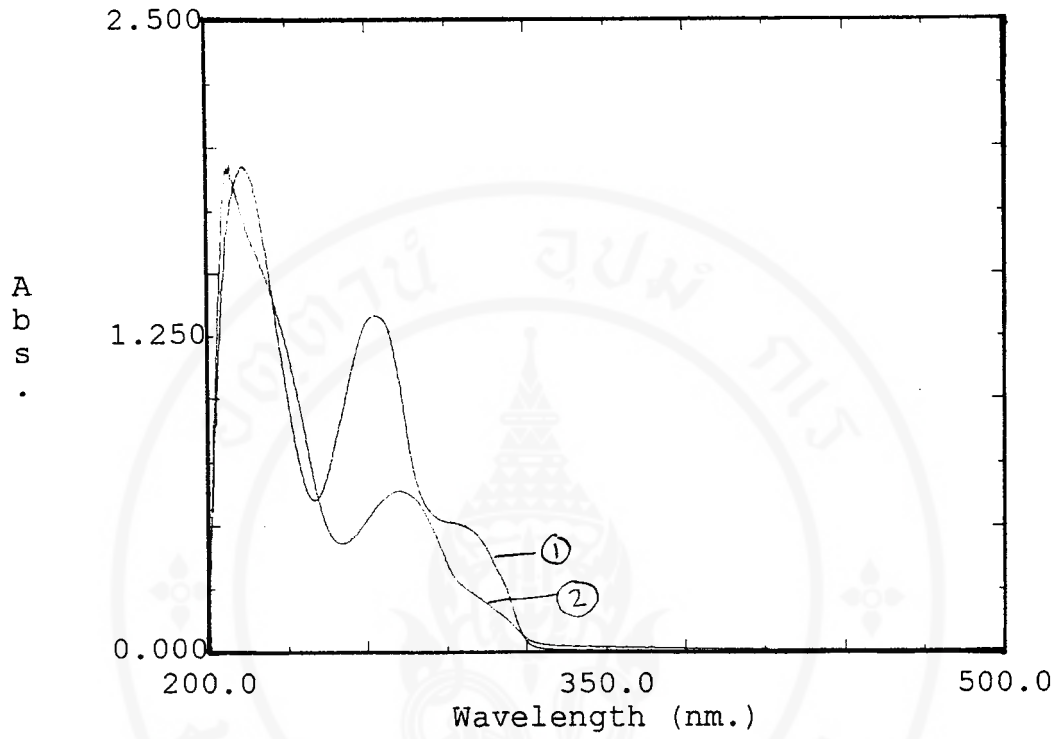


Fig. 18 The absorption spectrum in coniferyl alcohol.

1 = the absorption spectrum of coniferyl alcohol

2 = the absorption spectrum of its oxidation product

(peroxidase 0.5 μg in 3 ml +1 mM H_2O_2 + coniferyl alcohol)

Concentration of coniferyl alcohol = 7 μM

3.2.2.2 Amino acid composition

The amino acid composition of the purified cassava peroxidase was determined in duplicates as described in the section 2.15. The amount of each amino acid was calculated by comparing peak area to that of the standard amino acid. Amino acid composition of the enzyme is summarized in Table 5. HPLC chromotogram is shown in Fig 19. For the cassava peroxidase, the sum of the amino acids (Asx and Glx = 20.64 mole %) was greater than the sum of the basic amino acids (Lys, Arg and His = 13.33 mole %). The highest composition was Glx (about 13.6 mole %), followed by Gly (about 12.7 mole %).

3.2.3 Catalytic properties

3.2.3.1 pH optimum

The peroxidase activity of the purified enzyme was determined at different pH values as described in section 2.9. Fig. 20 shows that the cassava peroxidase had an optimum pH of 6. In the acidic pH range of 3.0-6.0, the activity increases rapidly. When pH increases from 6 to 10, the activity drops sharply reaching nearly zero a pH 10.

3.2.3.2 pH stability

The effect of pH on the activity of the cassava peroxidase was determined by incubating aliquots of the purified peroxidase in Universal buffer at various pH values (3 to 11) for 24 hr before being adjusted to pH 6.5 for assay as described in section 2.8. It was found that the activity of the cassava peroxidase was quite stable in the pH range of 4-11 while a loss of the activity was found at pH 3 (Fig. 20). The activity was most stable at pH 6.

Table 5 Amino acid composition of cassava leaf peroxidase

Amino acid	mole %	Residues/mole
Asx	7.03	36
Glx	13.61	69
Lys	6.62	34
Arg	5.45	28
His	1.26	6
Pro	5.08	26
Met	1.4	7
Trp	ND	ND
Phe	3.9	20
Ile	4.71	24
Leu	8.86	45
Val	6.11	31
Ala	8.21	42
Gly	12.74	65
Ser	7.49	38
Thr	5.21	26
Tyr	1.93	10
Cys	0.39	2
Sum	100	509
Molecular weight of protein = 56,000		

ND = not determined

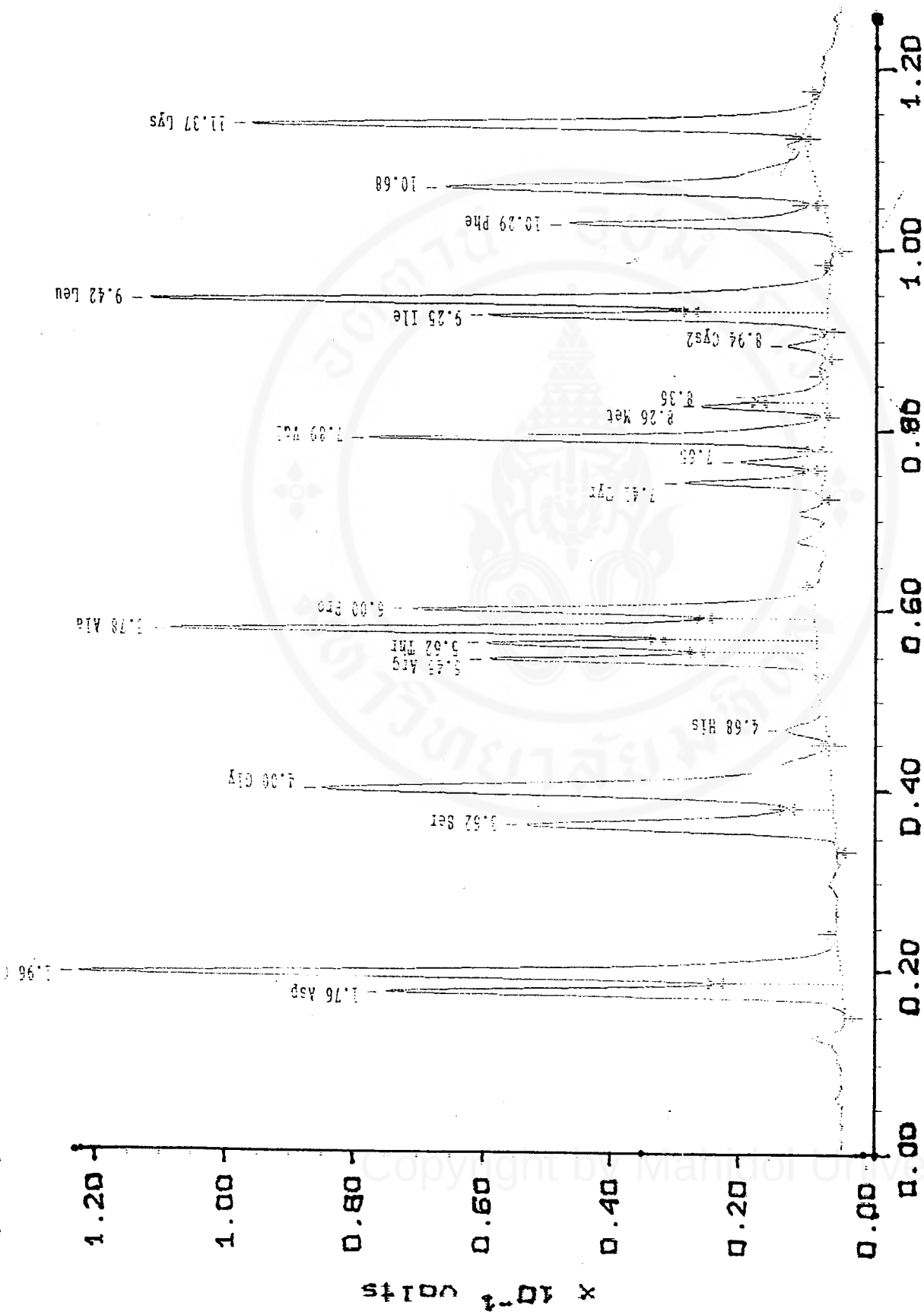


Fig. 19 HPLC chromatogram of amino acid composition of cassava leaf peroxidase. About 10 μg of purified peroxidase was hydrolyzed and derivatized, then reconstituted in 200 μl dilvent. 4 μl was injected to HPLC

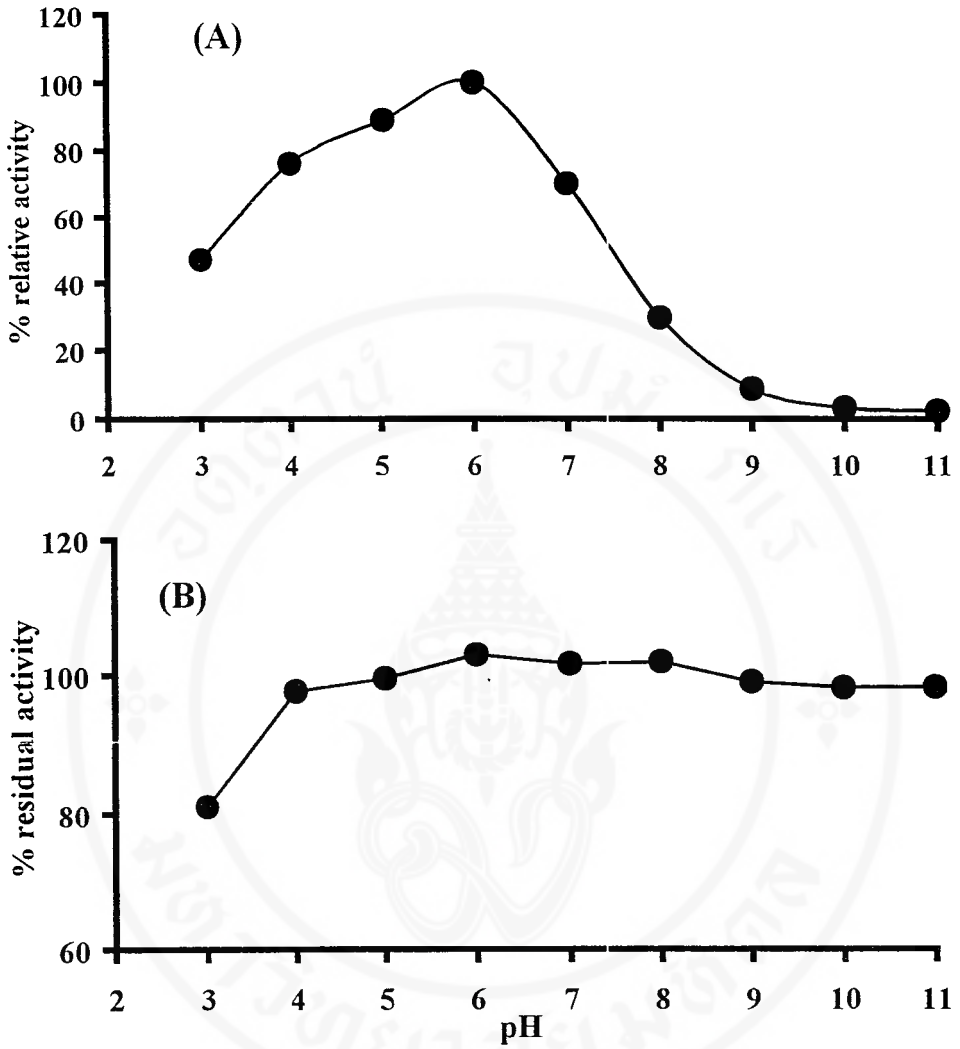


Fig. 20 **pH optimum and pH stability of cassava leaf peroxidase**

(A) Activity of purified enzyme was determined at various pH values, 3-11 by using DAB as substrate. The percentage of relative activity was calculated by using maximum activity of enzyme as 100%

(B) Aliquots of the enzyme were preincubated at 4°C for 24 hr at various pH values, 3-11 before being assayed for enzyme activity.

3.2.3.3 Temperature stability

The stability of the cassava bound peroxidase at different temperatures was studied as described in section 2.10. The cassava peroxidase was stable at temperature from 25-60°C for 24 hr (Fig. 21). The enzyme showed a slight loss of its activity to 80% when incubated at 65°C for 24 hr. The activity was completely lost when incubated at 70°C for 5 hr.

3.2.3.4 Optimum temperature

The optimal temperature for the purified peroxidase activity was also investigated as described in section 2.11. The result is shown in Fig. 22. The optimum temperture of cassava peroxidase was highest at 60°C. Increasing he assay temperature from 60°C to 70°C caused a gradually decreasing in peroxidase activity to 78%.

3.2.4 Kinetic constants of substrates

For the purified cassava peroxidase, the K_m values of different substrates, namely H_2O_2, DAB, guaiacol, o-dianisine, pyrogallol, coniferyl alcohol, syringaldazine and quercetin were determined as described in section 2.4. The reciprocal plots of Lineweaver-Burk were linear and they were used to determine the K_m values for these substrates. The calculation was made using a computer program Enzfitter as described in section 2.13. The estimated values of K_m are summarized in Table 6 and the curves are shown in Figs. 23-30. The enzyme also used ascorbate poorly as its substrate but the Km value cannot be determine from the abnormal saturation curves Fig. 31.

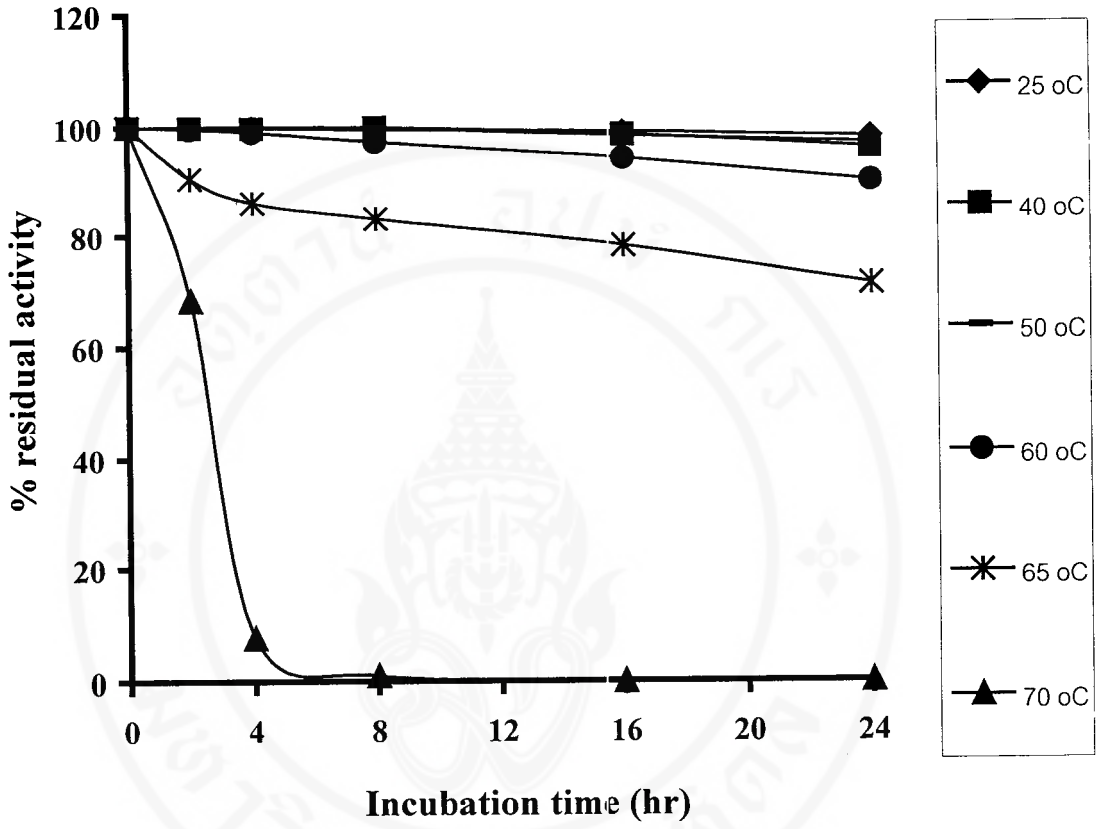


Fig. 21 Temperature stability of purified cassava peroxidase.

The purified enzyme was incubated in various temperature for 0-24 hr, then the enzyme activity was assayed.

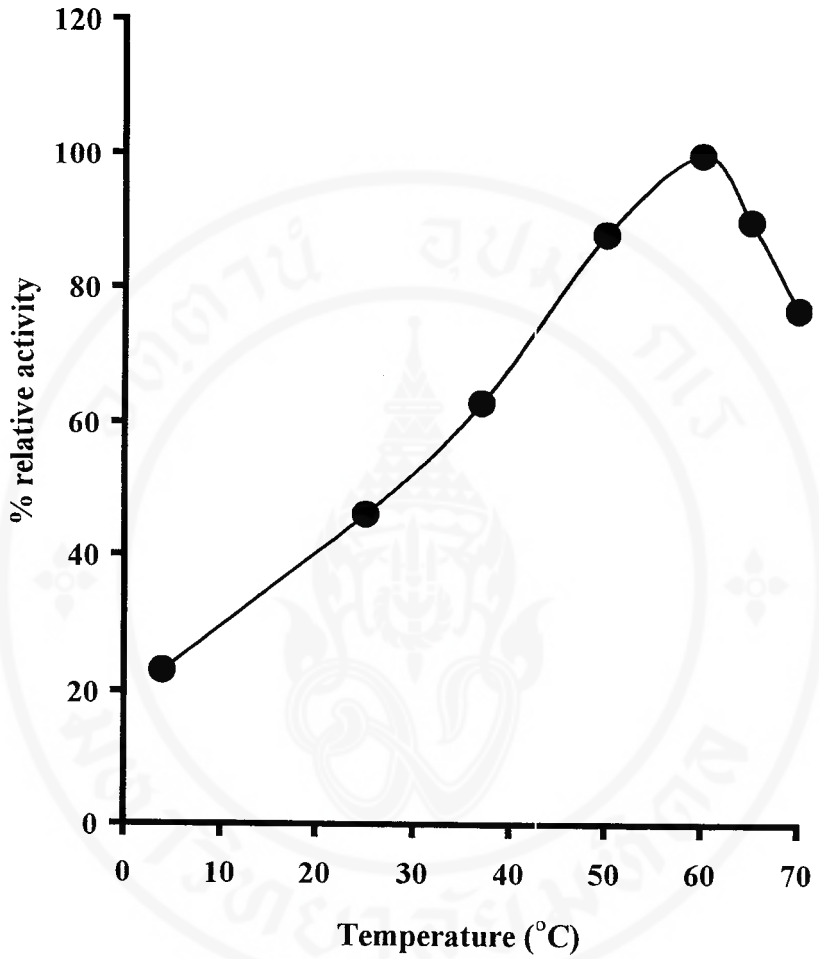


Fig. 22 Optimum temperature of purified peroxidase

Activity of purified enzyme was determined at various temperature by using DAB as substrate in phosphate buffer pH 6.5 . The percentage of relative activity was calculated by using maximum activity of enzyme as 100%

Table 6 K_m values and V_{max} values of cassava leaf peroxidase for various substrate

Substrate	V_{max} (U/ml)	K_m (mM)	V_{max}/K_m (U/mM/ml)
H_2O_2	0.87	0.09	9.7
DAB	0.92	0.092	10
Guaiacol	0.69	5.52	0.13
o-Dianisidine	0.99	0.037	26.8
Pyrogallol	1.21	0.89	1.4
Coniferyl alcohol	0.626	0.003	208.7
Suringaldazine	2.82	0.077	36.62
Quercetin	1.06	0.054	19.6

K_m for H_2O_2 was determined at a fixed concentration of DAB at 0.25 mM.

K_m values for other substrates were determined at a fixed concentration of H_2O_2 at 1 mM.

Specific activity of peroxidase assayed using DAB = 385.85 U/mg.

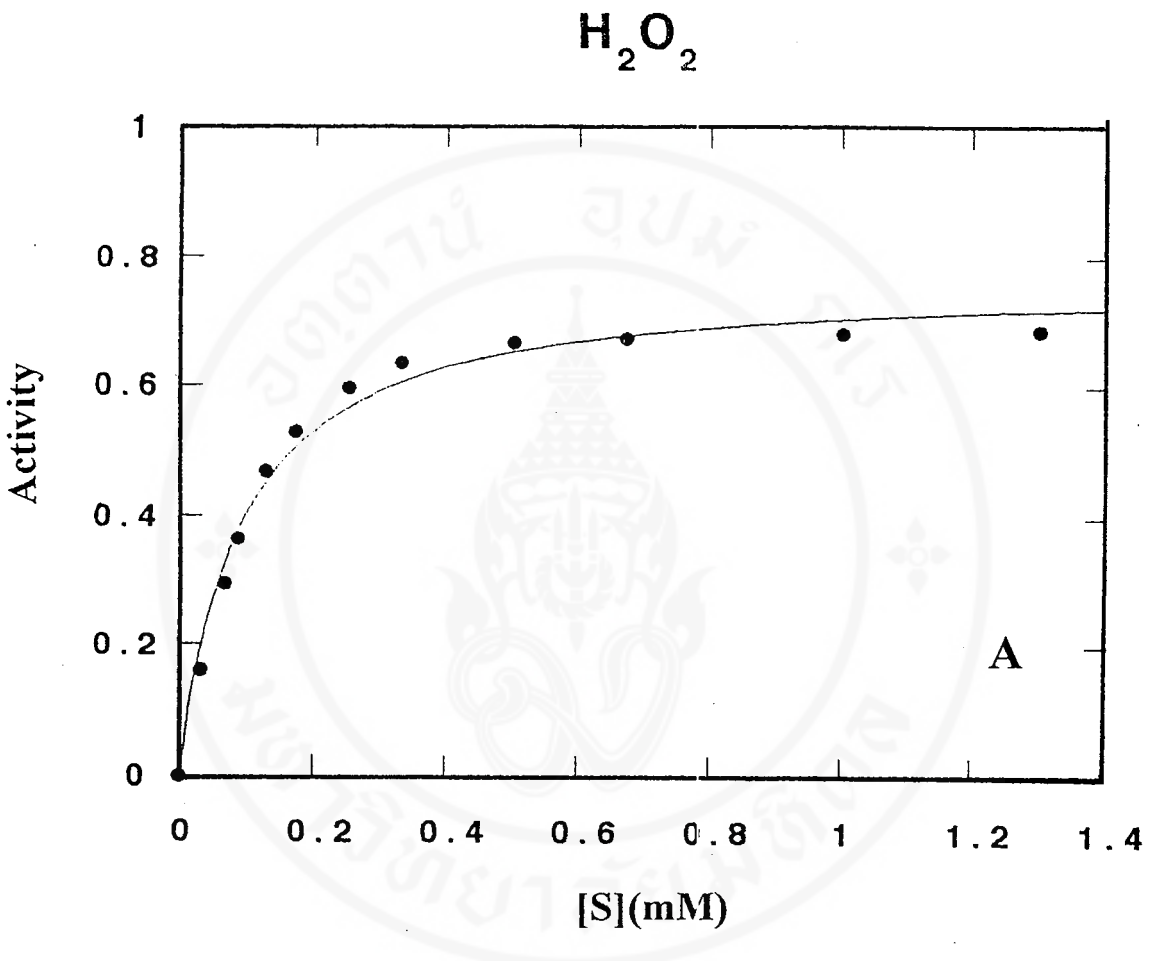


Fig. 23 **H_2O_2 saturation curve of cassava leaf peroxidase (A) and its Lineweaver-Burk Plot (B).**

Activity = Unit/ml

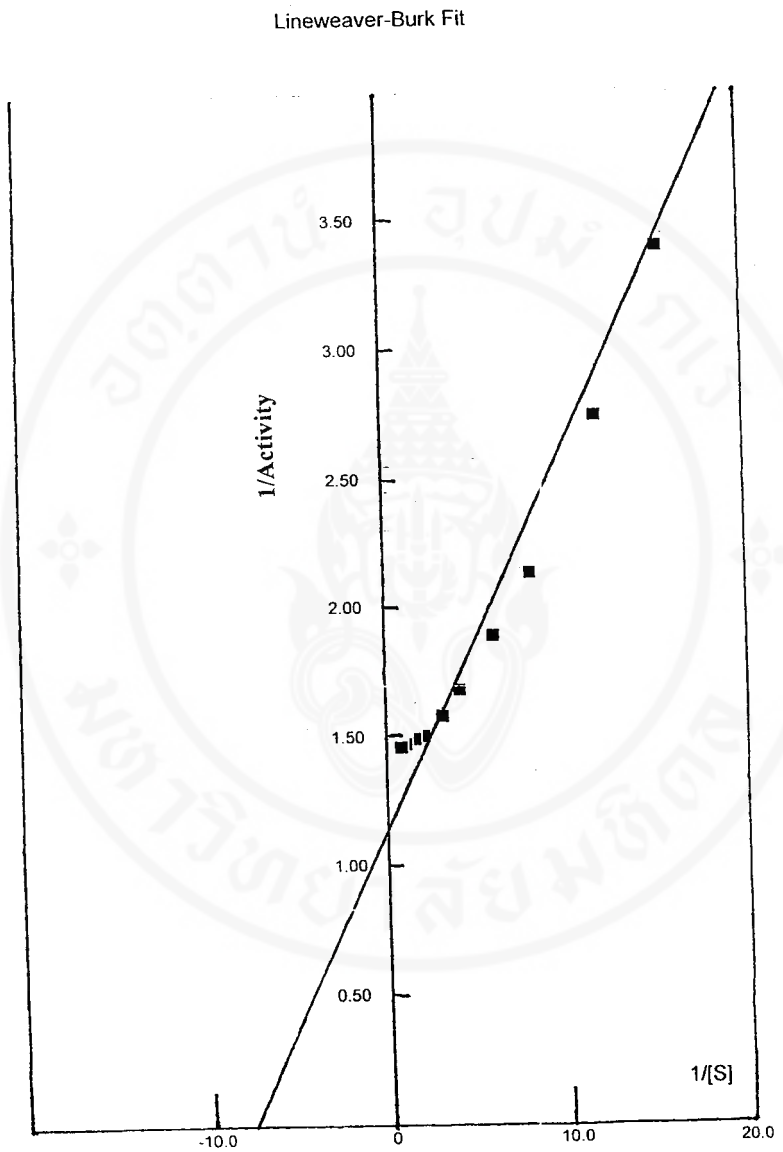


Fig. 23 (cont'd.)

The correlation coefficient = 0.99

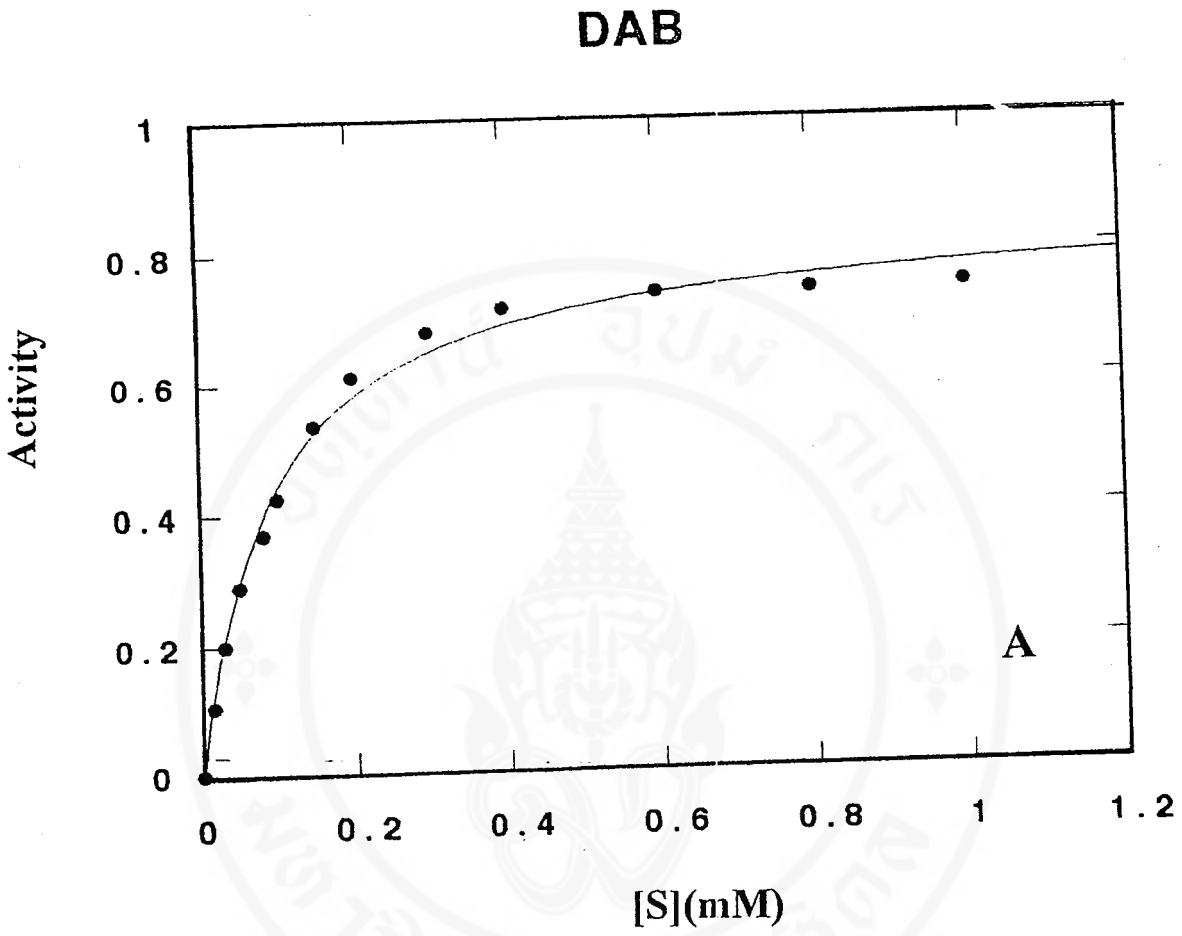


Fig. 24 DAB saturation curve of cassava leaf peroxidase (A) and its Lineweaver-Burk Plot (B).

Activity = Unit/ml

B

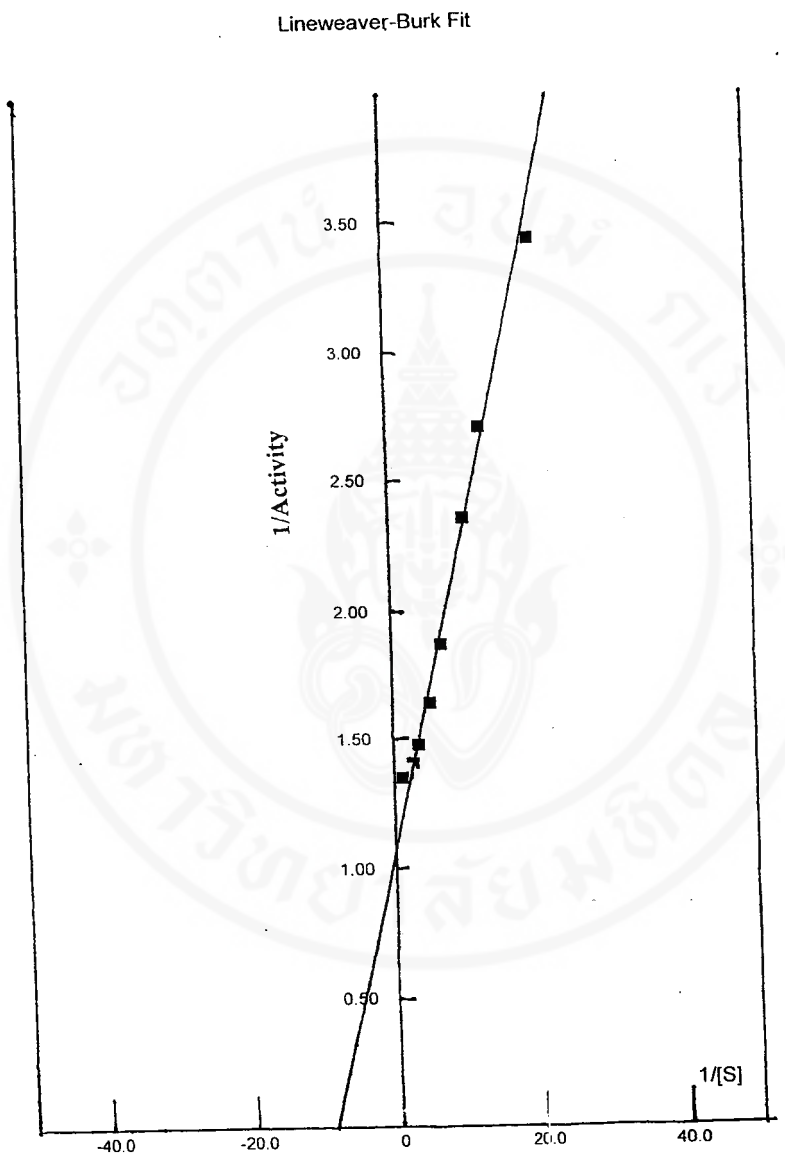


Fig. 24 (cont'd)

The correlation coefficient = 0.99

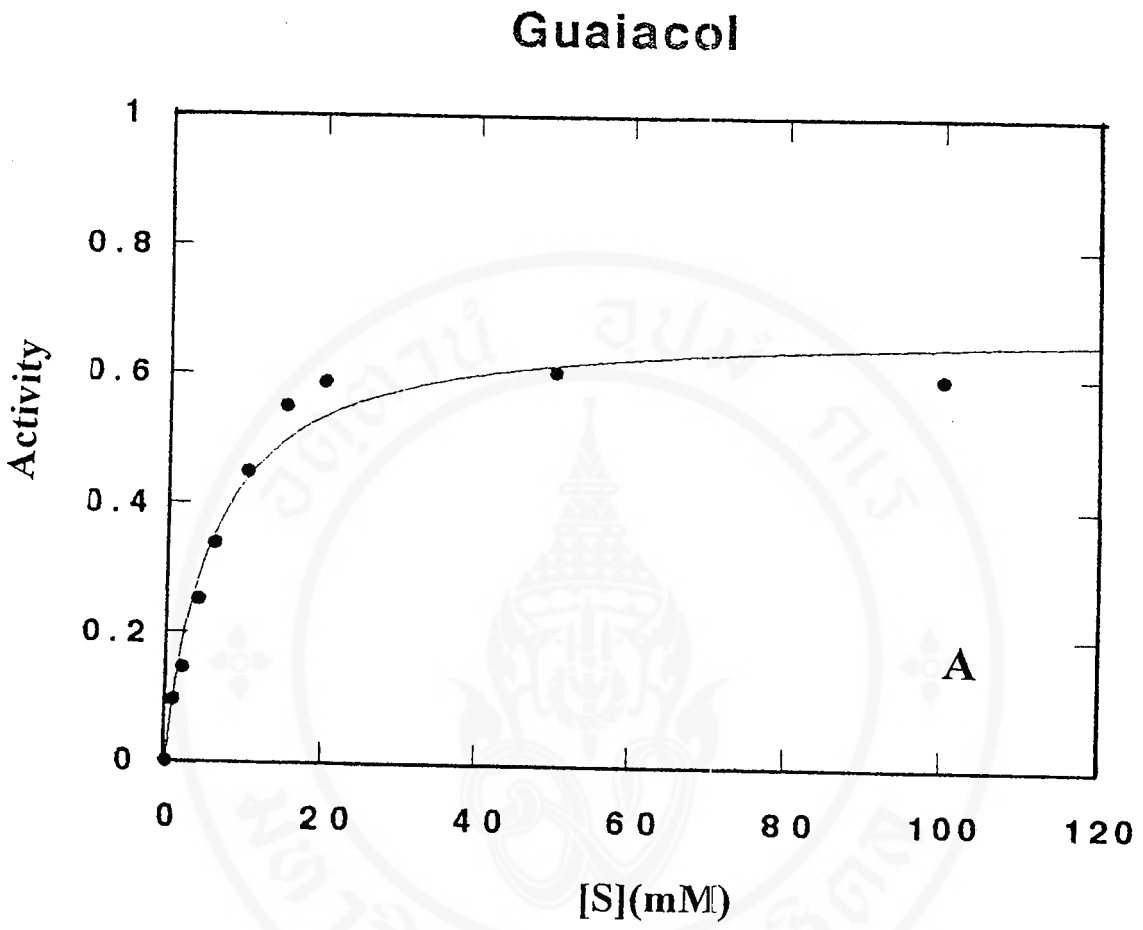


Fig. 25 Guaiacol saturation curve of cassava leaf peroxidase (A) and its Lineweaver-Burk Plot (B).

Activity = Unit/ml

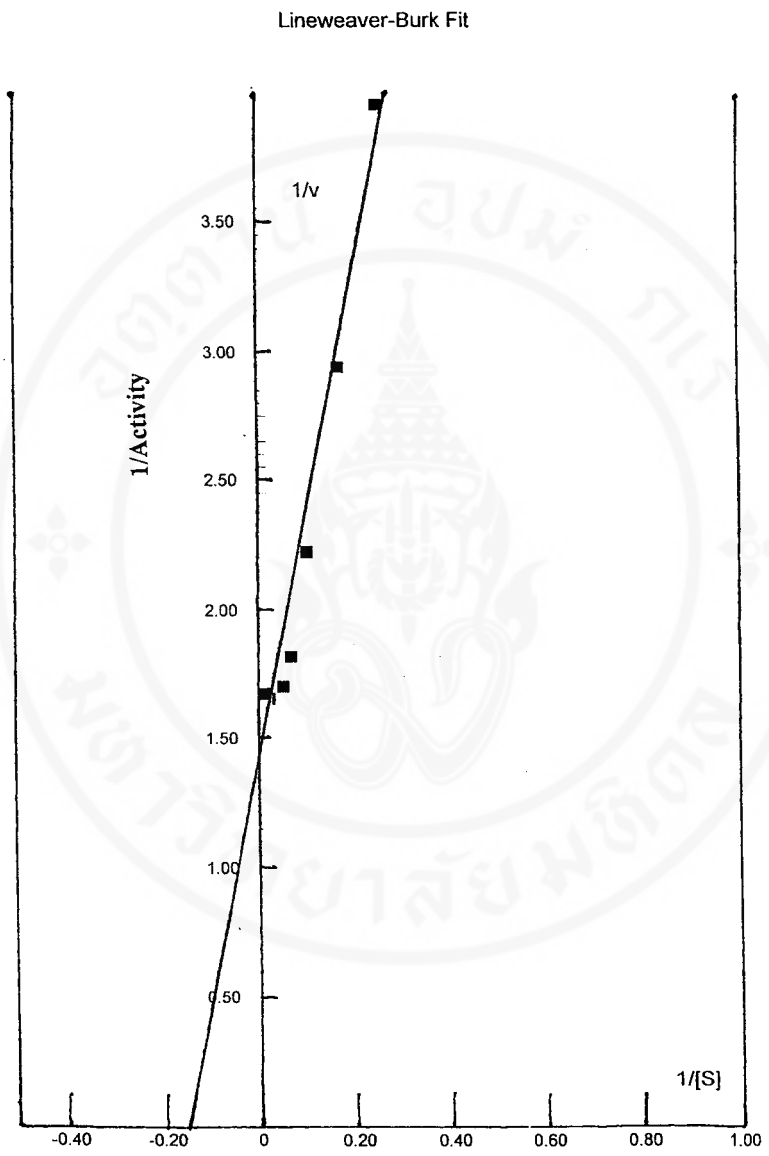


Fig. 25 (cont'd)

The correlation coefficient = 0.98

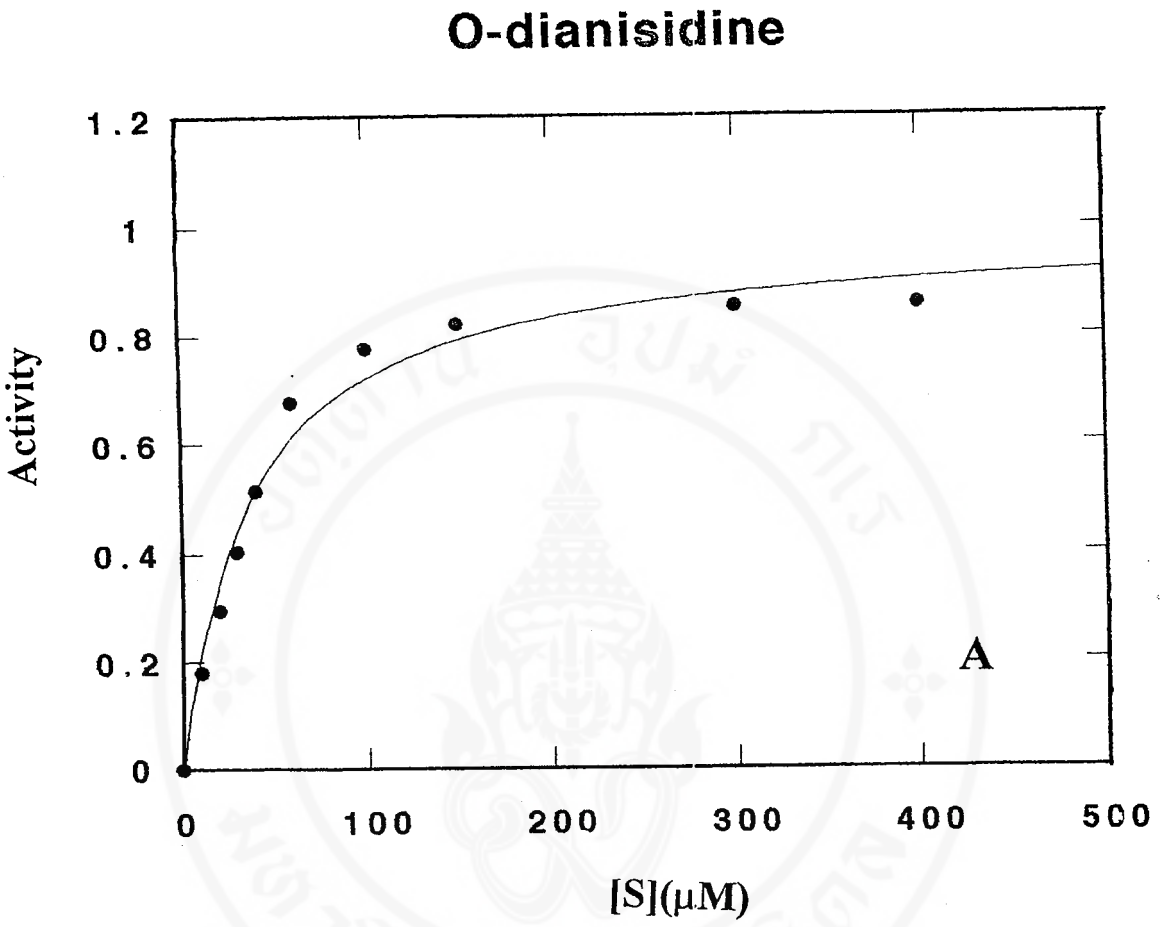


Fig. 26 **o-Dianisidine saturation curve of cassava leaf peroxidase (A) and its Lineweaver-Burk Plot (B).**

Activity = Unit/ml

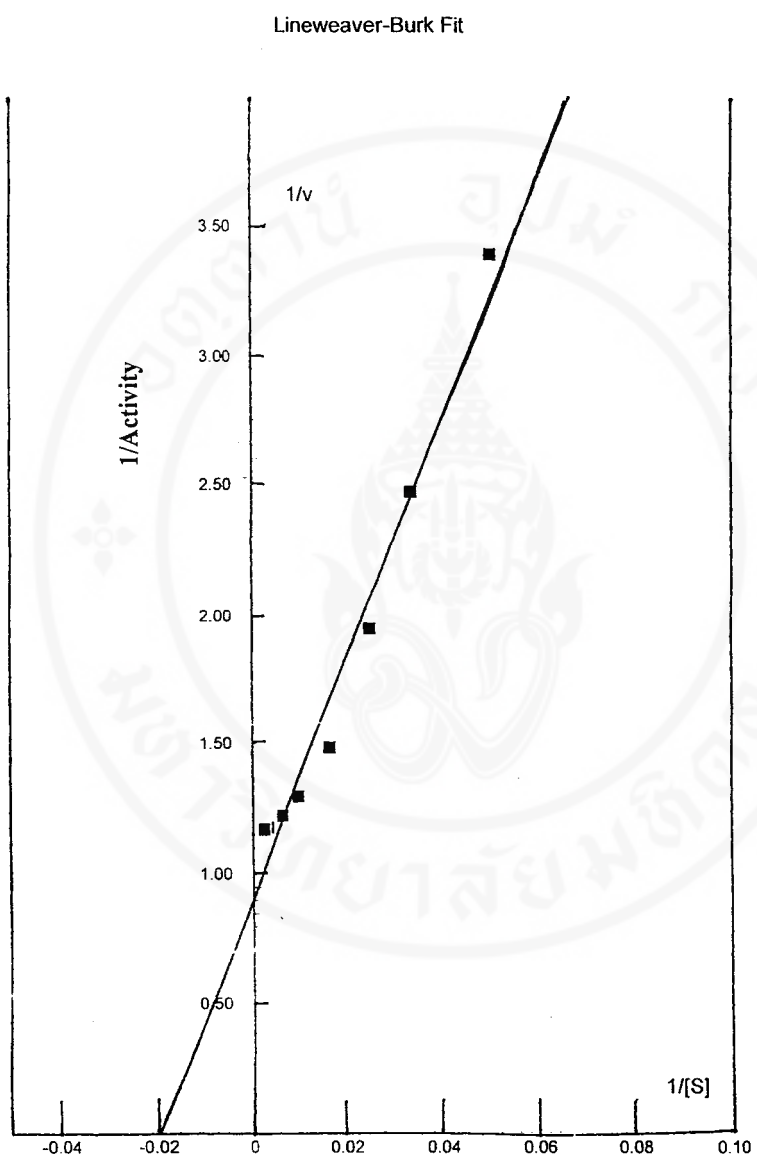


Fig. 26 (cont'd.)

The correlation coefficient = 0.99

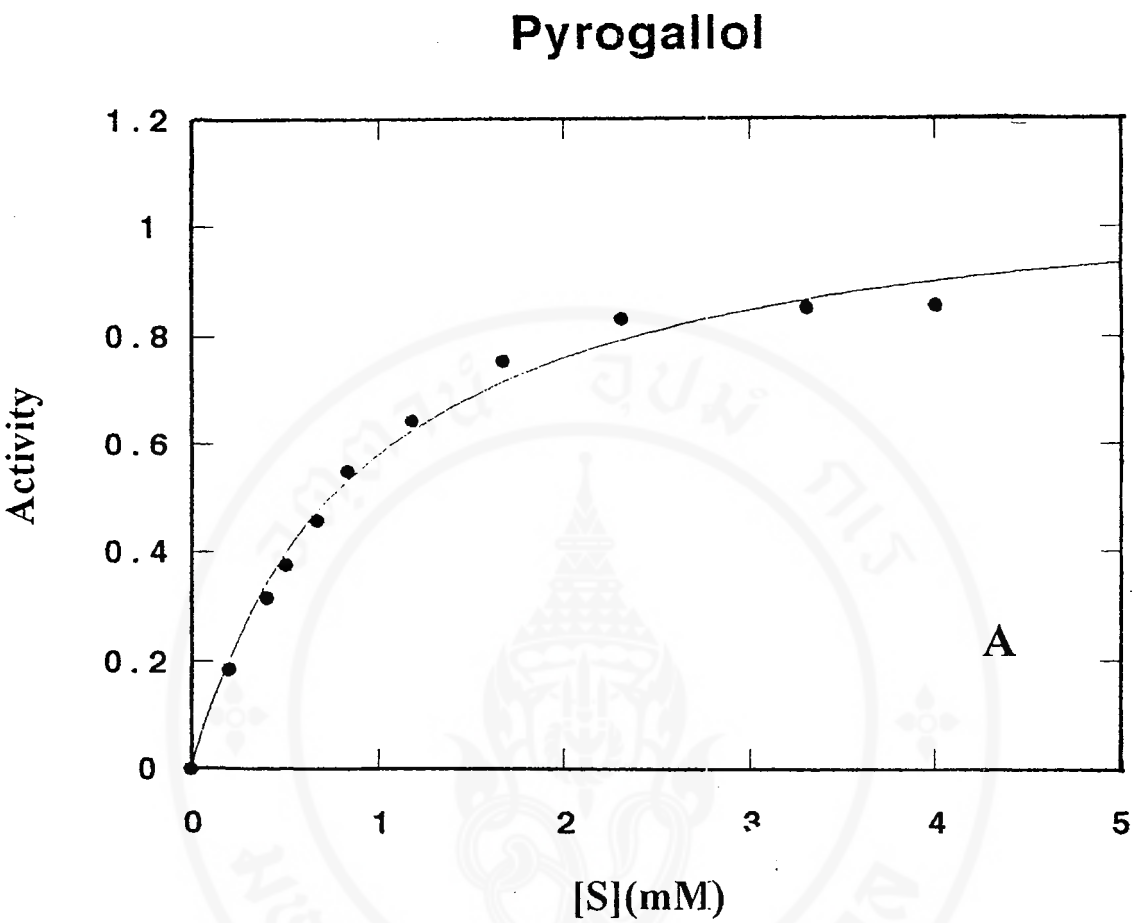


Fig. 27 Pyrogallol saturation curve of cassava leaf peroxidase (A) and its Lineweaver-Burk Plot (B).

Activity = Unit/ml

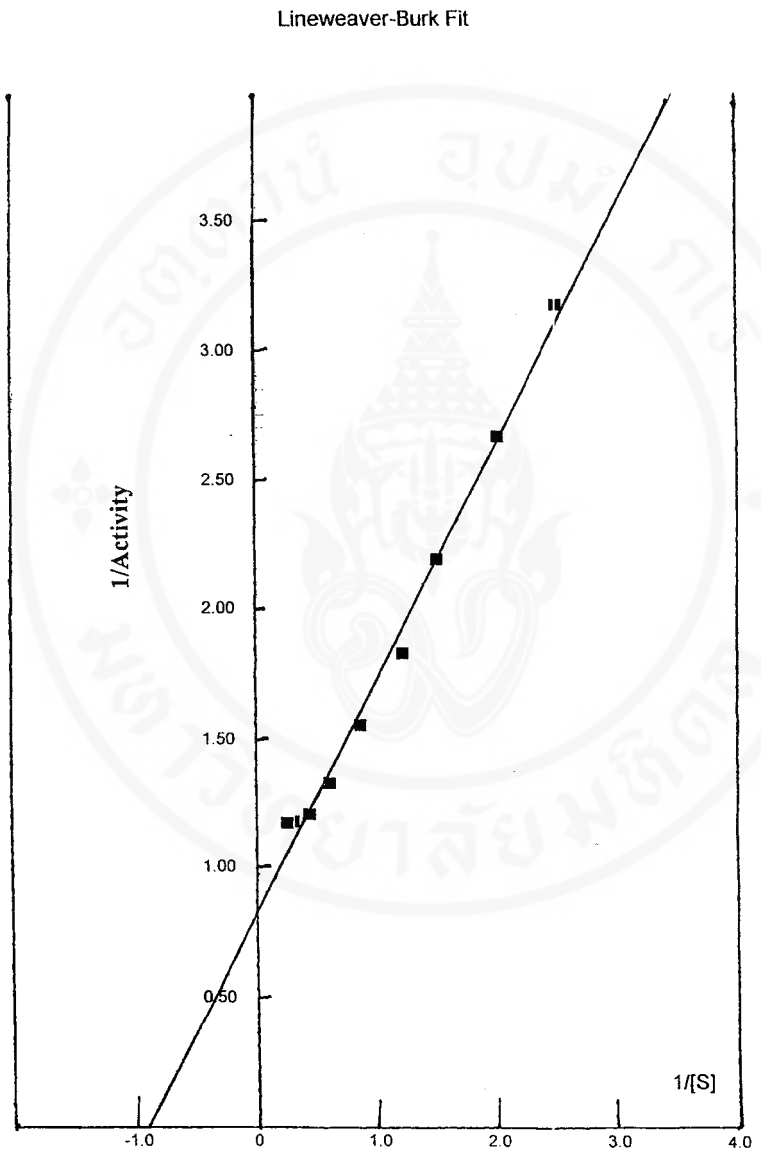


Fig. 27 (cont'd.)

The correlation coefficient = 0.99

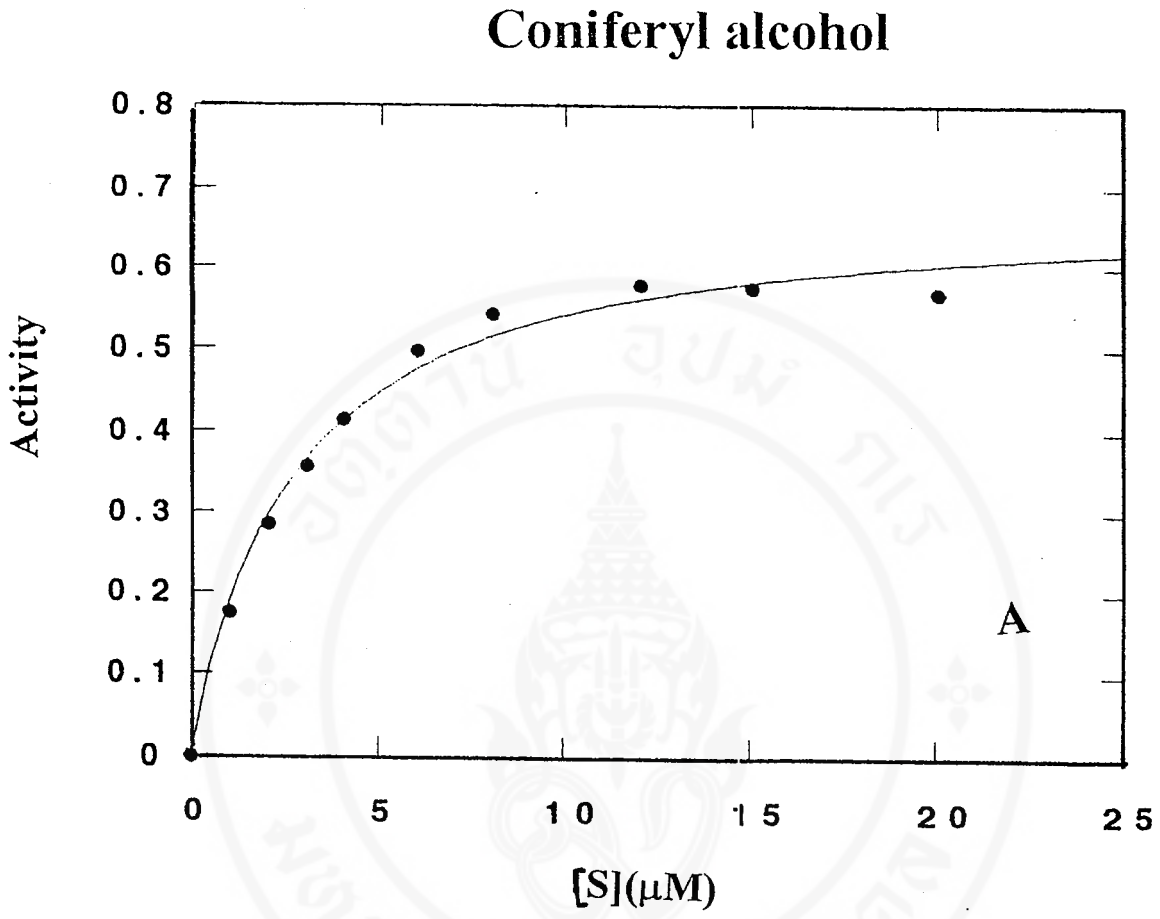


Fig. 28 Coniferyl alcohol saturation curve of cassava leaf peroxidase (A) and its Lineweaver-Burk Plot (B).

Activity = Unit/ml

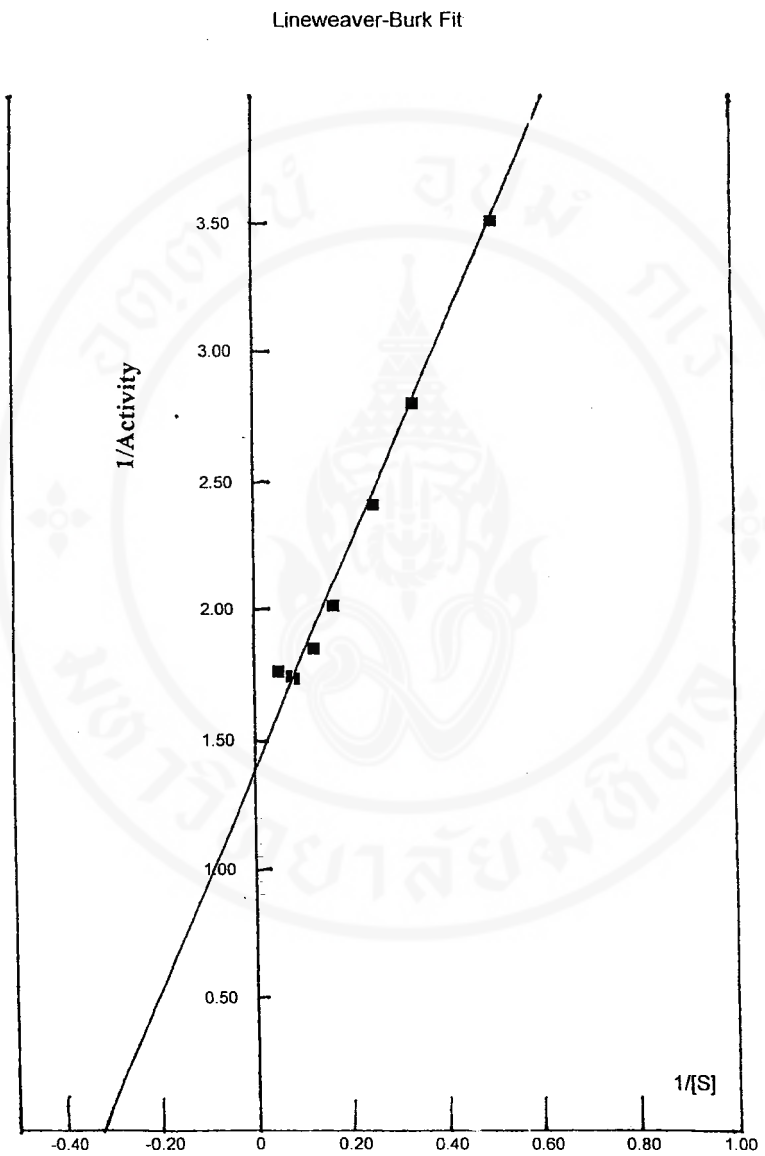


Fig. 28 (cont'd.)

The correlation coefficient = 0.99

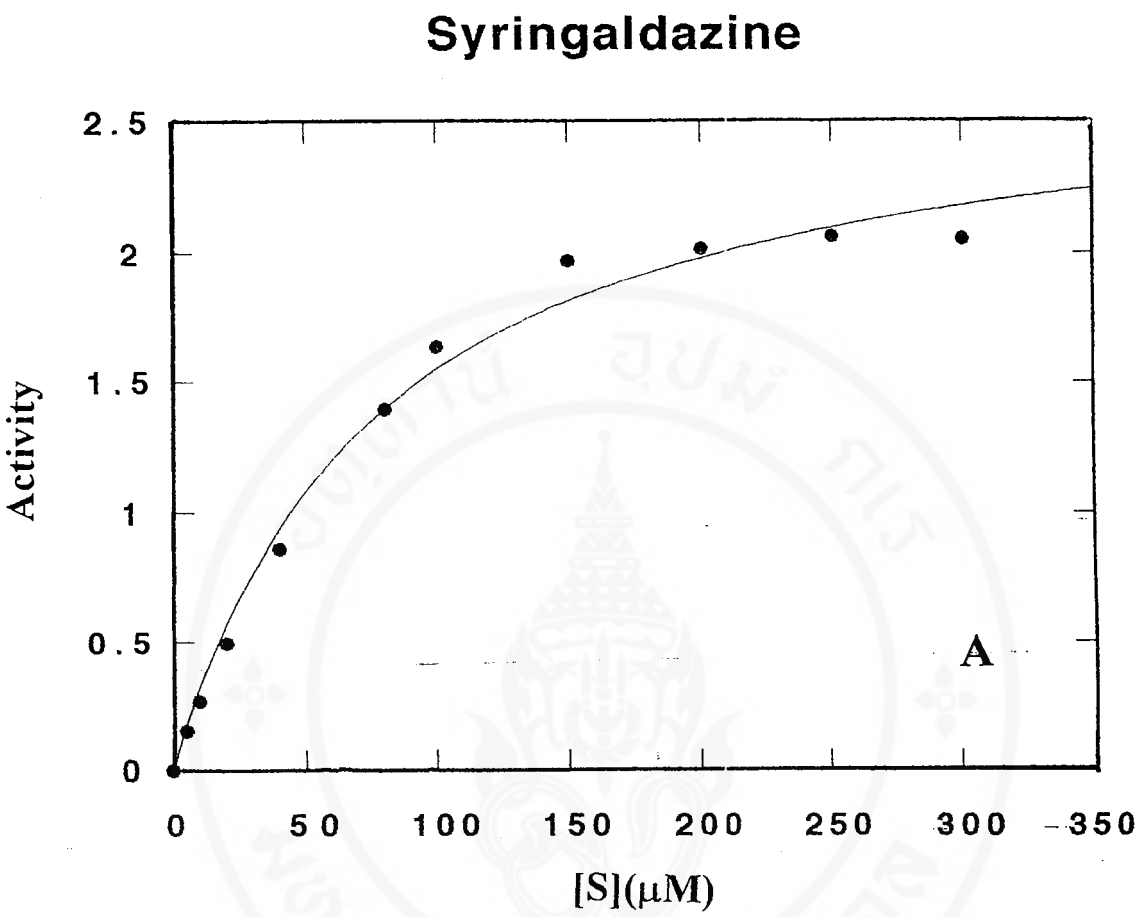


Fig. 29 **Syringaldazine saturation curve of cassava leaf peroxidase (A) and its Lineweaver-Burk Plot (B).**

Activity = Unit/ml

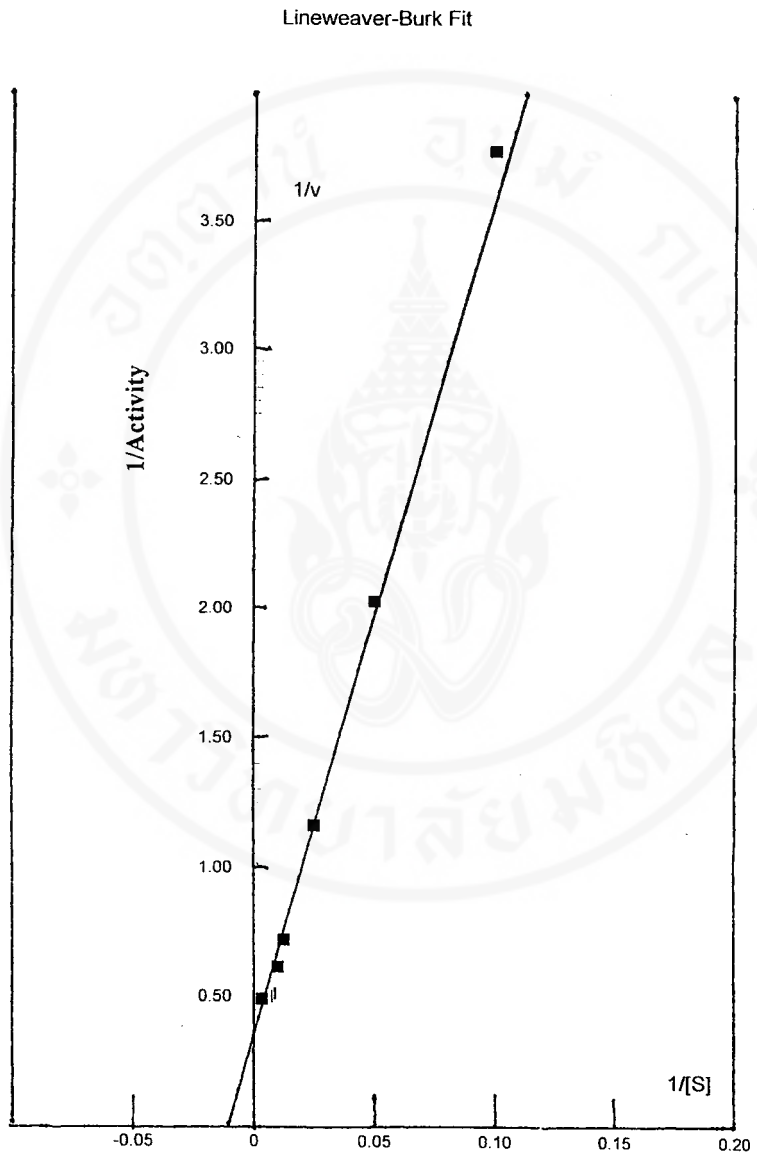


Fig. 29 (cont'd.)

The correlation coefficient = 0.99

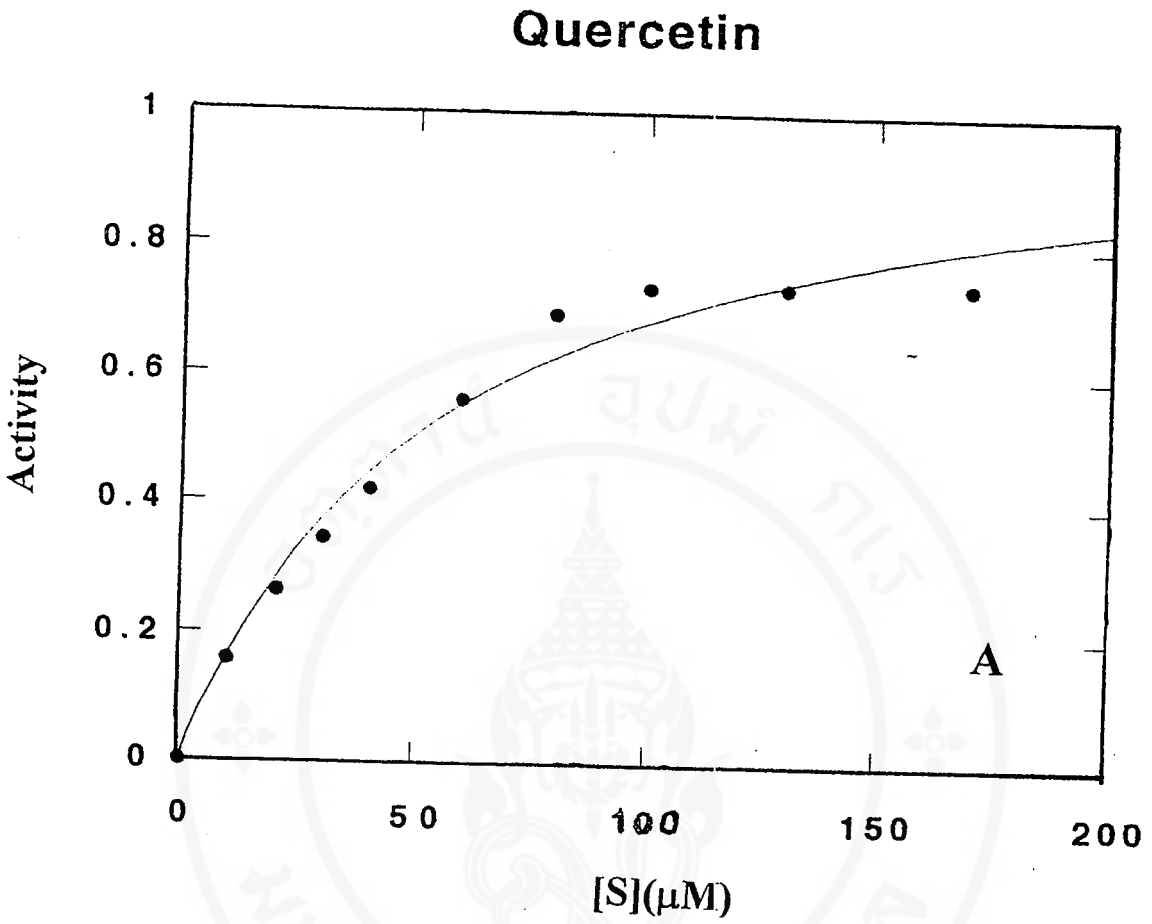


Fig. 30 Quercetin saturation curve of cassava leaf peroxidase (A) and its Lineweaver-Burk Plot (B).

Activity = Unit/ml

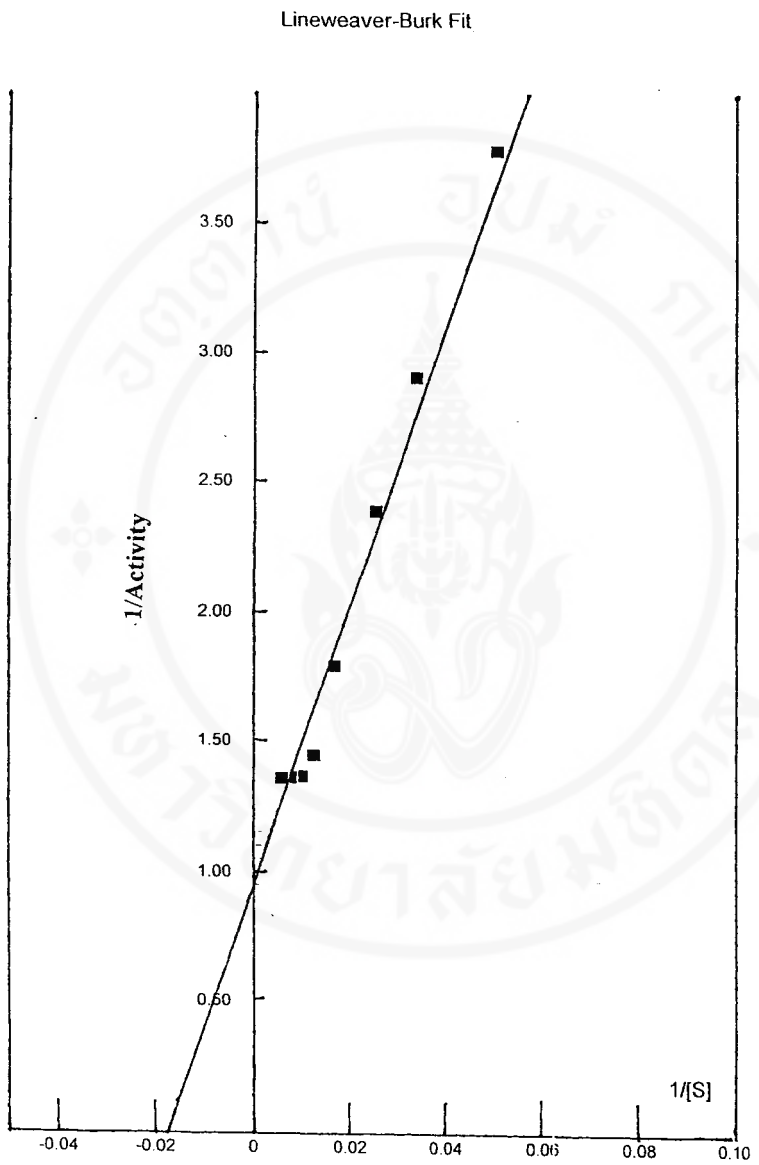


Fig. 30 (cont'd.)

The correlation coefficient = 0.99

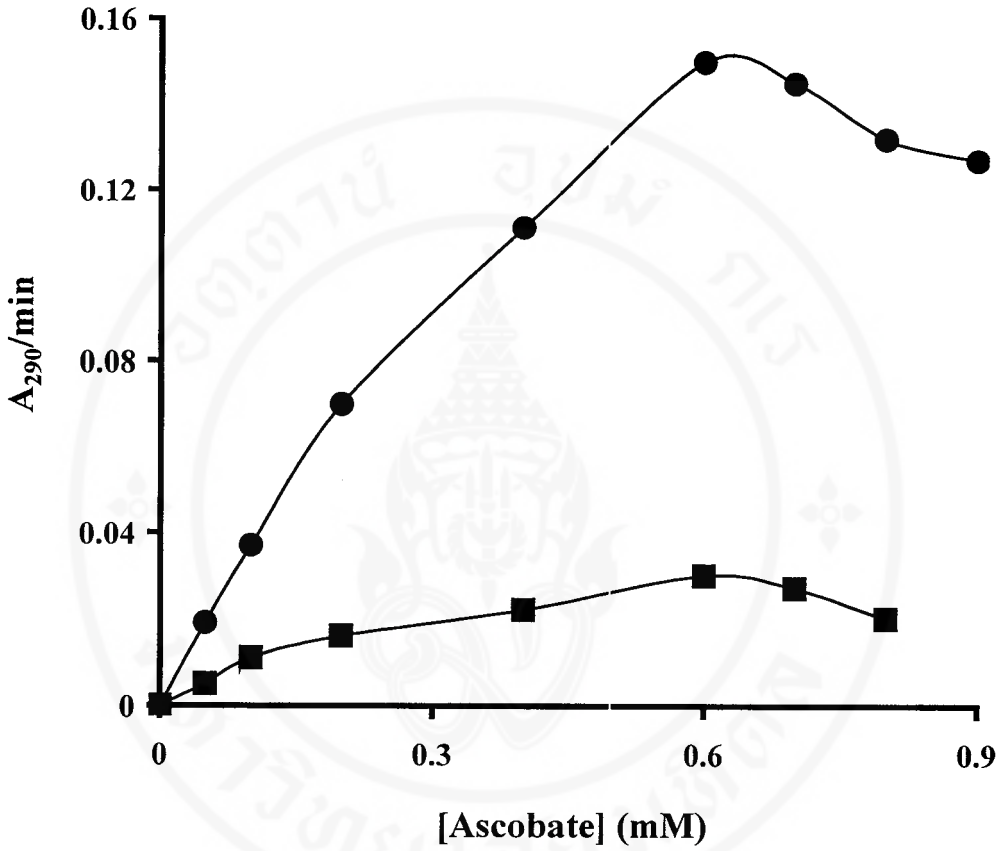


Fig. 31 Ascorbate saturation curves

= Horseradish peroxidase (2.5 μg in 3 ml)

= Cassava leaf peroxidase (1.75 μg in 3 ml)

3.3 Inhibitors of cassava peroxidase

The inhibition curve was obtained for each inhibitor as described in section 2.14. The 2% methanol and 6.5% DMSO (final concentration in the reaction mixture) were used to dissolve some substrates of the enzyme. Methanol (2%) was used to dissolve quercetin, o-dianisidine, coniferyl alcohol and 6.5% DMSO was used to dissolve syringaldazine. So, the effects of methanol and DMSO on the enzyme activity were also studied. They were found to inhibit the cassava peroxidase (Fig. 32). However, their inhibiting effects were slight at the concentration used to dissolve the substrates.

Effects of azide, cyanide and urea on the enzyme activity were also investigated Thiourea at 1.55 mM inhibited about 60% of peroxidase activity whereas the thiourea at 10 mM inhibited the activity completely (Fig. 32). At 150 mM, azide inhibited about 60% of the peroxidase activity (Fig. 33). Enzymes activity decreased as the concentration of cyanide increased (Fig. 33). At 25 mM cyanide inhibited about 60% of the peroxidase activity.

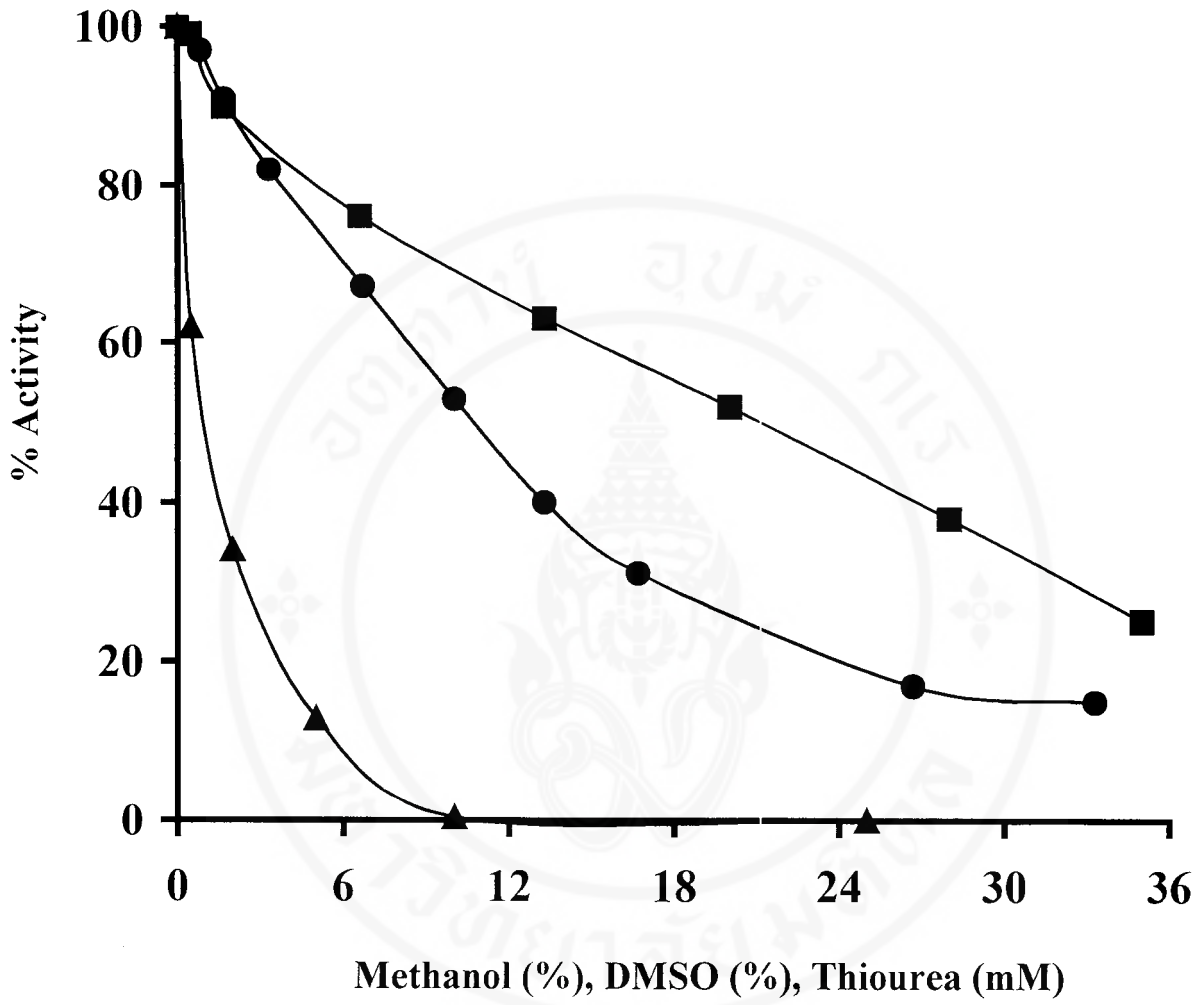


Fig. 32 Effects of methanol, DMSO and thiourea on cassava leaf peroxidase activity.

Cassava leaf peroxidase was incubated in various concentration of methanol, DMSO and thiourea. The remaining activity was determined in the reaction mixture. Activity was calculated in % relative activity of that in the absence of methanol, DMSO and thiourea.

▲ = thiourea, ● = DMSO, ■ = methanol

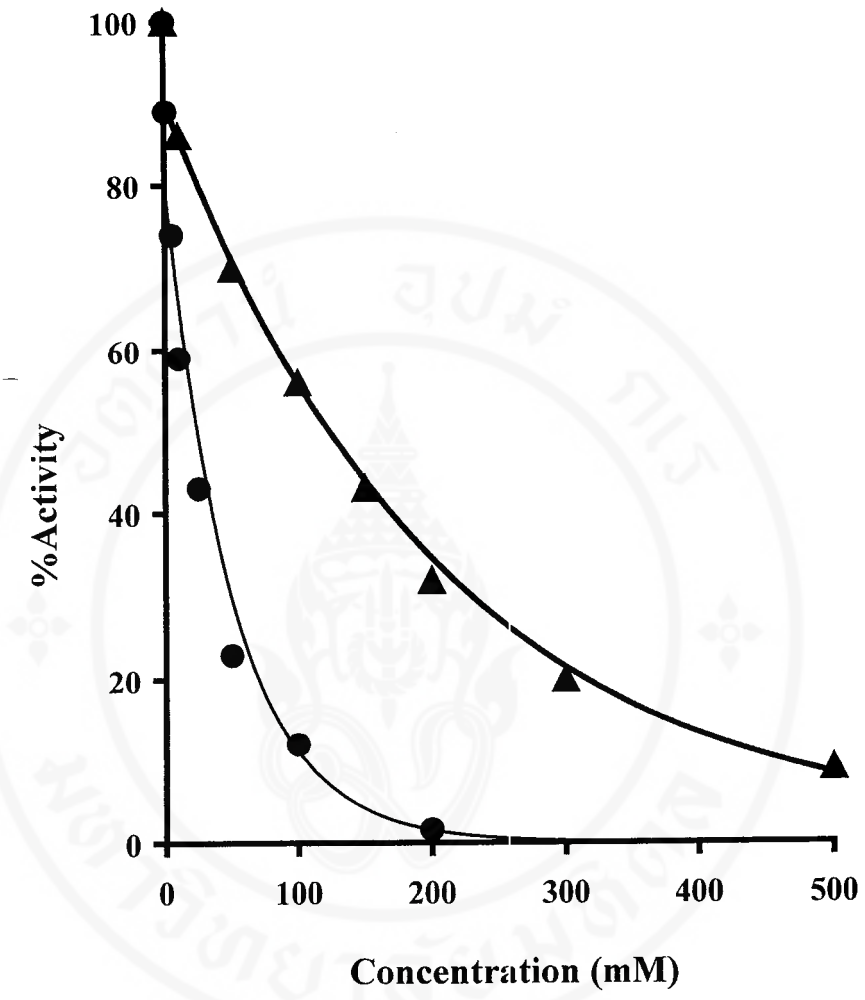


Fig. 33 Effects of cyanide and azide on the cassava leaf peroxidase activity.

Purified peroxidase was incubated in various concentration of cyanide and azide for 20 min. The remaining activity was determined in the reaction mixture. Activity was calculated in % relative activity of that in the absence of cyanide and azide.

● = cyanide ▲ = azide

CHAPTER IV

DISCUSSION

4.1 Purification of Cassava Peroxidase

Peroxidases are widely distributed in plants. Because peroxidases are widely used in immunological, histological and coupled enzyme assays, it is worthwhile to purify many enzymes from a cheap source like cassava. Based on previous work on purification of peroxidases from other sources which are summarized below, the purification procedure used here has been developed.

Patcharakorn Rattanapume *et al* (52), could purify peroxidase from *Hevea brasiliensis* leaves by using ammoniumsulfate precipitation followed by DEAE Sephacel and Sephadex G-75. The enzyme was purified about 115 folds. Maria Rosaria Elia (15) also purified the enzyme from potato tubers by using ammonium sulfate precipitation followed by DEAE Sephacel and hydroxylapatite. The purification was about 130 folds. Isina G. Gazaryan *et al.* (53) purified a peroxidase from tobacco leaf by using DEAE and gel filtration. They obtained 10 fold purification and its specific activity 4600 U/mg. Thakorn Sornwatana and Montri Chulavatnatol (34) purified a peroxidase from cassava parenchyma by using ammonium sulfate precipitation, followed by chromatography on Con-A Sepharose and Sephacryl S-300. The enzyme was purified about 67 folds. In this study, the peroxidase was purified from cassava leaf because the leaf enzyme had higher specific activity.

In the purification procedure, ammonium sulfate has been used to precipitate the enzyme. However, other compounds, particularly plant phenolic compounds, may be

trapped by precipitation as well. In the extracting solution, PVPP is used to remove the phenolic compounds. PMSF is used to inhibit protease. Further purification using an affinity column of Con A Sepharose (Fig. 7) separates the enzyme into 2 fractions: an unbound peak and bound peak. The unbound peak has a low peroxidase specific activity and hence is not of our interest. The binding of the cassava peroxidase to the Con A-Sepharose suggests that the enzyme probably was a mannose-bearing glycoprotein (54). The unbound peroxidase was likely to be another isozyme with no mannose-containing carbohydrates in the enzyme.

Since the cassava leaf peroxidase can be purified further to homogeneity by gel filtration, use of an ion exchange column such as Sephacel or absorption column such as hydroxyapatite becomes unnecessary.

4.2 Structural properties of cassava leaf peroxidase

The cassava leaf peroxidase is a dimer with a native molecular weight of 112 kD (Fig. 10) and a subunit molecular weight of 56 kD subunits (Fig. 12). Therefore, cassava leaf peroxidase is similar in size to cassava parenchyma with native molecular weight of 105 kD and subunit molecular weight of 54 kD (34). However, cassava leaf peroxidase is different from horseradish peroxidase which has a molecular weight of 42 kD (55), para rubber (*Hevea brasiliensis*) bark peroxidase (50 kD) (35) and *Araucaria araucana* seeds enzyme which exists as two cationic peroxidases with molecular weights of 83 kD and 145 kD (56).

The cassava leaf peroxidase exists in two forms with pI values of 6.40 and 6.25 (Figs. 13 and 14). So the cassava leaf enzyme is a neutral peroxidase similar to peroxidases from cassava root (pI = 6.8) (57) and barley leaf (pI 6.3, 6.8) (58) but

different from the enzymes from Heavea bark (pI = 3.5) (35), petunia leaf (pI = 3.8) (59), tobacco leaf (pI = 3.5) (53), Polar xylem (PXP3) (pI = 3.43 and 3.53) (32). The pIs of the horseradish peroxidases are reported to be in the basic range (pI = 10-12) (60). In carrot roots peroxidase had pI > 9.3 (61).

Concerning the amino acid composition (Table 7), cassava leaf peroxidase, barley leaf P1 peroxidase and Japanese-radish peroxidase (58) each has similar amount of amino acids (Glx + Asx). Basic amino acids of the cassava leaf peroxidase (Lys, Arg, and His) are not much different from those of Japanese-radish peroxidase. However, barley leaf peroxidase has a lower amount of basic amino acids than other enzymes. The cassava leaf peroxidase has a higher amount of amino acids (Glx + Asx) than the basic amino acids (Lys, Arg and His). The amino acid compositions of horseradish peroxidase C, turnip peroxidase, carrot root peroxidase, anionic potato peroxidase, Poplar xylem peroxidase and *Araucaria araucana* were different from that of the cassava enzyme.

Studies of the prosthetic group (Figs.16 and 17) clearly shows that the cassava leaf peroxidase contains heme with a Soret band at 404 nm. Several other peroxidases are also heme-enzymes. Horseradish peroxidase has a Soret band at 403 nm, a novel class III peroxidase from tea leaves has a Soret peak at 406 nm, barley leaf peroxidase showed absortion pesk at 403 nm, petunia leaf peroxidase shows a Soret band at 405 nm (59). It has been known that the chemical reactions catalysed by peroxidases take place on the heme prosthetic group. Removal of heme causes a total loss of the enzymatic activity (62).

Table 7 Comparison of amino acid compositions of peroxidases from different sources in mole%

Amino acid	Cassava leaf peroxidase (pI = 6.4)	BLP1 (pI 6.3)	JRP	*A. araucana* (basic pI) (56)		PXP_3	HRP C	PP Anionic	CRP Cationic	TP
				145 kD	83 kD	(pI = 3.53)	(pI = 8.6)		pI > 9.3	
Asx	7.03	10.4	11.7	4.52	4.87	14.80	16.1	16.3	6.5	13.6
Glx	13.61	9.6	7.8	7.03	8.6	8.08	6.7	9.4	3.3	4.9
Lys	6.62	3.5	2.6	4.92	5.37	2.17	2	4.1	0.8	3.5
Arg	5.45	4.4	9.7	4.12	2.26	4.48	7	4.5	7.1	5.9
His	1.26	1.9	1.3	1.73	2.93	1.13	1	1.6	1	1
Pro	5.08	4.3	5.2	5.87	4.92	4.41	5.7	4.7	7.3	3.8
Met	1.4	1	1	nd	nd	0.95	1.3	1.5	1.8	2.1
Phe	3.9	4.4	4.5	0	0.75	5.76	6.7	5.9	5.2	4.9
Ile	4.71	3.4	5.2	1.76	1.9	3.59	4.3	4.7	5.3	5.2
Leu	8.86	9.2	8.8	3.74	3.19	9.91	11.7	9.4	12.2	7.3
Val	6.11	6.4	8.8	0	3.97	5.2	5.7	7.2	6.3	6.6
Ala	8.21	12	9.1	6.86	10.23	9.35	7.7	6.8	10.5	11.1
Gly	12.74	11.3	11	32.91	36.16	7.31	5.7	5.2	11.9	8.4
Ser	7.49	9.6	7.5	18.84	15.65	9.95	8.4	5.9	8.6	14.6
Thr	5.21	6.5	5.2	4.32	2.5	9.05	8.4	7	9.3	5.6
Tyr	1.93	1.9	0.6	0	0	1.38	1.7	0.5	2.9	1.4
Cys	0.39			nd	nd	2.47		2.5	nd	

BLP1 = Barley leaf peroxidase (58), JRP = Japanese-radish peroxidase (58), PXP_3 = Polar xylem peroxidase (32), HRP = Horseradish peroxidase (58), PP = Potato peroxidase, CRP = Carrot root peroxidase (61), TP = Turnip peroxidase (56)

4.3 Catalytic properties of cassava leaf peroxidases

A simple characterization of the catalytic properties of an enzyme may be defined by the sensitivity to pH and temperature.

The slight acidic optimum pH of the cassava peroxidase (pH 6) is similar to *Hevea brasiliensis* leaf peroxidase (pH optimum at 5.4) (45), petinia leaf peroxidase pH optimum at 5 (50), tea leaf peroxidase (optimal pH in the range of 4.5-5.0) (19) and horseradish peroxidase (pH optimum at 4.3) (1). Hevea bark enzyme has a broad pH optimum in range 5-7 (35). The cassava peroxidase from leaf was stable in a broad pH range from 4-11 (Fig. 20). This is similar to barley grain peroxidase which is stable, in the pH range of 3-11 (63). Hevea bark peroxidase is active in the pH range of 5-9 (37).

The activity of the cassava peroxidase from leaf is rather stable (Fig. 21). The enzyme retains about 70% of its activity during incubation at temperatures up to 65°C for 24 hr. In comparison, Hevea bark peroxidase is stable up to 60°C with 100% activity while 75% of the activity still remains at 70°C (37), Fox-tail millet A_{3p} peroxidase is stable up to 65°C at pH 7.4 (64) and lignin peroxidase from Phanerochaete chrysosporium is relatively stable for 6 hr at 60°C (65).

The temperature optimum of the purified peroxidase from cassava leaves is 60°C (Fig. 22). This is similar to the peroxidase from Hevea leaves which has a temperature optimum in the range of 60-70°C (52). In Gracilaria B3, the optimum temperature of its peroxidase is 60°C also (66).

Therefore, the cassava leaf peroxidase should operate well in the extreme conditions (high pH and high temperature) in which most other enzyme may be in active. Both the heat and pH stabilities of the enzyme shoud widen the range of the peroxidase applications.

The binding of the peroxidase from cassava leaves to the Con A column suggests that it may be a glycoprotein enzyme. Plant peroxidases (classical) are glycoproteins and the carbohydrate chains may enhance the thermal stability of the enzymes (67). Partial deglycosylation of avocado peroxidase using N-glycosidase also causes a decrease in thermostability (68). Partial deglycosylation of an anionic peroxidase purified from peach seed using N-glycosidase results in a reduced activity, higher K_m for H_2O_2 and a decrease in enzyme stability at 4°C (69).

It remains to be shown if the stability of the cassava leaf peroxidase is due to the presence of carbohydrates attached to the enzyme.

Comparing the pH optimum curve and pH stability curve (Fig. 20) of the cassava leaf peroxidase points to a few suggestions. At low pH, the loss of the peroxidase activity is likely to be due to a structural denaturation of the enzyme. At high pH where the structure of the enzyme is stable, the decrease in the activity is probably due to deprotonation of a group at the active site. This group may have a pKa of about 7 (mid point in the alkaline range of the pH optimum curve).

4.4 Kinetic properties of cassava leaf peroxidase

The anionic peroxidase associated with the suberization response in potato tuber shows the following order of substrate preference : feruloyl (o-methoxyphenol) > caffeoyl > p-coumaryl ≈ syringyl. These compounds accumulate in tubers during

wound healing (33). This enzyme prefers for guaiacol over ascorbate as substrate. The peroxidase from Poplar xylem exists as isoenzymes PXP_1, PXP_2, PXP_3, PXP_4, PXP_5. They can oxidize ABTS and DAB where as only PXP_3, PXP_4, PXP_5 can oxidize the lignin monomer analog, syringaldazine. PXP_{3-4} and PXP_5 have been suggested to be involved in lignin polymerization (32). In *Hevea brasiliensis*, the peroxidase is more specific for o-dianisidine than ABTS and pyrogallol. K_m values for o-dianisidine and H_2O_2 are 0.12 mM and 1.10 mM respectively and it is inhibited by KCN and NaN_3 (52). In cassava root plantlet, peroxidase shows K_m value for DAB and H_2O_2 of 0.07 μM and 0.33 mM respectively (70). In pea cytosol, ascorbate peroxidase shows a very high preference for ascorbate as electron donor and is specifically inhibited by p-chloromercurisulfonic acid. Ascorbate peroxidase from potato tubers is labile in the absence of ascorbate and it has K_m for H_2O_2 of 30 μM and K_m for ascorbate of 55 μM. Ascorbate is specifically required as the electron donnor. The enzyme is inhibited by compounds reacting with thiol groups p-hydroxy-mercuribenzoate, p-choromercuriphenylsulfonate (15).

In the studies, cassava leaf peroxidase shows a good affinity for H_2O_2 (K_m = 0.09 mM) which is about 12.2 times higher than *Hevea brasiliensis* leaf enzyme (K_m = 1.1 mM), 3.7 times higher than cassava root plantlet enzyme (K_m = 0.33 mM) and 2.9 times higher than Hevea bark enzyme (K_m = 0.26 mM). However, its affinity for H_2O_2 is lower than ascorbate peroxidase from potato tubers by about 3 times (K_m = 0.03 mM). The affinity for DAB (K_m = 0.092 mM) of the cassava leaf peroxidase is higher than that of Hevea bark enzyme by about 18.2 times (K_m = 1.67 mM) but is lower than cassava root plantlet (K_m =0.07 μM). The cassava leaf peroxidase shows the K_m values for other substrate: coniferyl alcohol (K_m = 0.003 mM), O-dianisidine (K_m = 0.037 mM), quercetin

(K_m = 0.054 mM), syringaldazine (K_m = 0.077 mM), DAB (K_m = 0.092 mM), pyrogallol (K_m = 0.89 mM), guaiacol (K_m = 5.52 mM) (Table 6). For the enzyme, ascorbate serves as electron donor.

KCN and NaN_3 are strong inhibitors of cytosolic ascorbate peroxidase and horseradish peroxidase. In cassava leaf peroxidase 150 mM NaN_3 and 25 mM KCN led to 60% inhibition. The parenchyma of cassava root is more resistant to KCN and NaN_3 than the cassava leaf peroxidase (34). In potato tubers, the peroxidase (60% inhibition by 0.5 mM cyanide and 1 mM azide) is less resistant than cassava leaf peroxidase. In cytosolic ascorbate peroxidase from pea shows 80% inhibition by 0.5 mM KCN and 70% inhibition by 5mM NaN_3 (3). Horseradish root peroxidase is reversibly inhibited by cyanide and sulfide at a concentration of 5-10 M (20). KCN and NaN_3 are potent inhibitor of heme proteins. The KCN and NaN_3 inhibition supports the spectral data showing that the cassava leaf peroxidase is a heme enzyme. Urea at high concentration (6mM) is a detergent that can inactivate an enzyme by disrupting the hydrophobic interaction in the structure of the enzyme. However ,the concentration of thiourea that can inactivate the cassava peroxidase is about 10 mM (maximum inhibition)(Fig 32). So it probably acts by inhibiting the peroxidase reaction.

In conclusion, the cassava leaf peroxidase is a classical peroxidase because it preferentially oxidizes organic phenolic compounds and exhibits a very poor activity for ascorbate. It contains heme and it is a glycoprotein. The physiological role of cassava leaf peroxidase is presumably in lignin polymerization because it can oxidize coniferyl alcohol and the lignin monomer analog, syringaldazine.

CHAPTER V

SUMMARY

1. Peroxidase was purified from cassava leaf by ammonium sulfate precipitation, followed by chromatography on Concanavalin A-Sepharose 4B affinity column and Sephadex G-200.

2. The purified peroxidase had a specific activity of 386 unit/mg protein and the purification yield was 13%.

3. The native molecular weight of the purified peroxidase was estimated by Sephadex G-200 gel filtration to be 112 kD.. The subunit molecular weight of the peroxidase was estimated by SDS-PAGE to be 56 kD.

4. The isoelectric point (pI) of the purified peroxidase was estimated by polyacrylamide isoelectric focusing. There were at least 2 isoenzymes with pI values of 6.4 and 6.25.

5. The purified peroxidase was stable in the pH range of 4-11.

6. The optimum pH of the purified peroxidase was 6.

7. The peroxidase were remarkably thermostable, the enzyme retained about 70% of its activity during incubation at temperatures upto 65°C for 24 hr. At 70°C, activity was completely lost after 4 hr of incubation.

8. The optimum temperature of the purified peroxidase was 60°C.

9. The purified peroxidase showed a Soret band with an absorption maximum at 404 nm.

10. The K_m values of bound peroxidase with different substrates were as follows coniferyl alcohol (K_m = 0.003 mM), o-dianisidine (K_m = 0.037 mM), quercetin (K_m =

0.054 mM), syringaldazine (K_m = 0.077 mM), 3,3'diaminobenzidine (K_m = 0.092 mM), pyrogallol (K_m = 0.89 mM) and guaiacol (K_m = 5.52mM).

11. Both sodium azide and potassium cyanide were found to be inhibitory to the peroxidase at final concentration of 150 mM and 25 mM respectively, causing 60% inhibition. The peroxidase was completely inhibited by 10 mM thiourea.

12. The amino acid composition of the peroxidase from cassava leaves was determined.

CHAPTER VI

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RESEARCH GRANT Partially supported by a grant from the National Science and Technology Development Agency (NSTDA)