# Oxidoreductase Enzymes in *Hevea* Latex :

# NAD(P)H-Quinone Reductase and Polyphenol Oxidase

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**Doctor of Philosophy Thesis in Biochemistry**

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| ชื่อวิทยานิพนธ์ | เอนไซม์ออกซิโดรีดักเทสในน้ำยางพารา: เอ็นเอดี(พี)เอช-ควิโนน รีดักเทส และโพลีฟีนอลออกซิเดส |
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## บทคัดย่อ


อนุภาคที่ห่อหุ้มด้วยเมมเบรนใน bottom fraction (BF) ที่ได้จากการปั่นแยกน้ำยางธรรมชาติสด มักมีความเปราะบาง และไวต่อออสโมซิส (osmosensitive) อนุภาคดังกล่าวจะแตกสลายโดยง่าย หากมีการเปลี่ยนแปลงขององค์ประกอบทางเคมีรวมทั้งสภาวะรีดอกซ์ภายในน้ำยาง ความเสถียรของน้ำยางจะขึ้นตรงกับความคงตัวของอนุภาคแขวนลอยหรือความเสถียรของเมมเบรนที่ห่อหุ้มอนุภาคดังกล่าวใน BF เอนไซม์สำคัญ 2 ชนิดในกลุ่มออกซิโดรีดักเทสในน้ำยาง คือ เอ็นเอดี(พี)เอช-ควิโนนรีดักเทส (NAD(P)H-QR) และโพลีฟีนอลออกซิเดส (PPO) ถูกคาดว่ามีความเกี่ยวโยงกับเสถียรภาพของเมมเบรนของอนุภาคใน BF (d'Auzac and Jacob, 1989) การศึกษารายละเอียดของการทำบริสุทธิ์และคุณสมบัติของเอนไซม์ดังกล่าวในครั้งนี้ น่าจะนำไปสู่ความเข้าใจถึงบทบาท หรืออิทธิพลของเอนไซม์ที่มีต่อเสถียรภาพของเมมเบรนของอนุภาคใน BF ได้ดียิ่งขึ้น

NAD(P)H-QR ในน้ำยางของ *Hevea brasiliensis* Mull.-Arg. (Euphorbia ceae) ถูกทำให้บริสุทธิ์จาก B-serum ซึ่งได้จากการนำเอา BF มาทำการ freeze-thaw กระบวนการทำบริสุทธิ์ ซึ่งได้แก่การแยกส่วนโปรตีนโดยการตกตะกอนด้วยอะซีโตน และ

การนำไปอุ่นที่อุณหภูมิสูง, การทำคอลัมน์โครมาโทกราฟีแบบแลกเปลี่ยนประจุ และตามด้วย คอลัมน์โครมาโทกราฟีแบบจับจำเพาะ เป็นลำดับสุดท้าย จะได้ NAD(P)H-QR ที่บริสุทธิ์ จากการวิเคราะห์ด้วย SDS-PAGE พบว่ามีน้ำหนักโมเลกุลย่อยเท่ากับ 21 kDa และพบว่ามีน้ำหนักโมเลกุลรวมเท่ากับ 83 kDa โดยอาศัยการวิเคราะห์จากวิธี gel filtration ผลดังกล่าวบ่งชี้ว่า NAD(P)H-QR น่าจะประกอบด้วย homotetramer การศึกษาการทำงานของเอนไซม์ที่ pH ต่างๆ พบว่าสามารถทนต่อความเป็นกรด-ด่างได้ในช่วงกว้างตั้งแต่ 6 ถึง 10 โดยค่าความเป็นกรดด่างที่เหมาะสมในการทำงานเท่ากับ 8 NAD(P)H-QR ในน้ำยางเป็นเอนไซม์ตัวหนึ่งที่ทนความร้อนได้ดี โดยพบว่าความว่องไวของเอนไซม์ยังคงเหลืออยู่ถึงร้อยละ 70 หลังจากอุ่นที่อุณหภูมิ 80 องศาเซลเซียส เป็นเวลานาน 10 ชั่วโมง ในการศึกษาเปรียบเทียบความจำเพาะต่อสับเสตรทหลายชนิด (menadione, *p*-bezoquinone, juglone, duroquinone และ plumbagin) พบว่าสับเสตรทที่ใช้ศึกษาให้ค่าความว่องไวใกล้เคียงกัน มีเพียง duroquinone ที่ให้ค่าความว่องไวต่ำกว่าชนิดอื่น และยังพบความสัมพันธ์ในทางบวกระหว่างความว่องไวของ NAD(P)H-QR กับ ผลผลิตยาง (น้ำยางสด) ต่อครั้งกรีด ($r = 0.89$, $P < 0.01$) นอกจากนี้ยังพบความสัมพันธ์ในทางบวกระหว่างความว่องไวของ NAD(P)H-QR กับเนื้อยางแห้ง ($r = 0.81$, $P < 0.01$) รวมทั้งความสัมพันธ์ระหว่างความว่องไวของ NAD(P)H-QR กับเวลาในการไหลของน้ำยาง ($r = 0.85$, $P < 0.001$) อีกด้วย ซึ่งข้อมูลข้างต้นแสดงว่ามีความเป็นไปได้ที่จะใช้ระดับความว่องไวของเอนไซม์เพื่อเป็นตัวบ่งชี้ในการทำนายถึงศักยภาพในการให้ผลผลิตของสายพันธุ์ยางที่ถูกคัดเลือกได้

PPO เป็นเอนไซม์ที่พบใน BF เช่นเดียวกันกับ NAD(P)H-QR สามารถแยกทำให้บริสุทธิ์ โดยนำ B-serum มาผ่านขั้นตอนการทำบริสุทธิ์ ซึ่งประกอบด้วย การตกตะกอนโปรตีนด้วยอะซีโตน และ ตามด้วย การทำคอลัมน์ CM-Sepharose chromatography. ซึ่งให้ PPO 2 ตัว คือ PPO-I และ PPO-II จากการวิเคราะห์น้ำหนักโมเลกุลด้วย SDS-PAGE พบว่า PPO-I มีขนาด 32 kDa และ PPO-II มีขนาด 34 kDa ตามลำดับ เอนไซม์ PPOs ทั้งสองตัวมีค่า pI ที่เท่ากัน คือ 9.2, มีค่าความเป็นกรด-ด่างที่เหมาะในการทำงานคือ 7 และอุณหภูมิที่เหมาะสมในการทำงานเหมือนกันอยู่ในช่วง 35-45 องศาเซลเซียส เอนไซม์ทั้งสองสามารถทนต่อความร้อนได้ถึง 60 องศาเซลเซียส และสามารถทนต่อสภาพความเป็นกรด-ด่าง ในช่วง 4-9 อย่างไรก็ตามค่า $K_m$ ของ PPO-I เมื่อใช้ dopamine, L-dopa และ catechol เป็นสับเสตรทมีค่าเท่ากับ 2.08, 8.33 และ 9.09 mM ตามลำดับ ซึ่งต่างจากค่า $K_m$ ของ PPO-II เมื่อใช้ dopamine, L-dopa และ catechol เป็นสับเสตรทมีค่าเท่ากับ 2.12, 4.76 และ 7.14 mM ตามลำดับ ผลที่ได้บ่งบอกว่าเอนไซม์ทั้งสอง isoforms มีความแตกต่างในเรื่องความจำเพาะต่อสับเสตรท การศึกษาตัวยับยั้งการทำงานของ PPOs พบว่า 4-Hexylresorcinol เป็นตัวยับยั้งที่มีประสิทธิภาพที่สุดในจำนวนตัวยับยั้งหลายชนิดที่ได้ทำการทดสอบ นอกจากนี้ยังพบว่า anionic detergents สามารถทำหน้าที่เป็นตัวกระตุ้นการทำงานของเอนไซม์ได้ประสิทธิภาพสูงสุด ในขณะที่ cationic detergents และ nonionic detergents มีอิทธิพลกระตุ้นเพียงเล็กน้อยหรือไม่มีอิทธิพลต่อความว่องไวของเอนไซม์นี้เลย

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## Abstract

Membrane bound particles in the bottom fraction (BF) of centrifuged fresh *Hevea* latex are fragile and osmosensitive. They can be disrupted or bursted by a slight changes in chemical composition as well as the redox state within the natural latex. Colloidal stability of the rubber latex is dependent on and proportional to integrity of the BF membrane. Oxidoreductase enzymes are shown to have influence on the membrane integrity and hence the latex stability. It is postulated that two key oxidoreductase enzymes in the latex, NAD(P)H quinone reductase [NAD(P)H-QR] and polyphenol oxidase (PPO), are related to either stability or instability of the BF membrane (d'Auzac and Jacob, 1989). Therefore, detailed study on purification and characterization of there two enzymes are investigated for a better understanding of their roles in the BF membrane status, either the integrity or the disrupting nature of the membrane.

NAD(P)H-QR present in the latex of *Hevea brasiliensis* Müll.-Arg. (Euphorbiaceae) was purified to homogeneity from B-serum fraction obtained by repeated freeze-thawing of the bottom fraction obtained from ultracentrifuged fresh latex. The purification protocol involved acetone

fractionation, heat treatment, ion exchange chromatography and finally affinity chromatography. The $M_r$ determined by SDS-PAGE for the protein subunit was 21 kDa. The molecular mass of the native enzyme estimated by gel filtration was 83 kDa, indicating that the native enzyme is a homotetramer. The enzyme showed pH stability over a wide range of pH from 6 to 10. Optimum pH of the enzyme activity was at 8. Thermal stability of the enzyme was up to 80 °C, indicating it is quite a heat stable enzyme. High NAD(P)H-QR activity (70%) was still retained after 10 h of preincubation at 80 °C. A comparative study on substrates specificity for this enzyme was observed among several substrates (menadione, *p*-benzoquinone, juglone, duroquinone and plumbagin). Only duroquinone showed a lower activity while others showed similar activities. Positive correlations between latex NAD(P)H-QR activity and the rubber yield per tapping [fresh latex ($r = 0.89$, $P < 0.01$) was observed. Similar positive correlations were also found for the dry rubber content ($r = 0.81$, $P < 0.01$)] as well as the latex flow time ($r = 0.85$, $P < 0.01$). These highly positive correlations indicated that the level of enzyme activity could possibly be used as a marker to predict rubber yield potential of the selected rubber clones.

PPO activity was also located in the BF and was isolated from the B-serum obtained after repetitive freeze-thawing of the bottom fraction isolated from ultracentrifuged fresh latex. In purification steps, the B-serum was subjected to acetone precipitation and then CM-Sepharose chromatography. This purification protocol rendered two PPOs, PPO-I and PPO-II. SDS-PAGE analyses showed $M_r$ of PPO-I to be 32 kDa and PPO-II of 34 kDa, respectively.

Both PPOs possessed the same pI of 9.2. The optimum pH for both forms of the enzymes were the same at pH 7. Optimum temperature was also found to be similar for both forms at 35-45 °C. They are stable up to 60 °C and active at a broad pH range of 4-9. The $K_m$ values of PPO-I for dopamine, L-dopa and catechol as substrates are 2.08, 8.33 and 9.09 mM. The $K_m$ of these substrates for PPO-II are 2.12, 4.76 and 7.14 mM, respectively, indicating the two isoforms of the enzymes exhibited certain difference in substrates specificity. Among various PPO inhibitors tested, 4-hexylresorcinol was the most potent. Anionic detergents were among the most effective activators of the enzymes, while cationic and nonionic detergents showed little or no effect on the PPO activities.

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Nopphakaew Chareonthiphakorn

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## List of Abbreviations

| | | |
|---|---|---|
| $A$ | = | absorbance |
| BF | = | bottom fraction |
| BSA | = | bovine serum albumin |
| CM- | = | carboxymethyl- |
| CTAB | = | hexadecyl trimethyl-ammonium bromide |
| °C | = | degree celsius |
| DEAE- | = | diethylaminoethyl- |
| DOC | = | deoxycholate |
| EDTA | = | ethylenediamine tetraacetic acid |
| g | = | gram |
| $x\,g$ | = | gravitation acceleration |
| h | = | hour |
| IEF | = | isoelctricfocusing |
| kDa | = | kilodalton |
| $K_m$ | = | Michaelis-Menten constant |
| M | = | molar |
| mg | = | milligram |
| min | = | minute |
| ml | = | milliliter |
| mM | = | millimolar |
| $M_r$ | = | molecular mass or relative molecular weight |
| MW | = | molecular weight |

## List of Abbreviations (Continue)

| | | |
|---|---|---|
| NADH | = | β-nicotinamide adenine dinucleotide, reduced form |
| NADPH | = | β-nicotinamide adenine dinucleotide phosphate, reduced form |
| NAD(P)H-QR | = | NAD(P)H quinone reductase |
| NBT | = | nitroblue tetrazolium |
| nm | = | nanometer |
| PAGE | = | polyacrylamide gel electrophoresis |
| pH | = | -log hydrogen ion concentration |
| pI | = | isoelectric point |
| PMSF | = | phenylmethylsulfonyl fluoride |
| PPO | = | polyphenol oxidase |
| r | = | correlation coefficient |
| $R_f$ | = | relative mobility |
| SDS | = | sodium dodecyl sulfate |
| sec | = | second |
| TEMED | = | N,N,N',N' –tetramethyl ethylenediamine |
| Tris | = | Tris(hydroxymethyl)aminomethane |
| U | = | unit |
| µg | = | microgram |
| µl | = | microliter |
| % | = | percentage |

# Chapter 1

## Introduction

Tapping of rubber trees for latex collection was performed by making incision on the outer bark of rubber trees across latex vessels. The rubber tree has a mechanism to minimize its metabolites lost due to tapping by forming plug at the tapping site in order to retard or stopping the latex flow. An early electron microscopic study revealed the presence of rubber particles and lutoid debris at the plugging site or vessel ends to impede latex flow (Southorn, 1968). The biochemical process in latex vessel plugging of *Hevea brasiliensis* suggests the involvement of two cooperative processes. One is enzyme-dependent process leading to lutoid bursting. The other process is non-enzymatic involving specific aggregation between rubber particles induced by lutoid membrane lectin (Pasitkul, 2001).

Upon tapping, the opening end of latex vessel is exposed to atmospheric $O_2$ which in turn promotes activities of several oxidase enzymes leading to production of active oxygen species including superoxide ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$) and $H_2O_2$. These active oxygen species will cause damage on the lutoid membrane. The process may begin with reduction of quinone into hydroquinone and semi-quinone by NAD(P)H quinone reductase on the lutoid membrane. In the presence of $O_2$, the semi-quinone can auto-oxidize into quinone by producing $O_2^{\cdot-}$. The reaction between $O_2^{\cdot-}$ with

surrounding $H_2O_2$ results in the formation of $OH^{\cdot}$ (Fenton & Haber-Weiss reaction). The $OH^{\cdot}$ will cause damage to the unsaturated double bond of fatty acids in lutoid membrane leading to membrane breakage or disruption. Consequently, polyphenol oxidase in latex cytosol will utilize $O_2$ to oxidize hydroquinone substrate into its corresponding quinone product Therefore, the process will increase more and further chance of semi-quinone and active oxygen species production. It is the aim of this thesis to purify and characterize the latex NAD(P)H-dependent quinone oxidoreductase and polyphenol oxidase for further study on their roles and effect on the BF membrane. A possible enzymic influence of these two enzymes on latex stability and hence the rubber yield upon tapping is also explored.

# Literature Review

## 1. Rubber latex tree

The genus *Hevea* is a member of the family Euphorbiaceae and comprises of several species. Among them, *Hevea brasiliensis* which grows in the hot humid intertropical regions, is the best commercial rubber producing plant. The latex of this rubber tree species, is collected by tapping the bark tissues. It is a perennial rubber tree with a straight trunk covers with fairly smooth, whitish-gray bark surface. Native rubber trees can grow as high as 40 meters with a life span of almost 100 years. However, nowadays the height of rubber trees in most plantations rarely exceed 25 meters because its growth is retarded by tapping and they are usually being replanted every 23-35 years after the declining yield reaches an uneconomic level (Webster and Paardekooper, 1989).

Fresh latex from *Hevea brasiliensis* is a specialised cytoplasm containing a suspension of rubber particles and other organelles in an aqueous serum (the cytosol). The latex collected by regular tapping consists of the cytoplasm expelled from the latex vessels. Apart from water, it contains about 30-40 percent of rubber and about 3.5 percent of other substances. The structure and composition of fresh, tapped latex was firstly elucidated by high-speed centrifugation (Fig.1) (Mori, 1959). Depending on the centrifugation method employed but in general 3 major zones can clearly be distinguished. The top fraction consists almost entirely of latex rubber; the middle zone are made up of the watery phase of the latex, generally called C-serum; the relatively heavy

bottom fraction, normally yellowish, viscid and semi-liquid, consists mainly of the lutoids. The yellow, lipid-containing Frey-Wyssling complexes are normally found beneath the upper border rubber layer fraction.

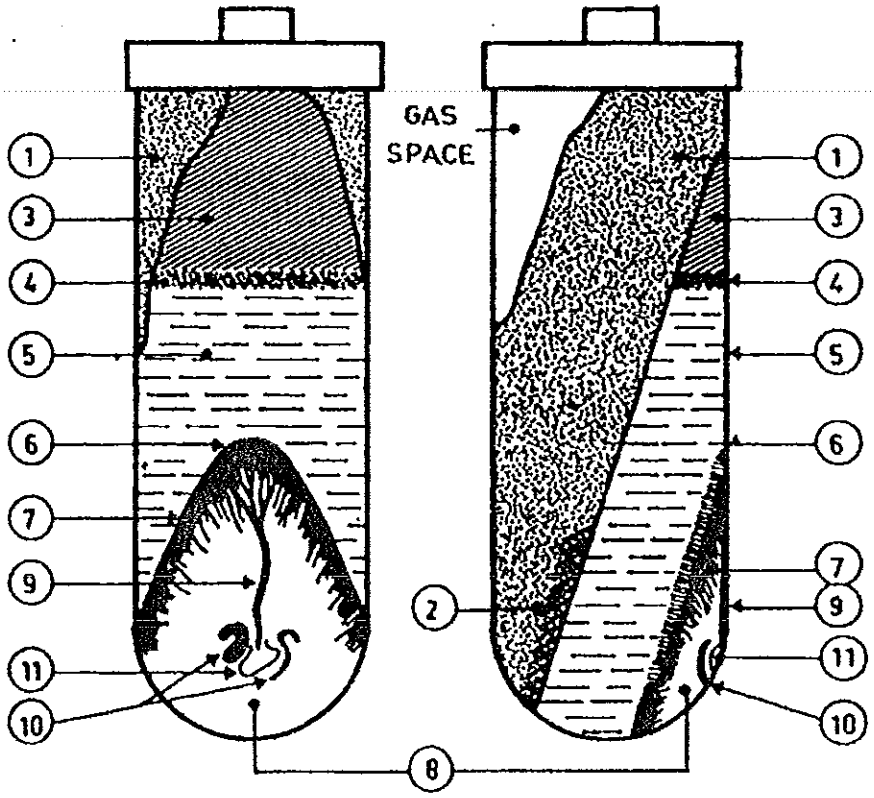


Fig. 1 Separation of fresh latex by ultracentrifugation (Moir, 1959).

Fraction 1-3 correspond to white rubber phase. Fraction 4 is a yellow orange layer constituted by Frey-Wyssling particles. Fraction 5 is an almost clear serum (C-serum) corresponding to the latex cytosol. Fraction 6 to 11 constitute the "bottom fraction" in which highest in quantity is the lutoid fraction.

## 2. NAD(P)H quinone reductase

### 2.1 Animal NAD(P)H quinone reductase

Animal NAD(P)H quinone reductase, also known as DT-diaphorase [NAD(P)H: (quinone acceptor) oxidoreductase, EC 1.6.99.2, NAD(P)H-QR] is widely distributed FAD-containing protein (Ernster *et al.*,1987) that catalyzes nicotinamide nucleotide-dependent reductions of quinones, quinoneimines, azodyes, and nitro groups (Ernster *et al.*,1987). The name DT-diaphorase arose because of it ability to use either NADH or NADPH as its cofacter in the reduction of quinone substrate. Induction of NAD(P)H-QR (Benson *et al.*, 1980 and Prochaska and Talalay, 1991) protects against the toxic and neoplastic effects of quinones. Measurement of inducer potency has been used to isolate anticarcinogens (Zhang *et al.*, 1992) and to design chemoprotectors (Posner *et al.*, 1994). Protection by NAD(P)H-QR is conferred by catalysis of obligatory two-electron reductions (Iyanagi and Yamazaki, 1970) that divert quinines from redox cycling, from reacting with critical nucleophiles, and from depleting sulfhydryl groups (Prochaska and Talalay, 1991 and Talalay, 1989). NAD(P)H-QR also reductively activates important chemotherapeutic quinines such as mitomycins and aziridylbenzoquinones (Ross *et al.*, 1993). Thus, since levels of animal NAD(P)H-QR are often elevated in tumors, the selective susceptibility of tumors to such agents (Schlager and Porwis, 1990 and Berger *et al.*, 1985) provides the opportunity for designing improved chemotherapeutic agents that are more efficiently activated by NAD(P)H-QR (Ross *et al.*, 1993)

DT-diaphorase has been crystallized from mouse and rat liver and the Walker rat tumor. Preliminary x-ray diffraction data have been reported

(Amzel *et al.*, 1986; Ysern and Prochaska, 1989; Skelly *et al.*, 1989 and Prochaska, 1988). The catalytic properties (Ernster *et al.*,1987; Prochaska, 1988 and Hosoda *et al*, 1974), physiological functions (Ernster *et al.*,1987 and Lind *et al.*, 1990) and transcriptional regulation of NAD(P)H-QR have been studied extensively (Favreau and Pickett, 1993 and Jaiswal, 1994). NAD(P)H-QR is a dimmer of identical subunits each comprising 273 amino acids; the FAD prosthetic group in each subunit is noncovalently attached but remains bound during catalytic cycling; NADH or NADPH cycle in and out of the enzyme and must be released from the enzyme before substrate can bind; no products of one-electron reductions arise; the enzyme is inhibited by Cibacron blue (Prochaska, 1988; Prestera *et al.*,1992 and Li *et al.*, 1995) and by dicumarol, warfarin, and other anticoagulants (Suttie, 1985).

In mammalian systems, glutathione S-transferases (GSTs) are classified as phase II detoxifying enzymes. Detoxification of xenobiotic and/or cancer-causing compounds often occurs via two steps. Phase I enzymes catalyze the metabolic activation of the compound. Phase II enzymes then detoxify it, often by catalyzing the conjugation of endogenous metabolites to the xenobiotic (Josephy *et al.*, 1997). The phase II enzymes also include NAD (P)H-QR that carry out obligate two electron reductions of quinones. These reactions may protect cells against oxidative stress by decreasing the rate of formation of semiquinones, which can contribute to the formation of reactive oxygen species (Ross, 1997).

## 2.2 Plant NAD(P)H quinone reductase

The presence of pyridine-nucleotide-dependent dehydrogenases using a quinone as the principal electron acceptor has been documented for plant cells since the pioneering work of Wosilait and Nason (1954). Who reported on the characterization of a quinone oxidoreductase of pea seeds (Wosilait and Nason 1954a), and the distribution of quinone oxidoreductase activity in higher and lower plant (Wosilait and Nason 1954b). Plant cell appear to contain several types of NAD(P)H-dependent quinone oxidoreductases in addition to those of energy-conserving reactions of plastids and mitochondria (Pupillo *et al.*, 1986; Valenti *et al.*, 1990 and Trost *et al.*, 1997). The purification of some of these plant proteins has been accomplished, and plant NAD(P)H-QR can be classified into three groups by using their biochemical characterization.

### 2.2.1 Soluble NAD(P)H quinone reductase

NAD(P)H-QR from plant tissues was purified and characterized by several researchers (Spitsberg and Coscia 1982; Rescigno *et al.*, 1995; Trost *et al.*, 1995 and Sparla *et al.*, 1996). This plant oxidoreductase represents a functional equivalent of animal DT-diaphorase, since it reduces short-chain quinones to quinols by two-electron donation with semiquinone intermediates, thereby resulting in enhanced quinone conjugation and low probability of formation of active oxygen species (Trost *et al.*, 1995).

A prominent catalytic feature of NAD(P)H-QR consists in the two – site ping pong reaction mechanism, where NAD(P)H and quinone are the substrates, and $NAD(P)^+$ and hydroquinone, rather than semiquinone, are the

reaction products (Trost *et al.*, 1995 and Sparla *et al.*, 1996). This two-electron reduction mechanism of quinoid acceptors is reminiscent of animal DT-diaphorase (or NAD(P)H: quinone reductase; EC 1.6.99.2) (Tedeschi *et al.*, 1995 and Li *et al.*, 1995) and contrasts with the one-electron reduction commonly performed by flavoreductases (Iyanagi and Yamazaki, 1970).

However, a number of enzyme properties found in plants are different from animal- type DT-diaphorase. For examples, the NAD(P)H-QR contains FMN, it has a mass of about 90 kDa with subunits of 21.4 kDa (by MS), and the hydride transfer from NAD(P)H to the flavin is B-stereospecific (Sparla *et al.*, 1996),whereas animal DT-diaphorase is an FAD-containing, A-stereospecific, dimeric dehydrogenase with 26 to 31 kDa subunit (Ernster, 1987 and Jaiswal *et al.*, 1990).

In spite of the similar reaction mechanism, animal DT-diaphorase and plant NAD(P)H-QR are structurally different. In fact DT-diaphorase is a dimer of 26 or 30 kDa subunits, each binding one molecule of FAD (Jaiswal *et al.*, 1988 and Jaiswal *et al.*, 1990). Also, DT-diaphorase is an A-stereospecific dehydrogenase (Lee *et al.*, 1965) whereas NAD(P)H-QR is B-stereospecific (Sparla *et al.*, 1996). Protein bearing clear structural relationships to DT-diaphorase have not been found in plants. Since quinones are heavily involved in the plant metabolism of lignin and other phenols, and in several allelopathic interactions (Smith *et al.*, 1996). NAD(P)H-QR may functionally replace DT-diaphorase in higher plants.

The basidiomycete *Phanaerochete chrysosporium* has also been shown to contain an FMN-binding oxidoreductase of the NAD(P)H-QR

type involved in the detoxification of quinones naturally produced by the fungal degradation of lignin (Brock *et al.*, 1995 and Brock and Gold, 1996).

The NAD(P)H-QR bears some structural and kinetic resemblance to some enzymes purified from plant plasma membrane (Luster and Buckhout, 1989 and Serrano *et al.*, 1994) and mitochondria, in the latter as rotenone-insensitive NAD(P)H dehydrogenases (Luethy *et al.*, 1991 and Rasmusson *et al.*, 1993). They are relatively unspecific toward pyridine nucleotides and use hydrophilic quinones efficiently, whereas Cyt *c*, oxygen and ferri-chelates are not reduced to a significant extent. With the exception of the 43 kDa NAD(P)H dehydrogenase purified from sugar beet mitochondria by Luethy *et al.* (1991), these dehydrogenases are composed of 25 to 27 kDa (Luster and Buckhout, 1989; Rasmusson *et al.*, 1993 and Serrano *et al.*, 1994), similar to the appearent molecular mass of 22 to 24 kDa of the NAD(P)H-QR, as calculated by SDS-PAGE (Brock *et al.*, 1995; Rescigno *et al.*, 1995; Trost *et al.*, 1995; Sparla *et al.*, 1996 and Trost *et al.*, 1997). Moreover, detergents are not required to extract the mitochondria dehydrogenases from the membrane (Luethy *et al.*, 1991 and Rasmusson *et al.*, 1993). Therefore, these proteins seem to be rather hydrophilic, like NAD(P)H-QR

### 2.2.2 Intrinsic NAD(P)H: quinone oxidoreductase

Another type of particulate pyridine nucleotide-dependent quinone oxidoreductase named NADH duroquinone reductase [NADH-DQ reductase] (Pupillo *et al.*, 1986 and Asard *et al.*, 1987) is widespread in plant microsomal membranes, although its purification has never been achieved. Unlike soluble NAD(P)H-QR, the NADH-DQ reductase is strongly bound to

the membrane (Valenti *et al.*, 1990) and the molecular mass is larger (300-340 kDa, according to Guerrini *et al.*, 1994 and Trost *et al.*, 1997). Whereas this form prefers NADH in sugar beet microsomes (Guerrini *et al.*, 1994) and zucchini (*Cucurbita pepo* L.) plasma membrane (Trost *et al.*, 1997).

### 2.2.3 Others

Other NADH-specific oxidoreductases are also found in plant microsomes and plasma membranes (Serrano *et al.*, 1994; Bagnaresi and Pupillo, 1995; Bérczi *et al*, 1995 and Bagnaresi *et al.*, 1997). Most of these preparations consist of proteins having prominent ferricyanide reductase activity with some capacity to reduce ferri-chelates, but they are inactive with duroquinone an artificial quinone commonly used in redox enzyme assays. These oxidoreductases are therefore distinct from both the intrinsic NAD(P)H: quinone oxidoreductase and the soluble NAD(P)H-QR.

## 2.3 Physiological function

Quinones are widely distributed in nature, being involved in many biochemical reactions (e.g. ubiquinone and plastoquinone), used as dyes (e.g. lawsone, found in henna), and sometimes as drugs (e.g. the anti-cancer quinones). They are also present in cigarette smoke tar and their widespread industrial use guarantees our exposure to a wide range of quinones as environmental pollutants.

Quinones can be toxic by at least two mechanisms. First, they or their semiquinones can often react with the –SH groups on essential molecules such as proteins and GSH (ubiquinone and plastoquinone cannot do this).

Second, like paraquat, they may create oxidative stress by redox cycling, i.e. For example, plumbagin and juglone, redox cycling quinones, are powerful

$$\text{Quinone} \xrightarrow[1e^- \text{ reduction}]{} \text{Semiquinone}$$

inducers of MnSOD activity in *E. coli*. In animal cells, both mitochondria and endoplasmic reticulum can catalyse one-electron reduction of quinones.

Reaction of the synthetic quinone menadione with oxyhaemoglobin causes oxidation and precipitation of the protein. It has been proposed that menadione first reacts with oxyhaemoglobin to give a semiquinone ($SQ^{\cdot-}$):

$$Hb(Fe^{2+})O_2 + Q \leftrightarrow Hb(Fe^{3+}) + O_2 + SQ^{\cdot-}$$

The semiquinone can both re-reduce methaemoglobin and can also convert oxygen to the superoxide radical. This is because a number of enzyme systems in liver, including NADPH-cytochrom P-450 reductase, catalyse the one-electron reduction of quinones into semiquinones that then react with oxygen to give $O_2^{\cdot-}$. Liver also contains high activities of the enzyme diaphorase, which, by contrast, catalyses a two-electron reduction of quinones into stable hydroquinones at the expense of NADH or NADPH.

Iyanagi and Yamazaki (1970) have suggested that the physiological function of diaphorase is as a quinone reductase, decreasing formation of $O_2^{\cdot-}$ *in vivo* by removing quinones and thus preventing their reduction to semiquinones by the other enzyme system (Fig.2). Consistent with this hypothesis, inhibition

of diaphorase in isolated hepatocytes by the anti-coagulant drug dicoumarol increases the toxicity of menadione to these cell.

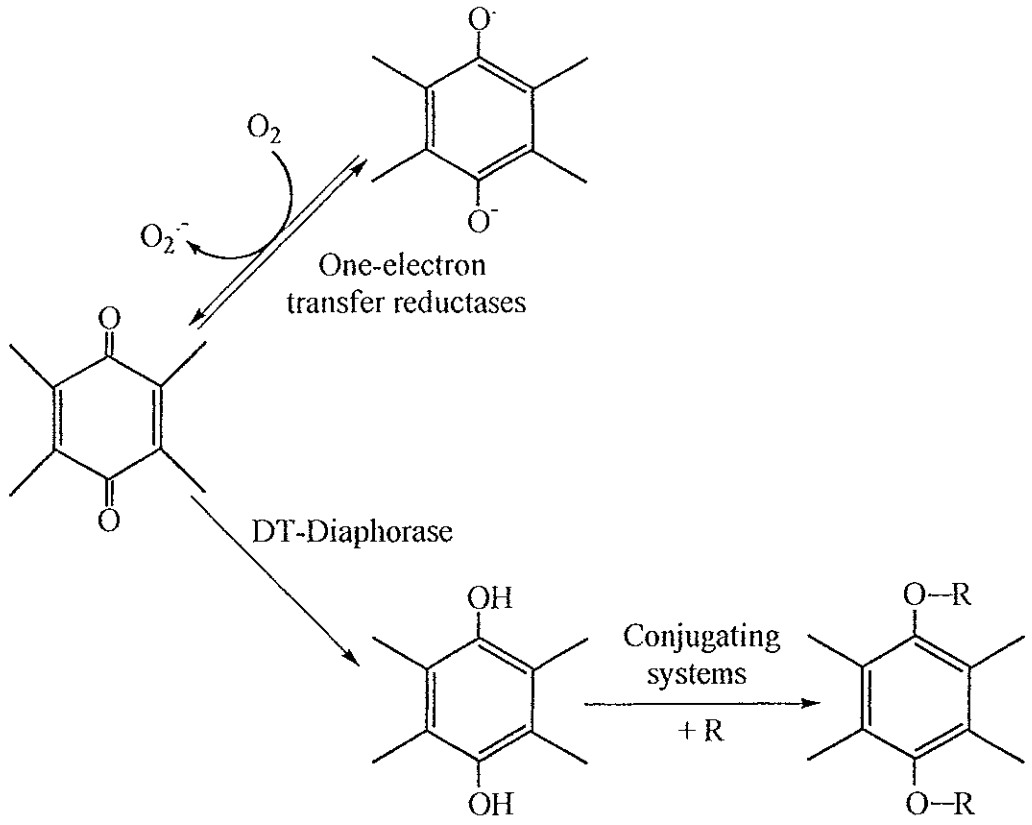


Fig.2 Role of DT-diaphorase in promoting hydroquinone formation and hydroquinone conjugation. R is UDP-glucuronic acid or phosphoadino--sine phosphosulfate (Iyanagi and Yamazaki, 1970).

## 2.4 Relationships between plant NAD(P)H quinone reductase and animal DT-diaphorase

The reaction mechanism and the elevated kinetic efficiency, as well as the difficulties in experimentally saturating the enzyme with substrate are common features of both plant NAD(P)H-QR and animal DT-diaphorase (Tedeschi *et al.*, 1995). Major differences between plant NAD(P)H-QR and animal DT-diaphorase refer to the polymerization state and the flavin coenzyme specificity.

Indeed, DT-diaphorase is a dimer of 26 or 30 kDa subunits which bind FAD (Ernster, 1987; Jaiswal *et al.*, 1988 and Jaiswal *et al.*, 1990). Moreover, sequence similarities between these proteins are limited. The best pairwise alignment was between *Arabidopsis thaliana* NAD(P)H-QR (or *Nicotiana tabacum* NAD(P)H-QR partial sequence) and a human DT-diaphorase of 231 amino acids (Jaiswal *et al.*, 1990), showing 18% identity. In a multiple alignment analysis by Sparla *et al.* (1999), conserved residues between *Arabidopsis thaliana* NAD(P)H-QR and mammalian DT-diaphorases were mainly located in the regions involved in the binding of the flavin coenzyme. Eight out of 11 residues which directly interact with either the isoalloxazine ring or the ribitol moiety of FAD in rat DT-diaphorase (Li *et al.*, 1995), are found conserved in *Arabidopsis thaliana* NAD(P)H-QR. According to this finding, Li *et al.* (1995) reported that the exact topology of the catalytic domain in rat liver DT-diaphorase resembles the topology of FMN-containing proteins without significant sequence identity. However, most crucial residues involved in pyridine nucleotide

Quinones are chemically reactive compounds which can oxidize important biomolecules such as glutathione, protein thiols and flavins. Resulting semiquinones undergo rapid auto-oxidation, leading to detrimental production of superoxide. On the other hand, hydroquinones are generally more stable, and in living plant tissues they are subjected to *o*-conjugation with loss of redox cycling properties (Harborne, 1980). Therefore one function of NAD(P)H-QR may be to protect plant cells from oxidative damage. Similarly, a role of DT-diaphorase in animal cells is believed to be the detoxification of redox active compounds such as *o*- and *p*-quinones, produced by the metabolism of carcinogenic aromatic hydrocarbons (Prochaska *et al.*, 1985 and Prochaska and Talalay, 1991). Accordingly, DT-diaporase expression is induced by a large number of redox active compounds (Ernster, 1987) and both of antioxidant response element (Favreau and Pickett, 1991) and an overlapping xenobiotic response element (Denison *et al.*, 1988) have been found in the promoter region of human DT-diaphorase.

In animal system DT-diaphorase might contribute to the reduced state of membrane quinones such as ubiquinone and tocopheryl quinone (Beyer *et al.*, 1996 and Siegel *et al.*, 1997), adds a further antioxidative function to this class of flavoenzymes. This might hold true for plant systems as well. In plant, NAD(P)H-QR has been shown to interact with the plasma membrane (Luster and Buckhout, 1989 and Serrano *et al.*, 1994) thereby assuming peculiar kinetic properties, including the sensitivity to diphenyleneiodonium and the capacity to interact with lipophilic ubiquinone homologs (Trost *et al.*, 1997).

## 3. Polyphenol oxidase

Polyphenol oxidase (PPOs; monophenol monooxygenase, *o*-diphenol oxygen oxidoreductase, EC 1.14.18.1 or EC 1.10.3.2), also known and reported under various names (tyrosinase, phenolase, PPO, catecholase, monophenol oxidase, *o*-diphenol oxidase, orthophenolase or monophenolase) based on substrates specificity (Mayer and Harel, 1979; Vaughn and Duke, 1984 and Mayer, 1987)

### 3.1 Assay of the enzymes

Polyphenol oxidase oxidize phenolic substrates, utilizing molecular oxygen. Since oxidized phenolic substances undergo many secondary reactions, both with each other and with protein, it is difficult to measure product formation in routine assays. Since some of products react with reagents for phenolic substances, it is also difficult to routinely assay for residual substrate. The most convenient, but in some ways inaccurate, method is to follow the initial rate of formation of the quinone spectrophotometrically (Carmona *et al.*, 1979 and Gauillard *et al.*, 1993).

It is, however, much more preferable to measure $O_2$ uptake directly again taking into consideration only initial rates, since the enzyme may undergo rapid inactivation during catalytic performance. It is important to note that the ratio oxygen consumed/substrate oxidized changes with time of the reaction and depends on substrate type and concentration and on the pH of the reaction mixture, the buffer used, etc.. An additional problem concerning the assay is the low affinity of phenol oxidases to oxygen. Most assays, and particularly the spectrophotometric ones, are therefore carried out at oxygen

concentrations far below the $K_mO_2$ of the enzyme. It is evident that true $V_{max}$ values are seldom determined. Some of these problems have been considered by Kean (1964), Mayer *et al.*,(1966) and Yamaguchi *et al.*, (1969).

### 3.2 Distribution and sources of polyphenol oxidase

Polyphenol oxidase is a copper enzyme which catalyses two-step reactions: (a) The hydroxylation of monophenols to *o*-diphenols by insertion of oxygen in a position *ortho* to an existing hydroxyl group, often referred to as monophenolase activity and (b) The oxidation of the diphenols to the corresponding quinone, with hydrogen abstraction, often referred to as diphenolase activity. Molecular oxygen participates in both reactions (Mueller *et al.*, 1996, Fig. 3).

Polyphenol oxidase is widely distributed in the plant kingdom. It has since been reported to occur in algae, bryophytes, polypodiophytes, lycopodiophytes, gymnosperms and angiosperms (Sherman *et al.*, 1991).

The presence of polyphenol oxidase has been reported from a variety of plant organs and tissues. Some particular cases which should be mentioned are pollen grains, the latex of some plants, crown-gall tissue, and a relatively high level in guard cells. Reports on differences in the properties of the enzyme from various parts of the same plant or from different organelles in same cells are widespread in the literature and the level of PPO often changes markedly during the development of the plant (Meyer and Biehl, 1980, 1981 and Broothaerts *et al.*,2000) and may be considerably affected by growth condition. Such effects have also been observed in tissue cultures of various plants.

### 3.3 Subcellular location

Plant PPO has been located in a variety of cell fractions, both in organelles, where it may be tightly bound to membranes (Mayer and Harel,1979; Harel, *et al.*,1964 and Stafford, 1969) and in the soluble fraction of the cell (Harel, *et al.*,1964; Sanderson, 1964 and Murata *et al.*, 1993). Tanning reactions, taking place after the disruption of tissues rich in phenols may cause binding of soluble PPO to a 'particulate' fraction.

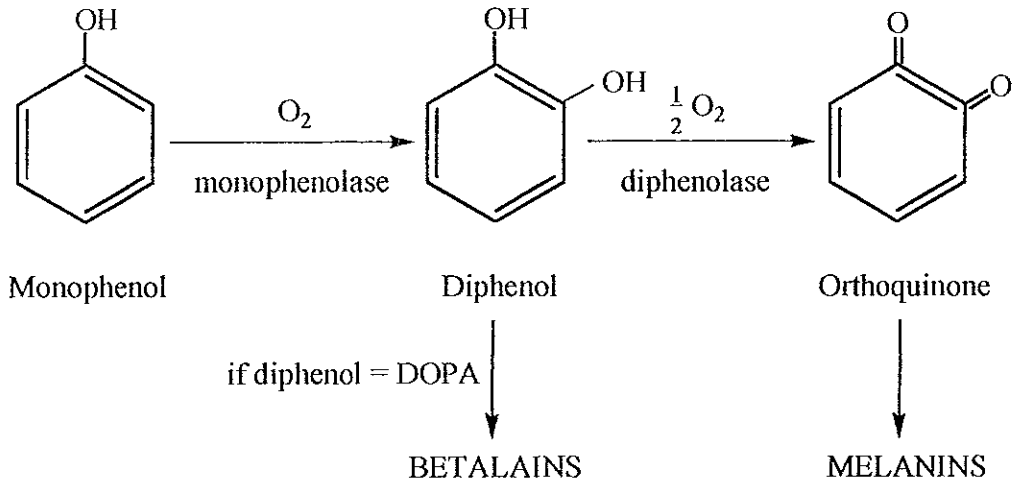


Fig. 3 The reaction of polyphenol oxidase (modified from Mueller *et al.*, 1996)

A monophenolic substrate is converted into a diphenol by the mono-phenolase activity of PPO and the diphenolic intermediate is further oxidized by diphenolase activity of PPO to yield *o*-quinones, which polymerize to form melanin-like pigments.

Craft (1966) concluded from a comparison of the properties of PPO in various cell fractions of potato tubers, that the enzyme was originally soluble and became non-specifically attached to 'particles' during grinding and fractionation (Alberghina, 1964). Sanderson (1964) showed that the enzyme from tea leaves remains soluble if isolation is carried out the presence of polyamide, which adsorbs endogenous phenols.

In spite of possible artifacts, the weight of evidence indicates the wide occurrence of membrane-bound PPO, particularly in chloroplasts. Although many reports localizing the enzyme in organelles are base merely on differential centrifugation, some more careful studies involving density gradient centrifugation and correlation with chlorophyll content and activities of 'marker' (Tolbert, 1973; Ruis, 1972 and Ben-Shalom *et al.*, 1977) enzymes reach the same conclusion. Furthermore, histochemical work employing dopa as a substrate and observations with the electron microscope showed the enzyme to be bound within chloroplast lamellae and grana (Katz and Mayer, 1969 and Parish, 1972) and in mitochondria (Czaininski and Catesson, 1974).

Apart from its localization in chloroplasts (Mayer and Friend, 1960; Harel *et al.*, 1964 and ; Harel *et al.*, 1965), PPO has been reported to be localized in mitochondria (Mayer and Friend, 1960) and in peroxisomes and 'microsomes'(Ruis, 1972).

The strength of binding of PPO to membranes appears to vary depending on the tissue and the stage of development of the plant. Thus, in tobacco, washing with buffer suffices to release the enzyme from chloroplast lamellae. In most cases, more drastic condition are required for the

solubilization of membrane-bound PPO, such as the use of detergent e.g. digitonin (Harel *et al.*, 1965; Harel and Mayer, 1968), Triton X-100 (Harel *et al.*, 1964 and Walker and Hulme,1966), Triton X-114 (Sánchez-Ferrer *et al.*, 1989 and Escribano *et al.*, 1997), SDS (Ben-Salom *et al.*, 1977 and Moore and Flurkey, 1990), Manoxol OT (Robb *et al.*, 1965), butanol (Stelzig *et al.*, 1972) or limited digestion with proteolytic enzymes (Mayer, 1966).

These treatments evidently cause changes in the enzyme's structure and/or conformation and are frequently accompanied by activation (Robb *et al.*, 1965 and Mayer, 1966) as well as changes in the substrate specificity, pH optimum and other properties of the enzyme (Harel *et al.*, 1965; Ben-Salom *et al.*, 1977; Robb *et al.*, 1965; Mayer, 1966 and Kenten, 1958). Apparently, conversion of the particulate forms of the enzyme to soluble ones occurs in tissue cultures of apple fruit following exposure to stress conditions such as low humidity and deficiency in nutrients or growth substances (Volk *et al.*, 1977). Solubilization occurs also under more natural condition, e.g. ripening of fruits or aging. Thus, apple (Harel *et al.*, 1966) and grape (Kidron *et al.*, 1978) PPOs become increasingly soluble during fruit ripening. The proportion of the soluble enzyme in tissue cultures of apple fruit increases with the age of the culture (Volk *et al.*, 1977). This is the case also with PPO in sugar beet leaves. The enzyme in green olives is tightly bound to membranes and requires drastic treatments for its solubilization. However, when the olive ripens and turns black the enzyme is completely soluble (Ben-Salom *et al.*, 1977).

### 3.4 Purification

PPO is a relatively difficult enzyme to purify. Tanning reactions, taking place during the isolation of enzyme, result in changes in its properties as well as in apparent multiplicity (Bendall and Gregory, 1963). Such reactions can be partially prevented by isolation under $N_2$ (Bouchilloux *et al.*, 1963), or in the presence of reducing agents or phenol absorbing agents such as polyethyelene glycol, polyamide or polyvinyl pyrrolidone (Sanderson, 1964; Loomis and Battaile, 1966; Anderson, 1968 and Benjamin and Montgomery, 1973). However, some of these agents inhibit the enzyme irreversibly (Harel *et al.*, 1964 and Walker and Hulme, 1965).

The binding of PPO to membranes in many tissues further complicates its isolation. Solubilization, usually achieved after preparation of acetone powder or extraction with detergents and other agents, undoubtedly result in modification of enzyme structure and properties. A widely observed phenomenon is the marked decrease or complete loss of the monophenolase activity upon solubilization of a membrane bound enzyme (Harel *et al.*, 1964; Harel *et al.*, 1965; Harel and Mayer, 1971 and Ben-salom *et al.*, 1977). An additional obstacle to purification is the extensive multiplicity of the enzyme and the inter conversions between forms which continually occur during purification steps and during storage of purified preparations.

### 3.5 Properties of polyphenol oxidase

#### 3.5.1 Molecular weight

The MW of higher plants polyphenol oxidase is even more complex. Most reports on the MW of the enzyme are based on estimates (e.g.

by employing gel filtration or acrylamide gel electyrophoresis) using partially purified preparations: the values reported cover a wide range. In many cases, crude or partially purified preparations show a multiplicity of forms, which may have resulted from association-dissociation. Thus, Harel and Mayer (1968) observed 3 forms of PPO from apple fruit, having MWs of 30-40, 60-70 and 120-130 kDa. These were shown to undergo interconversions. Demenyuk *et al.* (1974) observed 24, 67 and 13 kDa forms in apple, the high MW one being transformed into the lower MW forms during ripening and storage of the fruit. Samorodova-Bianki *et al.* (1977) reported values of 32-36 and 60 kDa from the same tissue.

Multiplicity of MWs has also been observed in PPOs from avocado,14, 28, 56, 112 kDa (Dizik and Knapp, 1970), potato tubers, aggregates of 2, 4, 8 and 16 subunits of 36 kDa MW (Balasingam and Ferdinand, 1970), sugar cane, 32 and 130 kD (Coombs *et al.*, 1974). This is the only report of such low MW of a PPO and it may well be the result of proteolytic degradation during isolation. Thus, even enzyme degraded by trypsin to a MW of less than 10 kDa retained activity (Mayer, 1966). The multiplicity observed being the result of partial degradation and tanning reactions.

Harel *et al.*(1973) reported that the predominant form of grape PPO (55-59 kDa MW) underwent 'dissociation' upon storage, dilution or exposure to acid pH or urea, forming a 30-33 and 20-21 kDa subunits. However, such dissociation could be imitated by a short exposure of the enzyme preparation to the action of pepsin or trypsin.

The molecular weight of monomeric PPO from many plant species has been reported as follows: tea leaf, 72 kDa (Halder *et al.*, 1998); sunflower seeds, 42 kDa (Raymond *et al.*, 1993); apple, 65 kDa (Murata *et al.*, 1992 and Murata *et al.*, 1993); banana, 41 kDa (Yang *et al.*, 2000) and 62 $\pm$ 2 kDa (Galeazzi *et al.*, 1981); cabbage, 39 kDa (Fujita *et al.*, 1995); field bean seed, 120 $\pm$ 3 kDa (Paul and Gowda, 2000) and Chinese Cabbage, 65 kDa (Nagai and Suzuki, 2001)

### 3.5.2 Substrate specificity

While PPO from animal tissues are relatively specific for tyrosine and dopa (Mason, 1955 and Vachtenheim *et al.*, 1985), the fungal and higher plants enzyme act on a wide range of mono- and *o*-diphenols. In addition, the specificity for optical isomers, which is clear cut in the mammalian enzyme (Pomeranz, 1963), is less evident in PPO from fungi (Harrison *et al.*, 1967) or higher plants (Palmer, 1963).

The rate of oxidation of *o*-diphenols by lettuce PPO increases with increasing electron withdrawing power of substituents in the *para* position (Mayer, 1962). The rate of oxidation corresponds to the substituent constant values in the Hammett equation. Similar observations were reported also for apple (Harel *et al.*, 1964) and cherry (Lanzarini *et al.*, 1972) PPOs. As the electron withdrawing ability of the substituent increased, $K_m$ and $k_{cat}$ decrease in the order H>SCN>$COCH_3$>CHO>CN>$NO_2$, using a purified enzyme from mushrooms (Duckworth and Coleman, 1970). Except for the $k_{cat}$ values for the two most slowly oxidized substrates, both $K_m$ and $k_{cat}$ comformed to Hammett relationships when the substituent parameter $\sigma$ was used.

*o*-Diphenol substituent at one of the positions adjacent to the –OH groups (e.g. by $-CH_3$ or and additional –OH) usually are not oxidized. It was suggested that these positions should remain free for oxidation to take place (Mayer, 1962). The *o*-diphenol might be undergoing ketonization in the process of its oxidation by the enzyme. Additional support for this suggestion may come from the observation that 2,3-naphthalenediol is not a substrate of PPO, but inhibits the enzyme competitively (Mayer *et al.*, 1964). This compound cannot undergo keto-enol tautomerism and does not form a quinone due to fixation of the double bonds near the hydroxyl groups (Donaldson, 1958).

There is a continuing argument on the physical relationships between the monophenolase and diphenolase functions. While some workers suggested that both functions are catalysed by a single site (Mason, 1956), others implied the participation of two sites, either on the same enzyme molecule (Dawson and tarpley, 1951 and Mason, 1966) or on different ones (Macrae and Duggleby, 1968). Contrary to the enzyme from animal tissues, different fractions or isozymes of which show a constant ratio of monophenolase to diphenolase activity (Brown and Ward, 1958), there are reports from fungi and higher plants showing different ratios among isozymes (Smith and Krueger, 1962 and Harel *et al.*, 1965). However, Long *et al.*(1971) did not find such differences in isozymes of mushroom PPO and Jolley *et al.* (1969) could not relate the differences they observed to differences in the primary structure of the isozymes.

Many preparations of polyphenol oxidase from plants are devoid of monophenolase activity (Palmer,1963 and Lanzarini *et al.*,1972), although it is difficult to assess whether these represent genuine native forms of the enzyme. The phenomenon of the liability of monophenolase activity result from changes in the structure of the protein during its purification (Mason, 1956 and Vaughan and Butt, 1969). Loss of monophenolase activity on solubilization of membrane-bound PPOs has been reported by Harel *et al.* (1964; 1965 and 1973) while Vaughan and Butt (1969) observed a sharp decrease in monophenolase activity upon aging. Activity could be restored by the addition of bovine serum albumin. On the other hand, monophenolase activity might be induced or increased after treatment with hormones, trypsin or urea or following infection (Taneja and Sachar, 1974 and Robb *et al.*, 1965).

More interesting in a way are cases in which certain isozymes show apparently only monophenolase activity. According to Taneja and Sachar (1974) the time of appearance of monophenolase isozymes differs from that of diphenolase isozymes during the development of wheat grains. The monophenolase isozymes were restricted to the endosperm while the diphenolase ones were found also in other parts of the seed. Separation of isozymes having only monophenolase activity was reported also from *Sorghum* (Stafford and Dresler, 1972).

The suggestions of different sites for the monophenolase and diphenolase function were base in some cases on differential effects of inhibitors or other treatments on the two activities (Kean, 1964; Vaughan and Butt, 1970 and Lerner *et al.*, 1974), including a specific protein inhibitor of

monophenolase. However, the interpretation of such observations is complicated by the lag in monophenolase activity and by the effect of mono- and diphenols, and of oxidizing and reducing agents on it.

Although the rate of oxidation of *para* substituted *o*-diphenols corresponds to the Hammett relationship, it appears that PPO from various sources show a preference for certain phenolic substrates. Yasunobu (1959) concluded from a comparison of the substrate specificity of various PPOs that although the enzyme could oxidize a wide range of phenolics, the individual enzymes tend to prefer a particular substrate or a certain type of phenolic compound. In some cases, the preferred substrate is also the most abundant phenolic in the particular tissue (Palmer, 1963 and Challice and Williams, 1970). Several authors reported the isolation of enzyme fractions or isozymes which differ in substrate specificity (Harel *et al.*, 1965; Alberghina, 1964 and Wong *et al.*, 1971).

The affinity of plant PPOs for the phenolic substrates is relatively low. The $K_m$ is high, usually around 1 mM (Harel *et al.*, 1964; Harel *et al.*, 1965; Palmer, 1963; Harel *et al.*, 1973 and Rivas and Whitaker, 1973). This value is higher than the values reported for fungi (Duckworth and Coleman, 1970 and Numbudiri *et al.*, 1972) and bacteria (Yoshida *et al.*, 1974) –*ca* 0.1 mM . However, several authors reported higher affinities for the phenolic substrates in PPOs from some sources-0.01-0.1 mM in potato tubers (Alberghina, 1964 and Patil and Zucker, 1965), cotton and banana (Palmer, 1963).

The affinity of PPO a to oxygen is also relatively low, similar to other copper containing oxidases. The values reported are in the range 0.1-0.5 mM. The affinity for oxygen depends on the phenolic substrate being oxidized and could vary also among different forms of the enzyme isolated from the same tissue (Harel *et al.*, 1965).

### 3.5.3 Inhibitors

There are two main types of PPO inhibitors-reagents which interact with the copper in the enzyme and compounds which affect the site for the phenolic substrate. With some inhibitors of the first type it is possible to show competitive inhibition with oxygen, inhibition being non-competitive toward the phenolic substrate. The reverse is true for some inhibitors of the second type. Thus Duckworth and Coleman (1970) showed, using mushroom oxidase, that cyanide was competitive to oxygen while benzoic acid competed with the phenolic substrate. In addition to cyanide, inhibitors which act on the copper the enzyme include diethyldithiocarbamate salicylaldoxime, K ethyl-xanthate and thiourea derivatives such as phenylthiourea. Other metal ion chelators, less specific for copper, also inhibit the enzyme in some cases –Na azide (Clayton, 1959) and EDTA (Wong *et al.*, 1971). 3,4-Dichlorophenyl-serine was reported to be a relatively effective inhibitor of PPO, being a specific reagent for copper (Volkmann and Beersteder, 1956). PPO copper is sometimes relatively inaccessible to inhibitors. Enzyme activated by urea or detergent was markedly more sensitive to inhibition by copper reagents (Swain, 1966).

The inhibition of PPO by –SH compounds and other reducing agents is well documented. Apart from the effect of compounds such as bisulfite, thiosulfate, GSH and cysteine, there are reported on effective inhibition by relatively low concentrations of certain thio-compounds, e.g. complete inhibition by Na mercaptobenzothiazole at 5 μM which is, however, slowly overcome due to the apparent oxidation of the inhibitor (Palmer and Roberts, 1967). It is generally assumed that these compounds too react with the enzyme's copper (Suzuki, 1959). It was suggested, however, that reducing agents can affect the enzyme reactions in several ways and that the observed effect would be dependent on the assay used (Mayer and Harel, 1979).

As competitive inhibitors of the phenolic substrate can serve compounds which are slowly oxidized substrates, e.g. 4-nitrocatechol (Lerch and Ettlinger, 1972), *p*-nitrophenol (Clayton, 1959 and Robb *et al.*, 1966), 4-chlorophenol (Robb *et al.*, 1966) or compounds which are not oxidizable but resemble the substrate's structure (e.g. benzoic acid, OH-benzoic acid (Duckworth and Coleman, 1970) and other aromatic carboxylic acids (Rivas and Whitaker, 1973 and Pifferi *et al.*, 1974)) and 2,3-naphthalenediol (Mayer *et al.*, 1964; Harel *et al.*, 1965 and Harel and Mayer, 1971). The interpretation of the mode of inhibition of PPO by various compounds is further complicated in relation to the monophenolase and diphenolase functions. Differential inhibition of the two activities by some compounds has been interpreted to indicated separate sites for mono- and diphenols (Vaughan and Butt, 1970).

The kinetics of inhibition of PPO by a series of substituted cinnamic acids indicated that the cinnamic acid derivatives do not act on the

site for the phenolic substrate but on a specific inhibitor site (Walker, 1975). A similar conclusion was reached earlier by Lerner *et al.*(1974) regarding the inhibition by phenylhydrazine. The action of this inhibitor is dependent on the presence of oxygen and its relatively specific for PPO from several sources (Lerner *et al.*,1974). SH-reagents may act as inhibitors, but this is apparently not a general phenomenon probably because PPOs usually have a low content of cysteine.

Polyvinylpyrrolidone (PVP) adsorbs phenols and has been widely used to protect plant enzymes from inactivation by phenolics and their oxidation products (Loomis and Battaile, 1966; Anderson, 1968). It also acts as an inhibitor of PPO. Hulme *et al.*(1964) suggested that PVP inhibits the enzyme by combining with a PPO-substrate complex, probably by attachment to the phenolic substrate moiety of such a complex. Harel *et al.*(1964) observed that PVP and its monomer, *N*-vinyl-2-pyrrolidone, inhibited the enzyme irreversibly and were able to act in the absence of added substrate.

### 3.5.4 pH optima

The pH optimum of most of the PPOs studied is between pH 5.0 and 7.0. However, even a scanty survey of the literature reveals conflicting reports on the pH optimum of the enzyme from the same source e.g. in potato tubers (Patil and Zucker, 1965) and in apples (Harel *et al.*, 1964 and Harel *et al.*, 1965). There are also many reports on the differences in the pH optimum, depending on the phenolic substrate being oxidized (Clayton, 1959 and Robb *et al.*, 1966).

For example, the optimum pH, for PPO activity in fruits, differs among plant sources. Differences in pH optima have been reported for partially purified PPOs from tea-leaf (Gregory and Bendall, 1966), strawberry (Wesche-Ebeling and Montgomery, 1990) and .field bean (Paul and Gowda, 2000). It is around pH 7–8.5 for Malatya apricot (Arslan *et al.*, 1998) and dog-rose fruit PPO (Sakiroglu *et al.*, 1996), pH 6–7 for PPO from kiwifruit (Park and Luh, 1985), apple (Murata *et al.*, 1992), pineapple (Das *et al.*, 1997), and longan fruit (Jiang, 1999) and pH 4–5 for eggplant (Fujita and Tono, 1988) and cherry (Fraignier *et al.*, 1995).

Changes in the form of the pH curve during development, or as a result of changes in growth conditions or treatment of the isolated enzyme with various agents have often been reported. These include changes in the pH optimum upon aging of tissue cultures, exposure of the tissue to stress conditions and treatment of the enzyme with denaturing agents such as detergents (Harel *et al.*, 1965; Ben-Shalom *et al.*, 1977 and Swain *et al.*, 1966), urea (Lerner *et al.*, 1972), elevated temperatures (Kenten, 1958 and Kenten, 1957) or a short exposure to acid pH (Lerner *et al.*, 1972). Such changes are sometimes accompanied by activation of the enzyme in the neutral to alkaline region of its pH curve. They might be explained by partial denaturation of the enzyme and/or conformational changes, which result in shifts in its pH optimum. It is not unlikely that similar changes in the pH curve take place also *in vivo* during aging of the tissue, following release of a membrane bound enzyme to the soluble fraction of the cell during fruit ripening (Ben-Shalom *et al.*, 1977) or upon damage incurred by stress conditions.

### 3.5.5 Multiple forms

Reports on multiple forms of PPO started to flood the literature as soon as methods of protein fractionation such as ion exchange chromatography, gel filtration, gel electrophoresis and isoelectric focussing were introduced to study the enzyme. Most reports on PPO mention multiple forms or isozymes (Vanneste and Zuberbühler ,1974). In addition, there is good evidence for differences in the primary structure between isozymes in mushroom PPO (Jolley *et al.*, 1969 including the characterization of different subunits, (Strothkamp *et al*, 1967). True isozymes differing in primary structure occur also in higher plants (Robb *et al.*, 1965; Taneja and Sachar,1974), although reports based on a thorough examination are relatively few.

The discussion of multiplicity obviously includes arguments as to whether it is a native, genuine phenomenon or results from the release of membrane bound forms, partial denaturation, fragmentation, proteolysis, activation of latent forms, tanning reactions (Robb *et al.*, 1965; Smith and Krueger, 1962, Harel *et al.*, 1973 and Jolley *et al.*, 1969), etc. Interconversion between forms, apparently due to association-dissociation phenomena, have been reported for the enzyme from several sources (Harel and Mayer, 1968 and Jolley *et al.*, 1969.). Such interconversions, which occur spontaneously can be accelerated by factors such as ionic strength, concentration or dilution, the presence of certain ions, urea, etc., and by presence of the phenolic substrate (Sato, 1976). The content of five hydrophobic residues (leucine, isoleucine, proline, valine and phenylalanine) in mushroom PPO is 30% (Jolley *et al.*,

1969). According to Van Holde (1966), a hydrophobic residue content of 30% is the approximate point of overlap between the single and multichain structure in globular proteins. Jolley *et al.* (1969) suggested that such an overlap is responsible for the dissociation-association phenomena, a view supported by calculations based on the relative side-chain hydrophobicities. These suggestions have implications with regard to PPOs in general, considering the close resemblance in amino acid composition in enzymes from various sources.

Differences in 'isozyme pattern' have been reported also in connection with subcellular organelles, the stage of development of the tissue and as a result of attack by pathogens or of treatment with plant hormones (Taneja and Sachar, 1977). Although such differences in isozyme patterns might have a physiological significance, the establishment of such significance requires further evidence.

### 3.5.6 Latency, activation and induction

Latency and activation of plant polyphenol oxidases were first studied by Kenten (1957, 1958) in *Vicia faba*. Activation was achieved by short exposure to acid (pH 3.0-3.5) or alkali (pH 11.5) or by incubation with ammonium sulphate at pH 5.0. The activation was ascribed to the removal of an inhibitory protein which was assumed to be attached to the enzyme. Activation by anionic detergents was interpreted to act by combining with cationic groups of the enzyme which result in dissociation of the 'enzyme inhibitor complex' (Kenten, 1958). Latency due to conbination of the enzyme with a diffusable inhibitor was suggested in spinach chloroplasts (Sato and Hasegawa, 1976). Mayer and Friend (1960) observed that various detergents

caused activation of membrane bound PPO in sugar beet chloroplasts without causing solubilization of the enzyme. Solubilization and activation were brought about by water saturated with butanol and by limited proteolysis with trypsin or carboxypeptidase (Mayer, 1966). A 30-fold activation due to treatment with trypsin was observed by Tolbert (1973) in isolated chloroplasts. He concluded that the activation was not due to proteolytic action since it took place even with denatured trypsin preparations. Activation by detergents has been observed also in mushroom PPO. It should be noted, however, that what might be considered as 'induced' latency is frequently observed after preparing acetone powders (Ben-Shalom, 1977 and Yamaguchi *et al.*, 1970). Such latency might well be a result of acetone-treatment rather than a native property of the enzyme.

The activation of broad bean PPO, was further studied by Swain *et al.*(1966). They interpreted the effects of detergents and short exposure to acid, alkali or urea as involving a limited conformational change rather than dissociation or aggregation. Activation was reversed upon removal of the denaturing agent and did not involve a change in the sedimentation behavior of the enzyme. Conformational changes as the cause of activation were also suggested by Lerner *et al.*(1972). They demonstrated that a short exposure to acid pH or urea caused an up to ten-fold activation which in 1-3 minutes. The activation was due primarily to an increase in the $V_{max}$ while affinity for the phenolic substrate decreased and that for oxygen increased. The process was reversible and could be repeated several times with the same preparation. Lerner and Mayer (1975) further showed that activation was

accompanied by a change in the Stoke's radius of the enzyme indicating the involvement of a conformational change. Conformational changes caused by a long exposure to pH 2-3 were observed also in purified mushroom PPO (Duckworth and Coleman, 1970). However, these changes were not reversed by dialysis against pH 7.0 buffer. Irreversible activation which apparently involves conformational changes were observed in grape PPO (Lerner *et al.*, 1972 and Lerner and Mayer, 1975) following long exposure to acid pH or urea.

The increase in activity or appearance of new isozymes in higher plants has also been ascribed to *de novo* synthesis. Hyodo and Uritani (1967) reported that the increase in PPO activity during the incubation of sliced sweet potato tissue was inhibited by actinomycin D, puromycin and blasticidin S. They concluded that the rise in enzyme activity resulted from *de novo* synthesis. Synthesis of the enzyme has also been suggested to be involved in the induction of PPO in germinating wheat seeds (Taneja and Sachar, 1977), which was affected by actinomycin D or cycloheximide. However, claims on the involvement of *de novo* synthesis should obviously be based on evidence more substantial than the effect of inhibitors of nucleic acid and protein syntheses. Appearance of, or marked rises in PPO activity following infection (Balasubramani *et al.*, 1971) or wounding (Cheung and Henderson, 1972) could be caused by activation rather than through re-synthesis. Infection by *Botrytis* was siad to cause activation of the latent PPO in leaves of *Vicia faba* (Balasubramani *et al.*, 1971). The effect of *Fusarium* in increasing enzyme activity in tomato plants could be imitated by the use of detergents. Activation was probably involved also in the increase in PPO activity observed with in 10-

20 hours of the exposure of apple tissue culture to low relative humidity (Volk *et al.*, 1977).

### 3.6 Physiological Function

Any attempt to ascribe a physiological function to PPO in green plants, from the algae to higher plants, must take into account a number of properties of the enzyme (Mayer and Harel, 1978). These properties are: (1) its subcellular location –the enzyme may be particulate or soluble and it often appears in several subcellular fractions; (2) the phenomenon of latency; (3) the activation of the enzyme under certain conditions due to conformational changes; (4) the presence of native inhibitors; (5) the enormous variation in enzyme level at different periods of growth and development; (6) the separation of the enzyme from most of its substrate due to compartmentation cellular or subcellular.

The oldest suggestions of the physiological role is that of synthesis of *o*-diphenols. This suggestion is base on the undoubted ability of many PPO preparations to oxidize monophenols to the corresponding *o*-quinone. An alternative possibility could be that the monophenol is oxidized to the corresponding *o*-quinone which is then reduced by a specific quinone reductase.

Although PPOs have been studied extensively due to their importance in the food industry (Mayer and Harel 1979; Mayer 1987), the physiological functions of PPOs in plant growth and development remain to be fully clarified. PPOs have been proposed to be involved in photosynthesis due to their localization in the thylakoid lumen (Vaughn et al. 1988). At present,

the most likely functions for PPO are its involvement in plant resistance against diseases (Bashan *et al.*,1985; Goy *et al.*, 1992; Ray and Hammerschmidt, 1998) and against insect herbivory (Felton *et al.*, 1989, 1992). While in the first case, an increase in PPO activity has been observed in incompatible interactions, in the second, the enzyme seems to induce an antinutritive defence. Upon wounding, the quinones formed by PPO induced oxidation of phenols can modify the plant proteins, reducing their nutritive value to herbivores.

For example, the covalent modification and cross-linking of nucleophilic substituents of amino acids and proteins by PPO-derived quinones are thought to exert an anti-nutritive defense against insects and pathogens (Duffey and Felton 1991). The active quinones produced by PPOs may possess direct antibiotic and cytotoxic activities to pathogens (Mayer and Harel 1979; Peter 1989). In addition, systemic induction of PPO expression in response to wounding and pathogens might provide an additional line of defense to protect plants against further attack by pathogen and insects (Bashan *et al.* 1987; Constabel *et al.* 1995; Thipyapong *et al.*, 1995 and Stout *et al.* 1999). In tomato the wound-inducible expression of PPO is regulated via the octadecanoid wound-signaling pathway (Constabel *et al.*, 1995 and Ryan, 2000).

For most plant tissues PPO is compartmentalized in plastids, whereas its phenolic substrates are located in the vacuoles (Vaughn and Duke, 1984; Vaughn *et al.*,1988). Action of PPO only occurs when this compart-mentation is disrupted after tissue wounding, as observed in diseased tissues or those damaged by insect attack. However, an increase in PPO mRNA has been

detected in artificially wounded tissues (Boss *et al.*, 1995 and Thipyapong *et al.*, 1995). In some plants the increase in PPO activity and PPO mRNA have been related to production of compounds (jasmonic acid and methyl jasmonate) of the octadecanoid signal transduction pathway (Constabel *et al.*, 1995 and Czapski and Saniewski, 1988), but this does not appear to be universal, as several plants had little or no induction of PPO by wounding or treatment with methyl jasmonate (Constabel and Ryan, 1998).

## Objective

The objectives of this study are :

1. To isolate and purify oxidoreductase enzymes : NAD(P)H-quinone reduct-ase and polyphenol oxidase from the latex of *Hevea brasiliensis*.
2. To study the biochemical characteristics and enzyme catalytic properties of NAD(P)H-quinone reductase and polyphenol oxidase from latex of *Hevea brasiliensis*.
3. To study the correlations between levels of NAD(P)H-quinone reductase and rubber yield , flow time per tapping.

# Chapter 2

# Materials and Methods

## Materials

### 1. Chemicals

#### 1.1 Chromatographic materials

CM-Sepharose CL-6B, DEAE-Sephacel, Blue Sepharose CL-6B and Blue dextran were purchased from Sigma. Sephadex G-100, Sephadex G-200, and standard moleccular weight markers of gel filtration were the products of Pharmacia Fine Chemicals.

#### 1.2 Chemicals for electrophoresis and isoelectricfocusing

Acrylamide, N,N'-methylene-bis-acrylamide, Coomassie brilliant blue R-250, β-mercaptoethanol, N,N,N',N'-tetramethylethylenediamine (TEMED) and riboflavin were the products of Fluka. Glycine, sodium dodecyl sulfate (SDS), Tris-(hydroxymethylaminomethane), ethylenediamine tetraacetic acid (EDTA), Bromophenol blue and IEF marker were from Sigma. Ammonium persulfate and glycerol were from BDH Chemicals Ltd. Methanol, isopropanol and acetic acid were from Lab-Scan. Standard molecular weight markers for denatured electrophoresis were purchase from Pharmacia Fine Chemicals. Bio-lyte ampholytes 3/10 (pH range 3-10) was from Bio-Rad.

#### 1.3 General chemicals

Pyridine nucleotide (NADH, NADPH), menadione, *p*-benzoquinone, plumbagin, juglone, dicumarol, propylgallate, pyrogallol, rutin, dopamine,

catechol, butyl-hydroxytoluene, nitro blue tetrazolium, dopamine, L-dopa, catechin, dithiothreitol, sodium azide, deoxycholate (DOC), hexadecyl trimethyl-ammonium bromide (CTAB), 4-hexylresorcinol, resorcinol and bovine serum albumin (BSA) were obtained from Sigma. Ascorbic acid, Folin-Ciocalteu phenol reagent, sodium metabisulphite, thiourea, sodium chloride (NaCl), Triton X-100 and Tween 20 were from Merck. Acetone and hydrochloric acid (HCl) were from Lab-Scan. Citric acid, monobasic sodium phosphate, dibasic sodium phosphate, sodium acetate, boric acid, borax, sodium carbornate, sodium hydroxide, potassium tartate, copper sulfate All other chemicals were of reagent grade.

**2. Instruments**

2.1 Ultracentrifuge model L8-70M (Beckman)

2.2 Centrifuge model J2-21 (Beckman)

2.3 Centrifuge model Avanti$^{TM}$ 30 centrifuge (Beckman)

2.4 Microcentrifuge model H-3 (Kokusan)

2.5 Deep-freeze refrigerator (Scientemp)

2.6 Speedvac concentrator model SVC 100 (Savant)

2.7 Fraction collector model 2110 (Bio-rad)

2.8 Vortex mixture model Vortex-genie-2 (Scientific Industries)

2.9 Reciprocating shaking water bath model RW 1812

2.10 Power supply model 1000/500 (Bio-rad)

2.11 Refrigerator model SJ-438F (Sharp)

2.12 Hot air oven model 630-7 (National applicance Co.)

2.13 Minigel IEF apparatus model 111 Mini IEF Cell (Bio-Rad)

## Methods

### 1. Preparation of plant materials

#### 1.1 Collection of latex from rubber tree

The latex used in this study was obtained from rubber trees of *Hevea brasiliensis* (Clone RRIM 600) under the age of 20 years, grown at Songkhla Rubber Research Institute, Hat Yai, Songkhla. The trees were tapped in a half-spiral fasion every other day at 6.00 a.m. Rubber tapping was performed with V-shape (V-bong) knife by stripping *Hevea* bark to make cut across latex vessels, located within the bark area 3-8 mm away from the outer cork layer of trees. The exuded latex was collected in plastic containers chilling on ice. Latex collection period of forty-five minutes was done on each tapping tree.

#### 1.2 Separation of fresh latex by ultracentrifugation

The chilled fresh latex was filtered through 4 layers of cheese cloth to remove the particulate materials and bark tissue debris. The filtrate was collected and centrifuged at 59,000 *x g* for 45 minutes at 4 °C in an ultracentrifuge (Beckman model L8-70M). After being centrifuged, the latex was separated into 4 distinct layers depend on different density of compound containing in the latex. The top layer was a white creamy layer of rubber, the next layer underneath was yellowish and called Frey-Wyssling, the middle layer was a clear solution called C-serum and the pellet was bottom fraction which mainly comprised with lutoid particles as shown in Fig.4

A spatula was used to make an opening in the rubber cream layer of the ultracentrifuged latex. The yellowish layer (Frey-Wyssling) was collected. The milky top layer of the serum phase was carefully sucked out and discarded

while the clear C-serum fraction was collected through a Pasteur pipette. A small volume of aqueous phase was discarded, and the rubber layer was then scooped out. A spatula was inserted to collected the pellet (bottom fraction)

**2. Preparation of protein fractions from acetone precipitation of B-serum**

The bottom (lutoid) fraction (50 g) was washed by suspending in a five volume of Tris-buffered saline (50 mM Tris-HCl pH 7.4 containing 0.9% NaCl) with gently stiring for 30 minutes at 4 °C and then separated from contaminated C-serum and rubber particles by ultracentrifugation 30,000 *x g* 30 minutes. The washing was repeated 3 times. The washed bottom fractions were pooled and then subjected to freezing and thawing (-20 and 37 °C for 4-5 times) until most of them were lysed. After ultracentrifuged on a Beckman model L8-70 M ultracentrifuge at 59,000 *x g* for 45 minutes at 4 °C, the yellow aqueous called B-serum was collected.

Cold acetone was added into B-serum up to percentage (v/v) that required, stirred in ice bath for 10 minutes and subjected to centrifuge (Beckman model Avanti™ 30 centrifuge) at 10,000 *x g* for 10 minutes at 4 °C. The supernatant was further extracted with cold acetone. The amount of acetone to be added to the solution to give desired final concentration was tabulated from Table 1. The pellet was blowed with nitrogen gas ($N_2$) for a minute to remove acetone, resuspended in 50 mM Tris-HCl pH 7.5 and used for further studied.

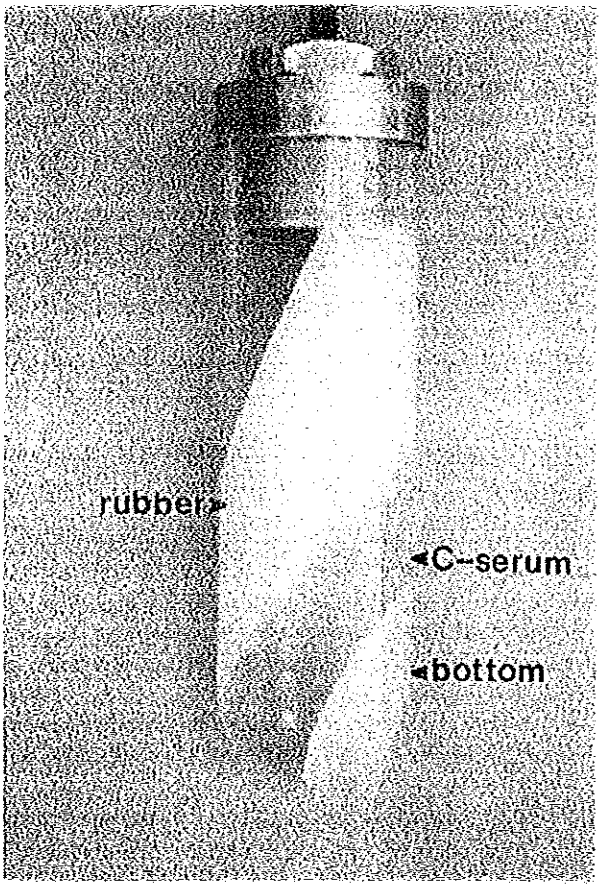


Fig. 4 Ultracentrifuged fresh latex.

Table 1 Volume of miscible solvent, ml to be added to 1 liter[a] (Scopes, 1994)

| From $C_1$% ↘ To $C_2$%→ | 5 | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 45 | 50 | 55 | 60 | 65 | 70 | 75 | 80 | 85 | 90 | 95 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **0** | 52 | 111 | 176 | 250 | 333 | 428 | 538 | 666 | 818 | 1,000 | 1,222 | 1,500 | 1,857 | 2,333 | 3,000 | 4,000 | 5,666 | 9,000 | 19,000 |
| **5** | | 55 | 117 | 187 | 266 | 357 | 461 | 583 | 727 | 900 | 1,111 | 1,375 | 1,714 | 2,166 | 2,800 | 3,750 | 5,333 | 8,500 | 18,000 |
| **10** | | | 58 | 125 | 200 | 285 | 384 | 500 | 636 | 800 | 1,000 | 1,250 | 1,571 | 2,000 | 2,600 | 3,500 | 5,000 | 8,000 | 17,000 |
| **15** | | | | 62 | 133 | 214 | 307 | 416 | 545 | 700 | 888 | 1,125 | 1,428 | 1,833 | 2,400 | 3,250 | 4,666 | 7,500 | 16,000 |
| **20** | | | | | 66 | 142 | 230 | 333 | 454 | 600 | 777 | 1,000 | 1,285 | 1,666 | 2,200 | 3,000 | 4,333 | 7,000 | 15,000 |
| **25** | | | | | | 71 | 153 | 250 | 363 | 500 | 666 | 875 | 1,142 | 1,500 | 2,000 | 2,750 | 4,000 | 6,500 | 14,000 |
| **30** | | | | | | | 76 | 166 | 272 | 400 | 555 | 750 | 1,000 | 1,333 | 1,800 | 2,500 | 3,666 | 6,000 | 13,000 |
| **35** | | | | | | | | 83 | 181 | 300 | 444 | 625 | 857 | 1,166 | 1,600 | 2,250 | 3,333 | 5,500 | 12,000 |
| **40** | | | | | | | | | 90 | 200 | 333 | 500 | 714 | 1,000 | 1,400 | 2,000 | 3,000 | 5,000 | 11,000 |
| **45** | | | | | | | | | | 100 | 222 | 375 | 571 | 833 | 1,200 | 1,750 | 2,666 | 4,500 | 10,000 |
| **50** | | | | | | | | | | | 111 | 250 | 428 | 666 | 1,000 | 1,500 | 2,333 | 4,000 | 9,000 |
| **55** | | | | | | | | | | | | 125 | 285 | 500 | 800 | 1,250 | 2,000 | 3,500 | 8,000 |
| **60** | | | | | | | | | | | | | 142 | 333 | 600 | 1,000 | 1,666 | 3,000 | 7,000 |
| **65** | | | | | | | | | | | | | | 166 | 400 | 750 | 1,333 | 2,500 | 6,000 |
| **70** | | | | | | | | | | | | | | | 200 | 500 | 1,000 | 2,000 | 5,000 |
| **75** | | | | | | | | | | | | | | | | 250 | 666 | 1,500 | 4,000 |
| **80** | | | | | | | | | | | | | | | | | 333 | 1,000 | 3,000 |
| **85** | | | | | | | | | | | | | | | | | | 500 | 2,000 |
| **90** | | | | | | | | | | | | | | | | | | | 1,000 |

[a] For 1 liter of solution: Volume (ml) = $10(C_2-C_1)/100-C_2$

## 3. Purification of NAD(P)H quinone reductase

### 3.1 B-serum and acetone precipitate preparation

B-serum was prepared from 80 g of bottom fraction by repetitive freeze-thawing with altering temperature between -20 and 37 °C for 4-5 times. The supernatant or B-serum was obtained after centrifugation at 10000 *x g*, 20 min. and brought to 30 % saturation with acetone solvent, under continuous stirring at 4 °C for 10 minutes. The mixture was centrifuged and the pellet discarded. The acetone was further added to the resulting supernatant to reach 50 % saturation and again stirred for 10 minutes at 4 °C. The acetone precipitate fraction was collected by centrifugation, blown under nitrogen gas to remove the remaining acetone residue and dissolved in a minimum volume of 50 mM Tris-HCl buffer, pH 8.0 and used further purification.

### 3.2 Heat treatment

The dissolved acetone precipitate solution was heated at 65 °C for 30 min, followed by immediate placing in an ice bath and centrifuged at 40,000 *x g* for 10 minutes to remove the pellet. The supernatant was concentrated by Speed Vac concentrator and used for further chromatographic purification steps.

### 3.3 DEAE-Sephacel chromatography

A DEAE-Sephacel column (1.7x20 cm) was pre-equilibrated with 50 mM Tris-HCl pH 8.0. After loading with heat-treated sample (20 mg), at flow rate of 12 ml/h, the column was washed with the same buffer until the absorbance at 280 was zero. The column was then eluted by using the same buffer containing 0.4 M NaCl and 3 ml fractions collected. The fraction

containing high NAD(P)H-QR activity were pooled, desalted and concentrated for further purification.

### 3.4 Blue-Sepharose affinity chromatography

The pooled active fraction obtained from DEAE-sephacel column was applied to a Blue Sepharose column (1.5x5 cm), pre-equilibrated with 50 mM Tris-HCl, pH 8.0. After washing at a flow rate of 6 ml/h with the same buffer until the $A_{280}$ of the effuent became zero, the bound proteins were eluted by the same buffer containing NaCl gradient (0 – 0.4 M) and 1 ml fractions were collected. Each fraction was assayed for NAD(P)H-QR activity and active fractions were pooled and used for further characterization.

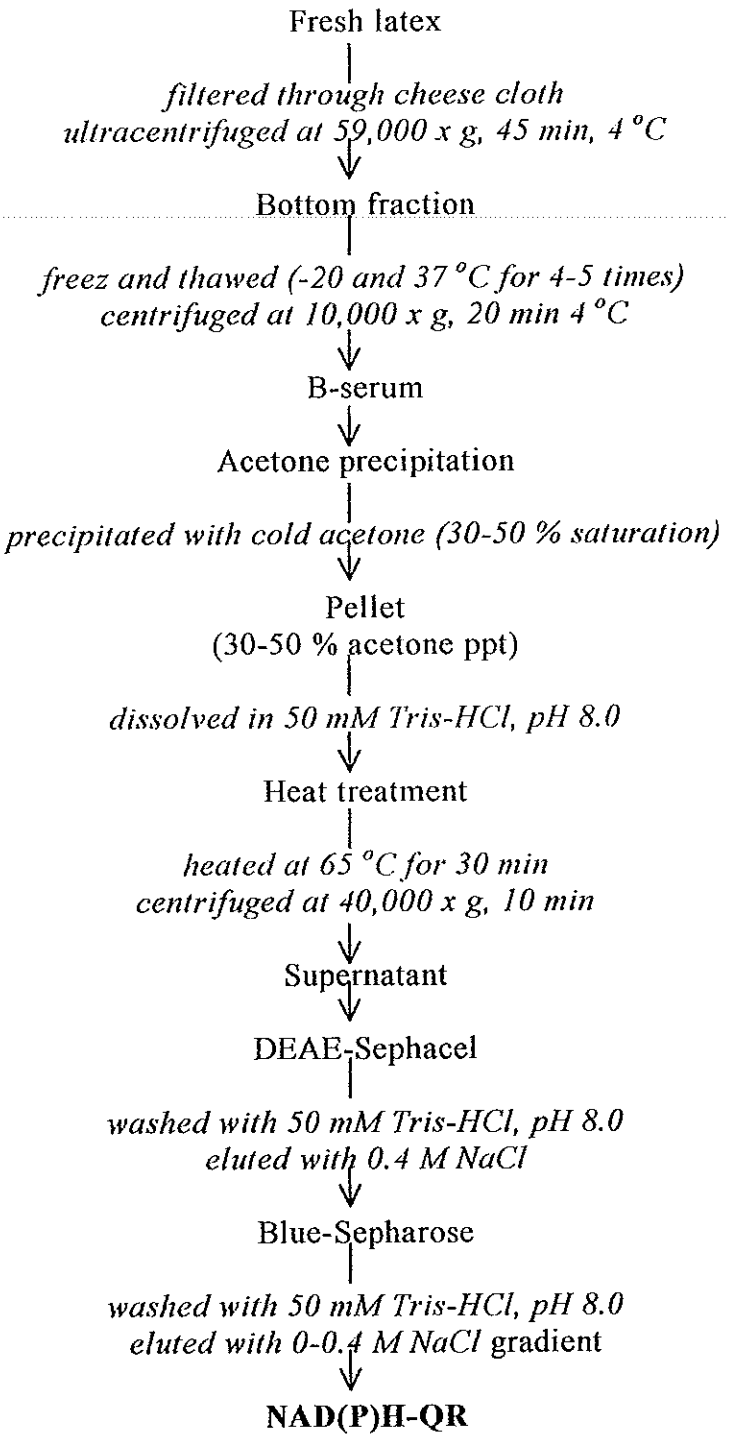


Fig.5 Summarized diagram for purification of latex NAD(P)H quinone reductase

## 4. Purification of Polyphenol oxidase

### 4.1 B-serum and acetone precipitate preparation

B-serum was prepared from 80 g of bottom fraction by repeatitive freeze-thawing with altering temperature between -20 and 37 °C for 4-5 times. The supernatant or B-serum was obtained after centrifugation at 10000 *x g*, 20 min. and brought to 30 % saturation with acetone solvent, under continuous stirring at 4 °C for 10 min. The mixture was centrifuged and the pellet discarded. The acetone was further added to the resulting supernatant to reach 50 % saturation and again stirred for 10 min at 4 °C. The acetone precipitate fraction was collected by centrifugation, blown under nitrogen gas to remove the remaining acetone residue and dissolved in a minimum volume of 50 mM Tris-HCl buffer, pH 7 and used further purification.

### 4.2 CM-Sepharose CL-6B chromatography

A CM-Sepharose CL-6B column (2.5x24 cm) was pre-equilibrated with 50 mM Tris-HCl pH 7.0 at a flow rate of 18 ml/h at 4 °C. After loading the resuspended acetone precipitate solution, the column was washed with the same buffer until the absorbance at 280 was below 0.005. The column was then subjected to stepwise-elution by using the same buffer containing 0.1 and 0.2 M NaCl, respectively. The fraction containing high PPO activity were pooled, desalted and concentrated for further characterization studies.

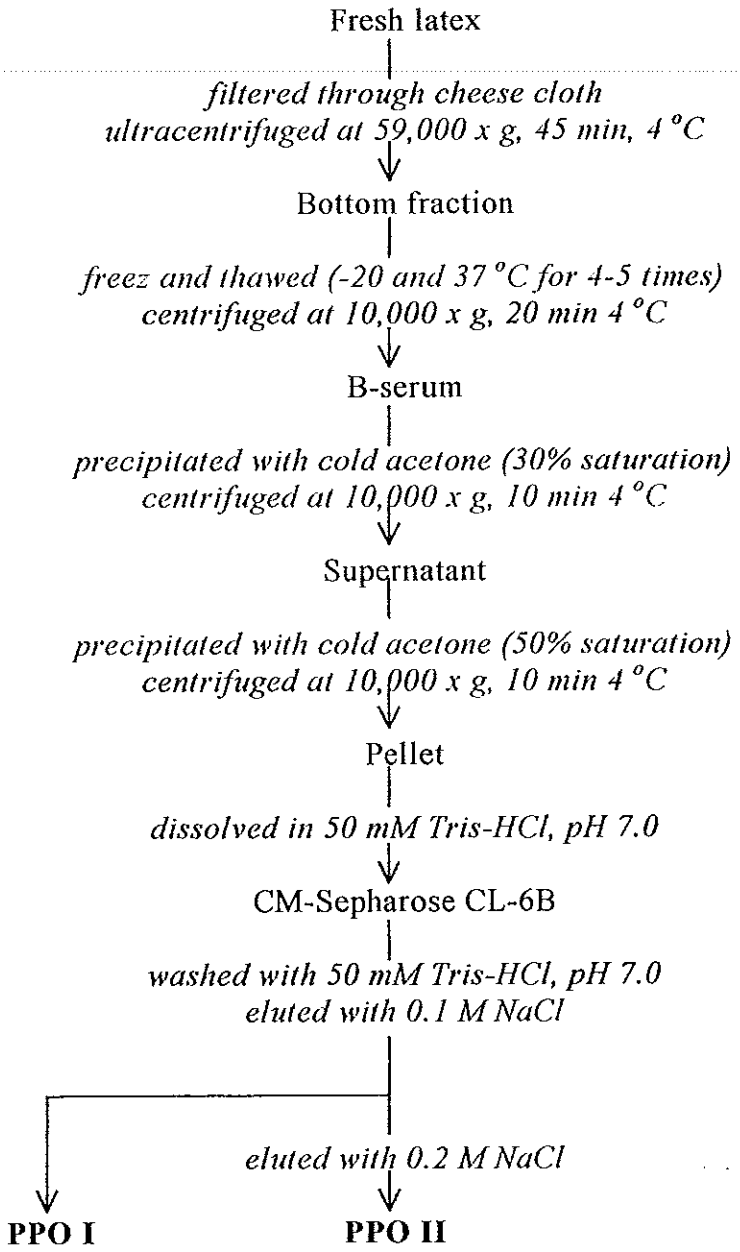


Fig. 6 Summarized diagram for purification of latex polyphenol oxidase

## 5. Quantiative analysis

### 5.1 Oxidoreductase assay

NAD(P)H-QR activity was measured under 2 procedures: a) from the disappearance of absorbance at 340 nm after addition of NAD(P)H (0.2 mM) to assay mixture containing NAD(P)H-QR sample in 50 mM Tris-HCl, pH 8.0 and 0.05 mM menadione or other quinone substrates as specified. An extinction coefficient at 340 nm of 6.23 $mM^{-1}$ for NAD(P)H was used for activity calculations. The NAD(P)H-QR activity is expressed a Katal in unit which equivalent to a conversion of one mol of NADH per sec. or b) from the appearence of formazan (at 540 nm) after addition of nitro blue tetrazolium (0.15 mM) to the assay mixture containing NAD(P)H-QR sample in 50 mM Tris-HCl, pH 8, 0.2 mM NADH, 0.05 mM menadione and NAD(P)H-QR inhibitors as specified in text.

PPO activity was spectrophotometrically monitored by following the oxidation of 5 mM substrate (dopamine ,470 nm; L-dopa, 475 nm; catechol ,410 nm or catechin, 380 nm) in 50 mM phosphate buffer, pH 7.0. The total assay volume was 1 ml. The linear portion of the absorbance vs time curve was used to determine the initial rates. One unit of enzyme activity equals to the amount of enzyme which caused a change in one absorbance per minute.

### 5.2 Effect of pH on enzymes activity

The effect of pH on NAD(P)H-QR activity, obtained after Blue Sepharose column, was determined by preincubating aliquots of NAD(P)H-QR sample at various pHs (3-10) for 1 h. The mixtures were adjusted back to pH 8 and assayed for NAD(P)H-QR activity. Enzyme activity, as a function of pH,

was determined at various pHs. Acetate, phosphate and Tris-HCl buffers were used for pH ranging from 3-6, 5-8 and 7-10, respectively.

PPO activity, as function of pH, was determined by using 100 mM buffer at pH ranging from 3 to 10 in the presence 10 mM of dopamine as substrate. pH stability was determined by pre-incubating the enzyme in 100 mM buffer (pH 3-10) for 1 h at room temp. PPO activity was assayed under standard condition with dopamine as the substrate. Acetate, phosphate and Tris-HCl buffers were used for the pH ranges of 3-6, 5-8 and 7-10, respectively.

### 5.3 Effect of temperature on enzyme activity

The effect of temperature on NAD(P)H-QR activity, obtained after Blue Sepharose column, was determined by preincubating aliquots of NAD(P) H-QR sample at various temperatures (10-100 °C) for 30 minutes. Each sample was adjusted back to 4 °C and assayed for NAD(P)H-QR activity. The heat stability of the latex NAD(P)H-QR was investigated by incubating aliquots of enzyme sample at 70, 80 and 90 °C for different intervals (1-10 h). After incubation, each sample was chilled on ice and NAD(P)H-QR activity determined as described under 5.1, procedure a.

The temperature optimum of PPO was screened at various temperature ranging from 20-60 °C in the presence of 10 mM dopamine. The thermal stability of PPO was tested at various temperature ranging from 20-80 °C. The PPO was preincubated at indicated temperature for 30 min and adjusted back to 4 °C before assay.

### 5.4 Effect of inhibitor on enzyme activity

The effects of several inhibitors (rutin, pyrogallol, propyl gallate, dicumarol, dopamine, catechol and butyl-hydroxytoluene) on latex NAD(P)H-QR were studied. The inhibition parameters ($I_{50}$) were obtained by titrating with various inhibitors as indicated, reaction mixture containing 0.2 mM NADH, 0.05 mM menadione, 0.2 mg of purified NAD(P)H-QR taken after DEAE Sephacel column and 0.15 mM NBT. The remaining enzyme activity was measured as described under 4.1, procedure b.

The effects of several inhibitors (ascorbic acid, sodium metabisulphite, dithiothreitol, thiourea, sodium azide, β-mercaptoethanol and sodium chloride) on latex PPO activity were determined in reaction mixtures containing 10 mM dopamine, various inhibitors as indicated and 20 µg enzyme taken from resuspended acetone precipitate solution.

### 5.5 Protein determination

Protein concentrations were measured by colorimetric assay described by Lowry, *et al* (1951). Under alkaline conditions, $Cu^{2+}$ forms a complex with the peptide bonds of proteins and becomes reduced to $Cu^{2+}$. The $Cu^{2+}$ as well as the R groups of tyrosine, tryptophan and cysteine residues then react with Folin reagent to give blue color. Proteins will produce different color intensities depending primarily on their tyrosine and tryptophan contents.

To a 100 µl sample, 3 ml of freshly prepared alkaline copper reagent (100 ml of 2% $Na_2CO_3$ in 0.1 M NaOH was mixed with 1 ml of 1% of potassium tartate and 1 ml of 0.5% $CuSO_4.5H_2O$) was added. The mixture was left at room temperature for 10 min before an addition of 0.3 ml of 1 M Folin-

Ciocalteu phenol reagent with rapid mixing. After standing for an additional 30 min at room temperature, the absorbance at 650 nm was measured using a BeckMan spectrophotometer (model DU® Series 650 i). The protein concentration was determined using a standard curve of crystalline bovine serum albumin (BSA; 20-100 μg) treated in the same manner.

## 6. Qualitative analysis

### 6.1 Gel filtration for native molecular weight estimation

Gel filtration for measuring the molecular weight of native NAD(P)H-QR was carried out on a Sephadex G-200 column (1.5x82 cm). A 1- ml sample of the DEAE-Sephacel-purified NAD(P)H-QR (5 mg) was load on Sephadex G-200 column equilibrated with 50 mM Tris-HCl, pH 8. Flow rate was 9 ml/h and 1.0-ml fractions were collected. The standard markers used were catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa) and chymotrypsinogen A (25 kDa). The NAD(P)H-QR molecular weight was calibrated from the curve plotted between relative log molecular weight of the standard markers against their $K_{av}$ values.

Gel filtration for measuring the molecular weight of native PPO was carried out on a Sephadex G-100 column (1x90 cm). A 1- ml sample of the CM-Sepharose-purified PPO was load on Sephadex G-100 column equilibrated with 50 mM Tris-HCl, pH 7. Flow rate was 9 ml/h and 1-ml fractions were collected and their $A$ at 280 nm and PPO activity measured. The standard markers used were albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa). The PPO-I and PPOII molecular

weight were calibrated from the curve plotted between relative log molecular weight of the standard markers against their $K_{av}$ values. Blue dextran (MW 2 x $10^6$) was used to determine the void volume (Vo) of the column. Total column volume (Vt) was determined by using potassium dichromate ($K_2Cr_2O_7$) (MW 294.19 g/mol). The $K_{av}$ values were obtained by

$$K_{av} = \frac{Ve - Vo}{Vt - Vo}$$

### 6.2 SDS-polyacrylamide gel electrophoresis for subunit molecular weight estimation

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a polyacrylamide slab gel according to the method of Laemmli (1972). Slab gels (10x10x0.1 cm) were prepared from a stock solution of 30% (w/v) of acrylamide and 0.8% (w/v) of N,N'-bis-methyleneacrylamide. Separating gel (10x8x0.1 cm) with a 10% acrylamide in 0.375 M Tris-HCl, pH 8.8 and 0.1% SDS was prepared. The gel was polymerized chemically by the addition of 0.025% (v/v) of tetramethyl-ethylenediamine (TEMED) and ammonium persulfate. A 2 cm high stacking gel of 3% acrylamide containing 0.125 M Tris-HCl, pH 6.8 and 0.1% SDS was polymerized chemically in the same way as for the separating gel. The composition of both separating and stacking gels were summarized in Table 2

The electrode buffer (pH 8.3) contained 0.025 M Tris, 0.192 M glycine and 0.1% SDS. Protein samples were mixed with sample buffer to give the final concentration of 0.0625 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol with or without 5% β-mercaptoethanol and 0.001% Bromophenol blue as the

tracking dye. The proteins were denatured by immersing the samples for 5 min in boiling water. Electrophoresis was carried out with a constant current of 14 mA until the Bromophenol blue marker reached the bottom of the gel.

Protein bands on the gel were fixed and stained overnight at room temperature with a 0.2 % Coomassie blue R-250 in 50 % methanol and 10% acetic acid. The gel was diffusion-destained by washing in the first destaining solution contained 50% methanol and 10% acetic acid for 2 h and repeated washing in the second destaining solution contained 5% methanol and 7% acetic acid until the background staining was clear. Relative molecular weight of the protein was estimated from the standard curve plotted between the logarithmic values of molecular weight of protein markers and the electrophoretic mobility ($R_f$).

$$R_f = \frac{\text{distance protein has migrated from origin}}{\text{distance from origin to reference point (the tracking dye)}}$$

The molecular weight markers included phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14.4 kDa).

Table 2 The composition of polyacrylamide separating and stacking gels

| Reagents (stocking solutions) (µl) | Stacking gel 3% | Separating gel 10% |
|---|---|---|
| 30% acrylamide + 0.8% bis-acrylamide | 300 | 2,000 |
| 1.5 M Tris-HCl, pH8.9 | - | 1,500 |
| 0.5 M Tris-HCl, pH6.8 | 750 | - |
| 10% SDS | 30 | 60 |
| Distilled water | 1,745 | 2,280 |
| 0.2 M EDTA, pH 7.0 | 20 | - |
| *1% Ammonium persulfate | 150 | 150 |
| *TEMED | 5 | 10 |
| Total volume | 3,000 | 6,000 |

* Added to chemically the gel within 10 minutes.

### 6.3 Polyacrylamide gel isoelectric focusing

Isoelectric focusing was performed in Bio-Rad minigel IEF apparatus (Model 111 Mini IEF Cell) by using the method described by Bio-rad company (mini IEF gel manual). The polyacrylamide slab gel (3.16 ml) contain 0.625 ml of monomer concentrated stock solution, comprising of 25% (w/v) acrylamide and 0.07% bis (N,N'-Methylene-bis-acrylamide), 0.625 ml of 25% (v/v) glycerol, 0.16 ml of Bio-lyte ampholytes 3/10 and 1.72 ml of deionized water. The gel mixture was degassed for 5 min before it was initially polymerized by adding 10 µl of 0.02 ammoniumpersulfate, 15 µl of 0.1% riboflavin and 5 µl of TEMED, and immediately poured into the casting tray. The gel was photo polymerized for 45 min at room temperature. After the gel had set completely, the casting tray was removed.

The purified PPOs sample and standard markers were directly applied to the gel surface and left to diffuse into the gel for 5 min. Focusing was carried out at constant voltage in a stepwised manner at 100 V for 15 min, 200 V for 15 min and finally 450 V for 60 min. After focusing was completed, the glass plate was removed from the gel supporting film. The protein band in IEF gel were visualized by staining with solution containing 0.04% (w/v) Coomassie Brilliant blue G250, 27% (v/v) isopropanol and 1% (v/v) acetic acid and followed by destaining with solution containing 25% (v/v) isopropanol and 7% (v/v) acetic acid until the background was clear.

The IEF markers (Kit for Isoelectric Focusing range 3.6-9.3) were used for determination pI value of oxidoreductase enzymes. The calibration curve was constructed by plotting the distances from anode (cm) of standard

protein markers [amyloglucosidase (pI 3.6), trypsin inhibitor (pI 4.6), β-lactoglubulin (pI 5.1), carbonic anhydrase II (pI 5.9), carbonic anhydrase I (pI 6.6), myoglobulin (pI 6.8 and 7.2), lentil lectin (pI 8.6 and 8.8) and trypsinogen (pI 9.3)] according to the method in sigma General Catalog, 1995. The relative pI of the oxidoreductase band was estimated from calibration curve.

**7. Correlation study among of levels of NAD(P)H-QR activity, flow time and rubber yield per tapping**

Forty rubber trees giving high, medium and low levels of rubber yield per tapping were used for the correlation study. Dry ruber yield obtained after each tapping was dried by hot air oven (National applicance Co. model 630-7) at 65°C to constant weight before weight measurement. Flow time is the amount of time that obtained from each rubber tree was tapped until stop flow. The activity of NAD(P)H-QR was determined from B-serum and expressed as total levels per tapping. Correlation curve among of levels of NAD(P)H-QR activity, flow time and rubber yield per tapping were constructed.

# Chapter 3

# Results

## Part 1. Purification and characterization of *Hevea* latex NAD(P)H quinone reductase

### 1.1 Distribution and protein fractionation of NAD(P)H quinone reductase

The activity of latex NAD(P)H quinone reductase in lutoids (B-serum) was 5.6- and 6.2-fold higher than that of the Frey-Wyssling particles and C-serum, respectively (Table 3). The protein in B-serum was precipitated with cold acetone in 10% serial fractionation. A 40-50% of acetone saturated precipitate fraction was found to contain highest amout of NAD(P)H-QR activity (Table 4).

Table 3 Distribution of NAD(P)H-QR activity in the ultracentrifuged fresh latex (100 ml).

| Sample fraction | Enzyme activity | |
|---|---|---|
| | NAD(P)H-QR (μkat)[a] | Yield (%) |
| Frey-Wyssling | 0.163 | 13.18 |
| C-serum | 0.149 | 12.05 |
| B-serum | 0.925 | 74.78 |

[a] The NAD(P)H-QR activity is expressed a Katal in unit which equivalent to a conversion of one mol of NADH per second.

Table 4 NAD(P)H-QR activity of protein fractions from B-serum (30 g bottom fraction), upon acetone fractionation.

| Fraction (% acetone saturation added) | Protein (mg) | NAD(P)H-QR activity (μkat) | Yield (%) |
|---|---|---|---|
| none | 1,114 | 3.51 | 100 |
| 0-10% | 6 | 0.02 | 0.57 |
| 10-20% | 12 | 0.03 | 0.85 |
| 20-30% | 24 | 0.04 | 1.14 |
| 30-40% | 58 | 0.55 | 15.67 |
| 40-50% | 171 | 1.75 | 49.86 |
| 50-60% | 422 | 0.08 | 2.28 |
| 60-70% | 90 | 0 | 0 |
| 70-80% | 41 | 0 | 0 |

[a] The NAD(P)H-QR activity is expressed a Katal (kat) in unit which equivalent to a conversion of one mol of NADH per second.

### 1.2 Purification of NAD(P)H quinone reductase

The initial purification step of NAD(P)H-QR involved sequential fractionations by employing acetone and heat treatment. Ion exchange chromatography on DEAE-Sephacel (Fig. 7) and affinity chromatography on Blue sepharose CL-6B (Fig. 8) were respectively followed. The data on purification was shown in Table 5. The B-serum protein precipitated between 30-50% of acetone was found to be a major fraction that could show most of NAD(P)H-QR activity (65%). Contaminated protein was removed from the solubilized active acetone fraction by means of heat treatment (incubated at 65 °C for 30 min.) and further purified by DEAE-Sephacel and Blue Sepharose CL-6B columns. The specific activity of purified NAD(P)H-QR was measured to be 1.227 μkat $mg^{-1}$ with 409 purification folds. A recovery yield of 17% was achieved for purified NAD(P)H-QR.

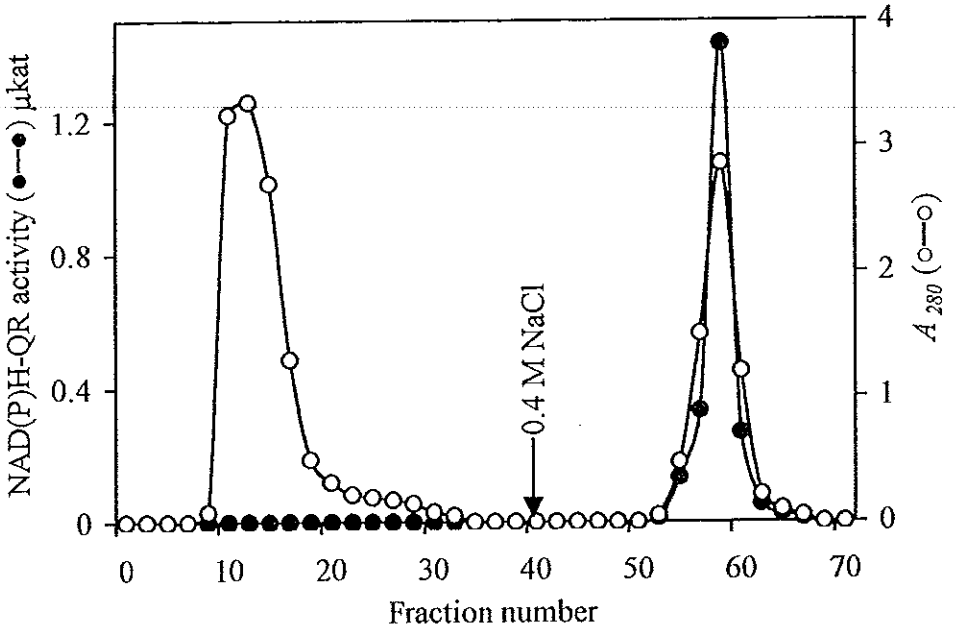


Fig. 7 Chromatographic elution profile of NAD(P)H-QR on DEAE-Sephacel column.

A DEAE-Sephacel column (1.7x20 cm) was pre-equilibrated with 50 mM Tris-HCl pH 8.0. After loading with heat-treated sample (20 mg), at flow rate of 12 ml/hr, the column was washed with the same buffer until the absorbance at 280 nm was zero. The column was then eluted by using the same buffer containing 0.4 M NaCl and 3 ml fractions collected. The fraction containing high NAD(P)H-QR activity were pooled, desalted and concentrated for further purification.

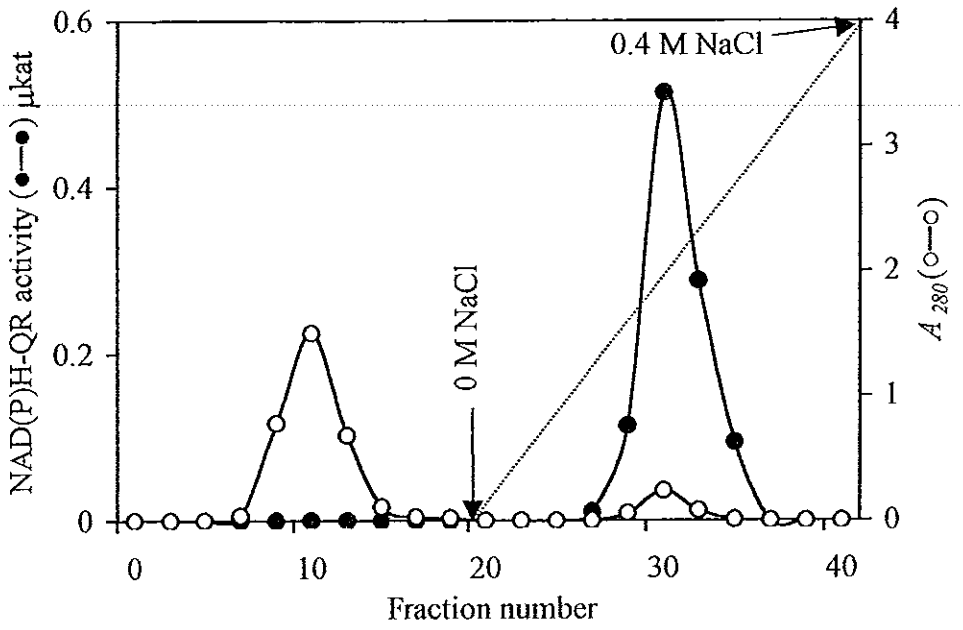


Fig. 8 Chromatographic elution profile of NAD(P)H-QR on Blue Sepharose CL-6B affinity column.

The pooled active fraction obtained from DEAE-sephacel column was applied to a Blue Sepharose column (1.5x5 cm), pre-equilibrated with 50 mM Tris-HCl, pH 8.0. After washing at a flow rate of 6 ml/hr with the same buffer until the $A_{280}$ of the effuent became zero, the bound proteins were eluted by the same buffer containing NaCl gradient (0 – 0.4 M) and 1 ml fractions were collected. Each fraction was assayed for NAD(P)H-QR activity and active fractions were pooled and used for further characterization.

Table 5 Purification protocol of latex NAD(P)H-QR for the homogeneous enzyme.

| Purification step | Total activity (μkat) | Protein (mg) | Specific activity (μkat $mg^{-1}$) | Yield (%) | Purification (-fold) |
|---|---|---|---|---|---|
| B-serum | 8.07 | 2,717 | 0.003 | 100 | 1 |
| Acetone ppt | 5.25 | 634 | 0.008 | 65 | 3 |
| Heat treatment | 4.32 | 308 | 0.014 | 54 | 5 |
| DEAE-Sephacel | 3.33 | 25.5 | 0.131 | 41 | 43 |
| Blue Sepharose | 1.35 | 1.1 | 1.227 | 17 | 409 |

### 1.3 Molecular properties of NAD(P)H Quinone reductase

The purified NAD(P)H-QR, obtained from DEAE-Sephacel column and commercial standard marker proteins, were subjected to gel filtration on Sephadex G-200 column (1.5x 82 cm) by using 50 mM Tris-HCl, pH 8 (Fig. 9). The plot between respective values on the $K_{av}$ of standard proteins and logarithm of their molecular weights was constructed (Fig. 10). The native molecular weight of NAD(P)H-QR was calibrated to be 83 kDa.

For determination of subunit molecular weight of NAD(P)H-QR, the purified NAD(P)H-QR obtained from Blue Sepharose CL-6B column chromatography and Pharmacia standard marker proteins were subjected to SDS-PAGE on 10% gel slab gel. The result, as shown in Fig. 11 indicated that the purified NAD(P)H-QR is composed of homomeric protein subunits seen as a single protein band. Its subunit molecular weight was estimated from a calibration curve presented in Fig. 12 to be 21 kDa. Thus, the native NAD(P)H-QR of 83 kDa is composed of 4 identical 21 kDa homosubunit.

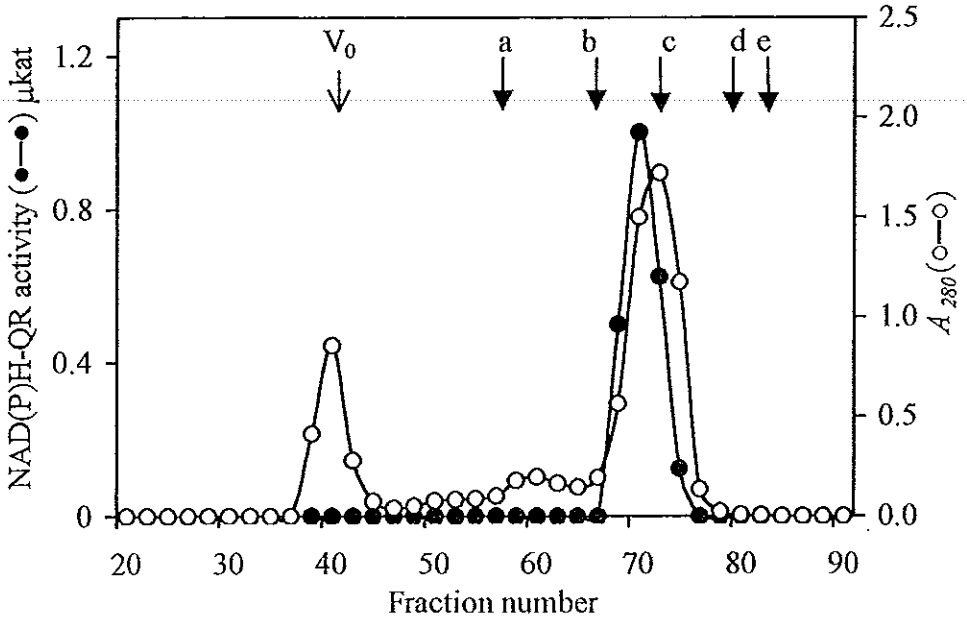


Fig. 9 Chromatographic elution profile on Sephadex G-200 column for native molecular weight determination of latex NAD(P)H-QR.

Positions of standard proteins run under identical conditions are indicated by the arrows : (a) catalase, 232 kDa; (b) aldolase, 158 kDa; (c) albumin, 67 kDa; (d) ovalbumin, 43 kDa; (e) chymotrypsinogen A, 25 kDa; $V_o$, Void volume.

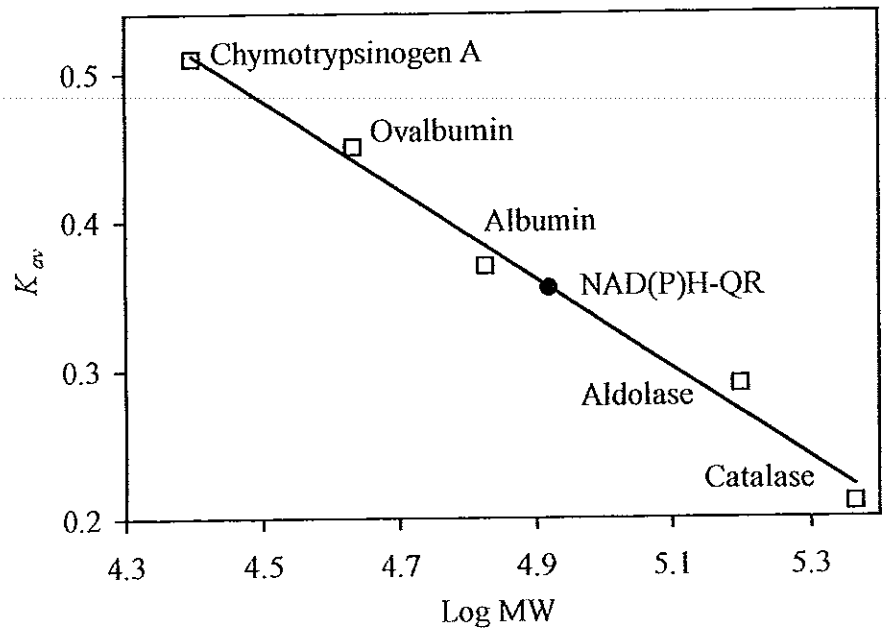


Fig.10 Calibration curve for determination of the native molecular weight of the NAD(P)H-QR after a Sephadex G-200 column chromatography.

The black circle indicated $K_{av}$ of the NAD(P)H-QR with corresponding to $M_r$ of 83 kDa. The calibration curve was plotted between respective values on the $K_{av}$ of standard proteins and logarithm of their molecular weights. The standard proteins employed were catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa) and chymotrypsinogen A (25 kDa).

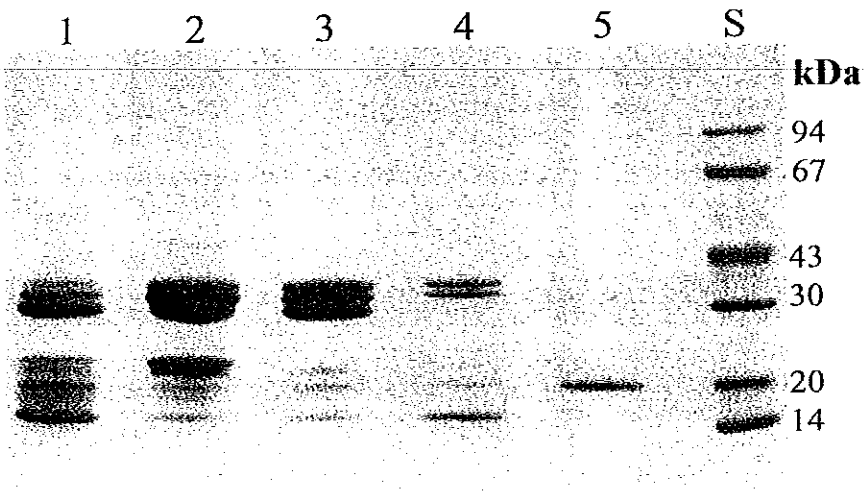


Fig.11 SDS-PAGE analyses on protein patterns of the purified latex NAD(P)H-QR;

Lane 1 = proteins obtained from B-serum (100 μg),

Lane 2 = 30-50% acetone pellet fraction (100 μg),

Lane 3 = As 2 but after heat-treatment (50 μg),

Lane 4 = peak fraction from DEAE-Sephacel column (15 μg),

Lane 5 = peak fraction from Blue Sepharose column (10 μg),

Lane S = standard protein markers (kDa).

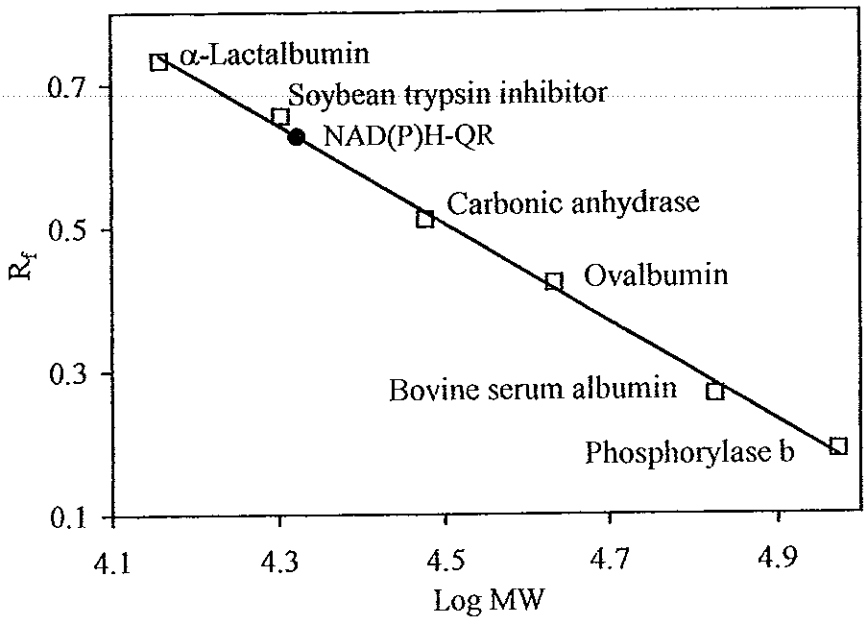


Fig.12 Calibration curve for determination of the subunit molecular weight for the NAD(P)H-QR after SDS-PAGE analyses.

The black circle represents the migration ratio of the NAD(P)H-QR with corresponding to $M_r$ of 21 kDa. The calibration curve was plotted between relative mobility of the standard proteins and the logarithm of their corresponding molecular weights. The standard protein references were phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14.4 kDa).

### 1.4 Effect of pH on NAD(P)H-QR activity and stability

The effect of pH on NAD(P)H-QR activity, as a function of pH, was determined at various pHs ranging of 3-10. The result was expressed as the percentage of relative activity as compared to the highest activity. The result revealed that NAD(P)H-QR activity has a sharp optimum pH of 8 (Fig. 13).

The pH stability of NAD(P)H-QR was performed by pre-incubating aliquots of NAD(P)H-QR sample at various pHs ranging of 3-10 for 1 h. The mixtures were adjusted back to pH 8 and assayed for remaining of NAD(P)H-QR activity. The result was expressed as the remaining activity percentage as compared to the control, sample kept at constant pH of 8, with 100% activity. The result revealed that NAD(P)H-QR activity was retained (more than 80%) at pH 6-10 as shown in Fig. 14. The effect of pH showed the broad ranges of pH stability and the sharp optimum pH of 8 for enzyme activity.

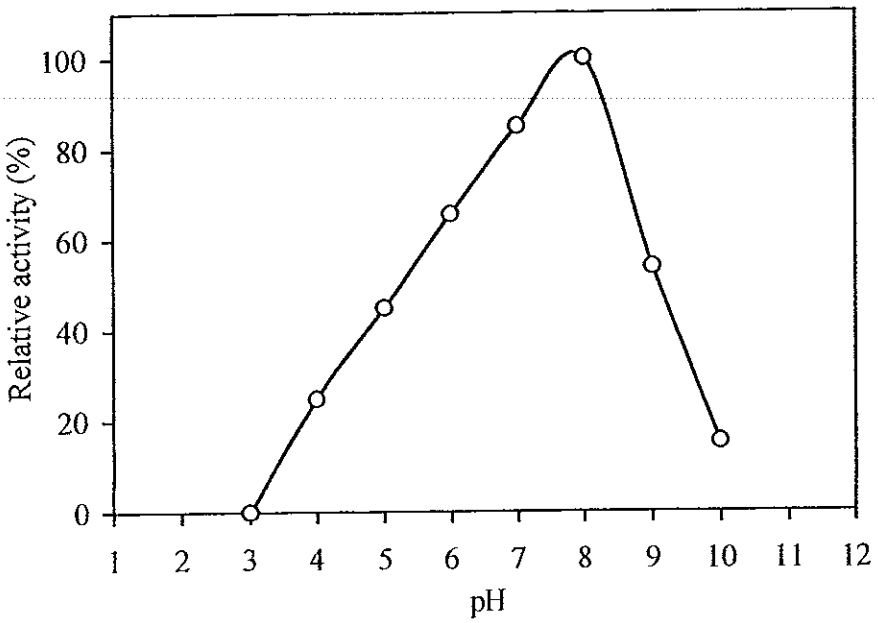


Fig.13 Optimum pH of Blue Sepharose-purified NAD(P)H-QR.

The purified NAD(P)H-QR was assayed at various pHs from 3 to 10 as indicated. The result was expressed as the percentage of relative activity as compared to the highest activity (100% relative activity).

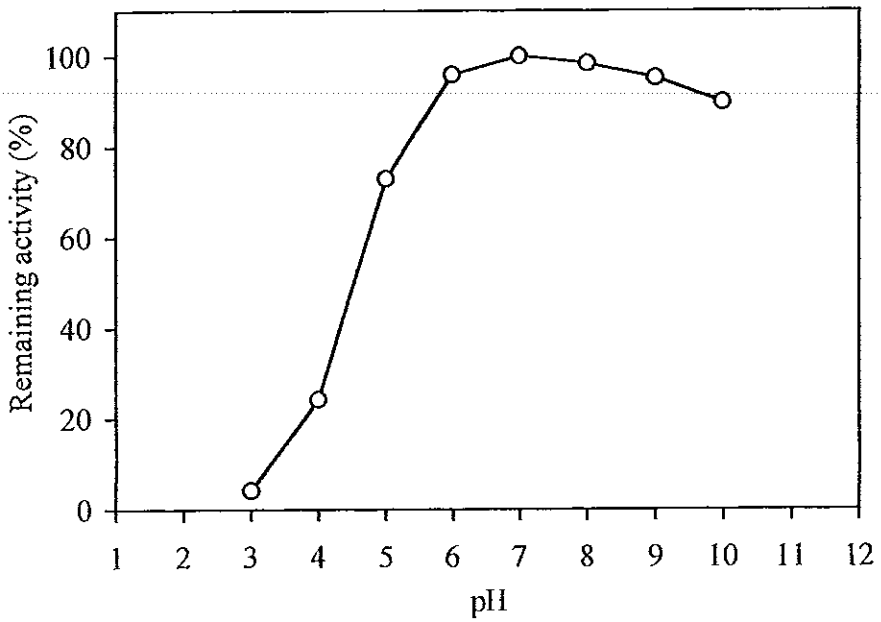


Fig.14 pH stability of Blue Sepharose-purified NAD(P)H-QR.

The purified NAD(P)H-QR was incubated at various pHs from 3 to 10 as indicated for 1 h. The sample pH was then readjusted back to pH 8 before measuring for the remaining activity. The result was expressed as the remaining activity percentage as compared to the control, sample kept at constant pH of 8, with 100% activity.

### 1.5 Thermal stability of NAD(P)H-QR

The effect of temperature on activity of NAD(P)H-QR was evaluated by preincubating purified NAD(P)H-QR at different temperature before measuring its remaining activity. It is shown to be highly heat-stable protein at up to 80 °C with little loss of the enzyme activity in the incubation mixture (Fig. 15). More than 80% and 70% of the latex NAD(P)H-QR activity retained after 10 hours of heat treatment preincubation at 70 and 80 °C, respectively (Fig. 16), indicating the highly unusual heat-stable property of this *Hevea* latex enzyme. The results showed the broad range of optimum temperature up to 80 °C (Fig. 15). On the other hand, heat pretreatment of the enzyme at 80 °C for 10 hours prior to the assay led to about 30% loss of the enzyme activity (Fig. 16).

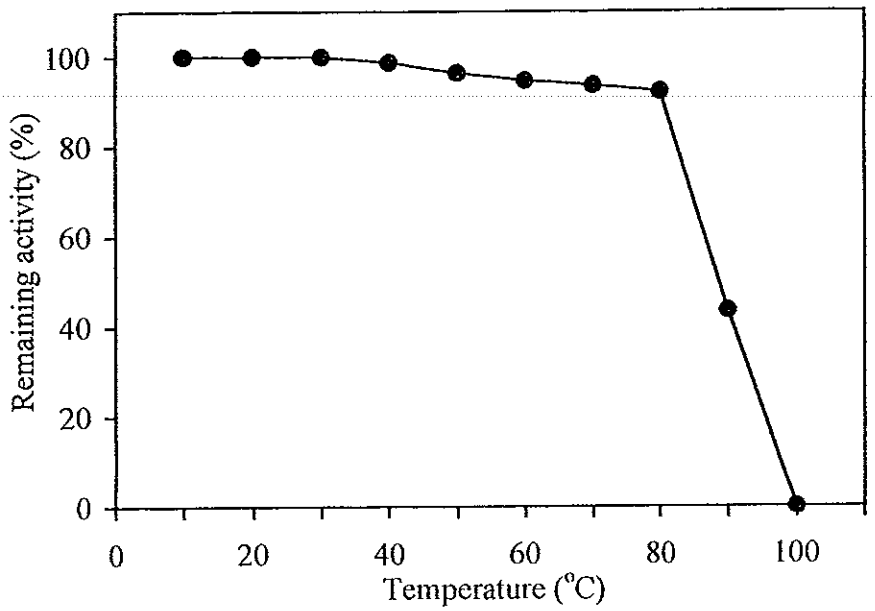


Fig.15 Thermal stability of Blue Sepharose-purified NAD(P)H-QR.

The remaining activities were determined after preincubations at various temperatures as indicated for 30 minutes. The result was expressed as the remaining activity percentage as compared to the control.

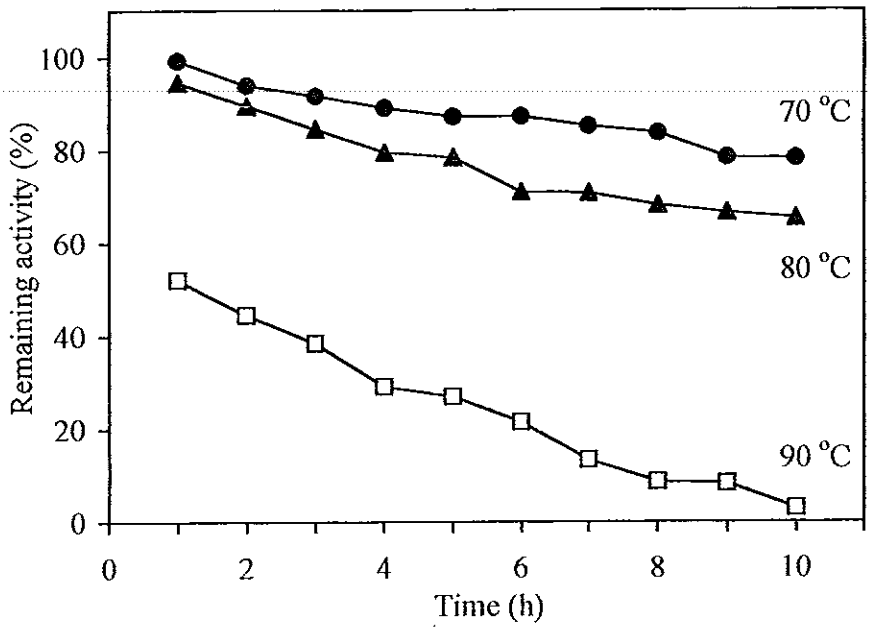


Fig.16 Thermal stability of Blue Sepharose-purified NAD(P)H-QR for up to 10 h.

The remaining activities were determined after preincubations at various temperatures from 70 to 90 °C as indicated for up to 10 h and was assayed at different points during the time course. The result was expressed as the remaining activity percentage as compared to the control.

### 1.6 Substrate specificity of NAD(P)H-QR

Comparative study on the preferential electron donors between NADH and NADPH was investigated (Table 6). A maximum activity was obtained when NADH used as a donor and hydrophilic short-chain quinones such as menadione and *p*-benzoquinone as acceptors compared to the results obtained with NADPH as donor (Table 6). The activity ratio between NADH and NADPH was found to be about 1.5, indicating the 50% higher activity with NADH as shown in Table 6. However, the enzyme showed very little different among the four quinone substrate acceptors (menadione, *p*-benzoquinone, juglone, and plumbagin), though a slightly lower level was noticible on duroquinone. A comparable $K_m$ for NADH and NADPH was observed, using any respective quinone acceptors, except for menadione where a lower level was found towards NADH (Table 6).

Table 6 Comparative and preferential studies on donor and acceptor substrates specificity of the purified NAD(P)H-QR[a].

| Acceptor | Donor (μkat $mg^{-1}$) | | $K_{m(donor)}$ (μM) | |
|---|---|---|---|---|
| | NADH | NADPH | NADH | NADPH |
| Menadione | 0.562 | 0.377 | 204 | 322 |
| *p*-Benzoquinone | 0.555 | 0.380 | 333 | 384 |
| Juglone | 0.542 | 0.368 | 250 | 286 |
| Plumbagin | 0.523 | 0.350 | 200 | 270 |
| Duroquinone | 0.317 | 0.250 | 375 | 435 |

[a] Assays were performed in 50 mM Tris-HCl, pH 8 in the presence of 0.2 mM pyridine nucleotide, 0.05 mM of each quinone as indicated and 10 μg of purified NAD(P)H-QR (after Blue Sepharose CL-6B column).

### 1.7 Effect of inhibitors on NAD(P)H-QR activity

Study of the enzyme affectors was carried out to investigate the inhibitory effect of various different reagents. The values for 50% inhibition of the enzyme by lowest concentrations of compounds as inhibitor were obtained (Table 7). Rutin was found to be the most potent inhibitor of the latex enzyme at 16 μM concentration for 50% inhibition. This was followed by pyrogallol, propyl gallate and dicumarol with significant inhibitory effect, while the others were only weak inhibitors for the latex enzyme.

Table 7 Comparative study on the potency degree of different inhibitors on NAD(P)H-QR activity.

| Compound | Conc. for 50% inhibition ($I_{50}$)[a] (μM) |
|---|---|
| Rutin | 16 |
| Pyrogallol | 39 |
| Propyl gallate | 58 |
| Dicumarol | 100 |
| Dopamine | 713 |
| Catechol | >1,000 |
| Butyl-hydroxytoluene | >1,000 |

[a] The inhibitor concentrations were increased to reach 90% inhibition before the determination of $I_{50}$.

## Part 2. Purification and characterization of *Hevea* latex polyphenol oxidase

### 2.1 Distribution and protein fractionation of polyphenol oxidase

Polyphenol oxidase (PPO) was earlier reported to be present in both *Hevea* latex lutoid and Frey-Wyssling particles (Coupé *et al.*, 1972). The activity of latex PPO in lutoids (B-serum) was 5- to 34-fold higher than that of the Frey-Wyssling particles (Table 8, Table 9) and minimum activity was detected in C-serum. The protein in B-serum was precipitated with cold acetone in serial 10% increment of acetone saturation. A 40-50% of acetone saturated precipitate fraction was found to contain highest amout of PPO activity (Table 10).

Table 8 Distribution of PPO activity in the ultracentrifuged fresh latex (100 ml).

| Sample fraction | Enzyme activity | |
|---|---|---|
| | PPO (unit)[a] | Yield (%) |
| Frey-Wyssling | 1.900 | 3.21 |
| C-serum | 0.301 | 0.51 |
| B-serum | 57.016 | 96.26 |

[a] One unit of enzyme activity equals to the amount of enzyme which caused a change in one absorbance per minute.

Table 9 Distribution of PPO activity in the ultracentrifuged fresh latex of various clones.

| Rubber clone | PPO activity[a] (units /ml latex) | |
|---|---|---|
| | Lutoid | Frey-Wyssling |
| RRIM 600 | 0.619 | 0.018 |
| GT1 | 0.778 | 0.055 |
| KRS 21 | 0.367 | 0.065 |

[a] Minimal PPO activity was detected in C-serum and rubber fractions.

Table 10 PPO activity of protein fractions from B-serum (30 g bottom fraction), 10% serial acetone fractionation.

| Fraction (% acetone saturation added) | Protein (mg) | PPO activity (unit)[a] | Yield (%) |
|---|---|---|---|
| none | 1,114 | 252.69 | 100 |
| 0-10% | 6 | 0 | 0 |
| 10-20% | 12 | 0 | 0 |
| 20-30% | 24 | 15.84 | 6.27 |
| 30-40% | 58 | 426.80 | 168.90 |
| 40-50% | 171 | 1,180.71 | 467.24 |
| 50-60% | 422 | 20.87 | 8.26 |
| 60-70% | 90 | 14.00 | 5.54 |
| 70-80% | 41 | 5.05 | 2.00 |

[a] One unit of enzyme activity equals to the amount of enzyme which caused a change in one absorbance per minute.

### 2.2 Purification of polyphenol oxidase

The PPO in crude lutoid extract, released from the bottom fraction by freezing and thawing, was subjected to further purification by acetone precipitated fractionation and ion-exchange chromatography on CM-Sepharose column. A 6-fold increase in PPO activity was obtained after the acetone treatment. The increase in PPO activity may result from limited proteolysis, by proteases present in the B-serum (Pujarniscle, 1968), during the PPO isolation. The activity of PPO in B-serum dialyzed against buffer containing protease inhibitors was lower than that containing no protease inhibitors, and much lower than the B-serum sample kept under the same condition at 4 °C for 24 h, respectively (Table 11) After the CM-Sepharose column chromatography, a major PPO-I peak (*ca* 70% of total PPO activity) and a minor PPO-II peak, were obtained from stepwise elutions with buffer containing 0.1 and 0.2 M NaCl, respectively (Fig. 17). The specific activity of PPO-I was measured to be 19.77 unit $mg^{-1}$ with 90 purification folds. The specific activity of PPO-II was measured to be 47.80 unit $mg^{-1}$ with 217 purification folds (Table 12).The specific activity of the eluted PPO-II was about 2.5-fold higher than that of the PPO-I.

Table 11 Limited proteolysis effect on B-serum PPO activity.

| Treatment | Relative PPO activity (%) |
|---|---|
| 1. 4 °C for 24 h | 100 |
| 2. Dialysed against 50 mM Tris-HCl, pH 7 at 4 °C for 24 h | 69 |
| 3. Same as for 2 but buffer contained 1 mM EDTA and 1 mM PMSF | 42 |

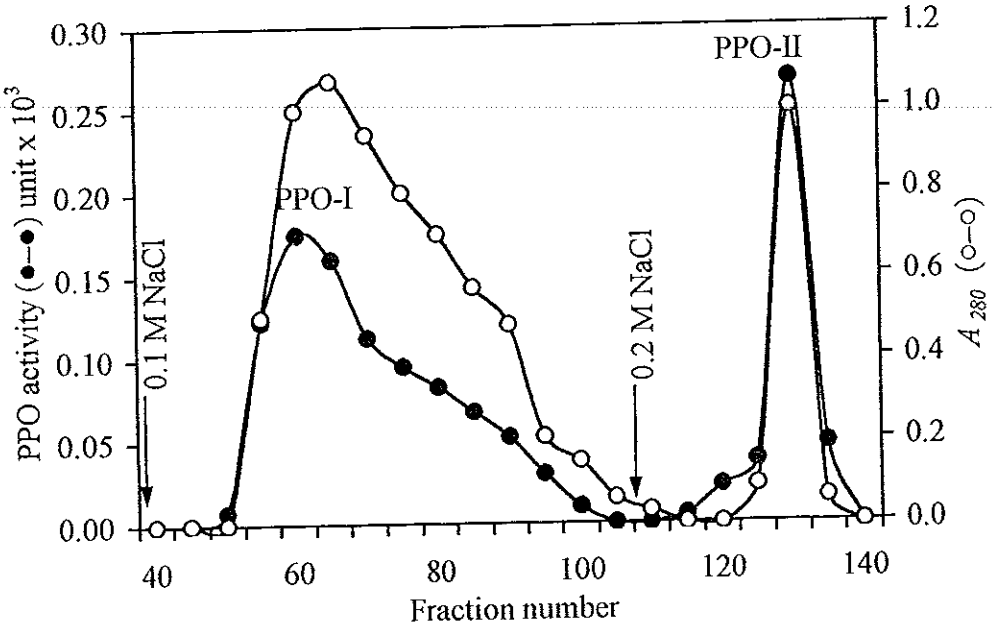


Fig.17 Chromatographic elution profile of PPOs on CM-Sepharose column.

A CM-Sepharose CL-6B column (2.5x24 cm) was pre-equilibrated with 50 mM Tris-HCl pH 7.0 at a flow rate of 18 ml/hr at 4°C. After loading the resuspended acetone precipitate solution, the column was washed with the same buffer until the absorbance at 280 was below 0.005. The column was then subjected to stepwise-elution by using the same buffer containing 0.1 and 0.2 M NaCl, respectively.

Table 12 Purification protocol of the latex PPO.

| Purification step | Total activity (unit)[a] | Protein (g) | Specific activity (unit.mg$^{-1}$) | Yield (%) | Purification (-fold) |
|---|---|---|---|---|---|
| B-serum | 603.25 | 2.72 | 0.22 | 100 | 1 |
| Acetone ppt | 3,627.00 | 0.63 | 5.72 | 601 | 26 |
| CM-Sepharose | | | | | |
| PPO-I | 1,977.36 | 0.10 | 19.77 | 328 | 90 |
| PPO-II | 860.43 | 0.02 | 47.80 | 143 | 217 |

[a] One unit of enzyme activity equals to the amount of enzyme which caused a change in one absorbance per minute.

### 2.3 Molecular properties of polyphenol oxidase

For the determination of native molecular weight of PPO-I and PPO-II, the purified PPOs obtained from CM-Sepharose column and standard marker proteins were subjected to gel filtration on Sephadex G-100 column (1 x 90 cm).in 50 mM Tris-HCl buffer pH 7.0. A plot between respective values on the $K_{av}$ of standard proteins and logarithm of their molecular weight was constructed. The native molecular weight of PPO-I and PPO-II obtained from Sephadex G-100 column were calibrated to be 32 and 34 kDa respectively (Fig. 18)

For the determination of subunit molecular weight, peak fraction of PPO-I and PPO-II obtained from CM-Sepharose column and Pharmacia standard marker proteins were subjected to SDS-PAGE on 10 % slab gel, The result shown in Fig 19 that PPO-I and PPO-II were revealed as partially purified and purified *ca* 32 kDa and 34 kDa enzymes, respectively (Fig. 20).

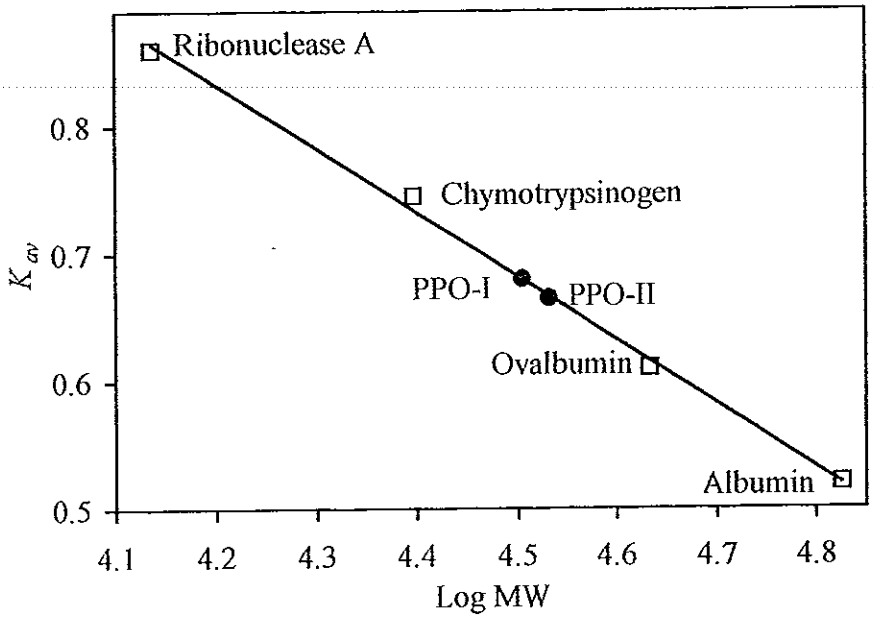


Fig.18 Calibration curve for determination of the native molecular weight of the PPOs after a Sephadex G-100 column chromatography.

The black circles indicated $K_{av}$ of the PPO-I and PPO-II, corresponding to $M_r$s of 32 and 34 kDa, respectively. The calibration curve was plotted between respective values on the $K_{av}$ of standard proteins and logarithm of their molecular weights. The standard markers used were albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa).

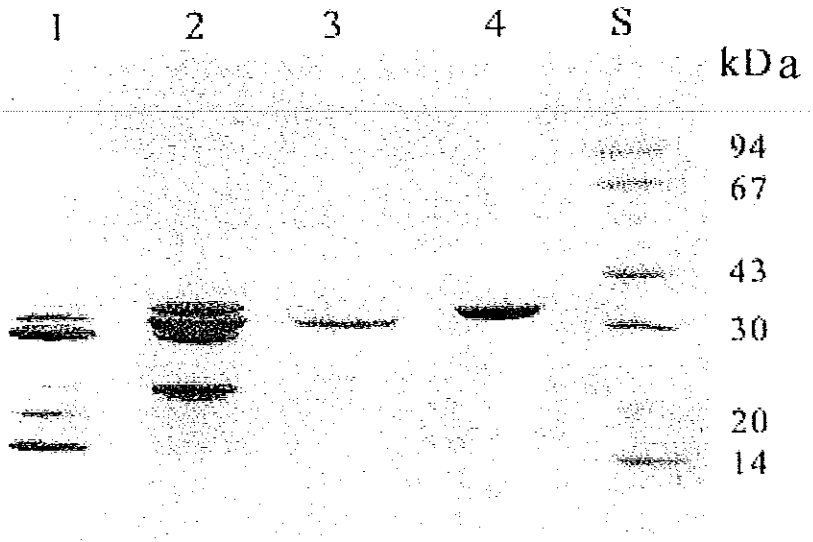


Fig.19 SDS-PAGE analyses on protein patterns of the purified latex PPOs;

Lane 1 = proteins obtained from B-serum (100 μg),

Lane 2 = 30-50% acetone pellet fraction (100 μg),

Lane 3 = peak fraction of PPO-I from CM-Sepharose column (20 μg),

Lane 4 = peak fraction of PPO-II from CM-Sepharose column (20 μg),

Lane S = standard protein markers (kDa).

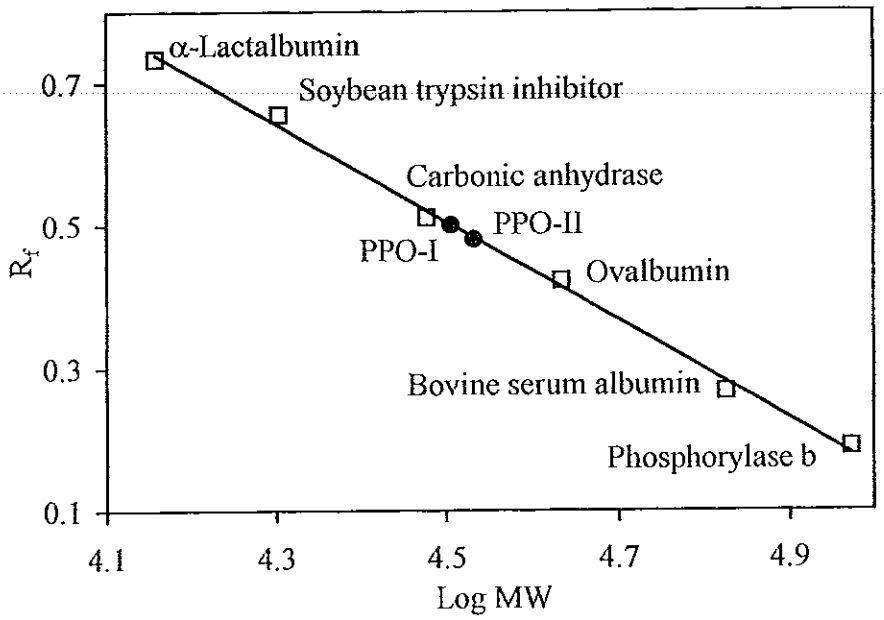


Fig.20 Calibration curve for determination of the molecular weight for the PPOs after SDS-PAGE analyses.

The black circles represent the migration ratio of the PPO-I and PPO-II, corresponding to $M_r$s of 32 and 34 kDa, respectively. The calibration curve was plotted between relative mobility of the standard proteins and the logarithm of their corresponding molecular weights. The standard protein references were phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14.4 kDa).

### 2.4 Isoelectric focusing of latex PPOs

The IEF was carried out in this experiment for determination the pI value of PPO-I and PPO-II by using the both PPOs sample from the peak fraction of CM-Sepharose column, as described in the Method. An amount of 4 μg of both PPOs were applied on polyacrylamide gel isoelectric focusing. After the focusing was completed the gel containing both PPOs and standard protein markers were removed from the supporting film. The PPO activity bands were stained by incubating or dipping gel in the PPO assay solution containing 10 mM dopamine for 10 min. The standard protein markers bands were stained for 1 hour and followed by destaining solution until the background was at minimum in order to visualize the protein staining pattern of the IEF gel. The relative pI of both PPOs activity band was estimated from the standard protein markers band. The band position of PPOs is very close to that of trypsinogen which served as standard pI of 9.3. Hence, the pI of both PPOs were estimated to be about 9.2 (Fig. 21).

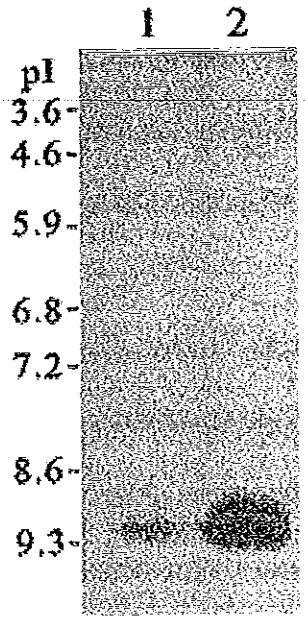


Fig.21 Isoelectric focusing gel electrophoresis for pI determinations of PPO-I and PPO-II.

Lane1 = PPO-I (4 μg)

Lane2 = PPO-II (4 μg)

### 2.5 Effect of pH on PPO activity and stability

The effect of pH on PPOs activity, as a function of pH, were determined at various pHs ranging of 3-10. The result was expressed as the percentage of relative activity as compared to the highest activity. The result revealed that PPO-I and PPO-II activity have the same sharp optimum pH of 7 (Fig. 22).

The pH stability of PPO-I and PPO-II were performed by preincubating aliquots of PPOs sample at various pHs ranging of 3-10 for 1 hr. The mixtures were adjusted back to pH 7 and assayed for remaining of PPOs activity. The result was expressed as the remaining activity percentage as compared to the control, sample kept at constant pH of 7, with 100% activity. The result revealed that both enzymes activity were retained (more than 80%) at pH 4-10 as shown in Fig. 23. The effect of pH showed the broad ranges of pH stability and the sharp optimum pH of 7 for both enzymes activity.

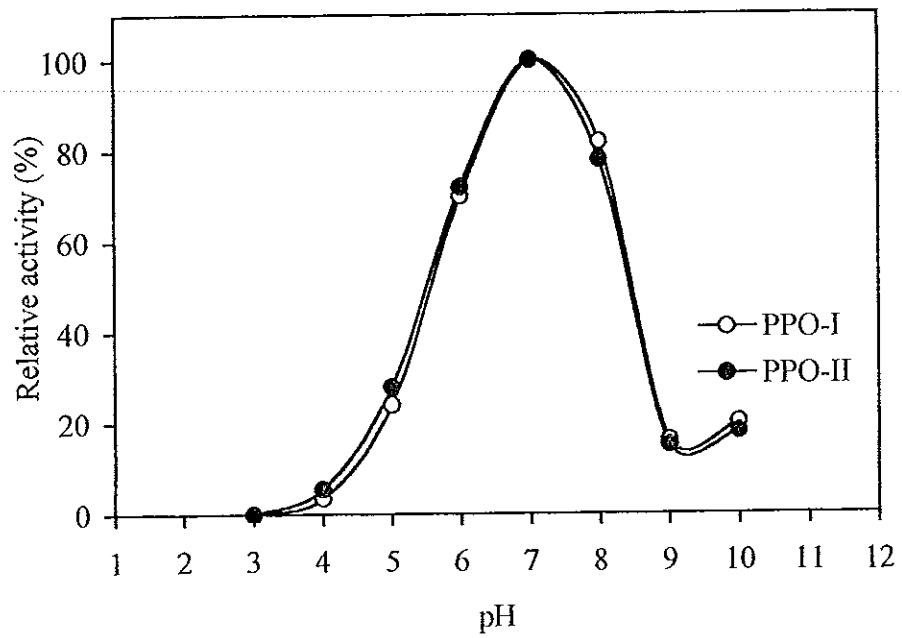


Fig.22 Optimum pH on the activity level of PPO-I and PPO-II fractions obtained from CM-Sepharose column.

The PPO-I and PPO-II fractions were assayed at various pHs from 3 to 10 as indicated. The result was expressed as the percentage of relative activity as compared to the highest activity (100% relative activity).

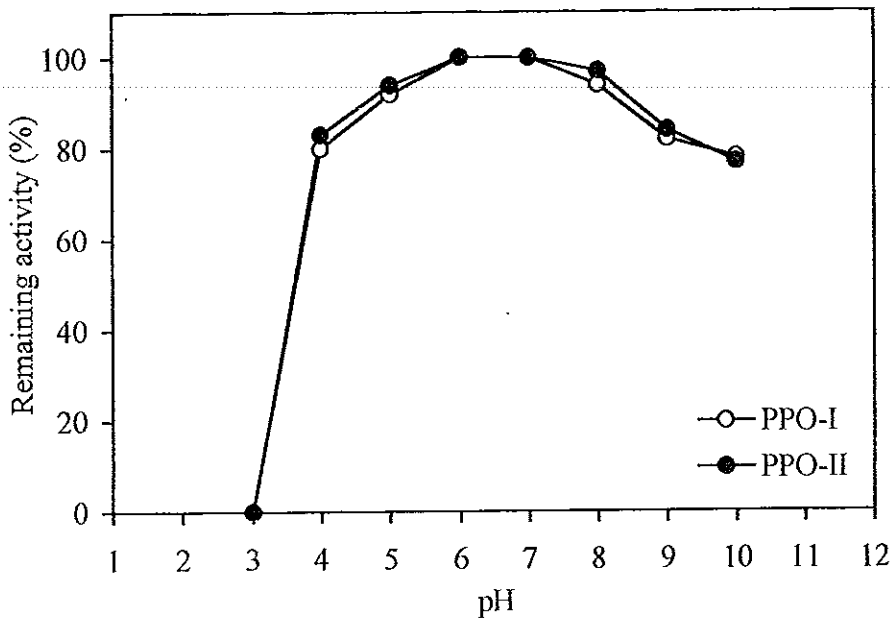


Fig.23 pH stability on the activity level of PPO-I and PPO-II fractions obtained from CM-Sepharose column.

The PPO-I and PPO-II fractions were incubated at various pHs from 3 to 10 as indicated for 1 h. The sample pH was then readjusted back to pH 7 before measuring for the remaining activity. The result was expressed as the remaining activity percentage as compared to the control, sample kept at constant pH of 7, with 100% activity.

### 2.6 Effect of temperature on PPO activity and stability

The temperature optimum of PPO-I and PPO-II were screened at various temperatures ranging from 20-60 °C. The same optimum temperature of 35-45 °C were found for both enzymes (Fig. 24)..

The thermal stability of PPO-I and PPO-II were tested at various temperatures ranging from 20-80 °C. The PPOs were pre-incubated at the indicated temperature for 30 min and adjusted back to 4 °C before assaying using 10 mM dopamine as the substrate. Both PPOs showed thermal stability up to 60°C with almost 80% activity remaining after heat pretreatments prior to the enzyme assays (Fig. 25).

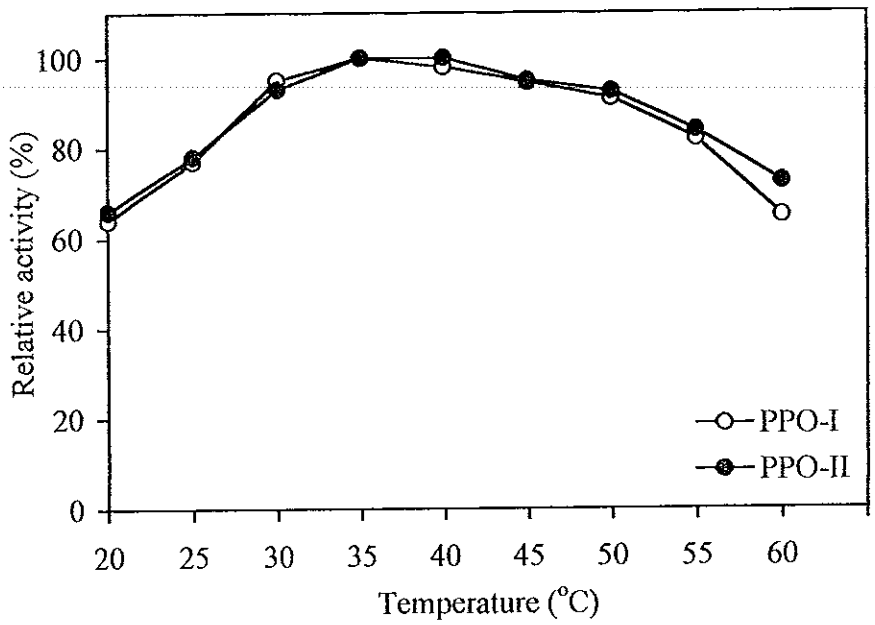


Fig.24 Optimum temperature on activity level of PPO-I and PPO-II fractions obtained from CM-Sepharose column.

The PPO-I and PPO-II fractions were assayed at various temperatures from 20 to 60 °C as indicated. The result was expressed as the percentage of relative activity as compared to the highest activity (100% relative activity).

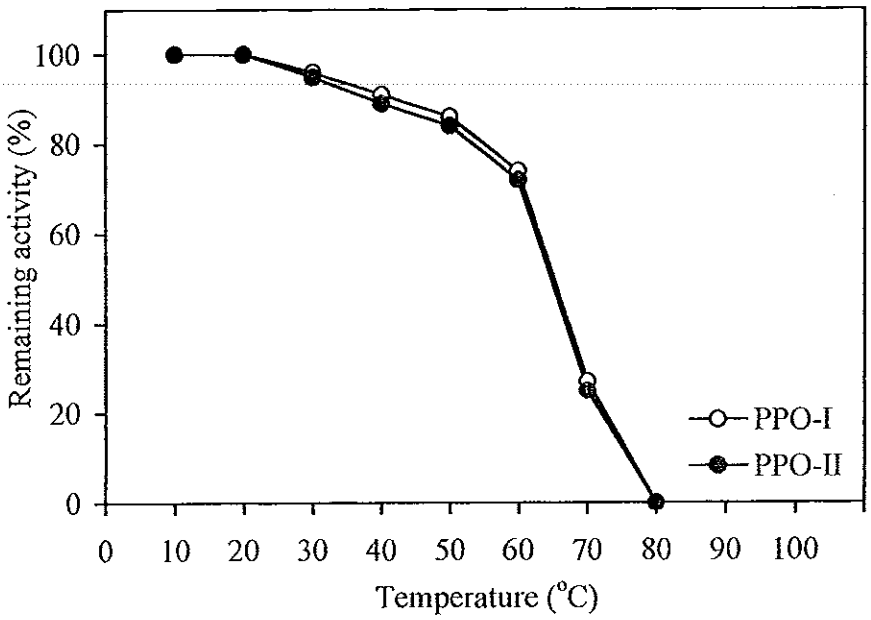


Fig.25 Thermal stability on activity level of PPO-I and PPO-II fractions obtained from CM-Sepharose column.

The remaining activities were determined after preincubations at various temperatures ranging from 20 to 80 °C as indicated for 30 minutes. The result was expressed as the remaining activity percentage as compared to the control.

### 2.7 Substrate specificity of PPO

Different $K_m$ values of PPO-I and PPO-II, using dopamine, L-dopa and catechol as substrates (Table 13), were obtained from the Lineweaver-Burk plots. The $K_m$ values of PPO-I for dopamine, L-dopa and catechol are 2.08, 8.33 and 9.09 mM, while those for PPO-II are 2.12, 4.76 and 7.14 mM, respectively (Table 13). Both enzymes have lower $K_m$ values of about 4-fold toward dopamine as compared to catechol substrate. Among several *ortho*-dihydroxyphenol substrates tested, PPO-II revealed its highest relative substrate specificity toward dopamine (0.214 unit) and lowest toward catechin of 0.037 unit (Table 14).

Table 13 Kinetic studies on the $K_m$ of PPO-I and PPO-II.

| Substrate | PPO-I | PPO-II |
|---|---|---|
| Dopamine (mM) | 2.08 | 2.12 |
| L-dopa (mM) | 8.33 | 4.76 |
| Catechol (mM) | 9.09 | 7.14 |

Table 14 Preference and suitable substrate specificity of the latex PPO-II.

| Substrate | [S] (mM) | Activity (unit) | Activity relative to dopamine (%) |
|---|---|---|---|
| Dopamine | 5 | 0.214 | 100 |
| L-dopa | 5 | 0.121 | 57 |
| Catechol | 5 | 0.102 | 48 |
| Catechin | 5 | 0.037 | 17 |
| Benzoic acid | 5 | 0 | 0 |
| *p*-Coumaric acid | 5 | 0 | 0 |
| Tyrosine | 5 | 0 | 0 |
| 3′,3′-Diaminobenzidine | 5 | 0 | 0 |

### 2.8 Effect of inhibitors and activators (detergents) on PPO activity

The effect of various inhibitors on latex PPO, obtained after acetone fractionation, with dopamine as substrate was determined (Table 15). 4-Hexylresorcinol, a specific inhibitor for PPO in antibrowning, was the most potent inhibitor and able to inhibit activity up to 84% at low concentration (0.1 mM). This was followed by dithiothreitol, sodium metabisulphite and 2-mercaptoethanol with significant inhibitory effect, while the others were only weak inhibitors for the enzyme. $NaN_3$ and NaCl, at 10 mM were unable to inhibit the latex PPO

The modulation of latex PPO activity by detergents was studied using anionic (SDS and DOC), cationic (CTAB) and nonionic (Triton X-100 and Tween 20) detergents (Table 16). Anionic detergents were found to be more effective in the activation of latex PPOs than the cationic one. The presence of SDS (10 mM) resulted in a maximum of 12-fold increase in the catalytic efficiency of the activated enzyme. Similarly, DOC (10 mM) was also able to activate the enzyme by 5.2-fold. The level of latex enzyme activation in the presence of cationic CTAB (10 mM) was insignificant (1.6-fold). In contrast to ionic detergents, the latex PPOs could not be activated by nonionic detergents.

Table 15 Comparative study on effects of various inhibitors on latex PPO activity[a].

| Inhibitor | mM | Inhibition (%) |
|---|---|---|
| None | 0 | 0 |
| 4-Hexylresorcinol | 0.1 | 84.10 |
| Dithiothreitol | 0.1 | 66.76 |
| Sodium metabisulphite | 0.1 | 20.89 |
| 2-Mercaptoethanol | 0.1 | 17.60 |
| Ascorbic acid | 0.1 | 3.71 |
| Thiourea | 1.0 | 33.41 |
| Resorcinol | 1.0 | 13.21 |
| Sodium azide | 10 | 0 |
| Sodium chloride | 10 | 0 |

[a]The enzyme (20 μg) was preincubated with various inhibitors as indicated at room temperature for 5 min before starting PPO assay by addition of dopamine (10 mM final concentration).

Table 16 Comparative study on effects of different detergents on latex PPO activity[a].

| Detergent | Units/ml | | % of control | |
|---|---|---|---|---|
| | 1 mM | 10 mM | 1 mM | 10 mM |
| None | 0.73 | 0.73 | 100 | 100 |
| SDS | 5.84 | 8.74 | 800 | 1,200 |
| DOC | 0.72 | 3.79 | 100 | 520 |
| CTAB | 1.16 | 1.08 | 160 | 140 |
| Triton X-100 (1%) | 0.67 | | 90 | |
| Tween 20 (1%) | 0.67 | | 90 | |

[a]Dialysed B-serum (0.2 mg) was used for assay.

**Part 3. Correlation study between levels of enzymes activity and rubber flow time, dry rubber concentration and flowtime, and between flow time and rubber yield per tapping**

The correlation between NAD(P)H-QR activity in the latex and the dry rubber content was investigated with the aim of establishing the effect of the enzyme on latex flow time and hence on latex yields. In this experiment, latex samples were collected from forty rubber trees giving high, medium and low levels of rubber yield. After centrifugation of fresh latex, the B-serum was separately prepared and used to quantitate for its respective NAD(P)H-QR activity. The result as shown in Fig. 26-28, revealed a positive correlation value between NAD(P)H-QR activity in the latex was obtained with flow time per tapping ($r = 0.85$, $P < 0.01$) but not latex rubber concentration ($r = -0.61$, $P < 0.01$) (Fig. 26). A positive correlations between rubber yield per tapping and latex NAD(P)H-QR activity [fresh latex ($r = 0.89$, $P < 0.01$), dry rubber ($r = 0.81$, $P < 0.01$)] (Fig. 27) together with flow time [fresh latex ($r = 0.80$, $P < 0.01$), dry rubber ($r = 0.68$, $P < 0.01$)] (Fig. 28).

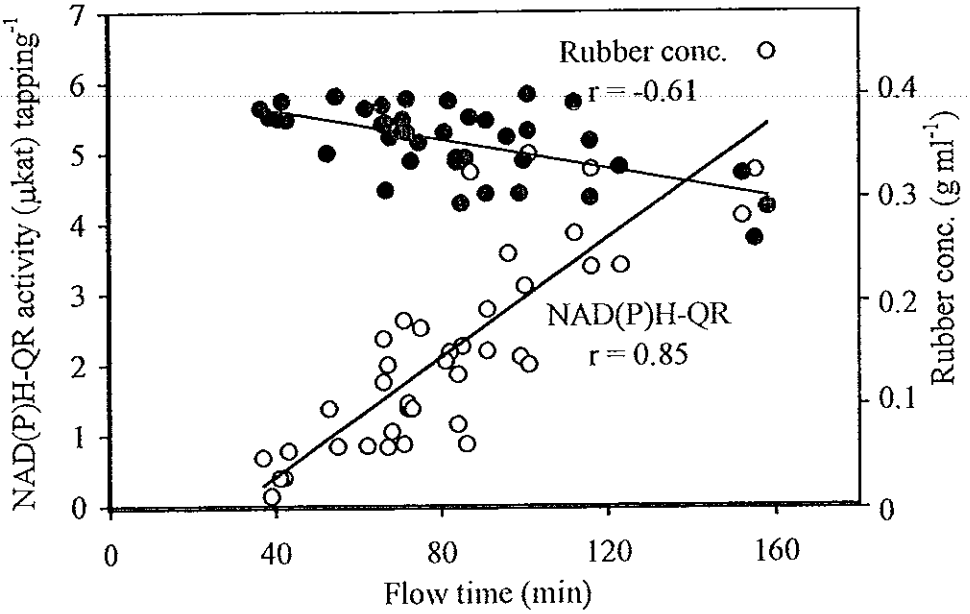


Fig.26 Correlation between latex NAD(P)H-QR levels and flow time and between latex dry rubber concentration and flow time per tapping (n = 40).

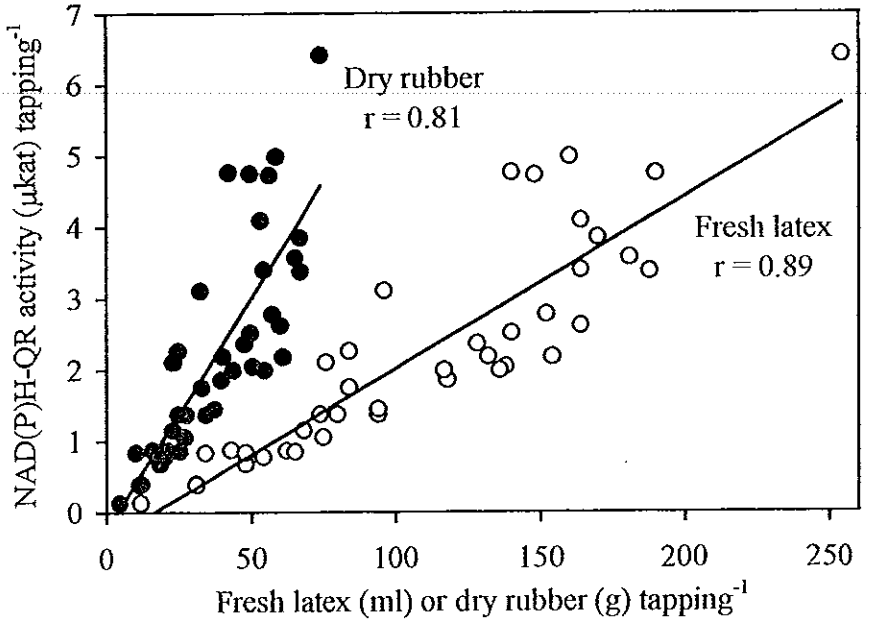


Fig.27 Correlation between latex NAD(P)H-QR levels and rubber yield per tapping (n = 40).

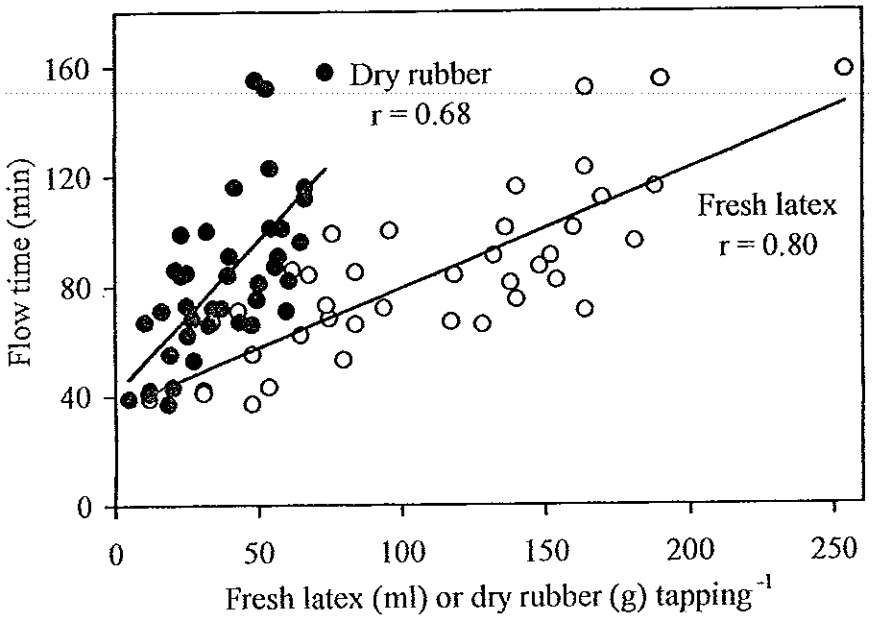


Fig.28 Correlation between latex flow time and rubber yield per tapping (n = 40).

# Chapter 4

# Discussion

## Part 1. Purification and characterization of *Hevea* latex NAD(P)H quinone reductase

### 1.1 Purification of latex NAD(P)H-QR

NAD(P)H-QR was purified from latex B-serum (obtained as described in Experimental) according to the protocol summarized in Table 1. The purification protocol included acetone fractionation, heat treatment and DEAE-Sephacel chromatography, followed by a final purification step on Blue Sepharose CL-6B to produce a homogeneous enzyme. The final step facilitated a 409-fold increase in purification to give an enzyme with a specific activity of 1.23 μkat $mg^{-1}$ (Table 1). The highest purification efficiency was obtained with the Blue Sepharose serving as affinity chromatography in the protocol final step.

### 1.2 Molecular weight determination

In the study on characterization of NAD(P)H-QR, its molecular mass for the native enzyme estimated from gel filtration was calibrated to be 83 kDa. The enzyme molecular weight as determined by SDS-PAGE was 21 kDa for the protein subunit. It thus seems that the 83-kDa native enzyme fraction is composed of four identical monomer homosubunits, with a molecular mass of 21 kDa. The above described molecular masses are in the same range as those

reported for the enzyme in sugar beet cells (Trost *et al.*, 1995), zucchini hypocotyls (Trost *et al.*, 1997) and tobacco leaves (Sparla *et al.*, 1996).

### 1.3 Effect of pH and temperature on activity and stability of latex NAD(P)H-QR

Latex NAD(P)H-QR exhibited pH stability over a broad range of 6 to at least 10, with a sharp optimum at pH 8. The enzyme exhibited good thermal stability up to 80 °C, with little loss of enzyme activity in the incubation mixture. More than 80% and 70% of the latex NAD(P)H-QR activity was retained after 10 h of preincubation at 70 and 80 °C, respectively, indicating the unusually high thermal stability as compared to pea seed NAD (P)H-QR which lost 95% activity at 50 °C within 5 min (Wosilait and Nason, 1954). The enzyme thus showed high degree of stability on the two key parameters for enzyme stability and activities, the pH and temperature. This make it practical for utilizing this enzyme under the harsh or extreme conditions.

### 1.4 Substrate specificity of latex NAD(P)H-QR

Maximal activity was obtained when NADH was used as the electron donor and a hydrophilic short-chain quinone, such as menadione or *p*-benzoquinone, as the acceptor. However, there was little difference on substrate specificity among the four quinone substrate acceptors (menadione, *p*-benzoquinone, juglone, and plumbagin), though a slightly lower level was noticeable on duroquinone. A comparable $K_m$ for NADH and NADPH was observed, using any respective quinone acceptors, except for menadione where a lower level was found towards NADH. The $K_m$ values (0.2-0.4 mM) of latex

NAD(P)H-QR are in the same range as those reported for tobacco leaves (Sparla *et al.*, 1996) but several folds higher than onion root plasma membrane (Serrano *et al.*, 1994). The results of this study thus showed low substrate specificities of latex NAD(P)H-QR on either the acceptor (quinone) or electron donor [NAD(P)H] substrates. The nature of this enzyme showed that it has a board range of substrates to serve as electron acceptors. Similar property can also be said of the electron donor, as shown by the similar activities either NADH or NADPH being used in the assays.

### 1.5 Effect of inhibitors on NAD(P)H-QR activity

Study of the enzyme affectors was carried out to investigate the inhibitory effect of various different reagents. Rutin was found to be the most potent inhibitor of the latex enzyme at 16 μM concentration for 50% inhibition. This was followed by pyrogallol, propyl gallate and dicumarol with significant inhibitory effect, while the others were only weak inhibitors for the latex enzyme. Dicumarol was found to be stronger inhibitor for latex NAD(P)H-QR, than those reported in zucchini (Trost *et al.*, 1997), tobacco leaves (Sparla *et al.*, 1996) and beet root tissues (Luethy, 1991). However, the dicumarol effect was with less potency than those observed with rutin, pyrogallol and propyl gallate with much stronger inhibitory effect as shown in Table 7. The results thus showed that the enzyme was most sensitive to the inhibition by rutin but least sensitive to the inhibitory effect of catechol or butyl-hydroxytoluene. This clearly showed that the Hevea NAD(P)H-QR is slightly different from those studied in other plant cells.

## Part 2. Purification and characterization of *Hevea* latex polyphenol oxidase

### 2.1 Distribution and possible function of the latex PPO

Polyphenol oxidase (PPO) was earlier reported to be present in both *Hevea* latex lutoid and Frey-Wyssling particles (Coupé *et al.*, 1972). We found that the latex PPO activity in lutoids was 5- to 34-fold higher than that of the Frey-Wyssling particles (Table 1). Since the latex bottom (lutoid) fraction was reported to contain several pathogenesis-related (PR) proteins; chitinases, β-1,3-glucanase and hevein (Churngchow *et al.*, 1995; Subroto *et al.*, 1996 and Van Parijs *et al.*, 1991), therefore this may also be the case for PPO as well. The transcripts of several lacticifer specific genes, highly expressed in *Hevea* lacticifers than in the leaves, were previously shown to include these PR proteins (Kush *et al.*, 1990; Broekaert *et al.*, 1990 and Chye and Cheung, 1995). The significant of the PR proteins accumulation in the lutoids is assumed to be wound response product from tapping of rubber trees for harvesting latex. The wound-induced specific genes activation, generally known to serve as part of the plant defense mechanisms, was also shown in *Hevea* system due to tapping wound (Kush, 1994). Hence, the PPO accumulated in lutoids as shown in this study may also serve as one of the wound-induced enzyme participating in defense against pests and pathogens. This was similar to those reported in tomato (Thipyapong and Steffens, 1997 and Thipyapong *et al.*, 1995) as has been generally suggested to play the defense function in plants (Mayer and Harel, 1979 and Mayer, 1987). Accordingly, highest level of latex PPO was found in latex of GT 1 clone which is the most disease resistant *Hevea* clone grown in Thailand. This

positive correlation thus suggested the possible role of PPO in the wound-induced defense function in *Hevea* in response to the tapping cuts. Moreover, the brown color development on the cut or sliced fruits and vegetables, is also similarly observed on dry rubber sheets prepared from milky white fresh latex. The white color of wet rubber sheet is gradually changed into brown color during the open-air drying period. This is presumably due to the latex PPO activity in catalyzing oxygen-dependent oxidation of phenols to reactive quinones which resulting in the brown color formation as occurred in the dry rubber sheets. Generally, the brown color of dry rubber sheet derived from GT 1 latex is relatively darker than that obtained from the RRIM 600. This study clearly showed that the PPO was the specific lutoid enzyme. It thus suggests a good possibility for utilizing this PPO enzyme as the marker enzyme for lutoid in the separation from Frey-Wyssling complex which sedimented together in the bottom fraction of centrifuged fresh latex.

### 2.2 Purification of the latex PPO

The PPO in crude lutoid extract (B-serum), released from the bottom fraction by freezing and thawing, was subjected to acetone precipitation and ion-exchange chromatography. A 6-fold increase in PPO activity was obtained after acetone treatment. Similar activation effects were previously observed with whole chloroplasts of Mayer and Friend (1960). The increase in PPO activity may result from limited proteolysis, by proteases present in the B-serum (Pujarniscle, 1968), during the PPO isolation. The activity of PPO in B-serum dialyzed against buffer containing protease inhibitors was lower than that containing no protease inhibitors, and much lower than the B-serum

sample kept under the same condition at 4 °C for 24 h, respectively, which further supports PPO activation by proteolysis (King and Flurkey, 1987). After CM-Sepharose column chromatography, a major PPO-I peak (*ca* 70% of total PPO activity) and a minor PPO-II peak, were obtained from stepwise elution with buffer containing 0.1 and 0.2 M NaCl, respectively. The specific activity of the eluted PPO-II was about 2.5-fold higher than that of the PPO-I. The results therefore suggested that the two PPOs are differentially expressed in the latex in response to the wounding as resulted from the tapping cuts. It is quite likely that PPO-II is playing more important role in the defense function for *Hevea* in wounding response augmented by the tappings.

### 2.3 Molecular weight of the latex PPO

Under SDS-PAGE, PPO-I and PPO-II were revealed as partially purified and purified *ca* 32 kDa (PPO-I) and 34 kDa (PPO-II) enzymes, respectively. Gel filtration chromatography showed both enzymes to be monomeric proteins with $M_r$ of 32 and 34 kDa. These $M_r$ values of latex PPO are slightly lower than that reported for the carrot cell culture (Soderhall and Soderhall, 1989). It is possible that the Hevea PPO is undergoing more and further elaborated modification of the translated protein precursor, thus yielding a lower molecular weight than those studied in other plant cells.

### 2.4 pI of the latex PPO

Both PPOs possessed the same pI of about 9.2. They therefore belong to the major class of positively charged B-serum basic proteins (Southorn and Yip, 1968), but are different from the majority of acidic PPO's, reported in other plant tissues as well as fungi (Soderhall and Soderhall, 1989;

Janovitz-Klapp *et al.*, 1989; Shin *et al.*, 1997 and Motoda, 1999). The basic nature of *Hevea* PPO with pI of 9.2 is in agreement with the discussion in the previous section. This might result from removal of the acidic amino acids residues to make it different from their acidic PPO in other plants.

### 2.5 Effect of pH and temperature on the latex PPO activity

The same optimum pH of 7 and optimum temperature of 35-45 °C were found for both enzymes, respectively. The latex PPOs' optimum pH value is higher than those found among the acidic PPOs (Shin *et al.*, 1997; Motoda, 1999 and Kader *et al*, 1997). Stability tests showed that they were stable in a broad pH range from 4-10, with more than 80% of the original activities retained at the extreme pH of 4 and 10, which is similar to that reported in the sunflower seeds (Raymond *et al.*, 1993). Both PPOs showed thermal stability up to 60°C with almost 80% activity remaining after heat pretreatments prior to the enzyme assays. Again, these results showed that PPO is another stable Hevea enzyme as has been observed for NAD(P)H-QR. It is broad range of pH stability (4-10) indicated that the enzyme can be active and functioning both within and outside the lutoid particles. This phenomenon might suggest its key important role in the latex vessels plugging upon being released from the lutoids during the flow of latex.

### 2.6 Substrate specificity and enzyme kinetics

Different $K_m$ values of PPO-I and PPO-II, using dopamine, L-dopa and catechol as substrates were obtained from the Lineweaver-Burk plots. Both enzymes have lower $K_m$ values of about 4-fold toward dopamine as compared to catechol substrate. Among several *ortho*-dihydroxyphenol

substrates tested, PPO-II revealed its highest relative substrate specificity toward dopamine and lowest toward catechin, similar to those reported in spinach chloroplast (Sheptovitsky and Brudvig, 1996). Moreover, the latex PPO-II exhibited no substrate specificity towards either monophenols or 3′,3′-diaminobenzidine, a suitable substrate for peroxidase and laccase. Hence, it is more likely to be a diphenol oxidase (EC 1.10.3.1) than a monophenol oxidase. Kinetic parameters difference between the two PPOs on the $K_m$ values might suggest that they play a cooperative role on the substrates preference. This might be a possibility to ensure that the PPO is always active and functioning pending on which substrates are available.

### 2.7 Effect of inhibitors and activators

The effect of various inhibitors on latex PPO, obtained after acetone fractionation, with dopamine as substrate was determined. Several compounds reported as PPO inhibitors (Raymond *et al.*, 1993; Anosike and Ayaebene, 1981 and Halim and Montgomery, 1978) were also shown to have inhibitory effect on the latex PPO. 4-Hexylresorcinol, a specific inhibitor for PPO in antibrowning, was the most potent inhibitor and able to inhibit activity up to 84% at low concentration (0.1 mM), although the results from inhibitor studies (Raymond *et al.*, 1993; Anosike and Ayaebene, 1981; Halim and Montgomery, 1978 and Golan-Goldhirsh and Whitaker, 1984) in other plant tissues (tuber, seed, fruit and mushroom) showed the thiol reagents as the most effective inhibitors for those enzymes. The level of latex PPO activity inhibited by 4-hexylresorcinol was, however, found to be higher than those with the thiol reagents either under equal or higher concentrations. These

results might suggest a small different on the amino acid residues at the active site of the *Hevea* enzyme than those other enzymes. Detailed studies on the protective function of substrates for enzyme active site against inhibitors would help revealing this observation. $NaN_3$, a broad spectrum peroxidase inhibitor, at 10 mM was unable to inhibit the latex PPO. NaCl, a browning inhibitor and observed to be the weakest PPO inhibitor in several plant tissues (Kavrayan and Aydemir, 2001; Yang *et al.*, 2000; Raymond *et al.*, 1993; Anosike and Ayaebene, 1981 and Halim and Montgomery, 1978), was also unable to inhibit latex PPO. The results on enzyme specificities towards specific inhibitors as well as substrates suggested that it is a PPO, not peroxidase or laccase. The lack of $NaN_3$ effect on PPO suggested that it is not a heme protein different from peroxidase which is very highly sensitive to the $NaN_3$ inhibition.

Anionic detergents were found to be more effective in the activation of latex PPOs than the cationic one. The presence of SDS (10 mM) resulted in a maximum of 12-fold increase in the catalytic efficiency of the activated enzyme, comparable to that described for banana and broad bean PPOs (Moore and Flurkey, 1990 and Sojo *et al.*, 1998), but higher than those described for table beet and spinach PPO (Escribano *et al*, 1997 and Sánchez-Ferrer *et al.*, 1989). Similarly, DOC (10 mM) was also able to activate the enzyme by 5.2-fold, which is higher than that described for sugar beet (Mayer and Friend, 1960). The level of latex enzyme activation in the presence of cationic detergent CTAB (10 mM) was insignificant (1.6-fold), which is similar to that described for banana PPO (Sojo *et al.*, 1998), but different from spinach PPO where CTAB acted as the enzyme inhibitor (Sánchez-Ferrer *et al.*, 1989).

In contrast to ionic detergents, the latex PPOs could not be activated by nonionic detergents, similar to those found with the latent broad bean and sugar beet PPOs (Kenten, 1958 and Mayer and Friend, 1960). The ability of SDS to activate the enzyme may involve alterations of its enzymatic and physical characteristics, as well as a limited conformational change due to binding of small amounts of SDS. This may induce or initiate the activation of the latent enzyme as earlier suggested (Moore and Flurkey, 1990). The differences in detergents activation suggested that it is the charge effect rather than the hydrophobic interaction of the enzyme with detergents. This is unlike some other hydrophobic enzymes which showed a wide spectrum for activation by various different detergents, particularly the prenyl transferase enzymes family.

**Part 3. Correlations between levels of NAD(P)H-QR activity and rubber flow time, dry rubber concentration and flow time and between flow time and fresh latex and dry rubber per tapping**

The correlation between NAD(P)H-QR activity in the latex and the dry rubber content was investigated with the aim of establishing the effect of the enzyme on latex flow time and hence on latex yields. A highly positive correlations between NAD(P)H-QR activity in the latex was obtained with flow time per tapping ($r = 0.85$, $P < 0.01$) but not latex rubber concentration ($r = -0.61$, $P < 0.01$) (Fig. 26). This finding may suggest a supportive role of NAD (P)H-QR in stabilization of the colloidal latex through its action in maintaining particle stability, particularly the integrity of the lutoid membrane. The contribution of the enzyme reaction to latex stability may be attributed to the

fact that it yields fully reduced quinols and not semiquinone intermediates, which can cause the build up of active oxygen species, leading to latex destabilization and hence vessel plugging. The results presented in Fig. 6 show that the higher the NAD(P)H-QR activity, the longer the flow times of the latex per tapping and hence the higher yields (Fig. 27, 28). This type of catalytic activity is similar to that reported for the enzyme in sugar beet cells (Trost *et al.*, 1995) or for DT-diaphorase in animal cells (Prochaska *et al.*, 1985; Tedeschi *et al.*, 1995). The practical implication of our results is that this latex enzyme activity may be used as an indicator of potential rubber yields in the early selection of superior seedlings with improved rubber yields.

These results provided several practical implications. The most obvious one is the key roles of NAD(P)H-QR for latex stability exerted by this enzyme in prolonging the latex flow. It should also be noted that the relative ratio between NAD(P)H-QR and PPO will determine the flow time as reflected for latex stability. High NAD(P)H-QR and PPO ratio will render a higher rubber yield, whereas the lower ratio will possibly result in a shorter latex flowing time. This aspect is certainly warrant further detailed investigation to study the relative ratio of the two enzymes and its impact on the rubber yields. This is base on the assumption that the comparing rubber clones have similar rubber synthetic capacity. It is therefore important to note that the NAD(P)H-QR is a good candidate for predicting the yield potential only if no significant difference on the PPO among the comparing rubber clones. Therefore, both NAD(P)H-QR and PPO have to be determined concurrently to make this observed results on NAD(P)H-QR to be valid. Verification of this outlook in

certainly attractive and could be a practical extended use on this enzyme study. This potential application and its impact on productivity in rubber plantations are certainly offering a good opportunity in the enzyme usage as yield marker. It therefore in certainly warranted for more and extensive detailed study for potential agroindustrial application that can lead to the improved productivity in rubber plantations.

# Chapter 5

## Summary

The result of this investigation could be summarized as follow :

1. NAD(P)H quinone reductase [NAD(P)H-QR] was extracted and homogeneously purified from B-serum obtained from bottom fraction of ultracentrifuged fresh latex.
2. The NAD(P)H-QR possesses native and subunit $M_r$ of 83 kDa and 21 kDa upon gel filtration chromatography and SDS-PAGE analyses, respectively.
3. The NAD(P)H-QR showed pH stability over a wide range of 6 to at least 10 (with an optimum at pH 8)
4. 70% of enzyme activity still remained after 10 hours of 80 °C pre-incubation. It shows that the NAD(P)H-QR was quite a thermostable protein enzyme.
5. NADH and menadione were good electron donor and acceptor for NAD(P)H-QR activity, respectively.
6. A comparable substrate spacificity for the NAD(P)H-QR was observed among menadione, *p*-benzoquinone, juglone, and plumbagin, with only duroquinone generating a lower activity.
7. NAD(P)H-QR activity could possibly be used as a marker to predict the yield potential of selected rubber clones since it was observed that the

highly positive correlations between the enzyme activity in latex and the rubber yield per tapping [Fresh latex (r=0.89, $P<0.01$] together with flow time (r=0.85, $P<0.01$) was with statistical significance with a high degree of confidence on data analyses.

8. Polyphenol oxidase (PPO) was isolated from B-serum obtained after repetitive freeze and thawing of bottom fraction isolated from ultra-centrifuged fresh latex.
9. Two PPOs, PPO-I and PPO-II, were obtained after purifiction step. Upon determenation by SDS-PAGE, the $M_r$ of 32 and 34 kDa were observed, for PPO-I and PPO-II, respectively.
10. Both PPO enzymes possessed the same pI of 9.2, indicating they are both basic proteins with a slight difference in enzyme properties.
11. The optimum pH of the PPOs was at pH 7. However, they were active at broad pH ranges from 4-9.
12. The PPOs optimum temperature was in the range of 35-45 °C and were stable up to 60 °C, indicating it is a heat stable enzyme. However, it was less stable than NAD(P)H-QR which can withstand the temperature up to 80 °C which still retained almost full activity.
13. The $K_m$ values of PPO-I for dopamine, L-dopa and catechol as substrates were 2.08, 8.33, and 9.09 mM, while those for PPO-II are 2.12, 4.76 and 7.14 mM, respectively. Catechol was a more prefers substrate for PPO-II than PPO-I.
14. Among various PPO inhibitors tested, 4-hexylresorcinol was the most potent inhibitors for both PPOs.

15. Anionic detergents were among the most effective activators of the enzymes, while cationic and nonionic detergents showed little or no effect on PPO activities, respectively. Charge effect was more important than hydrophobic interaction for the PPO activation.

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# VITAE

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**Educational Attainment**

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**Publications:**


1. Chareonthiphakorn, N., Wititsuwannakul, D., Golan-Goldhirsh, A. and Wititsuwannakul, R. 2002. Purification and characterization of NAD (P)H quinone reductase from the latex of *Hevea brasiliensis* Müll.-Arg. (Euphorbiaceae). *Phytochemistry* **61:** 123-128
2. Wititsuwannakul, D., Chareonthiphakorn, N., Pace, M. and Wititsuwannakul, R. 2002. Polyphenol oxidases from latex of *Hevea brasiliensis*: purification and characterization. *Phytochemistry* **61:** 115-121.
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