PURIFICATION AND STRUCTURAL CHARACTERIZATION OF A POLYPHENOL OXIDASE FROM FIELD BEAN *(DOLICHOS LABLAB)*

Thesis submitted to the
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(Doctor of Philosophy

in
BIOCHEMISTRY

By
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December, 2000

DECLARATION

I hereby declare that this thesis entitled **"Purification and structural characterization of a polyphenol oxidase from field bean** *(Dolichos lablab)",* submitted herewith, for the degree of **Doctor of Philosophy** in **Biochemistry of** the **University of Mysore,** Mysore, is the result of the work done by me in the Department of Protein Chemistry and Technology, Central Food Technological Research Institute (CFTRI), Mysore, India, under the guidance and supervision of **Dr. Lalitha R. Gowda,** during the period May 1996 - December 2000.

I further declare that the results of this work have not been previously submitted for any other degree or fellowship.

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CERTIFICATE

I hereby certify that this thesis entitled **"Purification and structural characterization of a polyphenol oxidase from field bean *(Dolichos lablab)"*** submitted by **Ms. Beena Paul** to the University of Mysore, Mysore, for the degree of Doctor of Philosophy (in Biochemistry) is the result of research work carried out by her in the Department of Protein Chemistry and Technology, Central Food Technological Research Institute (CFTRI), Mysore, under my guidance and supervision. This work has not been submitted either partially or fully for any other degree or fellowship.

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Preface

Polyphenol oxidase (PPO) is a copper-containing enzyme widely distributed in nature, which catalyzes the orthohydroxylation of monophenols and the oxidation of o-diphenols. Browning of fruits and vegetables is initiated by the enzymatic oxidation of phenolic compounds by PPO, and is reported to be one of the main causes of quality loss during post-harvest handling, processing and storage (Mathew and Parpia, 1971). Browning usually imparts a change in color, appearance and organoleptic properties, which are considered detrimental to the food quality. Hence, prevention of PPO activity in fruits and vegetables has enormous economic and quality benefits. However, PPO is sometimes important for the beneficial coloration of some foods such as prune, cocoa, raisin and black tea.

For many years the food industry world over used sulfites to prevent browning effectively and economically. However, because of the adverse health effects imparted by the chemical inhibitors and increased awareness in life styles related to food and health, there is an increased demand for more natural foods and fewer, safer added chemicals in processed foods. Hence alternate methods to prevent browning have been investigated. Through an understanding of the structural characteristics, mechanism of action of PPO and chemistry of enzymatic browning, targeted prevention methods can be developed.

The purification of PPO from higher plants is hampered by the occurrence of multiple forms and pigment contamination. Although the first PPO, a mushroom tyrosinase was discovered more than a century and half ago, the first three-dimensional structure of a plant PPO became available only in 1998. Pleminary investigations in our laboratory, earlier during the structural characterization of a lectin from the field bean seeds (Gowda et al., 1994)

revealed the presence of a single PPO in crude extracts. The single form of PPO in field bean seeds renders it ideal for primary structure determination and three-dimensional structure analysis, which in plant PPOs has been hindered by its multiplicity. As a primary step to understand the structure, regulation and function of seed PPO, the isolation and characterization of a PPO from field bean seeds was carried out.

In the last four and a half years (May 1996 - December 2000) of this investigation at CFTRI, the single form of PPO from field bean seeds has been purified and the kinetic and molecular properties were explored. In addition, due to the demand and need for natural compounds which inhibit PPO, a high molecular weight inhibitor of PPO was isolated from raw sapota extract and characterized.

The thesis entitled "Purification and structural characterization of a polyphenol oxidase from field bean *(Dolichos lablab)",* is a compilation of these results. The contents of this thesis are divided into eight chapters. An introduction to the PPO enzyme, the distinct reactions catalyzed by PPO and a detailed overview of published literature of plant PPOs are presented in Chapter 1. Chapter 2 describes the materials and detailed experimental procedures used in the present investigation. Chapter 3 deals with the results on the purification and properties of field bean PPO. In Chapter 4, the results on the effect of various phenolics, which are either inhibitors or activators of field bean PPO are presented. Chapter 5 reports the activation of monophenolase and diphenolase activities of field bean PPO, by diphenols. In Chapter 6, the activation of field bean PPO by SDS and pH is described. In Chapter 8, the major findings of the present investigation are summarized. The literature cited in the complete text is arranged at the end of the thesis.

CONTENTS

Abbreviations

A	absorbance
λ-max	absorption maximum (wavelength)
[I]	inhibitor concentration
[S]	substrate concentration
Ala	alanine
Å	Angstrom units
Arg	arginine
Asx	aspartic acid/asparagine
ATZ	anilinothiazolinone
BCIP	5-bromo-4-chloro-3-indolylphosphate
BSA	bovine serum albumin
ca	circa
CAPS	3 - [cyclohexylamino] -1 -propane
Cu	copper
Cys	cysteine
DOPA	dihydroxyphenylalanine
$E^{1\%}$	absorption coefficient of a 1% solution in 1cm path length cell at its absorption maximum.
eg	example
EXAFS	extended X-ray absorption fine structure
Fig	figure
g	gram
9	times acceleration due to gravity
Glx	glutamic acid/glutamine
Gly	glycine
h	hours
He	hemocyanin
HC1	hydrochloric acid
His	histidine
HPLC	high performance liquid chromatography
ibCO	*Ipomoea batatas* catechol oxidase
id	internal diameter

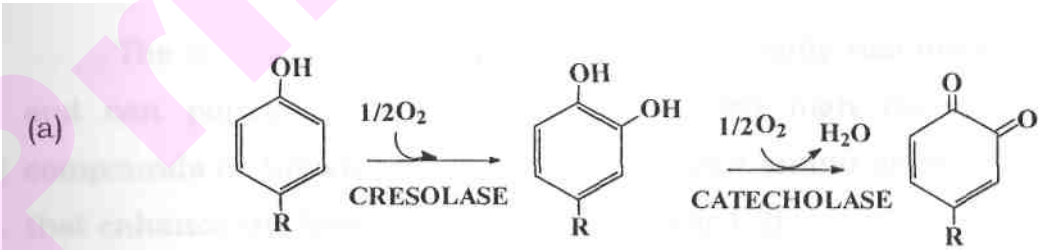
Ile	isoleucine
IU	inhibitory units
kDa	kilo Dalton
K_a	activation constant
Ki	inhibition constant
K_m	Michaelis-Menten constant
L	liter
Leu	leucine
Lys	lysine
M	molar concentration
Met	methionine
E	molar extinction coeffient (M^{-1} cm^{1})
µg	microgram
µL	microliter
µm	micrometer
µmol	micromole
min	minute
mL	milliliter
mM	millimolar
M_r	molecular weight
mtorr	millitorr
%	percent
Na acetate	sodium acetate
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide, reduce form
NaPi	sodium phosphate
NBT	nitroblue tetrazolium
NMR	nuclear magnetic resonance
°C	degree Celsius
ODS	octadecylsilane
PAGE	polyacrylamide gel electrophoresis
Phe	phenylalanine

PITC	phenylisothiocyanate
pmole	picomole
Pro	proline
PTC	phenylthiocarbamyl
PTH	phenylthiohydantoin
PVDF	polyvinylidene difluoride
PVP	polyvinyl pyrrolidone
PVPP	polyvinyl polypyrrolidone ,
RP	reverse phase
Rs	Stokes radius
S	svedberg constant
SDS	sodium dodecyl sulfate
sec	seconds
Ser	serine
TCA	trichloroacetic acid
TEA	triethylamine
TEMED	N,N,N',N''-tetramethylenediamine
TFA	trifluoroacetic acid
Thr	threonine
Tris	tris(hydroxymethyl)aminomethane
Tyr	tyrosine
UV	ultraviolet
V	velocity of reaction
v/v	volume by volume
Val	valine
V_e	elution volume
V_0	void volume
vs	Versus
w/v	Weight by volume
w/w	Weight by weight

Chapter 1
Introduction

Polyphenol oxidase (PPO) is the generic term for a group of enzymes that catalyze the oxidation of phenolic compounds, to produce brown color on the cut surfaces of fruits and vegetables. Browning of raw fruits, vegetables and beverages is a major problem in the food industry and is believed to be one of the main causes of quality loss during post harvest handling and processing (McEvily and Iyengar, 1992). The organoleptic and biochemical characteristics of the food items are profoundly modified by the appearance of brown pigments whose color is superimposed on the natural color. These changes result in a lowering of food quality both visually and with regard to taste and nutritional characteristics. The mechanism of browning in food and food products is well characterized and can be either enzymatic or non-enzymatic in origin. Non-enzymatic browning results from the polymerization of endogenous phenolic compounds as well as from the Maillard reaction that occurs when mixtures of amino acids and reducing sugars are heated.

PPO (1,2 benzene'.oxygen oxidoreductase, EC 1.10.3.1), which initiates enzymatic browning is also known as tyrosinase, phenol oxidase, monophenol oxidase, cresolase, catechol oxidase etc. PPOs are mixed function copper enzymes which catalyzes both the hydroxylation of monophenols to diphenols (monophenolase/cresolase) and also oxidation of o-diphenols to o-quinones (diphenolase/catecholase). Two kinds of enzymes are capable of acting upon diphenols in the presence of molecular oxygen, according to the reaction scheme shown in Fig 1.1.



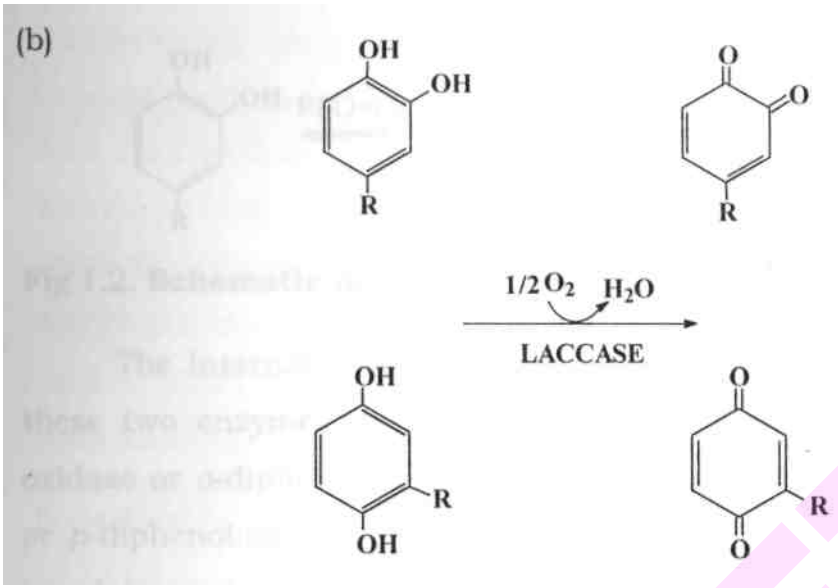


Fig 1.1. **Reactions catalyzed by PPO.** (a) Reactions catalyzed by polyphenol oxidase, (b) reactions catalyzed by laccase.

Both have the trivial name polyphenol oxidase, but are somewhat different in nature. The first class of enzymes, catalyze two distinct reactions (reaction a of Fig 1.1):

1) the insertion of a hydroxyl group in a position ortho to an existing one, often referred to as cresolase activity, usually followed by oxidation of the diphenol to the corresponding quinone.

2) the oxidation of the o-diphenol, to o-quinones, often referred to as catecholase activity. Molecular oxygen participates in both the reactions.

The second class, laccases (EC 1.10.3.2), oxidize o-diphenols as well as p-diphenols, forming their corresponding quinones (reaction b of Fig 1.1).

The o-quinones that are produced are highly reactive compounds and can polymerize spontaneously to form high molecular weight compounds or brown pigments, or react with amino acids and proteins that enhance the brown color produced (Fig 1.2).

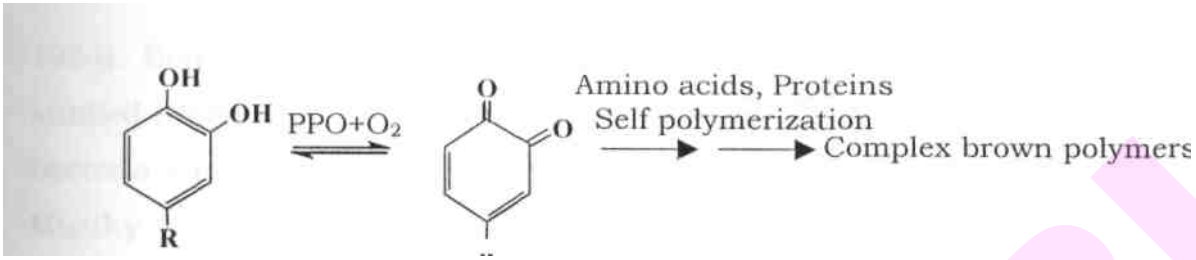


Fig 1.2. **Schematic diagram of the initiation of browning by PPO**

The International Commission on Enzymes originally referred to these two enzymes in the category of polyphenol oxidases; catechol oxidase or o-diphenol:oxygen oxidoreductase (EC 1.10.3.1) and laccase or p-diphenol:oxygen oxidoreductase (EC 1.10.3.2). The nomenclature was later revised, combining the two enzymes under the one heading: EC 1.14.18.1 which Mayer and Harel (1979) described as unfortunate. More recently the international nomenclature was changed once again (Enzyme Nomenclature, 1992). Cresolase activity was given the title monophenol monooxygenase (EC 1.14.18.1), catecholase activity became diphenol oxygen:oxidoreductase (EC 1.10.3.2) and laccase activity became labeled EC 1.10.3.1 which differentiates between cresolase and catecholase activity but does not distinguish clearly between distinct plant and fungal laccases. Owing to the prevailing confusion regarding the nomenclature, many of the authors continue to use the old biochemical titles and nomenclature (Osuga et al., 1994; Walker, 1995; Kader etal., 1997; Klabunde et al., 1998; Yang et al., 2000).

Occurrence

PPO was first discovered in 1856 by Shoenbein in mushrooms. He noted that something in mushrooms catalyzed the aerobic oxidation of certain compounds in plants. The enzyme is widely distributed in the plant kingdom (Sherman etal., 1991), in some fungi especially those that produce brown filaments (Osuga et al., 1994), in some higher animals including insects (Sugumaran, 1988) and humans (Witcop,

1984). Being easily detectable, it was among the first enzymes to be studied (Bertrand, 1896). It has since been reported to occur in several bacteria (Prabhakaran, 1968; Lerch and Ettlinger, 1972; Pomerantz and Murthy, 1974; Yoshida et al., 1974), numerous fungi, (Yopp, 1976), algae (Hoist and Yopp, 1976), bryophytes (van Poucke, 1967; Babbel, 1974), pteridophytes (Malesset-Bras, 1962), gymnosperms (Cambie and Bocks, 1966) and practically every order of angiosperms where it has been looked for.

Localization

In humans, PPO, which is responsible for melanin formation, is located in melanocytes, which are specialized dendrite pigment forming cells. In a few cases in fungi, tyrosinase has been reported to be excreted into the medium, (Bruchet, 1966; Kocher and Ettlinger, 1975). In plants, PPOs do not appear to be restricted to any particular part and their presence has been reported from a variety of plant organs and tissues. In spite of possible artifacts, the weight of evidence indicates the wide occurrence of membrane bound PPO, localized in the chloroplasts. Although many of the reports, localizing the enzyme in organelles are based merely on differential centrifugation, more careful studies involving density gradient centrifugation and correlation with chlorophyll content and activities of marker enzyme reached the same conclusion (Ruis 1972; Tolbert, 1973; Kato et al., 1976). 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; Parish, 1972; Czaininski and Catesson, 1974). The intracellular localization has been shown in chloroplasts of the olive fruit, particularly on the inner face of thylakoids (Shomer et al., 1979). PPO activity has also been observed in mitochondria of apple (Harel et al.,

1965), microbodies in avocado (Sharon and Kahn, 1979), or partly associated with the cell wall in banana (Jayaraman etal., 1987). In potato tubers, nearly all the subcellular fraction were found to contain PPO, in amounts proportional to the protein content (Craft, 1966). The localization of PPO in the plant cells depends on the species and age (Marques et al., 1995). In fruits and vegetables, the ratio of particle bound to soluble enzymes, varies with maturity, with more of the enzyme becoming soluble as the fruit matures. In unripe olive, PPO is tightly bound to the chloroplast, whereas in the ripe fruit, it is essentially soluble (Ben-Shalom et al., 1977). It is suggested that at maturity, chloroplast membranes disrupt, their lamellar structure disintegrates, thus facilitating solubilization of the enzyme. Similar, increased solubilization has been reported for other fruits (Macheix et al., 1991; Mayer and Harel, 1981) and has been observed in the tissue culture of apple fruit (Volke et al., 1977).

Physiological role

The function of PPO is best understood in humans where this enzyme is responsible for skin pigmentation including freckles. It is also responsible for the pigmentation of hair and eye. Tyrosine is the primary substrate in humans, justifying the trivial name, tyrosinase (Osuga et al., 1994). Absence of melanin biosynthesis, results in oculocutaneous albinism in humans (Witkop, 1984) and an over production of melanin by melanocytes may lead to toxic intermediates (Hochstein and Cohen, 1963). In insects, it is involved in sclerotization of the exoskeleton (Sugumaran, 1988) and in the protection against other organisms by encapsulating them in melanin (Sugumaran et al., 1990).

The physiological role in higher plants and fungi is not entirely certain or clearly understood (Osuga etal., 1994). One of the oldest

suggested physiological role of PPO in plants is in the synthesis of diphenols. This suggestion is based on the undoubted ability of many PPO to hydroxylate monophenols to the corresponding o-diphenols (Fig 1.1). In plants, cresolase activity is often very low or absent and is generally much lower than catecholase activity (Mayer and Harel, 1979). PPO is located exclusively in the plastids of healthy tissues and is apparently not even activated until it crosses the plastid envelope (Vaughn et al., 1988). The vast majority of phenolic compounds in higher plant cells are located in the vacuole, a cellular location isolated from the PPO. It is argued that the plastidic location of PPO is necessary to provide a strong reducing environment in order to prevent further oxidation of o-diphenols to o-diquinones by PPO and/or to provide the proper electron donor for the cresolase activity (Vaughn and Duke, 1984). Although some investigators have found relationships between phenolic compound content and extractable PPO activity from cells or tissues (Vaughn et al., 1981; Kojima and Conn, 1982), Vaughn and Duke (1981a) using cytochemical and *in vitro* enzymological methods, found that the complete loss of PPO activity had no effect on the phenolic metabolism of mung bean seedlings. Moreover, there is considerable evidence that PPO is not active as a phenol oxidase in chloroplasts, but is limited as a phenol oxidase by latency or lack of substrates (Golbeck and Cammarata, 1981). It has been claimed that catechol oxidase acitivity correlates with alkaloid content (Jindra et al., 1966), but attempts to show that PPO is required for or involved in the synthesis of morphine were unsuccessful (Roberts, 1971; Roberts, 1974).

A second long suggested role of PPO is in electron transport (Vaughn and Duke, 1984). Since quinones are powerful oxidizing agents, it is easy to envisage a role in which a diphenol is oxidized by catechol oxidase and the quinone then reduces some cell constituent,

such as a nucleotide, non-enzymatically. It has been often claimed that the cyanide-insensitive respiration of plant tissues might be mediated by PPO (Mayer and Harel, 1979). Against this suggestion is the high sensitivity of PPO to cyanide although admittedly it is much less sensitive than cytochrome oxidase (Duckworth and Coleman, 1970). An interesting finding by Rich and Bonner (1977) demonstrates that substituted hydroxamic acids are powerful inhibitors of mushroom PPO. The substituted hydroxamic acids are known inhibitors of the cyanide insensitive or alternate electron transfer pathways which suggests that, PPO after all, has some function in this pathway (Schonbaum et al., 1971). The thylakoid membranes of chloroplasts are sites of intense photochemical activity, which is linked to electron transport components, which are in turn coupled to ATP and NADPH synthesizing enzymes. As mentioned above, cytochemical studies strongly indicate that this membrane is the major site of the PPO molecule in green tissue (Vaughn and Duke, 1981b).

The only clear role that PPO has in phenolic metabolism is in those cases in which, plastid and vacuole contents are mixed *viz.* senescence and injury. PPO has been invoked to explain the development of pigmentation in black olives during senescence (Ben-Shalom et al., 1977) and other dark brown or black color usually associated with dead plant tissues. There is sometimes no correlation between extractable PPO activity and senescence (Patra and Mishra, 1977), the browning results from contact of pre-existing substrate and the enzyme. The increase in extractable PPO activity often seen during senescence is due to the activation of previously synthesized enzyme (Meyer and Biehl, 1981). There is no functional significance or survival advantage to this process.

Many researchers however believe that there is a functional significance in the rapid production of quinones caused by injury. (Szent-Gyorgyi and Vietorisz, 1931; Mukherjee and Ghosh, 1975; Butt, 1980). The disruption of the plastids results in the activation of latent PPO, which reacts with the phenolics released from the vacuole. The quinones produced by the PPO-phenolic compounds interaction, resulting from mechanical damage or cellular disruption from disease are very reactive, making them good candidates for involvement in protection from other organisms. Szent-Gyorgyi and Vietorisz (1931) suggested that PPO is a protective enzyme since it is responsible for forming a 'scab' of insoluble melanin over wounds in plants, sealing in the sap and sealing out insects and microorganisms and thereby preventing further insect attack. Wounding which occurs naturally or induced by pathogens has been shown to produce similar effects in plants (Monstafa and Wittenburg, 1970; Gentile et al., 1988). This may protect the plant in two ways. First, the melanins thus produced, seal off the site of infection or wound, forming a physical barrier to further infection. Secondly, the enzymatically produced o-quinones polymerize with both the host's proteins, and with the exo-enzymes produced by phytopathogens and hence negate their phytotoxicity (Walker and Ferrar, 1998). Zinkernagel (1986) has suggested that catecholase may be involved in the oxidative detoxification of pathogen-produced phytotoxins.

Isolation of PPO

Isolation of active **PPO** is fraught with problems because both the enzyme and **its** substrates are present in the cell. Even though they do **not** interact in the intact cell, as soon as **the** cell's internal organization is disrupted, the enzyme and substrates interact to yield reactive quinones. The quinones subsequently react with PPO and other

proteins and enzymes present, thereby causing their inactivation. Such reactions have been partially prevented by isolation under nitrogen (Kertesz and Zito, 1965; Bouchilloux et al., 1963) or in the presence of reducing agents or phenol adsorbing agents such as polyethylene glycol, polyamide or polyvinyl pyrrolidone (Sanderson, 1964; Anderson, 1968; Benjamin and Montgomery, 1974). However some of these agents also inhibit the enzyme irreversibly (Harel etal., 1964; Walker and Hulme, 1965). An additional obstacle for purification is the extensive multiplicity of the enzyme and the interconversions between forms, which continually occur during the purification steps and during storage of purified preparations.

The binding of catechol oxidase to membranes in many tissues further complicates its isolation. Solubilization, usually achieved after preparation of acetone powder or extraction with detergents and other agents, usually results in the modification of enzyme structure and properties (Mayer and Harel, 1979; Sojo etal., 1998b). A widely observed phenomenon is the marked decrease or complete loss of the cresolase activity upon solubilization of a membrane bound enzyme (Harel etal., 1964; Harel and Mayer, 1971). In recent years, temperature induced phase partitioning in Triton X-114 has been shown to be a suitable extraction method for PPOs. Bordier (1981) was the first to recognize that the low cloud point of Triton X-114 can be exploited as a means of separating hydrophilic and integral membrane proteins. This nonionic detergent, forms clear micellar solutions in water at low temperature (4 °C) but separates into two phases in equilibrium above 20 °C (temperature induced phase partitioning), to.a detergent rich (20% w/v) and the other detergent poor (0.03% w/v). When Triton X-114 was used for plant membranes, such as chloroplast membrane of broad bean (Sanchez-Ferrer et al., 1990) it completely removed the chlorophylls and phenols from the dark green extract. After

phase partitioning, the colored compounds moved into the detergent rich phase leaving a detergent poor phase, transparent and rich in PPO. This method has been successfully used to extract PPO with monophenolase activity from several sources including potato (Sanchez Ferrer etal., 1993a; Sanchez Ferrer et al., 1993b), pear (Espin et al., 1996), apple (Espin etal., 1995b), banana (Sojo etal., 1998a; Sojo etal., 1998b) and strawberry (Espin etal., 1997a). Triton X-114 can extract plant enzymes in its native form, at the same time, it is able to remove phenols and chlorophylls by centrifugation alone, rendering untanned latent enzymes, which has both cresolase (monophenolase) as well as catecholase (diphenolase) activity.

Procedures which have been employed in the purification of PPO include, conventional protein purification methods including fractionation with ammonium sulfate, ion exchange chromatography (Zhou etal., 1993; Chilaka etal., 1993; Zhang and Flurkey, 1999; Ridgway and Tucker, 1999), hydrophobic interaction chromatography (Takeuchi et al., 1992; Kader et al., 1997; Partington and Bolwell, 1996; Das et al., 1997) gel filtration chromatography, and often a combination of all these (Murao etal., 1993; Fujita etal., 1995; Shin etal., 1997; Ding et al., 1998).

Preparative isoelectric focussing has also been employed in some cases (Dubernet and Ribereau-Gayon, 1974; Kidren et al., 1978).

Enzyme assay

PPOs oxidize phenolic substrates, utilizing molecular oxygen (Fig 1.1). Since the unstable oxidized quinone products undergo many secondary reactions, both with each other and with other proteins, it is difficult to measure the product formation in routine assays. It is also difficult to routinely assay for residual substrate because, some of the

products react with reagents for phenolic substances (Mayer and Harel, 1979). The most convenient method is therefore to follow the initial rate of formation of the quinone spectrophotometrically. PPO activity is also measured polarographically, by measuring the oxygen uptake directly, which is also one of the substrates, again taking into consideration only the initial rates, since the enzyme undergoes rapid inactivation during catalytic performance. (Smith and Montgomery, 1985; Janovitz-Klapp etal, 1989; Wesche-Ebeling and Montgomery, 1990; Heimdal et al., 1994; Chevalier et al., 1999)

The commonly used substrates for assay of diphenolase activity are catechol (Galeazzi and Sgarbierri, 1981; Cosetang and Lee, 1987; Zhou et al., 1993; Ngalani et al, 1993), 4-methyl catechol (Siddiq et al., 1993; Wesche-Ebeling and Montgomery, 1990; Froderman and Flurkey, 1997) tertiary butyl catechol (Sanchez Ferrer et al., 1993a; Sojo et al., 1998a), and L-DOPA (Halim and Montgomery, 1978; Dawley and Flurkey, 1993; van Leeuwen and Wichers, 1999). Generally tyrosine is used to assay the monophenolase activity (Osaki, 1963; Friedman and Bautista, 1995).

Other methods are based on the reaction of corresponding o-quinones with coupling reagents such as ascorbate (Bayoumi and Frieden, 1957), NADH (Carlson and Miller, 1985), L-proline (Rzepecki and Waite, 1989), cysteine (Gauillard etal., 1993) and other chromogenic substances (Esterbauer etal., 1977; Leonowicz and Grzywnowicz, 1981; Shin etal., 1987). Radiometric discontinuous methods have also been used to assay monophenolase activity of PPO (Pomerantz, 1964; Husain etal, 1982; Winder and Harris, 1991). Such methods are very sensitive but are discontinuous, cumbersome and may require up to 30 min for each assay.

A continuous spectrophotometric method proposed by Espin et al. (1995a) is based on the coupling reaction between 3-methyl-2-benzothiazolinone hydrazone (MBTH) and quinone product of the PPO mediated reaction. MBTH, which is a potent nucleophile, traps the enzyme generated o-quinones, to render a soluble MBTH-quinone adduct with high molar absorptivity. The stability of the MBTH-quinone adducts and the rapid assays provide a reliable method for determining both the monophenolase and diphenolase activities of PPO. Of the different phenolic substrates used in conjunction with MBTH, monophenol p-hydroxyphenyl propionic acid (PHPPA) and diphenol 3,4-dihydroxyphenyl propionic acid (DHPPA) were found to be the best pair. The MBTH-quinone adducts of this pair are stable and do not have any solubility problems unlike the other substrates (Espin et al., 1995a; Espin etal., 1995b, Espin etal., 1997a; Espin et al., 1997b; Espin et al., 2000)

Despite the accuracy and high sensitivity of the MBTH method, continuous spectrophotometric assays using catechol and L-DOPA still remain the method of choice due to ease and convenience (Zhou et al., 1993; Das etal., 1997; Arslan etal., 1998; Zhang etal., 1999; Gonzalez, 1999).

General properties of plant PPOs

Molecular weight

The overall diversity of M_rs of PPO is not very clear. The M_rs of PPOs reported from plant sources is highly variable, probably due to the phenomenon of multiplicity observed in plant PPOs (Mayer and Harel, 1979). Sherman et al. (1991) reported that the M_rs of plant PPOs ranged from 33 to 200 kDa. The PPO from bacteria *Streptomyces glaucescens* and *Streptomyces antibioticus* have M_rs of 30.9 kDa and 30.7 kDa

respectively while the PPO from fungus *Neurospora crassa* has a M_r of 46 kDa. Zhang et al. (1999) found that tyrosinase from the cap flesh of Portabella mushrooms had a M_r of 41 kDa, which compares with that proposed by Wichers et al. (1996) for mushroom tyrosinase. But these M_rs are very much different from the quaternary structure proposed by Strothkamp et al. (1976) in which the mushroom holoenzyme (H2L2, 120 kDa) contained two heavy chains (H, 43 kDa each) and two light chains (L, 13.4 kDa each). PPO purified from cabbage (Fujita et al., 1995) had a M_r of 39 kDa, loquat fruit 55 kDa (Ding et al., 1998), oil bean, 110 kDa (Chilaka et al., 1993), potato 129 kDa (Partington and Bolwell, 1996) and pineapple, 104 kDa (Das et al., 1997). Sachde et al. (1989) reported a relatively low M_r of 17 kDa for date PPO.

Many of the higher plant PPOs are multi subunit proteins. Oil bean PPO is a tetramer of 28 kDa (Chilaka et al., 1993) and pineapple PPO a tetramer of 25 kDa (Das et al., 1997). PPOs from cabbage (Fujita etal., 1995), loquat fruit (Ding et al., 1998), cap flesh of Portabella mushroom (Zhang et al., 1999), are single subunit proteins whereas PPO from the dwarf variety of banana (Galeazzi and Sgarbieri, 1993) was found to be a dimer of 30 kDa.

Multiplicity

In many cases, crude or partially purified preparations of PPO show a multiplicity of forms, which may have resulted from association or dissociation. Thus Harel and Mayer (1968) observed three forms of PPO from apple fruit, having M_rs of 30-40, 60-70 and 120-130 kDa. These were shown to undergo interconversions. One of the major reasons for the discrepancy of M_r determinations lies in the fact that in many cases the proteins were not fully denatured, in order to preserve enzyme activity for staining of enzymes in partially denaturing gels

(Lax and Cary, 1995). The M_rs of the two isoforms of strawberry PPO were determined to be 11 and 34 kDa. (Wesche-Ebeling and Montgomery, 1990). Highbush blueberry PPO was also reported to have two isoforms (Kader et al., 1997). Crude extracts of mung bean leaf had six isoforms, M_rs ranging from 21-65 kDa (Shin et al., 1997). Plantain PPO had two, 30 and 70 kDa (Ngalani et al., 1993). The PPO isoforms in broad bean leaf differs in size, charge and the extent and type of carbohydrates attached (Ganesa et al., 1992). Raspberry fruit PPO (Gonzalez etal., 1999) is reported to have only one isoform. In lettuce, the single PPO isoform obtained from vascular tissue was different from that of the three isoforms isolated from photosynthetic tissue (Heimdal etal., 1994). Banana bud PPO had two isoforms (Oba etal., 1992) whereas Galeazzi etal. (1981) reported four isoenzymes for banana pulp. Zhang and Flurkey (1997) showed that at least ten isoforms were present in crude extracts of Portabella mushrooms by analytical isoelectric focussing. The distribution of isoforms was dependent upon the type of tissues that were examined and how well the tissues were separated from one another. There is also an, inter- and intra- species diversity in the number of PPO isoforms and their M_rs. In apple fruit, studies have described one form of 46 kDa (Janovitz-Klapp et al., 1989) or 26 kDa (Goodenough et al., 1983) or several forms ranging from 24 to 134 kDa (Demenyuk et al., 1974; Harel and Mayer, 1968). This may be related to genetic differences, stages of development, freezing temperatures, or the tissue studied. Tobacco shows a multiplicity of forms of PPO after electrophoresis of partially denatured chloroplasts (Lax et al., 1984), which appear to be products of a single gene family. There appear to be small gene families for PPO in all of the species in which the genes for the enzyme have been characterized (Lanker et al., 1987; Newman etal., 1993; Sommer etal., 1994; Hunt etal., 1993; Cary etal., 1992; Shahar etal., 1992). Steffens etal. (1994) have

extensively reviewed the reasons for the observed heterogeneity of forms upon electrophoresis, some of which are explained by the heterogeneity of the gene families but many of which are due to artifacts of protein isolation methods and electrophoresis.

Proteolysis has been implicated in the multiplicity of PPOs. Carboxy-terminal proteolysis during isolation of PPO from broad bean leaves yields an active peptide of 40-45 kDa and an inactive 18-20 kDa carboxy-terminal peptide (Robinson and Dry, 1992a). Proteolysis of PPO has also been observed in carrots (Soderhall, 1995), apples (Marques etal., 1995) and in the fruits of five prunus species (peach, apricot, almond, plums and cherry, Fraignier et al., 1995) in which the mature polypeptide can be converted into active fragments with M_r values of about 40 kDa to 45 kDa and also smaller in size. In plants, when protease inhibitors are added during extraction and fractionation of PPOs, the number of isoforms observed decreased (Soderhall, 1995; Flurkey and Jen, 1980; Ganesa et al., 1992).

Substrate specificity

The most important natural substrates of PPO in fruits and vegetables are catechins, cinnamic acid esters, L-DOPA and tyrosine (Baruah and Swain, 1959; Hermann, 1974; Walker, 1975). However some fruit PPOs use other phenolic substrates; for example, a relative of DOPA, 3,4-dihydroxyphenylethylamine (dopamine), is the major substrate in bananas (Vamos-Vigyazo, 1981). Grape catechol oxidase acts on p-coumaryl and caffeoyl-tartaric (caftaric) acids while dates contain an unusual combination of diphenol oxidase substrates including a range of caffeoyl-shikimic (dactylferic) acids, which are analogous to the ubiquitous isomers of chlorogenic acid (Walker, 1995). Among the cinnamic acid esters, chlorogenic acid (3-o-caffeoyl-D-quinic acid) is the most widespread natural PPO substrate. The caffeic acid

(3,4-dihydroxy cinnamic) acid moiety of chlorogenic acid has been reported to be hydroxylated from p-coumaric acid by PPO (Sato, 1962). The extent to which naturally occurring phenolic substrates contribute to enzymatic browning of individual fruits and vegetables depends on the localization and concentration of the phenol as well as on the color intensity of the macromolecular pigments obtained from the different quinones. Substrate specificity not only depends on the genus, but to a certain extent, also on the cultivar and on the part of the fruit or vegetable, the enzyme has been extracted from. The affinity of PPO towards a given substrate may vary within very wide limits, even if the isoenzymes of the same origin are concerned (Vamos-Vigyazo, 1981). The pH of the activity determination affects the suitability of the substrate as well. The selectivity of plant PPOs towards monophenols seems to be higher than for o-diphenols (Wilcox et al., 1985).

All o-diphenol oxidases require the basic o-dihydroxyphenol structure for oxidase activity, so that catechol is the simplest possible, but not necessarily the best substrate, 4-methyl catechol is usually the fastest (Walker, 1995). The structure of some natural and artificial PPO substrates are shown in Fig 1.3. However the nature and position of the substituent groups has profound effects on the rate of substrate oxidation (Passi and Porro, 1981; Janovitz-Klapp etal., 1990) and studies of these problems can shed light on the nature of the interaction between the substrate and the active site of PPO. p-Substituted 3,4-dihydroxy phenols are oxidized at a higher rate than 2,3-dihydroxy phenols. The basic requirement that allows a monophenol to act as a substrate for tyrosinase is the presence of an electron donor "R" group in the p-position, with respect to the OH group. A substituent with high affinity to donate electrons increased the PPO catalyzed reaction (Solomon et al., 1996; Espin et al., 1998b; Espin et al., 2000), the greater the electron donor nature, better the suitability

of the substrate. Substitutions in position 3 (3-methyl catechol, 2,3-dihydroxy benzoic acid) causes a decrease in the affinity of the enzyme for the substrate, probably owing to steric hindrance (Passi and Porro, 1981). Electron withdrawing side substituents in the aromatic ring of phenolic compounds caused their poor oxidation by PPO (Duckworth and Coleman, 1970).

It appears that PPO from various sources shows a preference for certain phenolic substrates. Yasunobu (1959) concluded from a comparison of the substrate specificity of various catechol oxidases that although, the enzyme could oxidize a wide range of phenolics, the individual enzyme tends 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; Challice and Williams, 1970). Other researchers have found that the best substrate of a PPO may not be a compound commonly occurring as a phenolic constituent of the plant (Tanfel and Voigt, 1963; Jen and Kahler, 1974).

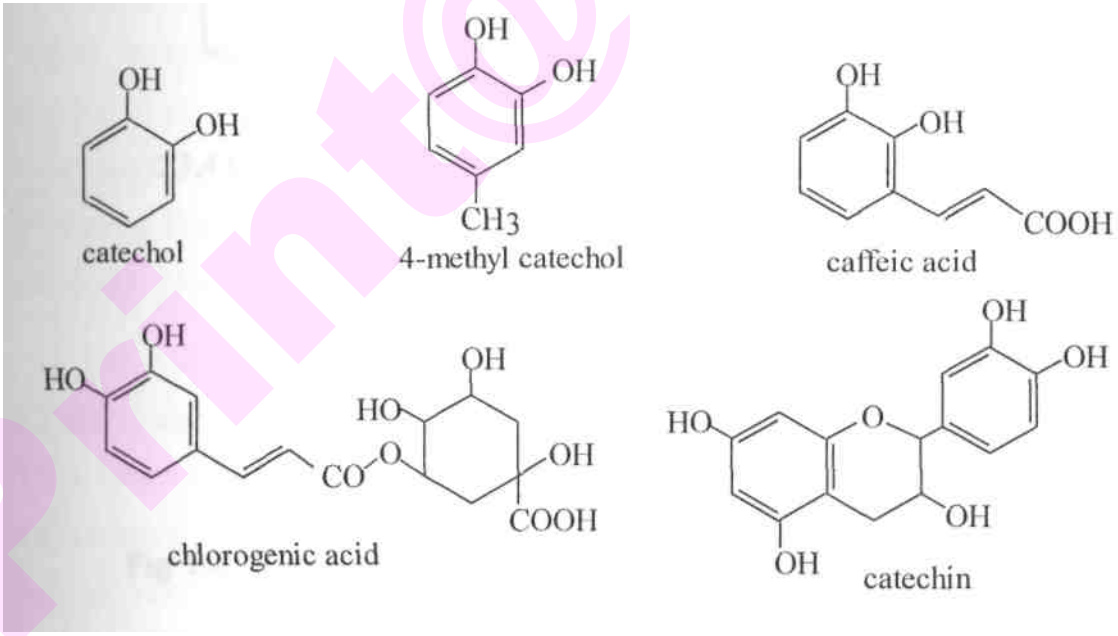


Fig 1.3. **Common natural and artificial substrates of PPO**

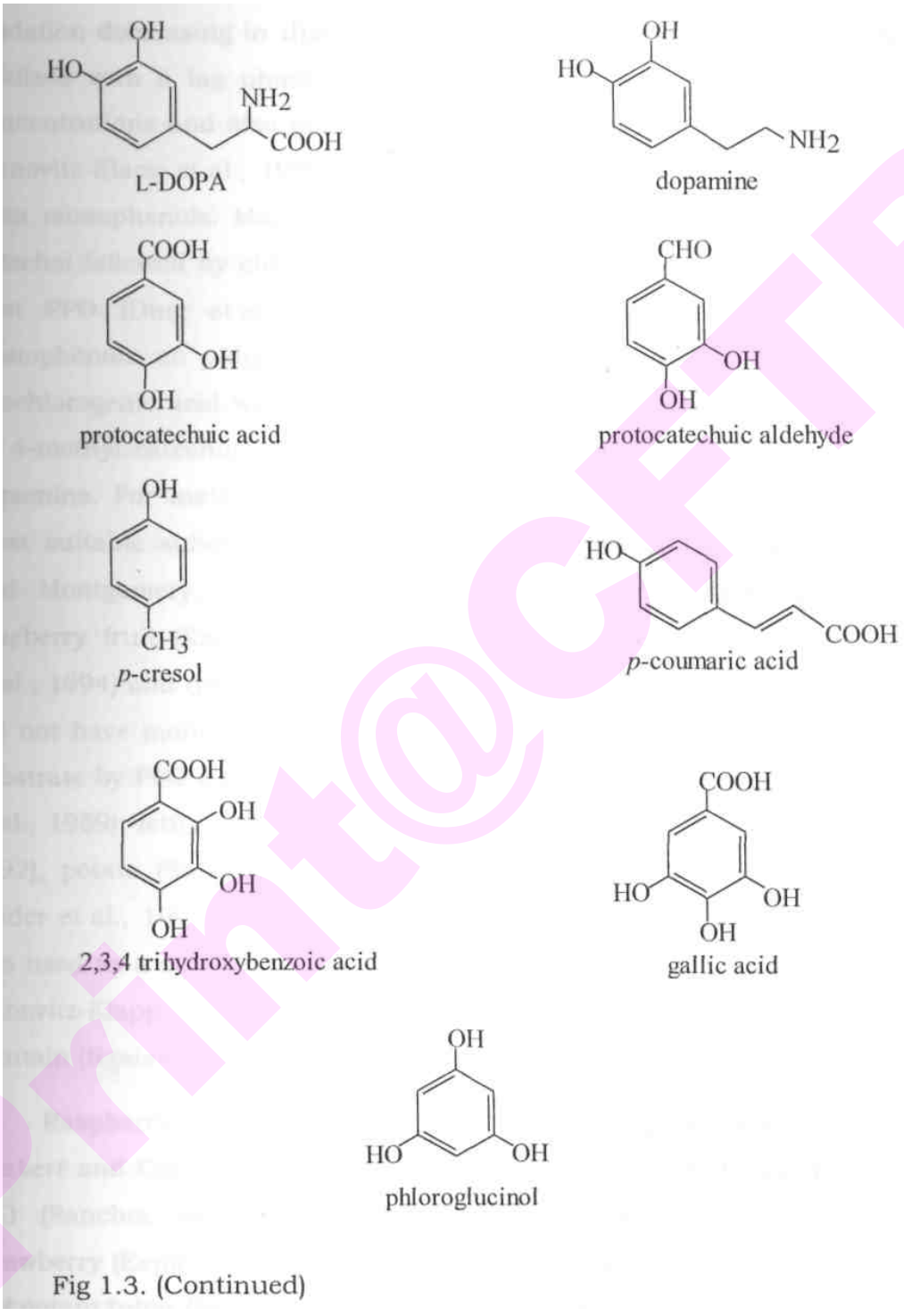


Fig 1.3. (Continued)

PPO from oil bean (Chilaka etal., 1993) oxidized pyrogallol, catechol, 4-methyl catechol and L-DOPA, with the intensity of the

oxidation decreasing in that order. Tyrosine, a monophenol was slowly oxidized with a lag phase of 5-10 min, which depended on enzyme concentrations and also presence of diphenols. Monroe apple peel PPO (Janovitz-Klapp et al., 1989) was more active towards diphenols rather than monophenols. Maximum activity was detected towards 4-methyl catechol followed by chlorogenic acid, catechol and D-catechin. Loquat fruit PPO (Ding et al., 1998) did not have any activity towards monophenols or p-diphenols but epicatechin, chlorogenic acid and neochlorogenic acid were oxidized significantly by the enzyme followed by 4-methyl catechol, catechol, pyrogallol, caffeic acid, D-catechin and dopamine. For malatya apricot (Arslan et al., 1998) catechol was the most suitable substrate. PPOs from strawberry fruit (Wesche-Ebeling and Montgomery, 1990), cocoa bean (Lee etal., 1991) highbush blueberry fruit (Kader etal., 1997), vascular lettuce tissue (Heimdal et al., 1994) and dwarf variety of banana (Galeazzi and Sgarbieri, 1981) did not have monophenolase activity. Chlorogenic acid was used as a substrate by PPO from several sources, including apple (Janovitz-Klapp etal., 1989), lettuce (Fujita etal., 1991), sweet potato (Lourenco etal., 1992), potato (Sanchez Ferrer et al., 1996) highbush blueberry fruit (Kader etal., 1997) and plums (Siddiq etal., 1996). 4-Methyl catechol was used as a substrate by sweet potato (Lourenco et al., 1992), apple (Janovitz-Klapp etal., 1990), grape (Sanchez Ferrer etal., 1992), plantain (Ngalani et al., 1993) and plums (Siddiq et al., 1996).

Raspberry fruit PPO (Gonzalez, 1999), eggplant PPO (Perez-Gilabert and Garcia-Carmona, 2000) and broad bean thylakoid bound PPO (Sanchez Ferrer etal., 1990) were active towards p-cresol. Strawberry (Espin et al., 1997a), Verdoncella apple (Espin et al., 1995b) and potato tuber (Sanchez Ferrer et al., 1993a) had cresolase activity.

The affinity of plant PPO for phenolic substrates is generally relatively low. The K_m is high, usually around 1-20 mM (Janovitz-Klapp etal., 1989; Lee et al., 1991; Lourenco et al., 1992; Zhou et al., 1993; Siddiq etal., 1996; Shin etal., 1997; Espin etal., 1997b; Das etal., 1997; Ridgway and Tucker, 1999). However several authors reported higher affinities for the phenolic substrates in PPO in the range of 0.01-0.9 mM from some sources, like in potato tubers (Alberghina, 1964), banana (Galeazzi and Sgarbierri, 1981), lettuce (Fujita etal., 1991), strawberry (Espin et al., 1997a) and pear (Espin et al., 1996). The affinity of PPO for oxygen depends on the phenolic substrate being oxidized (Duckworth and Coleman, 1970; Harel etal., 1964) and could vary also among different forms of the enzyme isolated from the same tissue (Harel et al., 1965). The affinity of PPO to oxygen is also relatively low, similar to other copper containing oxidases (Mason, 1955; Kobayashi, 1965; Frieden et al., 1965; Bull and Carter, 1973; Lerner and Mayer, 1976). The values reported are in the range 0.1-0.5 mM (Tocher and Meeuse, 1966; Bull and Carter, 1973; Rivas and Whitaker, 1973; Lerner and Mayer, 1976).

While catechol oxidase from animal tissues are relatively specific for tyrosine and DOPA (Mason, 1955), the fungal and higher plant enzymes act on a wide range of mono and diphenols. In addition the specificity for optical isomerism which is clear-cut in the mammalian enzyme (Pomerantz, 1963; Lerner, 1953) is less evident in catechol oxidase from fungi (Harrison et al., 1967) or higher plants (Palmer, 1963). A lack of stereospecificity was observed in the transformation step of the catalytic mechanism of pear and strawberry PPO, but the lower K_ms of L-isomers rather than the D-isomers, revealed a stereospecificity in the affinity of PPO towards its substrates (Espin et al., 1998a). The same effect was also observed in the monophenolase and diphenolase activities of mushroom tyrosinase (Espin et al., 1998c).

pH optima and stability

The optimum pH of most of the PPOs is in the range of pH 4.0-7.0. PPO from highbush blueberry fruit had an optimum of 4.0 (Kader etal., 1997), loquat fruit, 4.5 (Ding et al., 1998) broad bean, 5.0 (Jimenez and Garcia-Carmona, 1999), palmito, 5.2 (Robert et al., 1995) plantain, 6.5 (Ngalani etal., 1993), potato, 6.5 (Sanchez Ferrer etal., 1993a), cocoa bean, 6.8 (Lee etal., 1991) and cap flesh of Portabella mushroom, 7.0 (Zhang etal., 1999). PPO from apple, egg plant, pear and olives had an optimum pH of activity around 4-5 (Tono et al., 1986; Fujita and Tono, 1988; Murata et al., 1992) whereas PPO from kiwifruit, cherry and satsuma mandarin (Benjamin and Montgomery, 1973; Fujita and Tono, 1981) had an optimum of pH 7.0. The PPOs from both vascular and photosynthetic tissues of lettuce had the same broad pH optima of 5-8 (Heimdal et al., 1994) whereas PPO isoenzymes from mung bean leaf had an optimum of 5.5-6.5 (Shin et al., 1997). The isoenzymes of banana bud had optima of 6.8 and 5.5 (Oba et al., 1992).

Differences in pH optima with different substrates have been reported for PPO from different sources. pH optima of crude plum PPO (Siddiq et al., 1996) was of 5.8, 6.0 and 6.4 for caffeic acid, catechol and 4-methyl catechol respectively. Monroe apple peel PPO (Zhou et al., 1993) had optima of pH 5.0 and 4.6 with catechol and 4-methyl catechol respectively whereas for strawberry PPO (Espin et al., 1997b) it was 5.5 and 4.5 with catechol and 4-methyl catechol. Gregory and Bendall (1966) reported that the optimum pH of maximum activity of PPO from tea leaf varies depending upon the original material, extraction methods and substrates.

Stability of the PPO at different pH differs and depends on the source of the enzyme. PPO from vascular tissue of lettuce is stable at pH 4-8 whereas that from photosynthetic tissue is stable only from pH

5-8, for 20 h. At higher and lower pH the activity decreased slowly. Loquat fruit PPO (Ding et al., 1998) is stable between 4-8 and unstable below pH 3.0. Stability of monroe apple peel (Heimdal etal., 1994) increased from 2.5 to 8.0 and is most stable at pH 8.0.

Temperature optimum and stability

Many of the plant PPOs are optimally active between 25-40 °C. Both the vascular and photosynthetic tissues of lettuce had a temperature optimum of 25-35 °C (Heimdal etal., 1994). In PPO from parsnip root, an optimum temperature of 30 °C is reported using chlorogenic acid as substrate (Chubey and Dorrel, 1972). Temperature optimum for PPO in apple and grape using 4-methyl catechol as substrate is in the range of 25-45 °C (Valero et al., 1988; Trejo-Gonzalez and Soto-Valdez, 1991). The optimum temperature of activity for plum (Siddiq et al., 1992), Concord grape (Cash et al., 1976), peach (Jen and Kahler, 1974) and cabbage (Fujita et al., 1995) was 20 °C, 25 °C, 37 °C and 40 °C respectively. PPO from Koshu grape (Nakamura et al., 1983), Monroe apple peel (Zhou et al., 1993), palmito (Robert et al., 1995) and loquat fruit (Ding et al., 1998) had an optimum temperature of 30 °C.

PPO does not belong to the group of extremely heat stable enzymes. Short exposures, in the tissues or in the solution, to temperatures of 70-90 °C are in most cases sufficient for the partial or total irreversible destruction of its catalytic function. Exposures to temperatures below zero may also affect activity. Thermotolerance of PPO depends, similar to the substrate specificity, pH and temperature optima of activity, to a considerable extent on the source of the enzyme (Vamos-Vigyazo, 1981).

PPO in both vascular and photosynthetic tissue of lettuce, was stable for 5 min in temperatures ranging from 0-70 °C. At higher

temperatures, the activity decreased rapidly. In PPO from whole lettuce, a decreasing temperature stability using 30-90 °C for 5 min was stated by Fujita et al. (1991). Cucumber PPO is relatively stable for 10 min at 0-70 °C (Miller et al., 1990). Purified cabbage PPO (Fujita etal., 1995) was found to be highly heat stable. About 40% of the PPO activity remained after heat treatment at 100 °C for 10 min. Relatively high thermal stability was also found for PPO from kiwi fruit (Park and Luh, 1985) and mango (Park et al., 1980). Plum PPO seems to be relatively heat stable, because after heating for 30 min at 45 °C, it still retained 30% of activity. Loquat fruit PPO retained most of its activity (70%) over a wide temperature range (20-50 °C). Above 50 °C, the PPO activity rapidly declined as the temperature increased, but the enzyme was not completely inactivated even at 80 °C.

Inhibitors of PPO

Inhibitors of PPO may be grouped according to their mode of action although some compounds may belong to more than one group (Walker, 1995). Inhibition may be caused by (a) chelation of the prosthetic group, (b) competition for the substrate, or (c) interaction with the products of the reaction.

Since, Cu^{2+}, is the prosthetic group in PPOs , the enzyme can be inhibited by metal chelating agents such as cyanide, carbon monoxide, sodium-diethyl dithiocarbamate, mercaptobenzothiazol, azide, potassium methyl xanthate or thiourea (Walker, 1964; Robb et al., 1966; Palker and Roberts, 1967; Mathew and Parpia, 1971; Walker, 1975)

Substrate analogs are known to be good inhibitors of PPO (Walker, 1995). L-Mimosine, benzoic acid derivatives, benzhydroxamic acid and pyridones are a few of the substrate analogs, which inhibit

PPO activity (Lerch, 1995). Chemical and spectroscopic studies have shown that these compounds bind to the binuclear copper active site in the half reduced (half-mettyrosinase) or oxidized (mettyrosinase) state (Himmelwright etal., 1980; Winkler et al., 1981). Substrate analogs, tropolone, salicyl hydroxamic acid and 4-hexyl resorcinol are potent inhibitors of PPO. Tropolone in addition to being a structural analog is also an effective copper chelator. Tropolone inhibits both mono and diphenolase activity of mushroom tyrosinase (Kahn and Andrawis, 1985), the kinetic data indicating that it is a competitive inhibitor. Valero etal. (1991) found tropolone to be a slow binding competitive inhibitor of grape PPO. Slow binding inhibition of tropolone was also observed towards mushroom tyrosinase by Espin and Wichers (1999b). Inhibition due to 4-hexyl resorcinol is as effective as salicyl hydroxamic acid or tropolone against apple PPO (Monsalve-Gonzalez et al., 1995).

Klabunde etal. (1998) showed that the binding of the substrate analog, phenyl thiourea (PTU) to the binuclear metal centre of catechol oxidase from *Ipomoea batatas* leads to conformational changes which result in hydrophobic interactions between the aromatic ring of the inhibitor and Phe 261 at the active site of the enzyme. The sulfur of PTU also replaces the hydroxo-bridge, present in the Cu(II)-Cu(II) enzyme, and coordinates to both copper ions, thereby increasing the metal-metal separation from 2.9 A to 4.2 A, which contributes to the high affinity of the PTU for the enzyme.

Reducing agents such as sulfur dioxide, metabisulfite or ascorbic acid are also commonly used as inhibitors of enzymatic browning (Walker, 1995). These compounds prevent browning by reducing the enzymatically formed quinones back to their parent o-diphenols, the reducing agents being consumed in the process (Walker, 1995). The metabisulfite, although can act as a reducing agent, it can also react

with the quinone intermediates to form sulfoquinones and may irreversibly inhibit PPO (Zawitowski etal., 1991). By contrast, thiol compounds can combine chemically with the o-quinones to form a stable, colorless product thus preventing further oxidation to brown pigments (Walker, 1964). Among the reducing agents, sodium metabisulfite, ascorbic acid, thiourea and Cys have been commonly used to study the inhibitory effect on PPOs from several sources. PPO from monroe apple peel (Zhou et al., 1993), loquat fruit (Ding et al., 1998), banana (Galeazzi and Sgarbieri, 1981), strawberry (Wesche-Ebeling and Montgomery, 1990), plantain (Ngalani et al., 1993), malatya apricot (Arslan et al., 1998), blanquilla pear (Espin et al., 1996), potato (Sanchez-Ferrer etal., 1993b) and cocoa bean (Lee etal., 1991) are a few which were strongly inhibited by the inhibitors mentioned above.

Catecholases and laccases (Fig 1.1) are often distinguished by substrate specificity tests, which are not always unequivocal (Walker and McCallion, 1980). Both the types of diphenol oxidases have Cu^{2+} as the prosthetic group but the reaction mechanism are quite distinct (Walker, 1968). Selective inhibitors are often used as an additional measure to distinguish between catecholases and laccases other than their substrate specificity. Because of differences in the reaction mechanisms and the oxidation levels of copper in the active site, catecholase and laccase differ in their response to certain inhibitors (Walker, 1995). Catecholases are inhibited by substituted cinnamic acids (cinnamic acid, p-coumaric acid, and ferulic acids) and polyvinylpyrrolidone (PVP). Laccases are unaffected by cinnamic acids and PVP but are inhibited by cationic detergents such as cetyltrimethylammonium bromide (Walker and McCallion, 1980). 4-Hexyl resorcinol and salicyl hydroxamic acid selectively inhibited tyrosinase but not laccase (Dawley and Flurkey, 1993).

Latency of plant PPO

An unusual and intriguing characteristic of the PPO is its ability to exist, in some species, in an inactive or latent state. Most of the plant PPOs described seem to be latent in the mature form (Lanker et al., 1988; Cary et al., 1992; Robinson and Dry, 1992; Fraignier et al., 1995b). In this inactive form, the enzyme seems to be very stable (Soderhall, 1995) and upon activation becomes more sensitive to temperature. SDS-activated tyrosinase was shown to give rise to increased thermolability in *Vicia faba* tyrosinase (Moore and Flurkey, 1990). PPO has been activated by a variety of treatments or agents such as proteases (King and Flurkey, 1987; Soderhall and Soderhall, 1989), urea (Swain et al., 1966), polyamines (Jimenez-Atienzar et al., 1991), divalent cations (Jimenez and Garcia-Carmona, 1993), acid or base shock (Kenten, 1957) or anionic detergents such as SDS (Kenten, 1958; Moore and Flurkey, 1990). The other agents which induce activation of latent PPO include fatty acids (Hutcheson and Buchanan, 1980; Golbeck and Cammarata, 1981), alcohols (Asada et al., 1993; Espin and Wichers, 1999b) and pathogen attack (Soler-Rivas etal., 1997). Although *in vivo* regulatory mechanisms are as yet unknown, it is thought that endogenous protease(s) might be involved, based on the *in vitro* evidence (Harel et al., 1973; Burton et al., 1993).

The latency and activation of plant PPO was first studied by Kenten (1958), in broad bean *(Vicia faba)*. Activation was achieved by a short exposure to acid (pH 3.0-3.5) or alkali (pH 11.5) or by incubation with ammonium sulfate, at pH 5.0. The activation was ascribed to the removal of an inhibitory protein, which was assumed to be attached to the membrane. The activation of broad bean PPO further studied by Swain et al. (1966) was interpreted 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 behaviour of the enzyme. Lerner et al. (1972) demonstrated that a short exposure of grape PPO to acid pH or urea caused upto a ten fold activation within 1-3 min and was primarily due to an increase in the V_{max} while affinity for the phenolic substrates decreased and that for oxygen increased. This activation was attributed to a conformational change. Lerner and Mayer (1975) further showed that activation was accompanied by a change in the Stokes radius of the enzyme. Conformational change caused by a long exposure to pH 2-3 was observed in purified mushroom tyrosinase (Duckworth and Coleman, 1970). However these changes were not reversible. Mayer and Friend (1960) and Mayer (1965) observed that various detergents caused activation of the membrane bound PPO in sugar beet chloroplasts without causing solubilization of the enzyme. Activation by detergents has also been observed in PPOs from mushroom (Espin and Wichers, 1999c), iceberg lettuce, (Chazzara et al., 1997) table beet (Escribano et al., 1997) and William pear (Gauillard and Richard-Forget, 1997).

Angleton and Flurkey (1984) demonstrated that electrophoresis in the presence of SDS, or incubation of the gel with SDS, following electrophoresis, allows detection of less active or latent PPOs. Moore and Flurkey (1990) showed that the ability of SDS to activate the enzyme alters both its enzymatic and physical characteristics and suggested that a limited conformational change due to the binding of small amounts of SDS would have induced the activation of the latent enzyme. The binding of SDS would have opened the active site, which was otherwise blocked partially. Gauillard and Richard-Forget (1997), Chazzara et al. (1997) and Espin and Wichers (1999c) also support the theory of a limited conformational change involved in the activation process. According to the kinetic model proposed by Ricard et al. (1984)

to explain the pH response of enzymes bound to cell envelopes, the protein undergoes a slow conformational transition upon ionization or protonation of a strategic ionizable group.

Approximately 98-99% tyrosinase in mushrooms occurs in a latent form (Yamaguchi et al., 1970; van Leeuwen and Wichers, 1999). Active tyrosinase is the major factor responsible in enzymatic browning in mushrooms. Therefore, in mushrooms, the prevention of tyrosinase activation is a possible approach to avoid enzymatic browning. The increase in activity or appearance of new isozymes in higher plants has also been ascribed to *de novo* synthesis. Treatment with proteases such as trypsin **and** pancreatin has been reported to activate and increase solubilization of PPO (Tolbert, 1973). In case of the mold, *Aspergillus oryzae,* two proteases have been isolated which appear to be involved in activation of PPO (Ichishima et al., 1984). The rise in enzyme activity following wounding of Jerusalem artichoke tubers was inhibited by inhibitors of nucleic acid and protein synthesis (Bastin, 1968). A serine protease, which plays a role in senescence, has been isolated from mushrooms (Burton et al., 1993). Since browning is one of the major hallmarks of senescence, it could well be that this protease is important in regulating PPO activity.

Monophenolase activation

It has long been recognized that the monophenolase activity of PPOs require a reducing agent **for its** initiation (Pomerantz and Warner, 1967; Duckworth and Coleman, 1970). The oxidation of monohydric phenols by tyrosinase exhibits an induction time (or lag phase) during which the rate of oxidation accelerates (Lerner et al., 1949). This is due to the obligatory requirement of binding of molecular oxygen to the bicuprous active site, to enable the enzyme to convert phenolic substrates to the corresponding o-quinones (Cooksey et al., 1998). The

redox potential of the active site copper atoms result in a significant proportion of isolated tyrosinase having a bicupric active site unable to bind molecular oxygen and thus being incapable of phenol oxidation. This so-called met-tyrosinase requires a cofactor, able to reduce the active site copper atoms to give deoxy tyrosinase, which is able to bind O2 and take part in phenol oxidation (Lerch, 1981).

The hydroxylation step is slower than the oxidation of o-diphenols to o-quinones and is considered the limiting step of the turnover (Ros et al., 1994). The time required to reach the steady state depends on several factors, the enzyme source (Valero et al., 1988), the concentration of the monophenol, the lag phase being longer when monophenol concentration is increased (Osaki, 1963; Vaughan and Butt, 1972), the lag phase diminishing when the enzyme concentration is increased (Pomerantz and Warner, 1967; Duckworth and Coleman, 1970). The extent of induction time also depends on pH, substrate and a suitable hydrogen donor, with the lag phase increasing when the substrate concentration increases and decreasing when the concentration of the hydrogen donor increases (Palumbo etal., 1985). Even though o-dihydroxy phenols are the most widely studied and most effective activators of monophenolase activity (Pomerantz and Warner, 1967; Duckworth and Coleman, 1970; Hearing et al., 1978; Mclntyre and Vaughan, 1975; Cabanes etal., 1987), reducing agents such as ascorbic acid, NADH, dimethyl tetrahydropteridine (Osaki, 1963; Vaughan and Butt, 1972;), transition metal ions viz. Fe^{2+}, Cd^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+} (Palumbo et al., 1985), also have the same effect as diphenols in stimulating the monophenolase activity of PPO

Hearing and Ekel (1976) found that DOPA at low concentrations was not a competitive inhibitor of tyrosine hydroxylation but a non-

competitive activator of tyrosine binding, which results in an increased reaction velocity. Vaughan and Butt (1972) reported that low concentrations of o-dihydric phenols abolished the lag phase of p-coumaric acid hydroxylation by spinach beet PPO. When N,N-dimethyl tyramine was used as tyrosinase substrate, no significant oxidation took place unless the enzyme was primed with a trace amount of DOPA (Cooksey et al., 1997; Nayish-Byfield and Riley, 1998). There are two main mechanistic theories of tyrosinase activation a) allosteric activation by eliciting a conformational change in the enzyme b) recruitment hypothesis, which depends on the two electron reduction of the active site of the enzyme, transforming it into a catalytically active form (Cooksey et al., 1997).

The observation that DOPA has two K_m values supported the hypothesis that DOPA binds at different sites on the enzyme when functioning as a co-substrate or as a sole substrate apart from oxygen (Pomerantz and Warner, 1967). This idea of two different sites on the enzyme received additional support from the inhibition studies using diethyldithiocarbamate (Pomerantz, 1960) and rvpropyl gallate (Pomerantz and Warner, 1967), which was a much stronger inhibitor of DOPA oxidation than tyrosine hydroxylation. The same conclusion was also reached by Duckworth and Coleman (1970). These authors proposed a unified mechanism in, which the initial hydroxylating capacity of the enzyme shifts to an activated form, in the presence of a diphenol as soon as micromolar concentrations of the diphenol product appear. These allosteric models were later supported by the findings of Hearing and Ekel (1976) who found that low concentrations of DOPA non-competitively activated the oxidation of tyrosine. They suggested that tyrosinase had at least one site for tyrosine binding and hydroxylation, and at least one other site where DOPA could affect the affinity of the enzyme for tyrosine. Thus it was proposed that DOPA

induces a conformational change in normal tyrosinase, which not only makes the binding of tyrosine to the enzyme more effective, but also greatly increases the velocity of its transformation (Sanchez-Ferrer etal., 1995).

Sequence and structural features of PPO

PPOs are present in low concentrations in the sources studied so far. Furthermore, difficulty in purifying the enzyme and occurrence of multiple forms are also limiting factors in obtaining primary amino acid sequences of PPOs. Enzyme from only one species *(Neurospora crassa)* has been sequenced by classical protein chemical means (Lerch, 1982). With the advent of recombinant DNA technology, however, numerous aminoacid sequences have become available recently and are listed in Table 1.1.

Table 1.1. **List of organisms whose PPO sequences are determined.**

Species	References
Streptomyces glaucescens	(Huberetal., 1985)
Streptomyces antibioticus Aspergillus oryzae Neurospora crassa Rhizobium meliloti Agaricus bisporus Spinacia oleracea (spinach) *Phytolacea americana* (pokeweed) *Viciafaba* (broad bean) *Solarium tuberosum* (potato A) *Solarium tuberosum,* (potato B) *Vitis vinifera* (grape berry) *Malus domestica* (apple) *Lycopersicon esculentum* (tomato)	(Bernan etal., 1985) (Fujitaetal., 1995) (Lerch, 1982) (Mercado-Blanco et al., 1993) (Wichers etal., 1995) (Hind etal., 1995) (Joy etal., 1995) (Cary etal., 1992) (Hunt etal., 1993) (Thygsen etal., 1995) (Dry and Robinson, 1994) (Boss etal., 1995) (Newman etal., 1993)

Within different taxa, the sequence homology of PPOs is high, and conserved domains can be identified for each group. Potato B, tomato and broad bean proenzymes have 96.6, 92.2 and 38.1% strict homology, respectively when compared to potato A proenzyme. Tomato proenzyme has 91.4 and 40% strict homology to potato B and broad bean proenzymes respectively (Whitaker, 1995). The potato A, potato B, tomato and broad bean PPOs have nine, eight, eight and seven half-Cys residues respectively (Whitaker, 1995). There are 18, 18, 18 and 14 His residues in potato A, potato B, tomato and broad bean PPOs respectively. All 18 His residues are conserved among the potato A, potato B and tomato PPOs, whereas 10 His residues are conserved in the broad bean PPO among which, 6 are presumed to be in the active site of all the four PPOs (Whitaker, 1995). The PPO of S. *glaucescens* and S. *antibioticus* have 17.0 and 16.5% strict homology respectively with *N. crassa* PPO. There is 87.5% strict homology between S. *glaucescens* and S. *antibioticus* PPOs. Unlike the higher plant and animal PPOs, only the *N. crassa* PPO contains a single half-Cys residue (Whitaker, 1995).

Domains in tyrosinases

The tyrosinase sequence can be roughly divided into three domains, the amino-terminal, carboxy-terminal and central domain, of which the central domain contains the Cu-binding sites (van Gelder etal., 1997).

The cloning of several PPO genes from plants demonstrated that the enzyme is synthesized as a precursor with an amino-terminal extension of ca 10 kDa. It has been suggested that this extension serves as a transit peptide which post-translationally directs the protein to the chloroplast envelope (Shahar et al., 1992; Cary et al., 1992; Hunt et al., 1993; Newman etal., 1993). Limited proteolytic cleavage then leads to

the correct size of the mature enzyme. The peptide bond cleaved by the processing proteases was identified as Ala-Ser or Ala-Ala in all plant PPOs examined so far (Whitaker, 1995)

In the known fungal tyrosinases, no amino-terminal signal peptide was predicted, which is in agreement with the fact that no function similar to that of a transit peptide of higher plant PPO could be postulated, as the fungal tyrosinases are expected to be cytoplasmic (Mayer and Harel, 1991; Wichers et al., 1995). In human and mouse tyrosinases the amino-terminal 18 amino acids are putative signal peptides and it was suggested that they were involved in the transfer of the enzyme into the melanosome (Lerch, 1988). Another interesting region in the amino-terminal domain is a small Cys rich region which is conserved in both plant and animal tyrosinases and is located directly after the transit or signal peptide (van Gelder, 1997)

In higher plants, the enzyme is mostly membrane bound in non-senescing tissues (Mayer and Harel, 1991). Evidence for membrane tyrosinase in plants is based on the fact that detergent extraction of the tissue chloroplasts substantially increased tyrosinase activity and/or the release of tyrosinase from membranes (Rodriguez and Flurkey, 1992). However only in two of the seven tomato tyrosinase sequences, a transmembrane helix was suggested in the carboxy-terminal domain, as well as in a few other plant tyrosinases (Newman et al., 1993). Tyrosinase from *N. crassa* was found to be synthesized as a precursor with a very large carboxy-terminal extension of 200 amino acids (Giebel et al., 1991). It has been suggested that this extension could be involved in the activation of a protyrosinase by limited proteolysis.

The most important feature observed in all tyrosinase sequences are the two copper binding sites, called CuA and CuB, which are indicated in Fig 1.4. The active site of tyrosinase consists of a pair of

copper ions, which are each bound by three conserved His residues (Jackman et al., 1991). This copper pair is the site of interaction of tyrosinase with both molecular oxygen and its phenolic substrates (Fig 1.4).

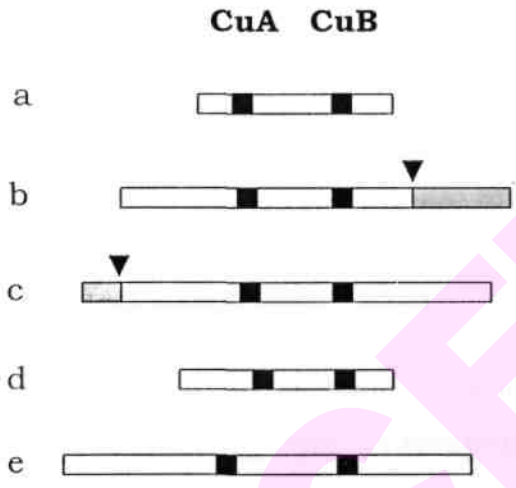


Fig 1.4. **Schematic representation of the polypeptide chains of tyrosinase from a) *Streptomyces glaucescens,* b) *Neurospora crassa, c) Lycopersicon esculentum,* hemocyanin from d) *Octopus dofleini* e) *Panulirus interruptus.*** The solid bars indicate the regions of the CuA and CuB sites. The alignment of the six polypeptide chains is based on conserved regions of CuB site. The shaded areas indicate extensions which are post-translationally removed by limited proteolysis (site of cleavage is shown by arrow). Reproduced with permission from (Lerch, 1995). Copyright (1995) American Chemical Society.

The regions in PPOs around the copper binding ligands also share sequence homology with hemocyanins (Hcs), which are copper containing oxygen carriers from the hemolymph of many molluscs and arthropods (Lang and van Holde, 1991). Sequence comparison in the CuB region with different tyrosinases and a molluscan He from *Octopus dofleini* (Lang and van Holde, 1991) shows a highly conserved region of 56 amino acids (Fig 1.4). The invariant and isofunctional residues comprise the three His known to be ligands to CuB in *Panulirus interruptus* He (Volbeda, 1989). Initially, the

understanding of tyrosinase structure was due to the solving of the X-ray crystal structure of He from the spiny lobster *Panulirus interruptus* (Gaykema et al., 1984). The recently available crystal structure of a catechol oxidase from sweet potato *(Ipomoea batatas)* complexed with a PTU at 2.5 A resolution now explains the similarities between He and PPOs (Klabunde et al., 1998)

The three-dimensional structure of catechol oxidase

The first structural report of a copper type-3 plant enzyme is that of catechol oxidase from sweet potato (Klabunde et al., 1998). Catechol oxidase from sweet potato *(Ipomoea batatas,* ibCO) lacks hydroxylase activity, but catalyzes a 2e– transfer reaction during the oxidation of a broad range of o-diphenols to the corresponding o-quinones, by molecular oxygen (Cary et al., 1992). The structure of the monomeric 39 kDa ibCO was solved and refined to 2.5 A resolution in the dicupric Cu(II)-Cu(II) state (Klabunde et al., 1998).

The enzyme is ellipsoid in shape, with dimensions of approximately 55 x 45 * 45 A (Fig 1.5). The secondary structure is primarily a-helical with the core of the enzyme formed by a four-helix bundle composed of oc2, a.3, a6, and a7. The helix bundle accommodates the catalytic binuclear copper centre and is surrounded by two a-helices, al, a4 and several short (3-strands. CuA is coordinated by the three His (88, 109 and 118) whereas, the CuB site is coordinated by His (240, 244 and 274). Two disulphide bridges (Cys 11-Cys 28 and Cys 27-Cys 89) help to anchor the loop rich amino-terminal region of the protein (residues 1-50) to helix a2. An interesting feature of the binuclear metal centre in ibCO is a covalent thioether bond formed between the *Ce* atom of His 109, one of the copper ligands, and the sulfur of Cys 92.

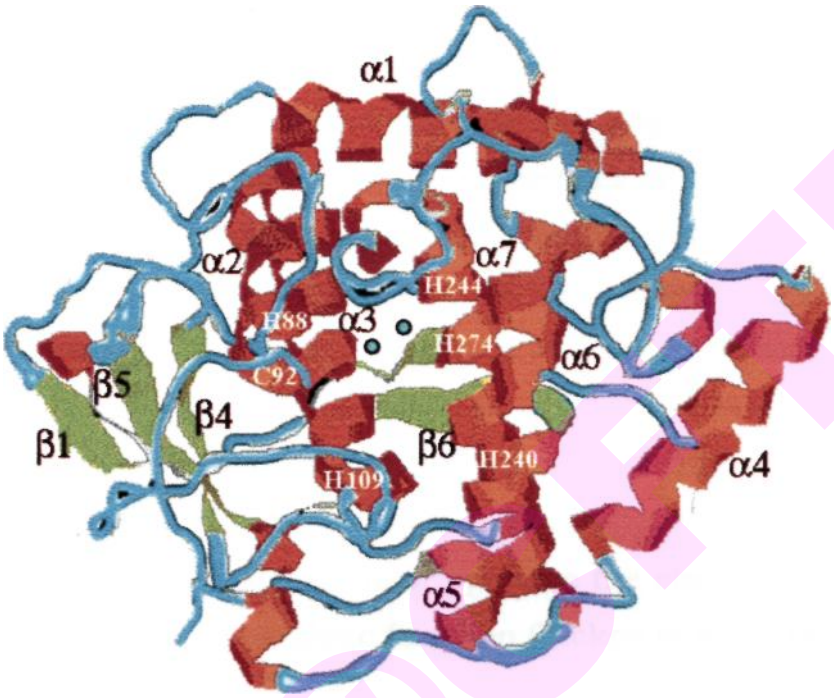


Fig 1.5. **Ribbon drawing of *Ipomoea batatas* catechol oxidase.** (Klabunde et al, 1998). © Cu atom. Reproduced with permission from the authors and from "Nature Structural Biology".

A similar thioether bridge is also present in *N. crassa* tyrosinase between the second His residue in CuA and a His residue located two amino acids towards the amino-terminus (Lerch, 1982). This thioether bridge was proposed to play a role in the regulation of tyrosinase activity (Lerch, 1987). Looking at the sequences of mushroom (Wichers etal., 1995) and *Aspergillus* tyrosinases (Fujita et al., 1995), it can be seen that such a thioether bridge is also possible. The recently published structure of the functional unit of octopus He also shows that one of the six His ligands is involved in a thioether bridge (Cuff et al., 1998), strengthening the similarity between the Hcs and PPOs. The restrained geometry confirmed by this structure would allow the fast binding of the dioxygen substrate or optimize the electronic structure of the metal needed for its catalytic function (Eicken et al., 1999).

As with ibCO, the locations of the dicopper centre and the ligating His in the He structures from *Panulirus interruptus* (Gaykema etal., 1984; Volbeda et al., 1989), *Limulus polyphemus* (Hazes et al., 1993) and *Octopus defleini* (Cuff et al., 1998) are nearly identical. In arthropodan Hcs, the aromatic ring of a Phe (Phe 49) for *Limulus polyphemus* He and Phe 75 for *Panulirus interruptus* He from the amino-terminal domain, shields access to the dimetal centre. This phenyl ring aligns perfectly with the aromatic ring of PTU in the ibCO-PTU inhibitor complex. The shielding of the dimetal centre by the Phe limits access of substrates to the dicopper centre and therefore, allows Hcs to function as oxygen transport proteins. Arthropodean Hcs show no or only very low, catalytic activity in their native form (Zlatova etal., 1996). Significant levels of monooxygenase and catecholase activity have been reported for the Tarantula He after removing the shielding Phe residue by limited proteolysis (Decker and Remke, 1998). Higher activity has been observed for the native molluscan He (Salvato etal., 1998) where the Phe residue is substituted by a less bulky He 2830 (Miller et al.,

1998). In addition a second residue, equivalent to Phe 261 (the gate residue of ibCO) is substituted by the smaller sidechain of Leu 2689.

The structural data on ibCO revealed both a close relationship between the active sites of ibCO and Hc's, and even extended homology to the molluscan Hc' s. Although the CuB site is strictly conserved for all copper type-3 proteins, the varying CuA environment seems to tune the proteins to specific activites (Eicken et al., 1999).

Functional states of PPO

Three different functional states of tyrosinases can be distinguished: met, deoxy and oxy. The interrelationship of the three forms and their active site structural models are shown in Fig 1.6. All the forms are EPR non-detectable (Lerch, 1995).

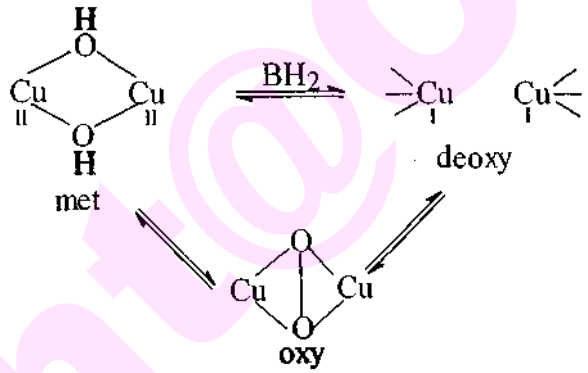


Fig 1.6. **Interrelation of the three functional states of PPO.** BH_2 is a hydrogen donor.

OxyPPO (E_{oxy})

E_{OX}y consists of two tetragonal Cu(II) atoms, each coordinated by two strong equatorial and one weaker axial NHIS ligand (Solomon and Lowery, 1993). Resonance Raman spectroscopy has shown that in the Eoxy (Eickman et al., 1978), dioxygen is bound as peroxide to the binuclear site. Solomon et al. (1992) also proved that the oxygen-oxygen bond in the oxy form is longer than in O_2 and resembles the bond in

hydrogen peroxide. These data imply that the two copper ions are in the cupric oxidation state. Investigations of inorganic and bioinorganic copper-dioxygen complexes have also added to structural knowledge of the active site of PPO (Kitajima and Moro-oka, 1994). E_{oxy} can be produced from E_met in the presence of reducing compounds (ascorbic acid, hydroxylamine, dithionite, o-diphenols) and hydrogen peroxide in the absence of O2 (Gutteridge and Robb, 1975). The circular dichroism and absorbance spectra are very similar to those of oxyhemocyanin (Lerch, 1983). EXAFS (extended X-ray absorption fine structure) study of *N. crassa* E_{oxy} showed that the Cu(II)-Cu(II) distances are 3.6 A, slightly larger than the one for E_met (Brown et al., 1980; Woolery et al.,1984). The oxidized ibCO and *Lycopus europeus* PPO has a Cu(II)-Cu(II) distance of 2.9 A as determined by EXAFS data (Rompel et al., 1995; Eickenetal., 1998)

MetPPO (Emet)

Emet, like the Eoxy form contains two tetragonal copper(II) ions antiferromagnetically coupled through an endogenous bridge. This derivative can be converted by addition of peroxide to $E_{OX}y$, which in turn decays back to Emet when the peroxide is lost. The resting form of tyrosinase, ie the enzyme as obtained after purification, is found to be a mixture of >85% Emet and < 15% E_{oxy} forms (Jolley et al., 1974; Wilcox etal., 1985). In the resting dicupric state, the geometric structure may help to impose the observed Cu(I) geometry (distorted trigonal bipyramidal coordination with a vacant apical position) on the Cu(II) in the CuA site. This entatic state may optimize the electronic sturcture (redox potential) of the metal needed for the oxidation of the o-diphenol substrate and may allow for a rapid electron transfer in the redox process (Klabunde et al., 1998).

DeoxyPPO {$E_{deox}y$)

In contrast to E_{met} and Eoxy, the Edeoxy form is devoid of spectral features in the visible region. Hence little information on the electronic and geometric structure is available. Consistent with the lack of an EPR signal, Edeoxy was assigned a bicuprous Cu(I)-Cu(I) structure, similar to deoxy He (Brown et al., 1980; Lerch, 1981). The crystal structure of the reduced ibCO revealed that although the metal-metal separation increased to 4.4 A, no other significant conformational changes were observed upon reduction of the protein. In the reduced state the coordination sphere of CuA (trigonal planar coordinated by the three protein ligands) thus matches a trigonal pyramid, whereas the coordination of CuB can be described as square planar with one missing coordination site.

Mechanism for substrate oxidation by PPO

Kinetic Mechanism

Kinetically, PPOs follow a Bi-Bi ordered sequential mechanism Fig 1.7, (Cleland, 1963a, 1963b, 1963c, Lerch and Ettlinger, 1972, Rivas and Witaker, 1973). The order in which the two products, water and benzoquinone are released from the enzyme is unknown.

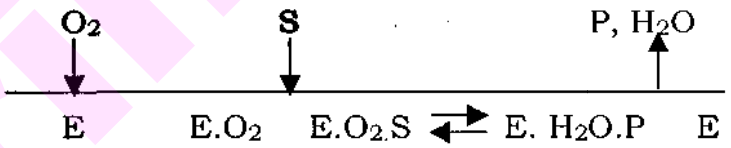


Fig 1.7. **Kinetic Mechanism for PPO according to Cleland nomenclature.** E=enzyme, S=monophenol or diphenol substrate, P=product.

Catechol oxidase from sweet potato lacks hydroxylase activity, but catalyzes a $2e^-$ transfer during the oxidation of a broad range of

o-diphenols (Klabunde etal., 1998). The dinuclear copper centre of ibCO catalyzes the oxidation of catechol through the $4e^-$ reduction of molecular oxygen to water (Fig 1.8). In the proposed catalytic pathway (Klabunde et al., 1998; Eicken et al., 1999) the catalytic cycle begins with the oxidized E_met form, which is present following isolation of the enzyme. Based on ibCO-PTU complex, the monodentate binding of the diphenolic substrate to CuB seems to be most likely to reduce the Cu(II)-Cu(II) form to the dicuprous state (Eicken et al., 1999). The observed binding mode for PTU and the modelled catechol binding mode, suggest that the binding of substrate and dioxygen is possible during the next step in the reaction.

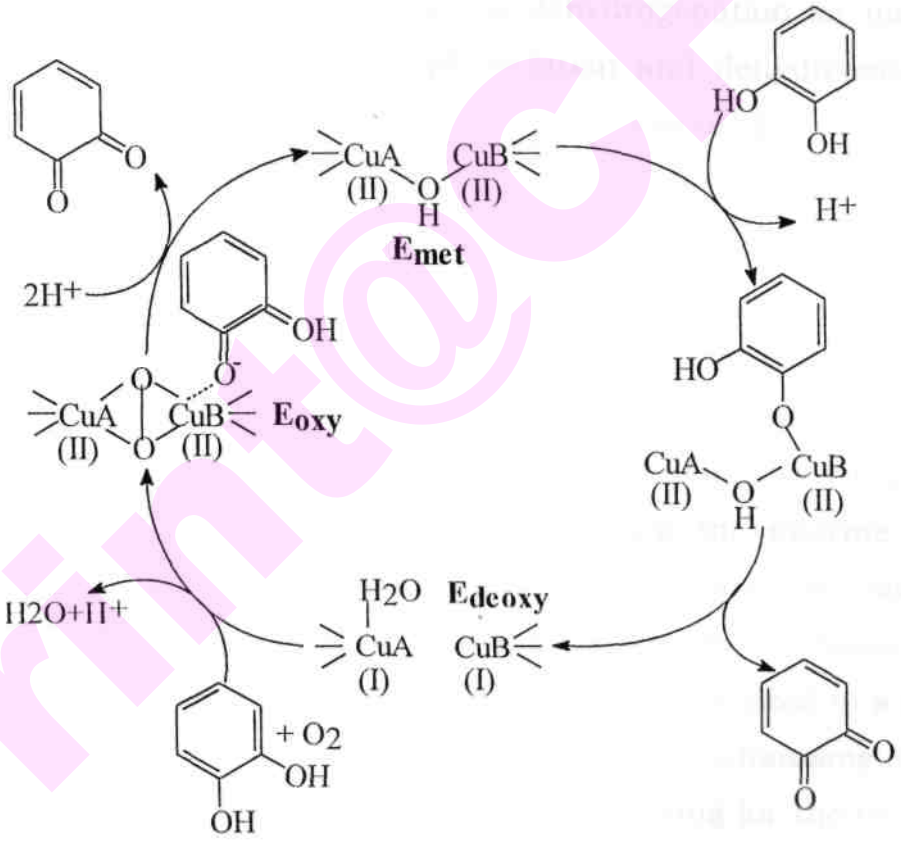


Fig 1.8. **Proposed kinetic scheme depicting the mechanism of oxidation of o-diphenol by *Ipomoea batatas* catechol oxidase.** Adapted from Eicken et al. (1999).

In the ternary ibCO-0_2-substrate complex, two electrons could be transferred from the substrate to the peroxide followed by the cleavage of the O-O bond, loss of water and departure of the o-quinone product. Binding of the catechol substrate to the reduced enzyme without binding of the oxygen seems less likely, as the incubation of reduced crystals did not show any catechol affinity indicating the low binding affinity of the substrate to the Cu(I)-Cu(I) centre. After oxidation of the second substrate molecule and loss of the bound water, the dicopper centre is in its met form again and ready to undergo another catalytic cycle.

The proposed mechanism of action of *N. crassa* PPO appears to fit the mechanisms of hydroxylation and dehydrogenation for most PPOs. The proposed mechanisms of hydroxylation and dehydrogenation are presented separately, but linked by a common $E_{0X}y$ intermediate. Proposed intermediates in the o-diphenol oxidation pathway are shown in a of Fig 1.9. Oxygen is bound first to the two Cu(I) groups of the Edeoxy to give $E_{0X}y$ in which oxygen has the characteristic of a peroxide (Solomon et al., 1992). The two Cu(II) atom groups of E_{oxy} then bind the two hydroxyl groups of catechol replacing the two water molecules or hydroxyl groups, to form the oxygen-catechol-enzyme complex. The catechol is oxidized to benzoquinone, leaving the enzyme as $E_m$et-Another molecule of catechol binds to E_{me}t and is oxidized to benzoquinone, in the process, reducing the enzyme to Edeoxy, thereby completing the cycle. Two catechol molecules are oxidized in a complete cycle, consuming two atoms of O_2. However the mechanisms of the two reactions appear to be different, with O_2 accounting for the oxidation of catechol in step 1 and the two Cu(II)s providing the oxidation of catechol in step 2.

For monophenol hydroxylation by PPO, the *in vitro* reaction begins with E_{met}t (b of Fig 1.9). Although Wilcox et al. (1985) indicated that the resting form of *N. crassa* PPO is a mixture of >85% E_{met}t and <15% Eoxy, it is of general experience that resting PPO has no activity on monophenols unless BH2 is added. BH2 has a unique binding site, converts Emet to Edeoxy (with oxidation of BH2), which can then bind O2 to give Eoxy. The E_{0X}y then binds the monophenol via one of Cu(II)s, displacing the water molecule.

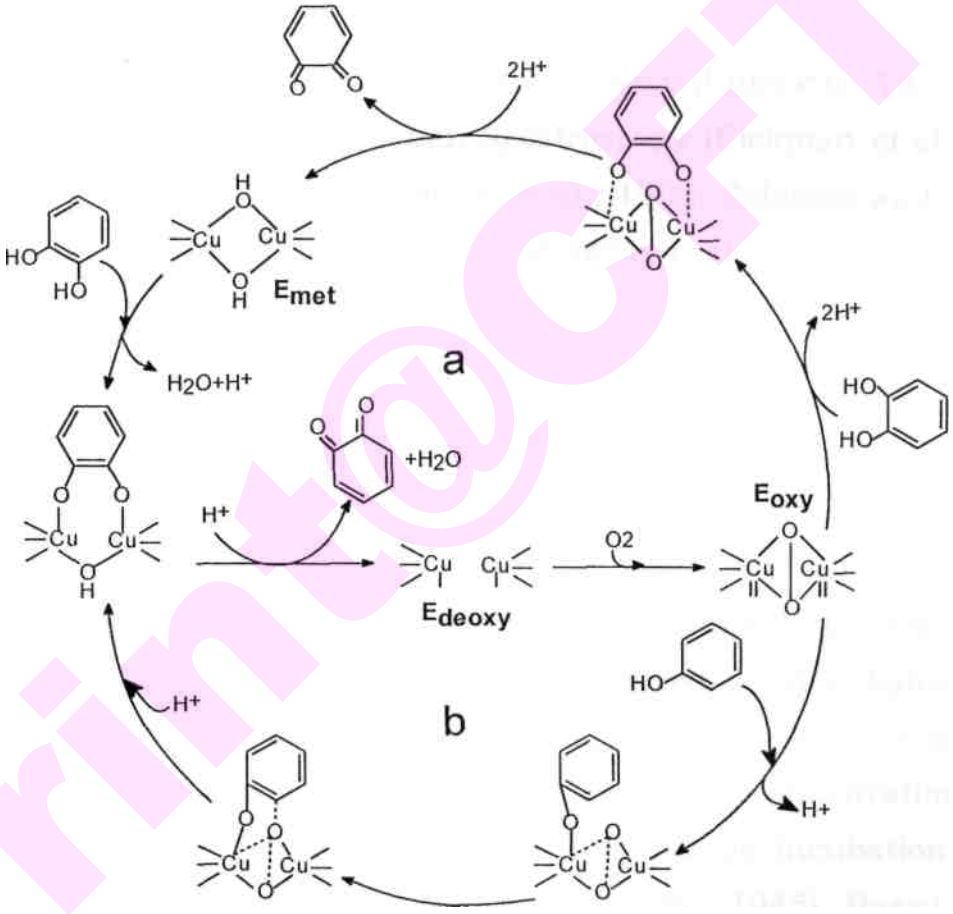


Fig 1.9. **Proposed kinetic scheme depicting mechanism of oxidation of substrates by *Neurospsora crassa* PPO.** (a) diphenol (b) monophenol. Reproduced with permission from (Whitaker and Lee, 1995). Copyright (1995) American Chemical Society.

In the subsequent steps, the steric orientation of the bound monophenol and O2 are altered to place the o-position of the monophenol adjacent to the second Cu(II) in E_{oxy}, so that the o-position is hydroxylated by the bound -O-O- moiety. The initial product of the monophenol is a catechol bound to both Cu(II)s. While still bound, it is oxidized to o-benzoquinone, and the enzyme is reduced to the Edeoxy and can again bind O_2 without cycling through the met form.

Evidence for the mechanisms of oxidation of monophenols and o-diphenols by *N. crassa* and *Agaricus bisporus* PPO are based on spectral changes (Mason, 1965), on the use of $^{18}0_2$ (Mason etal., 1955), essentiality of copper for activity of the enzyme (Kubowitz, 1938; Keilin and Mann, 1938) and on Raman spectroscopy (Eickman et al., 1978; Himmelwright et al., 1980; Solomon et al., 1992; Solomon and Lowrey, 1993). A combination of biochemical (Wilcox et al., 1995; Solomon et al., 1996), spectroscopic (Eicken et al., 1998) and the structural data (Klabunde et al., 1998) was used to elucidate the pathway of diphenol oxidation by ibCO.

Evidence for the role of copper in catalysis of oxidation of monophenols and diphenols came early on (Kubowitz, 1938; Keilin and Mann, 1938). It was shown initially that the addition of CUSO4 to plant extracts containing PPO increased the observed activity. Later it was shown that the copper can be removed in the presence of reducing compounds including ascorbate, leading to complete inactivation of the enzyme, and that the activity can be restored by incubation of the inactive PPO with $CuS0_4$ (Dawson and Mallette, 1945). Based on the primary structure of *N. crassa* PPO and resemblance of the sequence to an amino acid sequence of the 0_2-binding segment of He and from EPR studies, evidence accumulated that the active site must contain a binuclear copper complex. The elucidation of the three dimensional

structures of ibCO in the oxidized Cu(II)-Cu(II) state, the reduced Cu(I)-Cu(I) form, and of its complex with the inhibitor PTU provided further evidence regarding the three functional states (met, oxy and deoxy) of the binuclear copper centre (Klabunde et al., 1998).

Enzymatic chemical model

The enzymatic chemical model for the PPO mediated reaction, was first proposed by Cabanes et al. (1987), which takes into account the three forms, E_{met}t, E_{oxy} and Edeoxy of PPO and the chemical redox cycling of the o-quinones formed by the enzyme (Fig 1.10). During catecholase activity, o-diphenol binds to both the E_{oxy} and E_{met}t rendering E_{oxy}D and E_{met}tD intermediates, which give rise to o-quinones. These two o-quinones rapidly redox recycle to regenerate one diphenol
and one dopachrome in the chemical step (Fig 1.10).

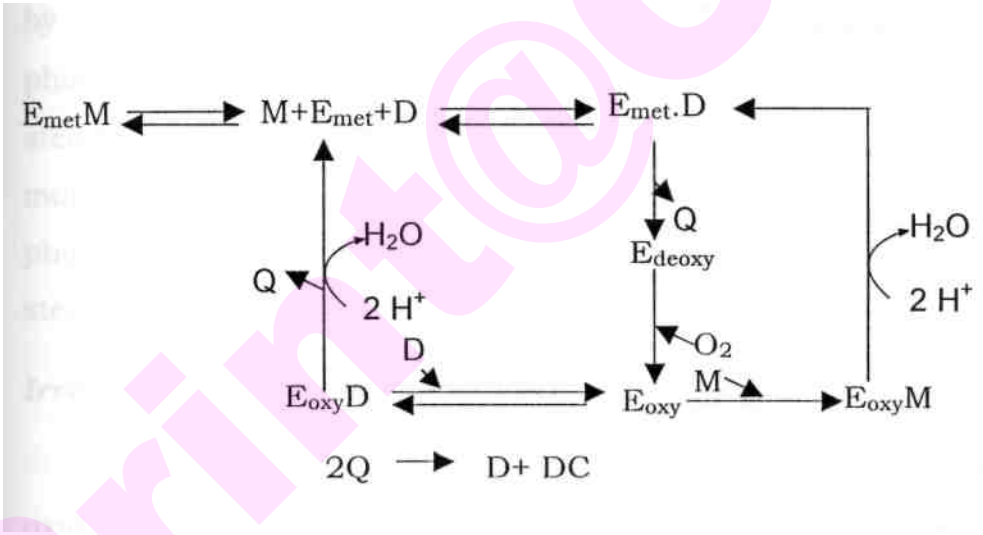


Fig 1.10. **Suggested kinetic mechanism for the enzymatic-chemical model for reactions catalysed by PPO.** D=diphenol, DC=aminochrome, Q=quinone, M=monophenol

In the cresolase cycle, the binding of monophenol to E_{0X}y renders EmetD which releases the quinone. Binding of the Emet to monophenol scavenges a portion of the PPO from the catalytic turnover as a deadend complex because Emet is inactive on monophenols. The resting form

of PPO, after isolation is found to contain <15% E_{oxy}, majority of the enzyme being in the E_{met} form (Lerch, 1981). The redox potential of the active site copper atoms result in a significant proportion of isolated PPO having a bicupric active site unable to bind molecular oxygen and thus being incapable of phenol oxidation. This met-tyrosinase requires a cofactor able to reduce the active site copper atoms to give Edeoxy which is able to bind oxygen and take part in the phenol oxidation. Although direct reduction may occur, eg., by electron donation from reduced metals (Palumbo et al., 1985), it is generally the consequence of diphenol oxidation by the enzyme. The acceleration of the phenol oxidation occurs because of the recruitment of met-tyrosinase by Cu(II) reduction, by the diphenolic substrate, which in turn is generated by the chemical step occurring after the quinone formation. The enzyme slowly reenters the catalytic cycle by means of the o-diphenol obtained by recycling in the chemical reactions. This puts an end to the lag phase and produces the level of diphenol necessary to maintain the steady state. This model explains the dependence of the enzyme, monophenol and diphenol on the lag phase of the enzyme. Thus, lag phase is the time required to reach the o-diphenol concentration in the steady state.

Irreversible inactivation of PPO

Another intermediate reaction occurs during PPO-catalyzed oxidation of o-diphenols and perhaps monophenols. In the oxidation of catechol to o-benzoquinone, there is a slow irreversible inactivation of PPO (Nelson and Dawson, 1944; Mason, 1965; Ingraham, 1955). It was shown that the inactivation was due to a free radical-catalyzed fragmentation of one or more of the active site His residues, which explains the loss of His and release of copper (Nelson and Dawson, 1944; Dietler and Lerch, 1982; Golan-Goldhirsh etal., 1992;

Whitaker, 1994). Golan-Goldhirsh (1992) and Whitaker (1984) calculated that the inactivation of mushroom PPO occurs at the rate of approximately one in 5000 turnovers of the enzyme. Therefore it appears that there is a free radical intermediate produced in the enzyme catalyzed reaction with o-diphenols (Fig 1.11) and that the free radical inactivates the enzyme (reaction inactivation mechanism). It has been impossible until recently to detect the semiquinone radical directly, possibly because it is converted rapidly to the o-quinone. Spin resonance stabilization NMR techniques have shown that semiquinones are formed during the PPO-catalyzed reaction (Peter et al., 1985; Korytowski et al., 1987). The mechanisms of monophenol and o-diphenol oxidation by PPO indicate that o-benzoquinone is the final product of the enzyme *in vivo and in vitro.* Further reactions occur because of high reactivity of the o-benzoquinones, further involvement of PPO, involvement of other enzymes and finally non-enzymatic reactions.

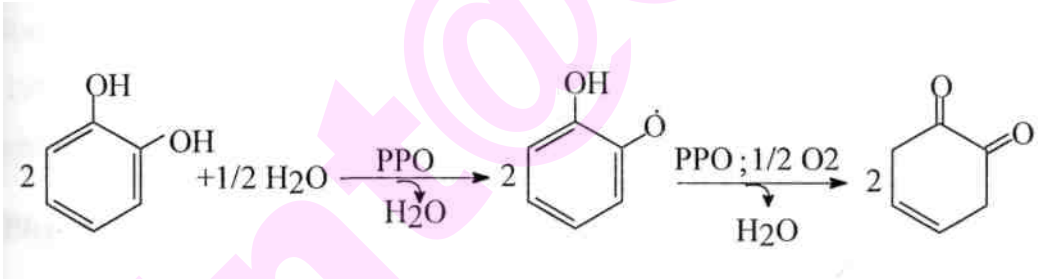


Fig 1.11. **Semiquinone formation during the PPO-catalyzed reaction.** Adapted from Whitaker, 1995.

Prevention of enzymatic browning

Endogenous PPO is present in foods that are particularly sensitive to oxidative browning eg. potatoes, apples, pears, peaches, bananas, mushrooms, fruit juices and wines. Browning is more severe when the food has been subjected to surface damage, which can result

from cutting, peeling, comminuting, pureeing, pitting, pulping or freezing. In uncut or undamaged fruits and vegetables, the natural phenolic substrates are separated from the PPO enzyme, by compartmentation, and browning does not occur. The loss of cell integrity results in the de-compartmentation of phenolic substrates and enzymes, and, then in the presence of molecular oxygen, the oxidative production of colored quinones (Macheix et al., 1990). Deleterious changes caused by browning results in shorter shelf life, decreased market value and in some cases, complete exclusion of the food from certain markets. PPO, thus is one of the most deteriorative enzymes in fresh fruits and vegetables, yet it is considered essential in the manufacture of tea, coffee, cocoa, raisins or cider where a specific degree of browning is desirable and is an essential part of the production process.

Because of the detrimental effects of the browning reaction in most commercially important foods, its control is of high priority to the food industry (Vamos-Vigyazo, 1981; Macheix et al., 1990; Sapers, 1993; Walker, 1995). Present methods of control use both physical and chemical methods, often in synergy (Almeida and Nogueira, 1995).

Physical methods

These involve removing or decreasing the activity of the enzyme and/or its substrate. The most effective method for controlling enzymatic browning in canned or frozen fruits and vegetables is to inactivate the PPO by heat treatment, such as by stream blanching, but this is not a practical alternative for treatment of fresh foods. Heat processes cannot be used in many systems because many fruit PPOs are relatively stable and also heat treatments often affect the sensory qualities of the product adversely, especially the texture, taste and

color. Freezing or refrigeration can be used to temporarily limit browning (Walker and Ferrar, 1998).

A variety of adsorbents have been used to remove the reactants involved in enzymatic browning. Cyclodextrins inhibit enzymatic browning by removing the soluble phenols from solution as inclusion complexes (Sapers et al., 1989; Iyengar and McEvily, 1992). Chitosan also inhibits phenolase probably by complexing with the phenols (Sapers et al., 1989; Iyengar and McEvily, 1992). Bentonite clay has protein adsorption qualities and has long been used in wine making to reduce PPO activity (Macheix et al., 1991). Phenolic adsorbents such as gelatin, activated carbon, soluble PVP and insoluble PVPP have also been used to remove soluble phenols from wines and beers (Walker and Ferrar, 1995).

Another method of removing PPO substrates is to limit oxygen availability, since oxyzen is one of the substrates for the reaction, although browning will occur rapidly when the oxygen is reintroduced. This can be achieved by packing under vacuum or in a carbon dioxide or nitrogen-enriched atmosphere, as has been done with pineapples (Macheix et al., 1990). Excluding of oxygen is also possible by immersion in deoxygenated water, syrup, brine or coating the food with surfactants (McEvily et al., 1992).

Chemical Methods

Chemical methods of controlling enzymatic browning involve adding browning inhibitors to the food product. This is often accomplished by dipping the product (usually fruit) in a solution of the inhibitor.

Sulfites

The most widespread methodology used in the food and beverage industries for control of browning is the addition of sulfiting agents *viz* sulfur dioxide, sodium sulfite, sodium and potasium bisulfites and metabisulfites (Mayer and Harel, 1991; Walker, 1995). These inhibit browning by a variety of mechanisms, they are potent non-specific reducing agents and hence can be used to inhibit browning by reduction of the quinones to the parent dihydroxyphenols (Kahn, 1985). Sulfites have also been reported to inhibit PPO directly (Embs and Markakis, 1965). They also react with intermediate products preventing their further participation in the non-enzymatic reaction to form brown pigments (Sayavedra-Soto and Montgomery, 1986). Finally and probably most importantly, sulfites are known to complex with quinones to form colorless sulfo-quinone adducts (Walker, 1975).

Although sulfites are very effective in the inhibition of both enzymatic and non-enzymatic browning reactions, there are several negative attributes associated with their use in foods and beverages. Several cases of sulfite related adverse reactions, including anaphylactic shock, asthmatic attacks, urticaria and angioderma, nausea, abdominal pain, diarrhea, seizures and death have been reported, some of them allegedly associated with restaurant food containing sulfites (Yang and Purchase, 1985). The adverse health effects, increased regulatory scrutiny and lack of consumer acceptance of sulfited foods have created the need for practical, functional alternatives to sulfiting agents.

Reducing agents

The major role of reducing agents or antioxidants in the prevention of browning is their ability to chemically reduce the enzymatically formed or endogenous o-quinones to the colorless

diphenol. Ascorbic acid and its derivatives are the common reducing agents used. These agents reduces o-quinones progressively as they are formed. In this process, the ascorbic acid itself gets oxidized to dehydroascorbic acid. PPO which indirectly controls the oxidation of ascorbic acid is virtually unaffected during the reaction, so that browning begins as soon as the reductant has been consumed. Even though ascorbic acid derivatives like the ascorbic acid-2-phosphate or ascorbic acid triphosphate are more stable, they release ascorbic acid when hydrolyzed by acid phosphatases present in the plant tissue (Seib and Liao, 1987).

Acidulants

The pH optimum of PPO activity varies with the source of the enzyme and the particular substrate but in most cases it has an optimum pH in the range of pH 6-7 (Aylward and Haisman, 1969). PPO preparations from several sources are reported to be inactivated below pH 4.0 (Weurman and Swain, 1955; Thomas and Janave, 1973). By lowering the pH of the media below 3, the enzyme is effectively inhibited. Hence, the role of acidulants is to maintain the pH well below that necessary for optimal catalytic activity.

The most widely used acid in the food industry for the prevention of browning is citric acid. Citric acid may have a dual inhibitory effect on PPO by reducing the pH and by chelating the copper at the enzyme active site. Other alternatives to citric acid are organic acids such as malic, tartaric, malonic and inorganic acids such as phosphoric and hydrochloric acids (McEvily et al., 1992).

Chelating agents

In the context of PPO-catalyzed browning, copper chelating agents are believed to either bind to the active site copper of PPO or reduce the

level of copper available for incorporation into the holoenzyme. Many chelating agents have been shown to inhibit PPO including diethyldithiocarbamate, ethylenediamine tetraacetate, azide and mercaptobenzothiazole (Walker, 1975), cyanide and thiourea (Mathew and Parpia, 1971) and carbon monoxide (Albisu et al., 1989).

Inhibition by substrate analogs

Various cinnamic acids were found to be powerful inhibitors of apple and inhibitory action decreased in the order cinnamic acid > p-coumaric acid > ferulic acid > m-coumaric acid > o-coumaric acid > benzoic acids (Kutner and Wagreich, 1953, Walker, 1976). Their inhibitory action stems from their close structural similarity to the enzyme's natural phenolic substrates. Cinnamic acids and their derivatives/homologs occur naturally in most plant foods, but absence of any possible health hazards associated with the use of these compounds as food additives has yet to be shown. In addition to cinnamic acids, many aromatic and aliphatic carboxylic acids inhibit phenolases. Potentially useful commercial inhibitors include sorbic, crotonic and kojic acids (Chen et al., 1991). Salicyl hydroxamic acid (SHAM) was found to be a potent inhibitor of catecholase (Allan and Walker, 1988). 4-Hexyl resorcinol has been proposed to be an effective and safe inhibitor of enzymatic browning (Iyengar and McEvily, 1992; Dawley and Flurkey, 1993).

Sulfhydryl compounds

Many sulfhydryl-containing reducing agents such as P-mercaptoethanol, dithiothreitol and thiourea will probably never be approved for food use as antibrowning agents. The primary mode of action of sulfhydryl compounds in the prevention of browning is to react with the o-quinones formed by enzymatic catalysis to produce stable,

colorless adducts (Mason and Peterson, 1965; Montgomery, 1983). Sulfur containing amino acids such as L-Cys, L-cystine and D,L-methionine are also used as PPO inhibitors (Walker, 1964). The naturally produced tripeptide glutathione (γ-L-glutamyl-L-cysteinyl-glycine) which acts as a natural browning inhibitor in grapes (Macheix etal., 1991) is also effective but expensive. The chief obstacle to the extended use of this group of compounds must be the possibility of sulfur-type off flavours and their higher costs compared with sulfites.

Blends

In the past few years, a number of custom designed formulations to control browning have become available. They use a blend combination of antibrowning agents that result in the enhancement of activity relative to the use of any single agent individually. 'Salad fresh' was introduced in 1983 as a method of preventing the browning of cut lettuce in salads, which includes citric acid, ascorbic acid, sodium ascorbate, phosphate and glucose (Walker and Ferrar, 1995). 'Everfresh' is a patented product, composed of 4-hexyl resorcinol as the active ingredient and sodium chloride as the carrier agent (Lambrecht, 1995).

Natural inhibitors of PPO

The use of chemical antibrowning agents in the food industry is constrained by considerations such as toxicity, effects on taste, flavor, color, texture and cost. The negative connotations associated with chemical inhibitors, has led to decreased consumer acceptance. Moreover, the recent changes in life style and an increased awareness of the relationship between food and health have led to a demand for more natural foods and safer and fewer added chemicals in processed foods. In this context inhibitors from natural sources has gained importance.

The most well-known of these is 4-hexyl resorcinol, which was synthesized following the discovery of a number of inhibitory 4-substituted resorcinols in fig extract (McEvily et al., 1992). 4-Hexyl resorcinol which is used to control enzymatic browning in shrimps can be synthesized chemically or isolated from fig latex and was first patented in 1991 (McEvily et al., 1991). The screening of plant extracts has led to the identification of the pseudostellarins, a group of inhibitory cyclic peptides from the roots of *Pseudostellaria heterophylla* (Morita etal., 1994). A low M_r compound with an absorption peak at 280-290 nm isolated from cultures of *Penicillium expansum* inhibited apple and tobacco catechol oxidase but did not affect fungal laccases (Avigad and Markus, 1965). Oszmianski and Lee (1990) demonstrated that honey contains a small unidentified noncompetitive peptide inhibitor with an approximate M_r of 600. Tan and Kubo (1990) reported that the roots of *Zea mays* secreted a 6.3 kDa protein which inhibited catechin oxidase activity. Ishihara et al. (1991) reported the discovery of melanostatin, a peptide inhibitor of melanin synthesis, found in culture filtrates of *Streptomyces clavifer*. However, this inhibitor did not inhibit mushroom catecholase in routine enzyme assay.

Antisense RNA approach for the control of PPO

A novel approach for the control of PPO *in vivo,* is the use of antisense techniques. According to this technique, a gene or a significant part of it, is introduced into the plant cells in a reverse orientation. The mRNA encoded by the antisense gene hybridizes with that encoded by the endogenous gene and thus the protein product is not made. The expression of PPO in potatoes has been decreased by using vectors, carrying antisense PPO cDNAs (Bachem et al., 1994). About 70% of the transformed plants had lower PPO activity than the controls.

AIM AND SCOPE OF THE PRESENT INVESTIGATION

PPO, a widely distributed copper-containing enzyme, is a key player in the oxidation of phenolic compounds to produce brown color on the cut surfaces of fruits and vegetables. The undesirable browning reaction occurs particularly during the processing of fruits and vegetables. Browning, apart from the color deterioration, also imparts off-flavours and loss in nutritional quality. Preventing PPO activity in post harvest fruits and vegetables has enormous economic and quality benefits, but current prevention methods (mainly chemical method) are not ideal. The adverse health effects, increased regulatory scrutiny and lack of consumer acceptance of added chemicals in food have created the need for other alternatives to prevent browning. Through an understanding of the structure and mechanism of action of PPO and the chemistry of enzymatic browning, better prevention methods can be devised.

Although the first PPO, a mushroom tyrosinase (Shoenbein, 1856) was discovered a century and half ago, the first three-dimensional structure of a plant PPO became available only recently (Klabunde etal., 1998). PPO has been isolated from a variety of sources since its discovery, but pigment contamination and the occurrence of multiple forms have frequently hampered its characterization. During the structural characterization of a lectin from field bean *{Dolichos lablab),* seeds, Gowda et al. (1994) observed severe browning of the crude extracts. The crude extract revealed the presence of a single PPO by polyacrylamide gel electrophoresis. The single form of PPO in field bean seeds renders it ideal for primary structure determination and three-dimensional analysis, which in plant PPOs has been hindered by its multiplicity. Therefore, in the present investigation, as a first step towards three-dimensional structure determination for the design of

novel and potent inhibitors, the purification and characterization of the PPO from field bean seeds was pursued with an emphasis on studying its kinetic and molecular properties. In addition, due to the demand and need for natural inhibitors of PPO, a natural inhibitor of PPO was identified in raw sapota *(Achras zapota)* and was investigated. The main objectives of the present investigation are:

1. Purification and characterization of polyphenol oxidase from field bean *(Dolichos lablab)* seeds.
2. Studies on the kinetic and molecular properties of the enzyme.
3. Purification and characterization of a natural inhibitor of polyphenol oxidase, from raw sapota *(Achras zapota)*.
4. Partial amino-terminal sequence analysis of the enzyme and comparison with other known plant polyphenol oxidase sequences.
5. Developing antibodies against the purified enzyme, and its cross reactivity with other plant polyphenol oxidases.

It is expected that the purification of the single form of PPO to homogeneity could facilitate, the determination of primary structure and crystallographic studies. These studies would lead to a better understanding of the structure-function relationships of PPOs, which could eventually lead to the design of potent inhibitors to prevent enzymatic browning. The polyclonal antibodies developed, could serve as useful tools in the localization of the enzyme and aid in the design of inhibitors. The natural inhibitor purified from raw sapota should serve as an antibrowning agent, for wide and varied application in the food industry.

Chapter 2
Materials and Methods

2.1. MATERIALS

2.1.1. Field bean powder

Dolichos lablab (field bean) seeds were procured from the local market, which served as the starting material (Fig 2.1). The moisture of the dry seeds was brought up to 8% and dried at 45 °C for 30 min. The seeds were dehulled and powdered to a mesh size of 60-80. Two hundred grams of the seed meal was delipidated with 1 L of carbon tetrachloride (1:5 w/v) by gently mixing at room temperature for 12 h. The suspension was filtered on a Buchner funnel using Whatman no.l filter paper and the bean meal was air-dried at room temperature and stored at 4 °C.

2.1.2. Chemicals

Catechol, 4-methyl catechol, L-3,4-dihydroxyphenylalanine (L-DOPA), chlorogenic acid, gallic acid, catechin, caffeic acid, vanillic acid, p-phenylenediamine, polyvinylpolypyrrolidone (PVPP), tropolone, DEAE-Sephacel, Phenyl Agarose, cysteine-HCl, pyrogallol, bovine serum albumin (BSA), tris (hydroxy methyl) amino methane (Trizma base), acrylamide, N,N'-methylenebis-acrylamide, Coomassie brilliant blue R-250, bromophenol blue, trifluoroacetic acid (TFA), triethylamine (TEA), Freund's complete adjuvant, Freund's incomplete adjuvant, N,N,N',N'-tetramethyl 1,2-diaminoethane (TEMED), catalase and mushroom tyrosinase were purchased from Sigma Chemical Co., St. Louis, MO, USA.

2,3,4-Trihydroxy benzoic acid was obtained from Aldrich Chemical Co. WI, USA. L-Tyrosine was from E. Merck, Germany.

Coomassie brilliant blue G-250 was from Eastman Kodak Co., Rochester, NY, USA.

Fig 2.1. **Field bean *[Dolichos lablab)* seeds.** *Chapter 2*

Nitrocellulose (0.45 μm) membranes were from Schleicher and Schuell, West Germany.

Ammonium sulfate, sodium chloride, sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate and sodium hydroxide were from Qualigens Fine Chemicals, Mumbai, India.

Glycine and sodium acetate were from E. Merck (India) Ltd., Mumbai, India.

Ammonium persulfate was from Hi Media Laboratories Ltd., Mumbai, India.

Benzoic acid, o-hydroxy benzoic acid, m-hydroxy benzoic acid, *p*-hydroxy benzoic acid, protocatechuic aldehyde and vanillin were purchased from S.D. Fine Chemicals Pvt. Ltd., Boisar, India.

Amino acid standards (Pierce H.), sodium dodecyl sulfate (SDS) and phenyl isothiocyanate (PITC) were from Pierce Chemical Company, Rockford, Illinois, USA.

β-mercaptoethanol was purchased from Fluka, Switzerland.

Pyrex™ brand hydrolysis and derivatization tubes were from Corning, NY, USA. The vacuum vials and resealable enclosures were from Waters Associate. Milford, MA, USA.

Immobilon-P (polyvinyldiflouride membrane PVDF; 0.45 |am) was obtained from Millipore Corporation, USA.

High performance liquid chromatographic (HPLC) grade solvents were obtained form Qualigens Fine Chemicals, Glaxo (India) Ltd., Mumbai and E. Merck (India) Ltd., Mumbai.

Sephadex G-50, Sephadex G-100, Sephadex LH-20, gel filtration low M_r calibration kit and blue dextran-2000 were from Pharmacia Fine Chemicals, Uppsala, Sweden.

SDS-PAGE markers, Goat Anti-Rabbit IgG alkaline phosphatase conjugate, 5-bromo-4-chloro-3-indolylphosphate (BCIP) / nitroblue tetrazolium (NBT), substrate for alkaline phosphatase were from Bangalore Genei Pvt. Ltd., Bangalore, India. All other chemicals used were of analytical grade.

Antibodies were raised against the purified field bean PPO in a New Zealand white rabbit raised in the Experimental Animal House Facility, CFTRI.

2.1.3. HPLC columns

Progel™-TSK G2000 SWXL (7.8 mm id x 30 cm) was obtained from Supelco, Sigma-Aldrich (India), Bangalore, India. Phenomenex ODS (4.6x250 mm, 5 jim) was from Phenomenex, Torrence, CA, USA, Zorbax GF-250 (250 x 9.4 mm) was from Dupont Company, Wilmington, DE, USA and Pico-Tag amino acid analysis column (150 x 3.9 mm, 4 ^im) was from Waters Associate Milford, MA, USA.

2.2. METHODS

2.2.1. Enzyme extraction

The dried defatted field bean powder (20 g) was extracted for 16 h at 4 °C with 100 mM Tris-HCl buffer, pH 7.0 (100 mL) containing 2% (w/v) PVPP and 1.2% NaCl (w/v). The extract was centrifuged at 8500 *g* for 30 min at 4 °C in a Beckman GS-15R centrifuge. The pellet was discarded and the supernatant was used as the crude extract.

2.2.2. Protein purification matrices

2.2.2a. *Preparation of DEAE-Sephacel*

DEAE-Sephacel obtained from Sigma Chemical Co. was provided as a suspension in 20% ethanol, which had a wet bead size of 40-160 . The gel matrix was washed several times in distilled water and equilibrated in 100 mM Tris-HCl buffer, pH 8.2 containing 1.2% NaCl (w/v) for 24 h.

The DEAE-Sephacel suspension in 100 mM Tris-HCl buffer, pH 8.2 containing 1.2% NaCl was packed in a glass column with dimensions 12 x 3.5 cm at a flow rate of 50 mL/h. The column bed was washed with the equilibrating buffer until the pH of the eluent was the same as the equilibrating buffer.

In ion-exchange chromatography one can choose whether to bind the substances of interest, or to adsorb the contaminants and allow the substance of interest to pass through the column. In our experiments field bean PPO was allowed to pass through the column while many of the contaminants were bound to the column.

Regeneration of DEAE-Sephacel ion exchanger. The bound contaminants were washed out after use, using 1 M NaCl in the equilibration buffer. More strongly bound substances were removed by washing the DEAE-Sephacel with 500 mL of 0.5 M NaOH in a Buchner funnel. The ion exchanger was washed with distilled water until the pH of the eluent was brought to neutral. The gel matrix was again washed with 500 mL of 0.5 M HC1, which was followed by washing with distilled water until the pH of the eluent was to neutral. The gel matrix was then washed in the equilibrating buffer, packed into the column and equilibrated in the same buffer until the pH of the eluent was 8.2. When not in use, the

column was stored in equilibrating buffer containing 0.05% (w/v) sodium azide.

2.2.2b. *Preparation of Phenyl Agarose column*

Phenyl Agarose purchased from Sigma Chemical Co. was supplied as a suspension in 0.5 M NaCl containing 0.02% thimerosal. The Phenyl Agarose medium was washed extensively with distilled water and then equilibrated in 25 mM Tris-HCl buffer, pH 7.0 containing 1 M (NH4)2S04 and 1.2% NaCl (w/v). The medium was packed in a glass column of dimensions, 11.5 x 3.5 cm at a flow rate of 50 mL/h. The column was washed with 5 bed volumes of the equilibrating buffer and the pH of the eluent was checked before loading the sample on the column.

Regeneration of Phenyl Agarose: After each purification cycle, bound substances were washed out from the column to restore the original function of the column. Hydrophobic interaction chromatography adsorbents can normally be regenerated by washing with distilled water after each run. To prevent slow build up of contaminants on the column over time, after every three cycles, a more rigorous cleaning protocol was applied (Pharmacia, 1993). According to this sanitization protocol, the Phenyl Agarose column was washed with 4 bed volumes of 0.5 M NaOH to remove any precipitated proteins, followed by 2-3 bed volumes of distilled water. The column was further washed with 6 bed volumes of 30% isopropanol to remove strongly bound hydrophobic proteins, lipoproteins or lipids. The column was then washed with 3-4 bed volumes of distilled water. The column matrix after sanitization was equilibrated in the starting buffer and was reused. The column was stored in starting buffer containing 0.05% sodium azide when not in use.

2.2.2c. *Preparation of Sephadex G-100*

Sephadex G-100 of particle size of 40-120 μ*m,* which gives a bed volume of 15-20 mL per gram of dry gel was used. The exclusion limit of Sephadex G-100 is 4 kDa-150 kDa for globular proteins.

Fifteen grams of Sephadex G-100 dry powder was allowed to swell in 500 mL of 25 mM Tris-HCl pH 7.0 containing 1.2% NaCl (w/v) for 72 h. After three changes of the buffer, the slurry was packed into a glass column (100 x 2 cm) at a flow rate of 18 mL/h. Size exclusion chromatography on Sephadex G-100 was used as the final step in the purification of field bean PPO. The column was stored in buffer containing 0.05% sodium azide.

2.2.3. Enzyme assay

PPO (catecholase or diphenolase activity) was assayed according to the spectrophotometric procedure of Cosetang and Lee (1987). All the spectrophotometric readings were performed on a Shimadzu UV-Vis recording spectrophotometer, Model UV-160A. The assay mixture consisted of 0.9 mL of 50 mM Na acetate buffer, pH 4.0, 0.1 mL of 0.5 M catechol prepared in distilled water and (10-100 fig) of enzyme. The reference cuvette contained all the compounds except the enzyme in a final volume of 1 mL. The increase in absorbance at 420 nm was measured as a function of time for 3 min. The reaction rates were estimated by drawing tangents to the slopes of the time dependent recordings of absorbance. One unit of enzyme activity is defined as the amount of the enzyme that causes an increase in absorbance of 0.001 /min at 25 °C.

Cresolase (monophenolase) activity of the PPO is characterized by a lag phase, when monophenols L-tyrosine and ferulic acid are used as substrates. The lag phase was estimated by the extrapolation of the

linear zone of the product accumulation curve to the abscissa axis. Cresolase activity in the steady state rate was calculated from the linear part of the product accumulation curve after the lag phase.

For PPO, activated by acid pH and SDS, the enzyme assay was performed as described above except that the buffer used was 50 mM Na acetate, pH 6.0.

2.2.4. Oxygen consumption measurements

Oxygen consumption was followed using a dissolved oxygen meter (EDT Instuments, UK), based on a Clark's electrode. The sample cell contained 4.9 mL of 50 mM Na acetate buffer pH 4.0/4.5, 0.1 mL of substrate and 50-500 ug of the enzyme. Oxygen was bubbled through the buffer to reach saturation levels and then the enzyme was added and the oxygen consumption up to 3 min was noted.

2.2.5. Protein estimation

Protein concentration was determined by the dye binding method of Bradford (1976) and Zor and Selinger (1996). BSA was used as the standard.

2.2.6. Intrinsic fluorescence measurement

Fluorescence measurements of field bean PPO under different conditions were conducted at 27 °C using a Shimadzu (Model RF 5000) recording spectrofluorimeter. Protein solution of 0.05-0.1 mg/mL concentrations was taken in a cuvette and the spectra were recorded between 300-400 nm after exciting at 280 nm.

2.2.7. Effect of pH

PPO activity as a function of pH was determined using 50 mM of catechol, 50 mM 4-methyl catechol and 10 mM of DOPA as substrates.

The buffers used were Mcllvaine (0.1 M citric acid-0.2 M Na2HP0$_{4)}$ pH 2.5-7.5), glycine-HCl (pH 2.5-3.5), Na acetate (pH 3.5-6.0) and NaPi (pH 6.0- 8.0) at 25 °C.

The effect of pH on the activated field bean PPO was determined by pre-incubating the enzyme in the respective activating agents and then assaying the enzyme at different pH (Mcllvaine buffers pH 2.5-7.5) using 50 mM catechol as substrate. The pH at which the enzyme had optimal activity was also determined using ferulic acid (0.2 mM), caffeic acid (0.2 mM) and L-tyrosine (10 mM) as substrates in the presence of catechol as the activator.

2.2.8. pH stability

The stability of the native and activated isoforms of PPO at different pH were determined by incubating the enzyme at different pH, Mcllvaine (0.1 M citric acid-0.2 M Na_2HP0_4, pH 2.5-7.5) buffers, for different time intervals and assaying the residual activity in 50 mM Na acetate assay buffer, pH 4.0, for the native form, and 50 mM Na acetate assay buffer, pH 6.0, for the activated form. Catechol, 50 mM was used as the substrate in all the assays.

2.2.9. Effect of temperature

The optimum temperature of the PPO reaction was determined at various temperatures ranging from 4-75 °C, using 50 mM catechol as the substrate in 50 mM Na acetate buffer, pH 4.0.

2.2.10. Temperature stability

The field bean PPO was incubated at temperatures ranging from 4-75 °C in a water bath preset to the appropriate temperatures. Aliquots were removed at different time intervals and assayed for the residual

activity. The assays were carried out at room temperature using 50 mM catechol in 50 mM Na acetate buffer, pH 4.0.

2.2.11. Substrate specificity

Catechol (2.5-100 mM), 4-methyl catechol (0.25-20 mM), chlorogenic acid (0.5-50 mM), L-DOPA (2.5-12.5 mM), catechin (0.5-50 mM), caffeic acid (0.5-50 mM), protocatechuic acid (0.5-50 mM), protocatechuic aldehyde (0.5-50 mM), 2,3,4-trihydroxy benzoic acid (2.5-25), gallic acid (0.5-50 mM), tyrosine (0.5-50 mM), p-cresol (0.5-50 mM) and pyrogallol (2.5-50 mM), were used to monitor PPO activity. The rate of the reaction was measured in terms of the increase in absorbance at the absorption maxima of the corresponding quinone products for each substrate at 25 °C (Zhou et al., 1993). The values for K_m and V_{max} were obtained by evaluation of Lineweaver-Burk plots (Lineweaver and Burk, 1934) of kinetic measurements.

2.2.12. Effect of inhibitors

Tropolone (0.2-1 µM), potassium metabisulfite (2-10 µM), ascorbic acid (2-10 µM) and cysteine-HCl (5-50 mM) were evaluated for their effectiveness as inhibitors of PPO activity using catechol as the substrate. The enzyme (10-100 µg) was pre-incubated with 0.1 mL of the inhibitor in 0.9 mL of 50 mM Na acetate buffer pH 4.0, for 3 min in the sample cuvette. Reaction was started by adding the substrate to the reaction mixture, and the increase in absorbance at 420 nm for 3 min was recorded. The assays were repeated, by varying the inhibitor and substrate concentrations, keeping the enzyme concentration constant. The type of inhibition was deduced by analyzing the Lineweaver-Burk plots of 1/V vs 1/[S] at different inhibitor concentrations. The inhibitory constant K_t was determined from the Dixon plot (Dixon, 1942) where 1/V vs [I] were plotted at different substrate concentrations.

2.2.13. Polyacrylamide gel electrophoresis (PAGE)

Vertical slab gel electrophoresis was carried out on a Broviga mini slab gel electrophoresis unit, at room temperature.

2.2.13a. *SDS-polyacrylamide gel elecrophoresis (SDS-PAGE)*

SDS-PAGE at pH 8.3 was carried out according to the method of Laemmli (1970) in a discontinuous buffer system.

Reagents

A. Acrylamide (29.2 g) and bisacrylamaide (0.8 g) were dissolved in water (100 mL), filtered and stored in a dark brown bottle at 4 °C.

B. *Separating gel buffer.* Tris (18.15 g) was dissolved in water, the pH of the solution was adjusted to 8.8 with HC1 (6 N), solution made upto 100 mL and stored at 4 °C.

C. *Stacking gel buffer.* Tris (3 g) was dissolved in water, pH of the solution was adjusted to 6.8 with HC1 (6 N), made upto 100 mL in water and stored at 4 °C.

D. Sodium dodecyl sulfate (SDS, 10 g) was dissolved in water (100 mL).

E. Ammonium persulfate was freshly prepared by dissolving 50 mg in 0.5 mL of distilled water.

F. *Tank buffer.* Tris (0.3 g), glycine (1.44 g) and SDS (0.15 g) were dissolved in 150 mL of water.

G. *Staining solution:* Coomassie brilliant blue R-250 (0.2 g) was dissolved in a mixture of methanol: acetic acid: water (25:15:60 v/v). The reagent was filtered and stored at room temperature.

H. *Destaining solution:* Methanol: acetic acid: water (25: 15: 60, v/v)

I. Sample buffer was prepared in solution C diluted 1:4, containing SDS (4% w/v), ß mercaptoethanol (10% v/v), glycerol (20% v/v) and bromophenol blue (0.1% w/v).

Preparation of separating gel (7.5% T, 2.7% C)

Solution A	2.0 mL
Solution B	1.5 mL
Distilled water	2.4 mL
Solution D	0.06 mL
TEMED	0.01 mL
Solution E	0.03 mL

The contents were mixed, degassed and poured between the assembled glass plates with edges sealed with agar (2% w/v). The gels were layered with 0.5 mL of distilled water and allowed to polymerize at room temperature for 30 min.

Preparation of stacking gel (5% T, 2.7% C)

Solution A	0.83 mL
Solution C	1.25 mL
Distilled water	3 mL
Solution D	0.05 mL
TEMED	0.01 mL
Solution E	0.03 mL

The contents were mixed and poured above the polymerized seperating gel. The gels thus prepared were of the size 10.5 × 9 cm and thickness 0.8 mm.

Samples were prepared by dissolving protein (10-25 µg) in solution T (50 µL). The samples were heated in a boiling water bath for 5 min. Cooled samples were then loaded into the wells immersed in

solution 'F' (tank buffer) and were run at constant voltage (60 V) for 3-4 h or until the tracking dye, bromophenol blue was just (0.5 cm) above the lower end of the gel. Medium range protein M_r markers, phosphorylase b (97.4 kDa), BSA (66.3 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa), soyabean trypsin inhibitor (20.0 kDa) and lysozyme (14.3 kDa) were used. The markers were supplied as a solution having each protein at a concentration of 0.5 to 0.8 mg/mL. The markers were diluted 1:1 with solution T and boiled prior to use.

Staining: The gels were stained for protein with reagent 'G' for 6 h at room temperature followed by destaining in reagent ´H'

2.2.13b. ***Native PAGE (Non-denaturing gel for acidic proteins)***

Polyacrylamide gel electrophoresis under native conditions was carried out to evaluate the purity of PPO and also to check for the presence of isoforms if any. Separating gels (7.5% T, 2.7% C) were prepared as described earlier (section 2.2.13a) by mixing solutions 'A' (1.5 mL) and 'B' (1.5 mL) with water (2.9 mL) without the addition of SDS.

Stacking gels (5% T, 2.7% C) was prepared similar to that for SDS-PAGE excluding SDS from the gel.

Sample buffer (solution I) was prepared minus SDS and ß-mercaptoethanol. Tank buffer was also prepared just as in SDS-PAGE minus SDS. About 5-20 µg of protein was dissolved in 20 µL of the sample buffer and layered on the gel. The gel apparatus was connected to the power supply with the lower electrode connected to the positive power supply lead. After the electrophoretic run, proteins were visualized using Coomassie brilliant blue R-250.

PPO activity staining: Staining of PPO in gels was performed following the procedure of Lee (1991). Following electrophoresis, the gel was

immersed in 50 mM Na acetate buffer, pH 4.0 for 5 min. The gel was then stained in the same buffer containing 50 mM catechol and 0.05% p-phenylenediamine until the protein became clearly visible. The gel was rinsed with water, immersed in a 1 mM ascorbic acid solution for 5 min, to stop the reaction, soaked overnight in distilled water and stored in 30% ethanol. The PPO activity bands appear as dark brown bands against a clear background.

2.2.13c. *Acid PAGE {Non-denaturing gel for basic proteins)*

Non-denaturing gels for basic proteins were prepared by a modified procedure of Reisfeld et al. (1962). All the solutions were similar to that of the electrophoresis of acidic proteins except for solutions 'B', 'C and I.

B. Potassium acetate was dissolved in water, the pH of the solution was adjusted to 4.3 with acetic acid, made upto 100 mL in water and stored at 4 °C.

C. Potassium acetate was dissolved in water, pH of the solution was adjusted to 6.8 with acetic acid, made upto 100 mL in water and stored at 4 °C.

I. Sample buffer was prepared in solution C (diluted 1:4), containing glycerol (20% v/v) and bromocresol green (0.1% w/v).

Separating gels 7.5% T, 2.7% C were prepared as described earlier (section 2.2.13b) substituting Ά' (1.5 mL) and 'B' (1.5 mL) with water (2.9 mL). TEMED 50 µL and solution Έ' 150 µL was added. The gel was layered with buffer saturated n-butanol.

Stacking gel (5% T, 2.7% C) was prepared similar to that for native PAGE for acidic proteins, substituting the solution 'C'.

Tank buffer was prepared by dissolving 1.42 g β-alanine in water, pH adjusted to 4.5 with glacial acetic acid and made upto 100 mL. About 5-20 fag of protein was layered on the gel, after diluting 1:1 in sample buffer. The gel apparatus was connected to the power supply with the lower electrode connected to the negative power supply lead. After the electrophoretic run, proteins were visualized using coomassie brilliant blue R-250. PPO activity was detected by incubating the gel for 15 min in 50 mM Na acetate buffer, pH 4.0 containing 50 mM catechol and 0.5% p-phenylenediamine.

2.2.14. Capillary electrophoresis

Purified PPO was electrophoresed on a Prince Technologies capillary electrophoresis system equipped with a coated capillary (Prince Technologies B. V., The Netherlands) (id=75 µm, length =100 cm), at 28 °C by applying a voltage of 30 kV. The running buffer was 25 mM Tris-192 mM glycine (pH 8.3) or 50 mM Na acetate (pH 4.0). Prior to analysis, the capillary was flushed for 2 min with running buffer. The samples (5 µg protein in the running buffer) were injected at 20 mBar pressure for 10 sec. The PPO was detected at 280 nm with an online lambda 1010 Bishoff detector set at 280 nm.

2.2.15. Molecular weight determination

The apparent M_r of the native enzyme was determined by analytical HPLC gel filtration. The Shimadzu LC-6A chromatography system, which was used, consisted of two separate solvent delivery systems with a system controller (SCL-6A), UV-Vis spectrophotometric detector (SPD-6AV) and data processor (C-R4A). The column used was a Progel™-TSK G2000 SWXL, (7.8 mm idx30 cm) and the eluent was 0.1 M NaPi pH 7.0 containing 0.1 M sodium sulfate, at a flow rate of 1 mL/ min. The proteins were detected at 230 nm. The column was calibrated

using thyroglobulin (660 kDa), ferritin (450 kDa), aldolase (158 kDa), BSA (66.3 kDa), ovalbumin (43.5 kDa), p-lactoglobulin (36.8 kDa), chymotrypsinogen (25.7 kDa), carbonic anhydrase (25.0 kDa), myoglobin (16.9 kDa) and ribonuclease (13.7 kDa). Hydrodynamic dimensions (Stokes radius, R_s) in different conformational states of field bean PPO were also measured by gel filtration on Progel™-TSK G2000 SWXL column. A set of globular proteins with known R_s values was used (Uversky, 1993). The gel filtration column was calibrated in terms of 1000/Ve vs R_s dependence, where V_e is the elution volume of the given protein (Uversky, 1993, Ackers, 1970).

The apparent M_r was estimated using a calibrated Sephadex G-100 column according to the method of Andrews (1964). The column was calibrated using ferritin (450 kDa), catalase (232 kDa), aldolase (158 kDa), BSA (66.3 kDa), ovalbumin (43.5 kDa) and chymotrypsinogen (25.7 kDa).

2.2.16. Preparation of polyclonal antisera

A control serum was prepared from the blood drawn from the marginal ear vein of a New Zealand white rabbit, prior to the immunization. For the primary immunization, a suitable water-in-oil emulsion was prepared by thoroughly mixing 1 mL of Freund's complete adjuvant and 1 mL of antigen solution containing 300 µg of purified field bean PPO. The emulsion was injected intradermally into the rabbit at several sites. On the 21st day, a booster dose of 150 µg of the same antigen emulsified in Freund's incomplete adjuvant was administered intradermally. This was followed by a second booster dose of 150 µg of the antigen after another 20 days. Thirty days after the last injection, the rabbit was bled from the marginal ear vein. Blood (20 mL) was allowed to clot at 4 °C overnight and serum collected by low speed centrifugation and stored at -10 °C.

2.2.17. Electroblotting of PPO

The PVDF membrane cut to the required size (slightly larger than the gel) was soaked in methanol for 5 min before use. The membranes were then immersed in transfer buffer (10 mM CAPS pH 11.0 containing 8% methanol) and equilibrated for 10 min. (Matsudaira 1987, Speicher 1989).

Following electrophoresis, the gel was immediately rinsed in transfer buffer for 15 min. Semi-dry electroblotting was carried out using a Novoblot apparatus (Pharmacia) (Towbin et al., 1979). The surface of the bottom of the graphite electrode was moistened with transfer buffer. Six strips of Whatman no. 3 filter paper were placed on the electrode. The size of the Whatman no. 3 filter paper strips was ^{1}A cm longer on each side of the membrane. The activated PVDF membrane was placed on the moistened Whatman strips. The gel was carefully placed on the membrane. Another 6 strips of Whatman no. 3 filter paper cut as before and soaked in transfer buffer were placed on the gel. During these manipulations air bubbles between the layers were avoided, and the bubbles, if present were removed by rolling a glass rod. The gel was at the centre of the apparatus where the electrical field was uniform. The gel was placed at the cathodic side and PVDF at the anodic side of the electrode. The upper electrode was connected to the power supply and the transfer was carried out for 120 min using a current of 0.8 mA/cm^2 of the filter paper.

Staining of the electroblotted protein on the PVDF membrane

a) Staining solution: 0.5% Coomassie blue R-250 in 50% methanol

b) Destaining solution: 50% methanol

The PVDF membrane was immersed in staining solution for 4 h and later destained with solution 'b'

For amino acid analysis and amino-terminal sequencing, the PPO bands were cut out from the membrane, destained in 70% methanol, dried and used.

2.2.18. Extraction of PPO from other plant sources

Crude PPO was extracted from different fruits, vegetables and defatted legume powders. Apple *(Pyrus malus),* pear *(Pyrus communis),* potato *(Solanum tuberosum)* and brinjal *(Solarium melangena)* were deskinned and minced into small pieces. Ten g of each was homogenized in 50 mL of 100 raM Tris-HCl buffer, pH 7.0 containing 2% PVPP (w/v) and 1.2% NaCl (w/v) in a Waring blender. Acetone powder prepared from raw banana *(Musa paradisiaca)* was extracted with the above buffer for 6 h. Each of the extracts were filtered through a cheese-cloth and then centrifuged at 8500 *g* for 30 min in a Beckman GS-15R centrifuge. The pellet was discarded and the supernatant was used as the crude extract. Mushroom tyrosinase used was obtained from Sigma Chemical Co.

Ten grams each of dehulled, powdered and defatted chick pea *(Cicer arietinum),* mung bean *(Vigna radiata),* horse gram *(Dolichos biflorus),* pigeon pea *(Cajanas cajan),* soybean *(Glycine max)* and field bean *(Dolichos lablab)* were extracted for 6 h with 50 mL of of 100 mM Tris-HCl buffer, pH 7.0 containing 2% PVPP (w/v) and 1.2% NaCl (w/v). The extracts were centrifuged at 8500 *g* for 30 min. The pellet was discarded and the supernatant was used as the crude PPO preparation for dot blot analysis.

2.2.19. Dot-blot analysis

Approximately 5-40 μ*g* of the protein was immobilized on a marked spot on a nitrocellulose membrane. Applications were repeated,

employing a current of dry air to accelerate the drying, until the required amount was spotted.

Immunodetection of PPO: Following immobilization, the nitrocellulose membrane was washed several times in immunoblot buffer (5% skim milk powder in phosphate buffered saline, pH 7.0). The membrane was incubated overnight in immunoblot buffer containing antibodies raised against field bean PPO (1:1000 dilution). After repeated washes in the immunoblot buffer, the membrane was incubated with alkaline phosphatase conjugated goat anti-rabbit immunoglobulins for 1-2 h at room temperature. After several washes in immunoblot buffer and finally in substrate buffer (100 mM Tris, 0.5 M NaCl, 5 raM $MgCl_2$, pH 9.5), the alkaline phosphatase activity was detected with a mixture of BCIP and NBT in substrate buffer. After the color developed, washing the blot in distilled water stopped the reaction.

2.2.20. Amino acid analysis

Amino acid analysis was performed according to the method of Bidlingmeyer et al. (1984), using a Waters Associate Pico-Tag amino acid analysis system.

Hydrolysis of protein: An aliquot of 20 µ*L* of purified protein (-20 *xg)* was pipetted into a tube (6 x 50 mm Pyrex™) and placed in the special vacuum vial. The vial was then attached to the Waters Associates Picotag Work station manifold and the samples were dried under vacuum to 50-60 mtorr. After drying, the vacuum was released and 200 µL of constant boiling HC1 (6 N) containing phenol (1% v/v) was pipetted into the bottom of the vacuum vial. The vacuum vial was again reattached to the manifold, evacuated and sealed under vacuum. Samples were hydrolyzed in the workstation at 110 °C for 24 h. After hydrolysis, residual HC1 inside the vacuum vial was removed under vacuum.

Standard free amino acids as a mixture (Pierce H) containing upto 25 nmol of each amino acid were placed in the tubes (6x50 mm) and dried under vacuum. Free amino acids and hydrolyzed samples were dried down again under vacuum after adding a re-drying mixture (10-20 µ*L)* containing ethanol:water:triethylamine (2:2:1) to each tube. When the vacuum reached 50-60 mtorr, the samples were ready for derivatization.

The derivatization agent was made fresh each time and consisted of ethanol:triethylamine:water:PITC (7:1:1:1). The PITC was stored at -20 °C under nitrogen to prevent the formation of break down products. To make 300 |.iL of reagent, sufficient for 12 samples, 210 (.iL of ethanol was mixed thoroughly with 30 uL each of PITC, TEA and water. PTC amino acids were formed by adding 20 µ*L* of the reagent to the dried samples and sealing them in the vacuum vials for 20 min at room temperature. The excess reagents were then removed under vacuum using the workstation. When the vacuum reached 50-60 mtorr, the samples were ready for analysis by RP-HPLC.

Chromatography: The HPLC was carried out using a Shimadzu LC-6A chromatography system consisting of two separate solvent delivery-systems with system controller (SCL-6A), UV-Vis spectrophotometric detector (SPD-6AV) and data processor (C-R4A). The temperature was controlled at 38 °C±1 °C with a column heater. Samples were injected in volumes ranging from 5-50 (µL. The column was an application specific Pico Tag column (150 x3.9 mm).

The solvent system consisted of two eluents, (A) an aqueous buffer (0.14 M Na acetate containing 0.5 mL/L TEA, titrated to pH 6.4 with glacial acetic acid):acetonitrile (94:6) and (B) 60% acetonitrile in water. The gradient run for the separation consisted of 0% B traversing

to 46% B in 10 min. After the run, a washing step in 100% B is included so that the residual sample components are removed.

Table 2.1: **The gradient programme for amino acid analysis**

Time (min)	Flow (mL)	%A	%B	Curve
Initial	1.0	100	0	-
10	1.0	54	46	-2
11	1.0	0	100	0
13	1.0	0	100	0
14	1.0	100	0	0
25	1.0	100	0	0

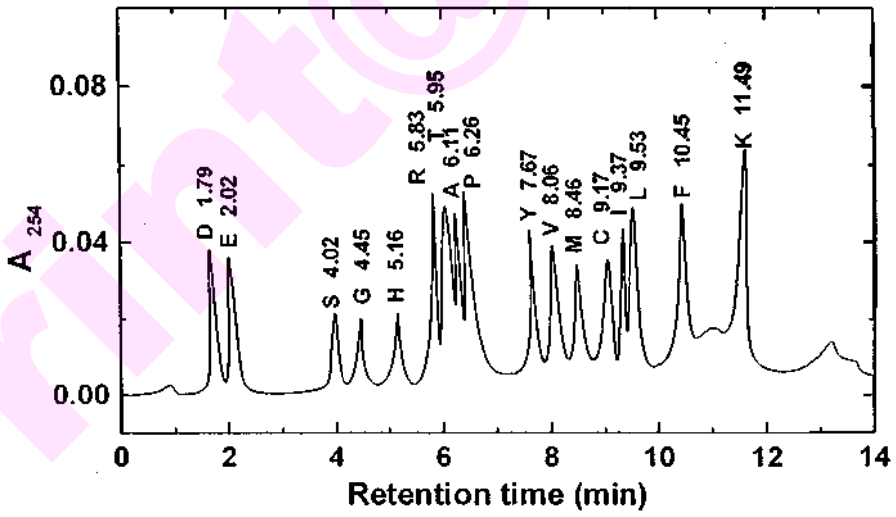


Fig 2.2: **Elution profile of PTC-amino acids with retention times, using a Pico-Tag amino acid analysis system.** The amino acids are represented by the single letter code. Column: Pico-Tag™ (4 |j.m, 150x3.9 mm); flow rate: 1.0 mL/min; wave length: 254 nm; temperature: 38 °C; injection volume: 5 \x*L* (312.5 pmole of each amino acid); solvent A: 140 mM Na acetate, 3.6 mM triethylamine (TEA), pH 6.4/acetonitrile (94/6); solvent B: 60% acetonitrile.

The PTC amino acids were detected at 254 nm. The gradient elution programme is shown in Table 2.1. The separation of a standard amino acid mixture is shown in Fig 2.2.

2.2.21. Automated gas phase protein sequencing

Automated gas phase sequencing was carried out on the protein sequenator PSQ-1, (Shimadzu). This sequenator carries out Edman degradation by supplying gaseous reagents for the coupling and cleavage reactions. A flow diagram of the steps involved in automated sequencing is shown in Fig 2.3. The protein or peptide, 100 pmol , was spotted on a glass fiber disc previously coated with polybrene and washed for 3 cycles. Alternatively, the cut portion of the PVDF membrane which contains the electrotransferred protein band is directly placed on the glass fiber disc. The coupling reaction is carried out with phenyl isothiocyanate (Rl) in the presence of gaseous trimethylamine (R2). Excess reagents and by-products are washed with n-heptane (SI) and ethyl acetate (S2). The cleavage reaction is carried out with gaseous TFA to form an anilino thiazolinone (ATZ) derivative. Both the coupling and cleavage reactions are performed in a temperature controlled reaction chamber. The free ATZ-amino acid is extracted to the conversion flask by n-butyl chloride (S3). The ATZ-amino acid is converted to the more stable PTH-amino acid by reaction with 25% TFA (R4). The PTH-amino acid is dissolved in acetonitrile (S4) and automatically injected into the HPLC. The residual PTH-amino acid is collected in a sample tube by a fraction collector. The PTH-amino acids are separated by RP-HPLC. Fig 2.4 depicts the separation of the PTH amino acids using an isocratic elution. The PTH-amino acid in each cycle is identified, quantified and recovery percentage calculated using an online CR4A system. The results are displayed and recorded.

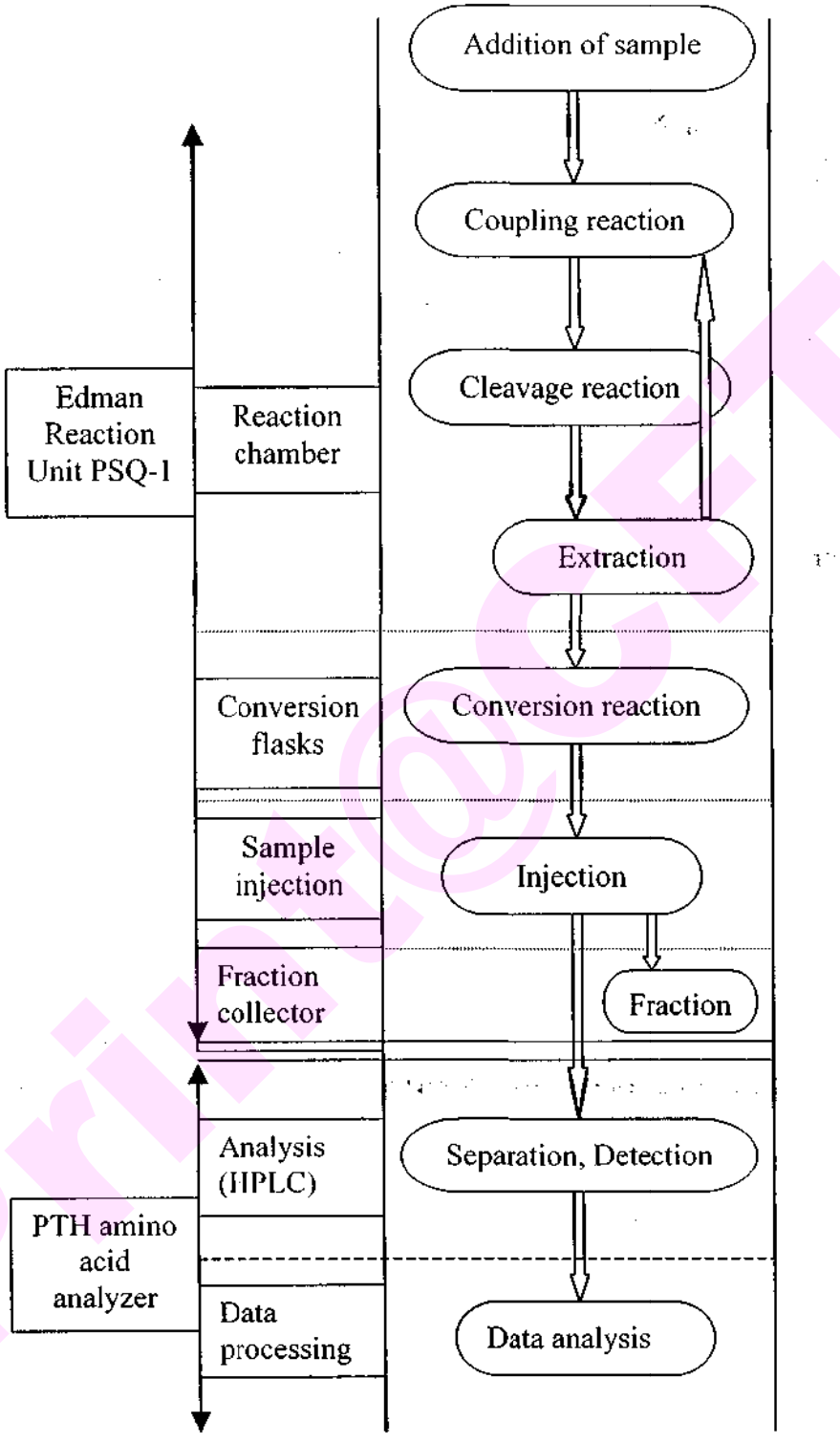


Fig 2.3. **Flow diagram of the reactions that occur during the gas phase sequencing on a PSQ-1 (Shimadzu) sequenator.**

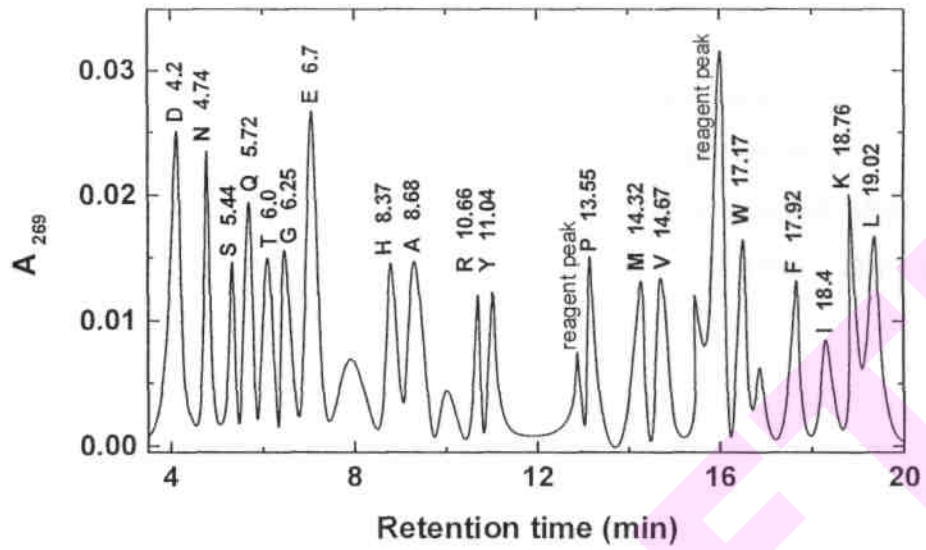


Fig **2.4. RP-HPLC seperation of PTH-amino acid standards on the automated protein sequenator, PSQ-1.**

2.2.22. Isolation of a PPO inhibitor from unripe sapota *(Achras zapota)* fruit

Fruits of uniform maturity, 2 months from fruit set, were collected from the Institute Orchard from marked trees. The young fruits were de-skinned, seeds removed and minced into small pieces. Fifty grams of the minced tissue was macerated in a Waring blender, adding 50 mL of 10 mM NaPi pH 7.0. The extract was centifuged at 8500 *g* at 4 °C and the clear supernatant was used as the crude extract. The crude extract was dialyzed extensively against water (4 × 1000 mL). The dialyzed extract was freeze dried in a Virtis freeze mobile 12 lyophilizer, at -60 °C and 15 mtorr vacuum. The dry flesh colored powder was stored at 4 °C and used for further studies.

2.2.23. Purification of proanthocyanidins (PA)

Fifteen grams of Sephadex G-50 dry powder (40-120 μm) was allowed to swell in 500 mL of 10 mM NaPi, pH 7.0 for 5 h. After three changes of the buffer, the slurry was packed into a glass column

(100 x 2 cm) at a flow rate of 32 mL/h. One hundred milligram of the lyophilized powder of the crude extract was dissolved in lmL of 10 mM NaPi buffer pH 7.0 and loaded onto the Sephadex G-50 column and eluted with the same buffer at a flow rate of 18 mL/h. Fractions of 3 mL were collected. The active fractions were pooled, dialyzed against water, freeze-dried and dissolved in 50% methanol. This sample was subjected to Sephadex LH-20 chromatography.

Five grams of Sephadex LH-20 (25-100 um) was swollen in 200 mL of 50% methanol for 24 h and equilibrated in the same solvent with three changes. The gel was packed into a glass column of dimensions (20 x 1 cm) and equilibrated in 50% methanol. The sample was loaded to the column in the same solvent. Unbound materials were removed by washing the column with aqueous methanol (40%). The condensed tannins were eluted with 70% aqueous acetone (Jones, 1976) at a flow rate of 12 mL/h and fractions of 2 mL were collected. The active fractions obtained from Sephadex LH-20 column were pooled and after removal of acetone by dialysis, was concentrated and used.

2.2.24. PPO inhibitor assay

The effect of the sapota inhibitor on the enzyme was determined spectrophotometrically. Since the mode of inhibition depends on both the substrate and the inhibitor concentration, inhibition of diphenolase activity observed was in relation to catechol as the substrate and the inhibition of monophenolase activity was based on the oxidation of the monophenol, using ferulic acid as the substrate in the presence of catechol as the activator.

Inhibition of diphenolase activity: The PPO assay was performed as previously described (Section 2.2.3). For studying the effect of the inhibitor, the assay was performed by adding the inhibitor

(120-320 µg) to the assay buffer (50 mM Na acetate, pH 4.0) containing the enzyme. After 3 min incubation, the reaction was started by the addition of the substrate, and the increase in absorbance at 420 nm was monitored. Inhibitory activity was calculated as the difference in the PPO activity in the presence and the absence of the inhibitor.

Inhibition of monophenolase activity: The assay for monophenolase activity was performed using ferulic acid as described in Section 2.2.3, in the presence of catechol as co-substrate. The enzyme was preincubated with 50-280 µg of the inhibitor in the assay buffer (50 mM Na acetate pH 4.5) for 3 min prior to the assay. Reaction was started by adding the substrate, ferulic acid and co-substrate catechol (10 mM) into the reaction mixture, and the increase in absorbance at 480 nm for 3 min was recorded.

2.2.25. Trichloroacetic acid (TCA) precipitation

Crude extract (10 mg/mL, section 2.2.22) was treated with 10% TCA and kept at 4 °C for 10 min. The treated sample was centrifuged at 10,000 *g* at 4 °C for 10 min to remove the precipitated protein.

2.2.26. Trypsin digestion

Trypsin (0.5 mg/mL) was prepared in 1 mM HC1. The crude extract (10 mg) was dissolved in 1 mL of 10 mM NaPi pH 7.0 and was incubated with trypsin (1% w/w) at 37 °C for 24 h. The reaction was stopped by adding 50 µL of 98% formic acid to reduce the pH.

2.2.27. Estimation of PA

PA content was determined spectrophotometrically. The anthocyanidins produced by heating 0.5 mL of the sample with 3 mL of 5% n-butanolic-HCl for 2 h at 90-95 °C were measured at 550 nm. In the absence of precise information regarding the degree of

polymerization of PAs, the value of $E^{1\%}$ is assumed to be 150 for the hydrolyzed product (Swain and Hillis, 1959; Bate-Smith, 1973)

2.2.28. Two-Dimensional paper chromatography of PA

The fractions under investigation were spotted (25 µL) at a distance of 2 cm from both the edges of the lower left hand corner of a square (25.5 × 25.5 cm) Whatman no. 1 filter paper. 2-D chromatograms of PA fractions were obtained by downward development of the chromatograms, first in butanol:acetic acid:water (6:1:2) and then in 2% aqueous acetic acid in the second direction (Jones et al., 1976). The chromatograms were developed in vanillin-HCl (5% vanillin in 12 N HC1), as recommended by Hath way (1958) and Swain and Hillis (1959). The PA appeared as bright red spots at the origin.

2.2.29. Identification of the monomers

The hydrolysis of PA to monomers was carried out as described by Bate-Smith (1954). Twenty milligrams of the PA in 5% butanolic-HCl was heated in a boiling water bath for 20 min. The hydrolyzate was subjected to ascending chromatography on Whatman no. 3 filter paper in Forestal solvent consisting of water:acetic acid:conc. HC1 (10:30:3) (Bate-Smith, 1954) to separate the different anthocyanidins. The anthocyanidins were identified by their color and retention times.

RP-HPLC of the hydrolyzed condensed tannins was also carried out on a Phenomenex C_8 column (250 × 4.6 mm) using water:acetic acid:methanol (71:10:19) as the solvent (Wilkinson et al., 1977). The detector was set at 530 nm and the flow rate was 1.5 mL/min.

2.2.30. Molecular weight determination of PA

The purity and $M_Г$ of the PA was determined by HPLC on a Zorbax GF-250 gel filtration column using 0.1 M NaPi buffer at a flow rate of 2 mL/min and were detected at 280 nm. The standards used for determination of $M_г$ were ribonuclease (13.7 kDa), chymotrypsinogen (25 kDa), ovalbumin (43 kDa) and BSA (66.3 kDa).

2.2 31. pH stability of PA

Stability of the PA at different pHs was studied by incubating the purified PA in different pH buffers (glycine-HCl, pH 2.5-3.5), Na acetate (pH 3.5-6.0) and NaPi (pH 6.0-8.0) and determining the residual inhibitory activity and PA content after 24 h.

2.2.32. Temperature stability of PA

The PA inhibitor at different pHs was incubated at different temperatures ranging from 20-100 °C for a period of 1 h. Aliquots were withdrawn at different time intervals and checked for residual inhibitory activity against PPO as well as for PA content.

Chapter 3
Purification and characterization of a polyphenol oxidase from the seeds of field bean *[Dolichos lablab)*

In this laboratory, during the affinity purification of a glucose-mannose-specific lectin from field bean *{Dolichos lablab)* seeds (Gowda et al., 1994), severe browning of the extracts was observed. Preliminary investigations revealed the presence of a single PPO, in crude extracts of field bean seeds. The problem of purification of PPOs from higher plants is compounded by the presence of multiple isoforms. The single PPO form in field bean seeds is ideally suited for structural characterization and X-ray crystallography studies. As a primary step to understand the structure, regulation and function of seed PPO, the isolation and characterization of a PPO from field bean seeds was carried out. In this chapter the results on the purification and characterization of the single form of PPO from field bean seeds and its molecular properties are presented and discussed.

RESULTS

Extraction and Purification

Plant PPOs have been located in a variety of cell fractions, in organelles, where it may be tightly bound to the membranes, and also in the soluble fractions of the cell. The strength of this binding appears to vary depending on the tissue and the stage of development of the plant. In many cases, drastic conditions are required for the solubilization of membrane bound PPO. Extraction of field bean PPO was done in different pH buffers to determine the optimal extraction conditions. Buffers of pH 2.5 (glycine-HCl, 100 mM), pH 4.0 (Na acetate, 100 mM), pH 6.0 (Na acetate, 100 mM), pH 7.0 (Tris-HCl, 100 mM) and pH 8.0 (Tris-HCl, 100 mM) were used for extraction. The total enzyme activity extracted by these buffers was almost similar. However the specific activity of the enzyme extracted in pH 7.0 buffer was the highest (Table 3.1).

Table 3.1. **Extraction of field bean PPO under different conditions.***

Extraction buffer	Total activity $\times 10^4$ (U)	Total protein (mg)	Specific activity (U/mg)
Glycine-HCl, pH 2.5	33	340	970
Na acetate, pH 4.0	34	227	1495
Na acetate, pH 6.0	34.2	181	1892
Tris-HCl, pH 7.0	34.5	170	2029
Tris-HCl, pH 8.0	28	180	1555
0.1% SDS	34.9	270	1292
0.1% Triton X-100	35.3	290	1217
8% Triton X-114 (aqueous phase)	30.3	158	1917
8% Triton X-114 (detergent phase)	6	111	540

* Three grams of defatted field bean powder was used as the starting material. All the buffers (15 mL) were used in 100 mM concentration. Extraction buffers contained 2% PVPP (w/v). Buffers containing SDS, Triton X-100 and Triton X-114 were prepared in 100 mM Tris-HCl, pH 7.0.

Detergents have been commonly employed for the solubilization and isolation of PPO. Digitonin (Alberghina, 1964), Triton X-100 (Harel et al., 1964; Ngalani et al., 1993; Zhou et al., 1993), SDS (Ben-Shalom et al., 1977) and more recently Triton X-114 has been used for the extraction of PPO from several sources (Sanchez Ferrer et al., 1993a; Espin et al., 1995b; Sojo et al., 1998; Espin et al., 1997a). Field bean PPO was extracted in the presence of detergents, Triton X-100 and SDS at 0.1% concentration in 100 mM Tris-HCl buffer, pH 7.0. Although the total PPO activity extracted in the presence and absence of detergents remained the same (Table 3.1), the specific activity of the extracted

enzyme was higher in the absence of the detergent. Therefore, field bean PPO appears not to require detergents for solubilization.

Temperature induced phase partitioning in the presence of Triton X-114 has been used to extract PPO from several sources (Sanchez-Ferrer et al. a&b; Espin et al., 1993a; Sojo et al., 1998a). The extraction was carried out in 100 mM Tris-HCl buffer, pH 7.0 containing 8% Triton X-114. The aqueous and detergent phases were separated by centrifugation at 4 °C. The aqueous phase was found to contain 80% of the total activity whereas the detergent phase contained 20% of the total activity. The specific activity of the aqueous phase 1917 U/mg was lower than the enzyme extracted in buffer alone. Therefore extraction of PPO from the defatted flour for further purification studies was carried out using 100 mM Tris-HCl buffer, pH 7.0.

PVPP was added to the extraction medium to adsorb the endogenous phenols present in the crude extract and prevent irreversible binding of phenols with proteins. At very high protein concentrations, field bean PPO exhibited a tendency to associate in solution which dissociated upon addition of 1.2% NaCl (w/v) with the regain of complete activity. Hence 1.2% NaCl was included in all the buffers used for extraction and purification.

After extraction, $(NH_4)_2S0_4$ precipitation was investigated to partially remove inactive proteins. To the crude extract, solid $(NH_4)_2S0_4$ was added slowly to obtain varying levels of saturation at 4 °C and allowed to stand for 2 h at 4 °C. The precipitated protein was removed by centrifugation at 8500 *g* for 30 min at 4 °C. Both the supernatant and precipitates were assayed for PPO activity. The 0-40% precipitate contained 24% of the total activity but the specific activity was 2 fold less than the crude. The 40-60% precipitate contained 55% of the total activity with a 2.2 fold increase in specific activity whereas the 60-80%

precipitate contained 19.7% of the total activity with a 1.8 fold increase in the specific activity. No activity was detected in the supernatant. Hence a 40-80% ammonium sulfate fractionation was used as the next step of purification. The precipitate obtained by saturating the crude extract to 40-80% exhibited the maximum PPO activity and also allowed a ~2 fold purification.

To the crude extract, solid $(NH_4)_2S0_4$ (22.6 g/100 raL) was added to obtain 40% saturation at 4 °C. The precipitated protein was removed by centrifugation at 8500 *g* for 30 min and discarded. Solid $(NH_4)_2S0_4$ (25.8 g/100 mL) was added slowly to the supernatant at 4 °C and allowed to stand overnight. The precipitate thus obtained was re-dissolved in 10 mM Tris-HCl buffer, pH 8.2 containing 1.2% NaCl (w/v) and dialyzed against the same buffer (3x500 mL).

Anion exchange chromatography

The 40-80% $(NH_4)_2S0_4$ precipitate after extensive dialysis against the equilibration buffer, was subjected to anion-exchange chromatography on DEAE-Sephacel which was effective in removing inactive compounds. The dialyzed solution was loaded onto a DEAE-Sephacel column (12 x 3.5 cm) previously equilibrated with 10 mM Tris-HCl buffer, pH 8.2 containing 1.2% NaCl (w/v). The column was developed in the same buffer at a flow rate of 35 mL/h. The PPO did not bind to DEAE-Sephacel at this pH and eluted as a single peak unbound to the ion-exchange matrix, in the column wash (Fig 3.1). The fractions that exhibited activity were pooled. The pooled fractions (Fig 3.1) had a specific activity of 11,556 U/mg. A purification of 5.7 fold was obtained in this step with an 88% yield from the previous step.

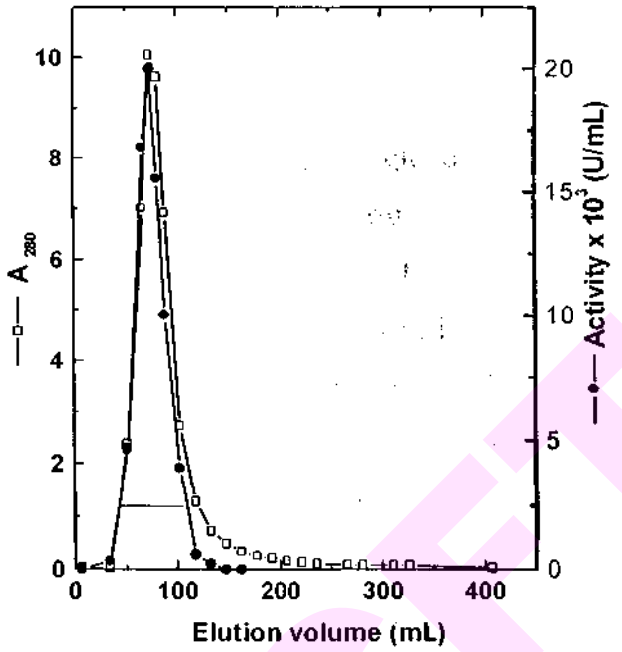


Fig **3.1. DEAE-Sephacel chromatography elution profile of field bean seed PPO.** The dialyzed fraction of ammonium sulfate precipitation was loaded on a DEAE-Sephacel column (12 × 3.5 cm) equilibrated with 10 mM Tris-HCl, pH 8.2 containing 1.2% NaCl (w/v). Elution was carried out in the same buffer at a flow rate of 35 mL/h. Fractions of 3 mL were collected and active PPO fractions were pooled as indicated (—).

Hydrophobic interaction chromatography

The PPO was further purified by including hydrophobic interaction chromatography on Phenyl Agarose as the next step. Phenyl Sepharose CL-4B chromatography has been successfully used for purification of PPO from various plant sources (Wisseman and Lee, 1980; Janovitz-Klapp et al., 1989; Zhou et al., 1993; Das et al., 1997). The pH of the pooled fractions obtained from the DEAE-Sephacel fractionation was adjusted to 7.0 and solid $(NH_4)_2S0_4$ added to a final concentration of 1 M and loaded onto a Phenyl Agarose column (11.5 × 3.5 cm), previously equilibrated with 25 mM Tris-HCl buffer, pH 7.0 containing 1 M $(NH_4)_2S0_4$ and 1.2% NaCl (w/v) and washed with the same buffer. In this step, an inactive protein eluted in the void volume. The bound PPO was eluted using the same buffer minus the $(NH_4)_2S0_4$.

The elution profile of field bean PPO from Phenyl Agarose is presented in Fig 3.2.

All the PPO activity eluted as a single symmetrical peak with a low ionic strength elution buffer, containing no $(NH_4)_2S0_4$, with a 3 fold increase in specific activity. Seventy percent of the total activity loaded on the Phenyl Agarose column was recovered (Table 3.2). The protein fractions containing PPO were appropriately combined and precipitated by 80% $(NH_4)_2S0_4$ fractionation.

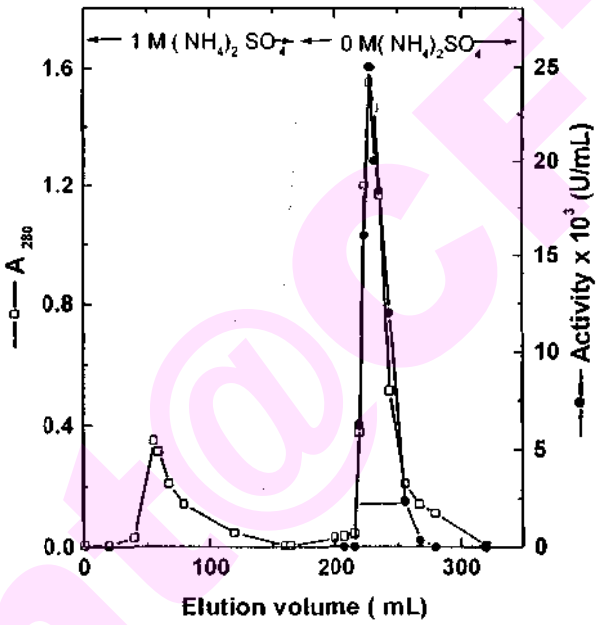


Fig 3.2. **Phenyl Agarose elution profile of field bean seed PPO.** The active PPO fraction obtained from the DEAE-Sephacel column was loaded on a Phenyl Agarose column (11.5 x 3.5 cm) equilibrated with 25 mM Tris-HCl containing 1.2% NaCl (w/v) and 1 M $(NH_4)2SC>4$. The PPO was eluted with the same buffer minus the $(NH_4)2S04$ at a flow rate of 40 mL/h. Fractions of 3 mL were collected and the active fractions were pooled as shown (—).

Size exclusion chromatography

Size exclusion chromatography, which separates molecules, based on molecular size and useful in desalting was used as the next step. The 80% precipitate was loaded onto a Sephadex G-100 column

(100 x 2 cm) equilibrated in 25 mM Tris-HCl buffer pH 7.0 containing 1.2% NaCl (w/v). Elution of the sample was carried out at a flow rate of 8 mL/h. The PPO eluted as a single peak on the descending shoulder of the major protein peak (Fig 3.3). A 68% recovery of the activity and a 2.2 fold increase in the specific activity from the previous step was observed. The final recovery of PPO was -30%, after a 34 fold purification, with a specific activity of 68,077 U/mg (Table 3.2).

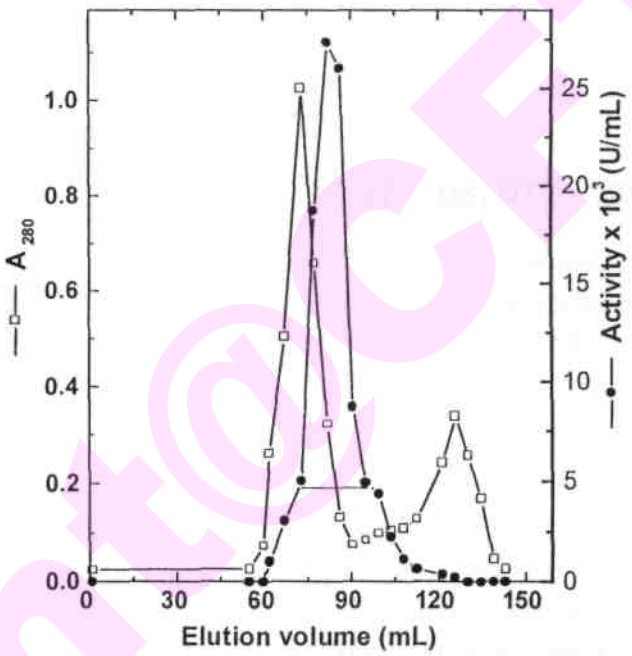


Fig **3.3. Sephadex G-100 chromatography elution profile of field bean seed PPO.** The 80% ammonium sulfate precipitate of Phenyl Agarose pool was dissolved in 25 mM Tris-HCl, pH 7.0 containing 1.2% NaCl (w/v) and loaded onto a Sephadex G-100 column (100 × 2 cm). The column was developed with the same buffer. Fractions of 2 mL were collected at a flow rate of 8 mL/h. The active fractions were pooled as shown (—).

The field bean seed PPO was purified to apparent homogeneity after three steps of purification by column chromatography. The results of the purification are summarized in Table 3.2. The pure PPO was stored at 4 °C, and used for further studies.

Table 3.2. **Purification of field bean seed PPO'**

Purification step	Total activity $\times 10^5$ (U)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Fold Purification
Crude extract	24.3	1207	2013	100	-
$(NH_4)_2SO_4$ (40-80%)	17.7	431	4176	72.8	2.1
DEAE-Sephacel Chromatography	15.6	135	11, 556	64.2	5.7
Phenyl Agarose Chromatography	10.9	36.5	29, 863	45	14.8
Size exclusion chromatography (Sephadex G-100)	7.4	10.87	68, 077	30.5	33.8

* These are the results of a typical purification starting from 20 g of defatted field bean flour. These values were reproduced in five separate purifications.

Criteria of Homogeneity.

The homogeneity of the purified protein was assessed by native PAGE, acid PAGE, HPLC gel filtration and capillary electrophoresis. In the native PAGE, the purified enzyme was electrophoresed in a 7.5% polyacrylamide gel in Tris-glycine buffer pH 8.3 and located by enzyme activity staining. The purified PPO revealed a single band (Fig 3.4, lane a) both by protein staining using Coomassie brilliant blue R-250 and by specific enzyme staining with catechol (Fig 3.4, lane b), indicating the presence of a single isoform and also the homogenous nature of the purified PPO. The pure enzyme also migrated as a single species in acid-PAGE when electrophoresed in 7.5% polyacrylamide gel in ß-alanine-acetic acid buffer pH 4.5 indicating its homogeneity (Fig 3.5). The diffused bands of field bean seed PPO is due to the general glycoprotein nature of PPOs.

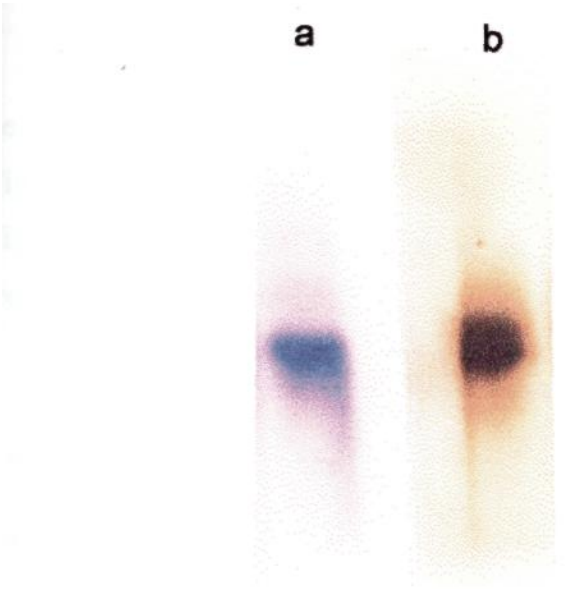


Fig 3.4. **Native-PAGE (7.5% T, 2.7% C) of field bean PPO.** Run in Tris-glycine buffer pH 8.3. The gels were stained for protein (lane a) and for PPO activity (lane b).

Fig 3.5. **Acid-PAGE (7.5% T, 2.7% C) of field bean PPO.** Run in ß-alanine-acetic acid buffer pH 4.5. The gels were stained for protein (lane a) and for PPO activity (lane b).

The purified PPO electrophoresed as a single peak by capillary electrophoresis in Tris-glycine buffer pH 8.3 at 20 mbar pressure (Fig 3.6a) as well as in Na acetate buffer, pH 4.0 at 100 mbar pressure (Fig 3.6b). At both the pH, only one peak was observed confirming the presence of a single isoform.

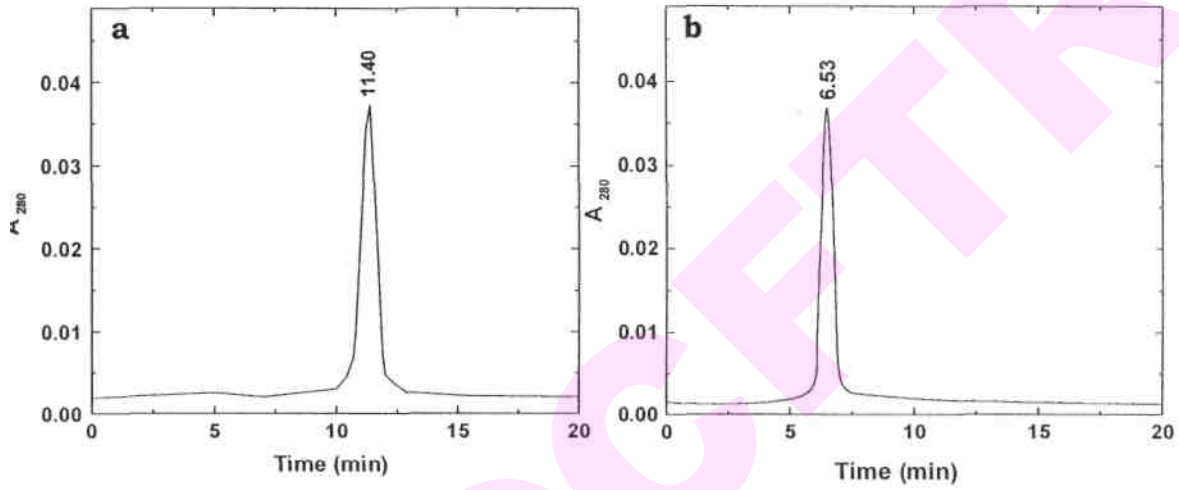


Fig 3.6. **Capillary electropherograms of field bean seed PPO.** Capillary electrophoresis was conducted in a) Tris-glycine buffer (25 mM Tris, 192 mM glycine, pH 8.3) at 20 mbar pressure b) Na acetate buffer (50 mM, pH 4.0) at 100 mbar pressure. The detector was set at 280 nm.

The release of a single amino-terminal amino acid, asparagine of both the native enzyme (Fig 3.7a) and the denatured enzyme (Fig 3.7b) indicated the enzyme to be homogenous. Further sequence analysis of the native enzyme, showed the following sequence from the amino-terminus, NH_2-NNLISFT....

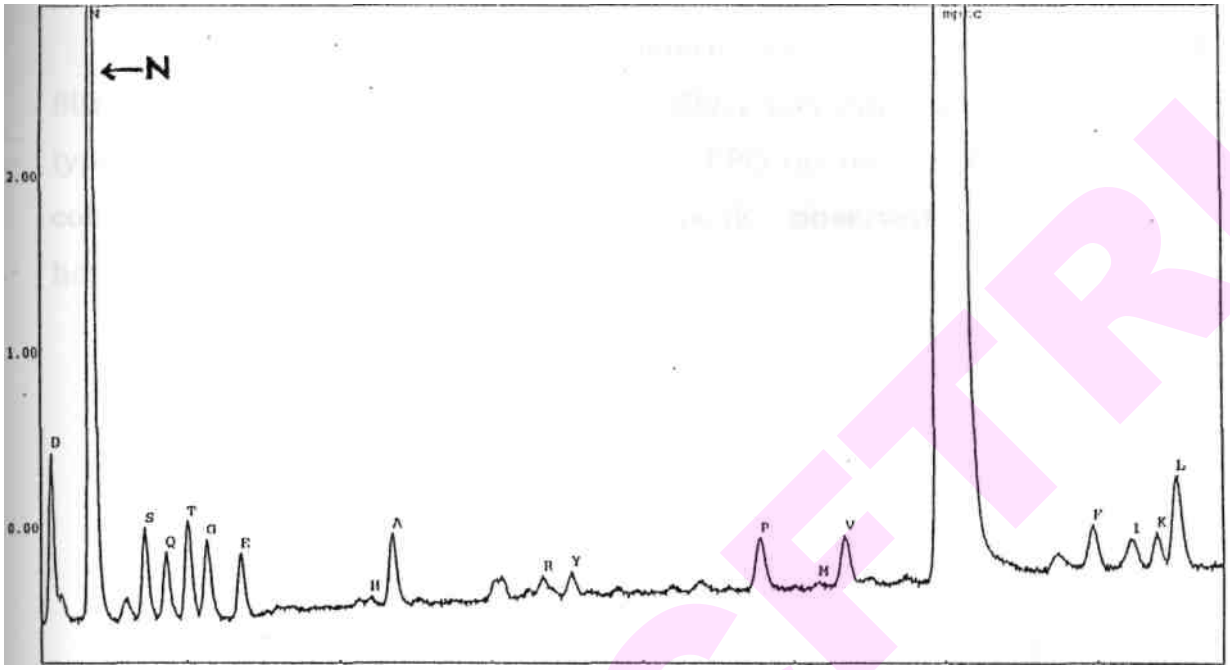


Fig 3.7a. RP-HPLC of the amino-terminal amino acid (asparagine) of native PPO obtained using automated sequenator.

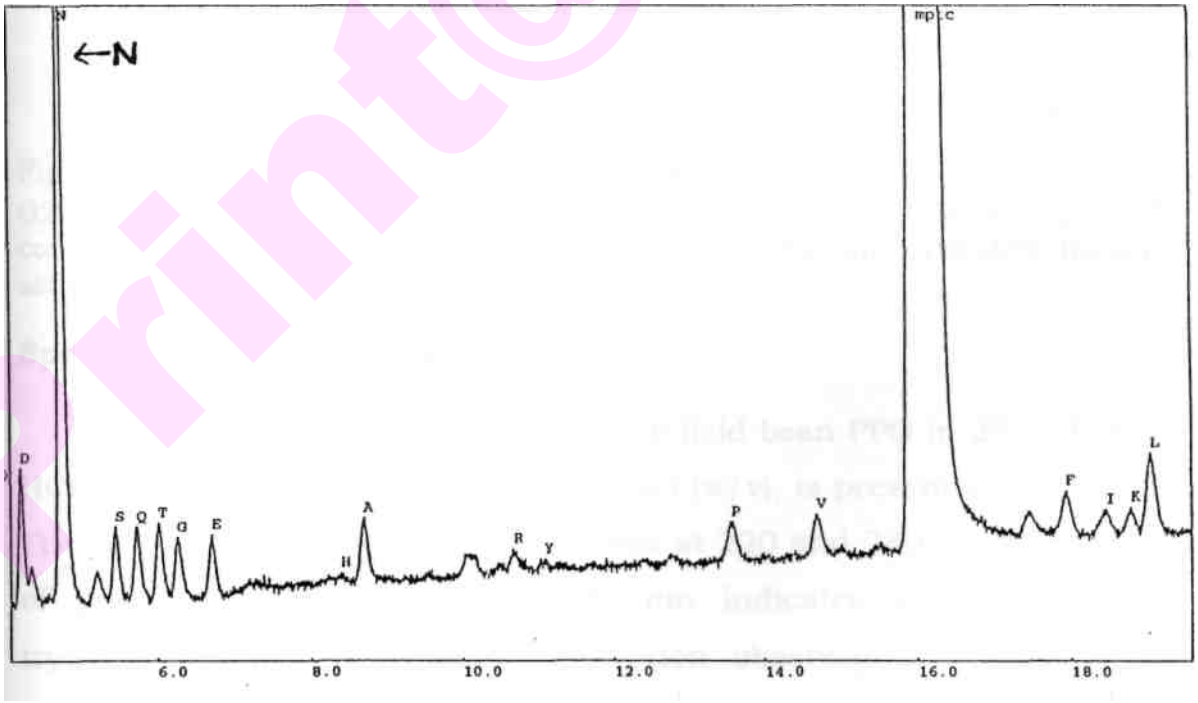


Fig 3.7b. RP-HPLC of the amino-terminal amino acid (asparagine) of denatured field bean PPO obtained using automated sequenator.

The purity of PPO was also determined by analytical HPLC gel filtration using a Progel™-TSK G2000 SWXL column. Fig 3.8 shows the typical elution pattern of the field bean PPO on the HPLC gel filtration column. The single symmetrical peak observed indicates the homogenous nature of the enzyme.

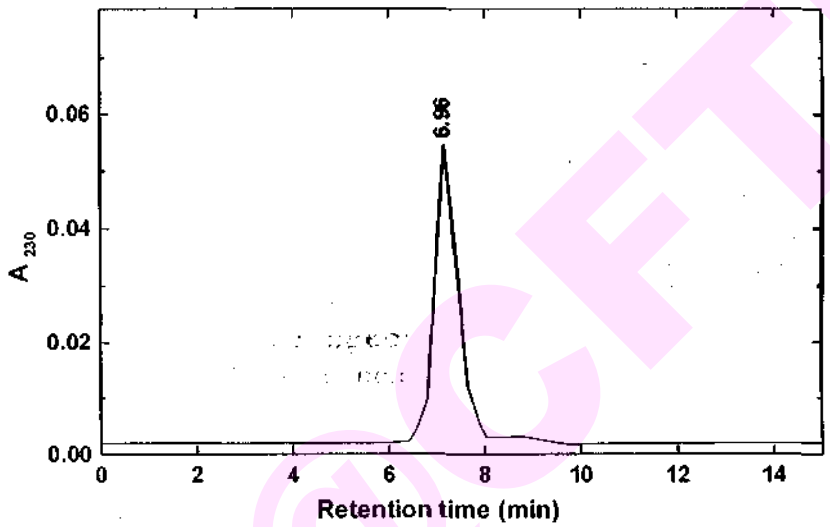


Fig 3.8. **HPLC (gel filtration) profile of PPO.** Column used:. Progel™_TSK G2000 SWXL (7.8 mm idx30 cm). The eluent used was 0.1 M NaPi pH 7.0 containing 0.1 M sodium sulfate, at a flow rate of 1 mL/min. The detector was set at 230 nm.

Spectroscopic properties

The UV-Vis spectrum of the native field bean PPO in 25 mM Tris-HC1 buffer pH 7.0, containing 1.2% NaCl (w/v), is presented in Fig 3.9. The spectra with an absorption maxima at 220 and 280 nm are typical of proteins. The shoulder at 292 nm indicates the presence of tryptophan residues. A weak absorption observed in the range of 310-330 nm is characteristic of all type 3 copper enzymes, like sweet potato catechol oxidase (Eicken et al., 1998).

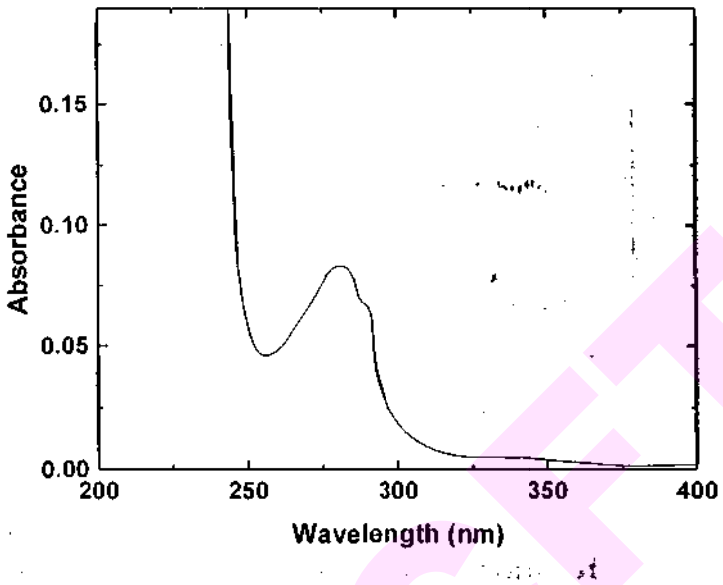


Fig **3.9. UV-Vis Absorbance spectrum of purified field bean PPO.**
Purified PPO (0.1 mg/mL) of field bean in 25 mM Tris-HCl containing 1.2% NaCl (w/v) scanned between 200-400 nm.

Molecular Weight Determination.

The apparent M_r of field bean PPO was determined by analytical gel filtration on a Progel™-TSK G2000 SW_{XL} HPLC column, conventional gel filtration on a Sephadex G-100 column (Andrews, 1964), and also by SDS-PAGE (Laemmli, 1970). The $M_r$of the purified enzyme estimated by HPLC gel filtration was l23±3 kDa from a plot of log M_r versus retention time (Fig 3.10a). The M_r estimated by Sephadex G-100 size exclusion chromatography is l2O±3 kDa (Fig 3.10b) when extrapolated from a plot of log Mr versus V_e/V_0 and is in close agreement to a general M_r of 110.0 kDa for most plant PPOs (Mayer and Harel, 1979).

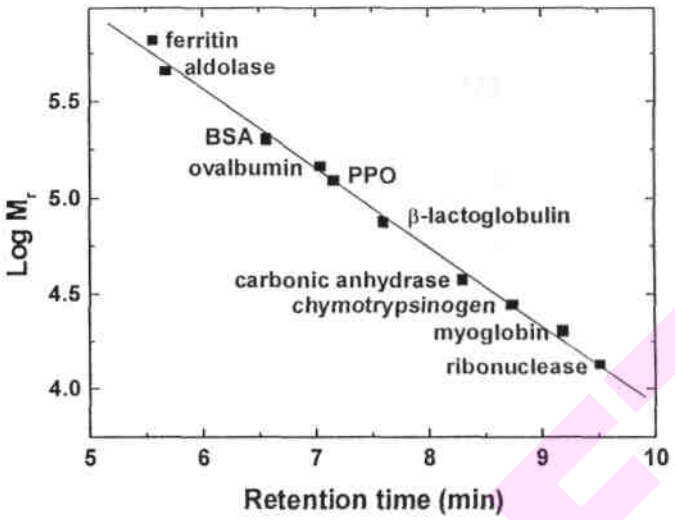


Fig 3.10a. **Molecular weight determination of field bean PPO by HPLC gel filtration using Progel™-TSK G2000 SWXL column.** Fig shows the plot of retention time vs log M_r of standard proteins and PPO.

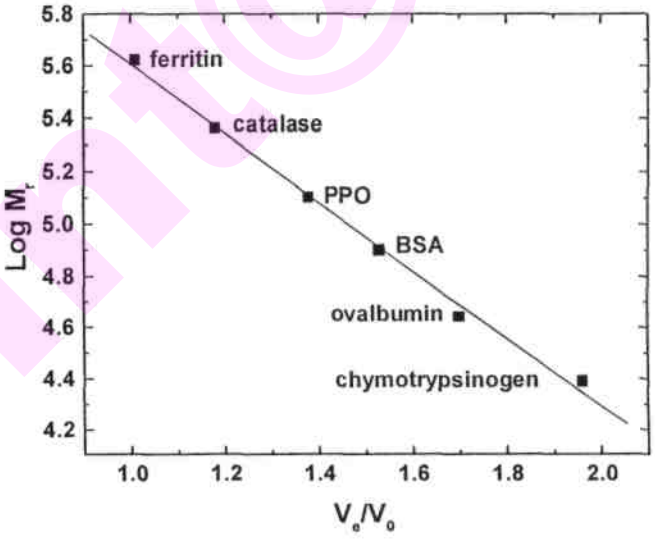


Fig 3.10b. **Molecular weight determination of field bean PPO by gel filtration on Sephadex G-100.** Fig shows the plot of V_e/V_0 vs log M_r of standard proteins and PPO.

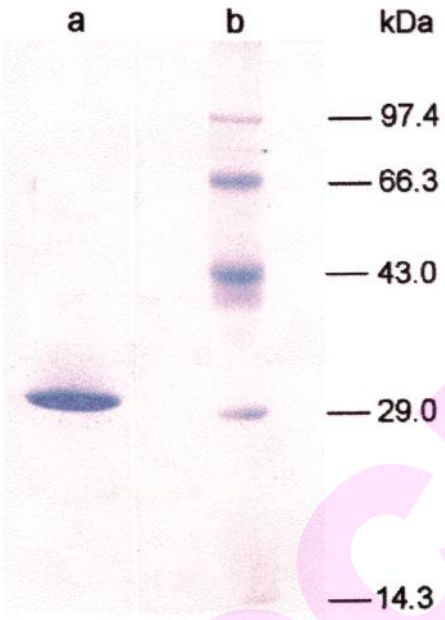


Fig 3.11. **SDS-PAGE (10% T, 2.7% C) of field bean seed PPO.** (lane a) field bean PPO and (lane b) M_r standard proteins: phosphorylase b (97.4 kDa), BSA (66.3 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa), soyabean trypsin inhibitor (20.0 kDa), lysozyme (14.3 kDa).

SDS-PAGE (10% T, 2.7% C) of the purified PPO was carried out in a discontinuous buffer system. The M_r markers used were phosphorylase b (97.4 kDa), BSA (66.3 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa), soyabean trypsin inhibitor (20.0 kDa) and lysozyme (14.3 kDa). The protein staining using Coomassie blue R-250 showed a single subunit of 30+1.5 kDa (Fig 3.11). The apparent subunit size was the same irrespective of the enzyme being reduced with p-mercaptoethanol or not (results not shown), which suggests that the enzyme is a tetramer of identical subunits with no intermolecular disulfides.

Amino-terminal Sequence

SDS and heat denatured PPO were transferred from SDS-PAGE to PVDF membrane and subjected to amino-terminal sequencing on a Shimadzu PSQ-1 gas phase sequenator. The sequence obtained after 17 cycles of automated Edman microsequencing was NH2-NNLISFTMKEFSXTIIA Native PPO was sequenced by loading the purified enzyme directly to the polybrene washed filter of the sequenator. The amino-terminal sequence obtained upto 11 cycles, NH2-NNLISFTMKE.... is identical to that of the denatured PPO. These results reflect the identical status of the subunits of field bean seed PPO.

Amino Acid Composition.

Post column derivatization of the acid hydrolysate of PPO to phenyl thiocarbamyl amino acids followed by RP-HPLC, was used to determine the amino acid composition (Section 2.2.20). A comparison of the amino acid composition of the purified field bean PPO to that of pineapple (Das et al., 1997), grape (Kidren et al., 1977) and glandular trichomes of *Solanum* and *Lycopersicon* species (Yu et al., 1992) is given

in Table 3.4. Glu and Gin are grouped as Glx, and Asp and Asn are grouped as Asx. The contribution of Trp residues was not determined as they are destroyed by acid hydrolysis.

Table 3.4. **Amino acid composition of PPO.**

Amino acid	Field bean* (mole%)	Pineapple[1] (mole%)	Grape[2] (mole%)	Solanum berthaultii[3] (mole%)	Lycopersicon esculentum[4] (mole%)
Asx [a]	12.6	8.0	13.0	11	12.8
Glx[b]	7.9	16.0	10.2	9.6	10.5
Ser	10.3	12.0	6.4	5.2	6.9
Gly	12.6	14.0	5.2	8.8	7.4
His	1.1	2.0	2.6	2.6	2.2
Arg	4.5	6.0	4.9	4.4	4.9
Thr	8.5	7.0	5.6	5.2	5.5
Ala	7.8	5.0	5.7	5.6	6.3
Pro	4.4	2.0	7. 7	7.4	9.8
Tyr	2.2	3.0	8.1	6.9	4.3
Val	6.8	5.0	4.9	6.3	5.5
Met	1.0	1.0	-	1.6	1.6
Cys	0.2	1.0	<1	2.3	2.5
lie	4.0	4.0	5.3	5.2	4.7
Leu	7.8	6.0	7.9	6.9	7.4
Phe	5.8	4.0	5.1	5.2	4.7
Lys	3.0	4.0	6.9	5.8	3.0

a Aspartate and asparagine

b Glutamate and glutamine

*Average of duplicates

Data taken from (1) Das et al., 1997 (2) Kidren et al., 1997, (3) & (4) Yu et al., 1992.

The high content of aspartate, serine, glycine, threonine, alanine and leucine of field bean PPO are similar to that of grape PPO (Kidren et al., 1977), cabbage (Fujita et al., 1995) and PPO in glandular trichomes of *Solanum* and *Lycopersicon* species (Yu et al., 1992). The amino acid composition also showed considerable similarities to pineapple PPO (Das et al., 1997). Sulfur containing amino acids, cysteine and methionine are relatively low in field bean, cabbage, pineapple and grape PPOs.

Comparison of field bean PPO sequence to other PPO sequences

A comparison of the field bean PPO sequence with other known PPO sequences obtained after 17 cycles is given in Table 3.3. The amino-terminal sequence of field bean, is compared with the amino-terminal sequences of potato A (Hunt et al., 1993), potato B (Thygsen et al., 1995), tomato A and B (Newman et al., 1993), broad bean leaf (Cary et al., 1992), apple (Boss et al., 1995) and grape berry (Dry & Robinson, 1994) which have been obtained by cDNA techniques.

Table 3.3. **Comparison of the amino terminal sequence of field bean PPO with other PPO sequences.**

	1										10
Field bean	**N**	**N**	**L**		**I**	**S**		**F**	**T**		**M**	**K**	**T**
Potato-A	T	T	**L**	P	L	C	N	N	K	S	L	S	S
Potato-B	S	S	S	S	T	T	T	I	P	L	C	T	N	K	S	L	S	S
Tomato-A	M	A	S	L	C	S	**N**	S	S				S	**T**	S	L	**K**	**T**
Tomato-B	M	A	S	V	V	C	**N**	S	S	S	S	T	T	T	**T**	T	L	**K**	**T**
Broad Bean	M	T	S	I		S	A	**L**	S	F	**I**	**S**	T	I	N	V	S	S	N
Apple	M	T	S	L		S	P		P	V	V	T	T	P	**T**	V	P	N	P
Berry	M	A	S	L			P	W	S	L	T	T	S	T	A	I	A	N	**T**

Table 3.3. (Continued)

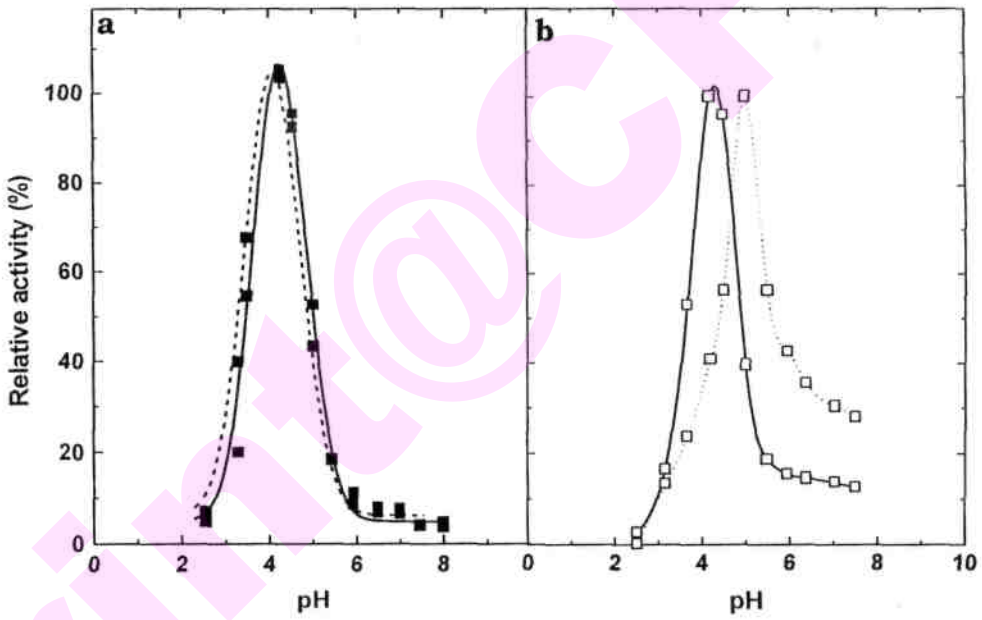
									20							
Field bean			**T**	**M**	**K**	**E**	**F**	**S**	**X**		**T**	**I**	**I**		**A**	
Potato-A	S	F	P	T	N	N	S	**S**	F	L	S			K	P	S
Potato-B	S	F	**T**	T	N	N	S	**S**	F	L	S			K	P	S
Tomato-A	P	F	**T**	S	S	T	T	C	L	S	S	T	P	T	**A**	S
Tomato-B	P	F	**T**	S				L	G	S	**T**	P	K	P	S	
Broad Bean	S	K	I	S	H	S	S	V	Y	P	F	L	Q	K	Q	H
Apple	A	T	K	P	L	S				P	F	S	Q	N	N	S
Berry	T	N	I	S	A	F	P	P	S	P	L	F	Q	R	**A**	S

An alignment of the sequences of PPO proteins from various sources have been made. Comparison of field bean PPO has been made with potato *(Solanum tuberosum),* tomato *(Lycopersicon esculentum),* broad bean *(Vicia faba),* apple *(Malus domestica)* and grape berry *(Vitis vinifera)* PPOs. The conserved amino acids are shown in bold.

pH optima and pH stability.

The optimum pH of PPO was determined by measuring the activity at various pH using different buffers. The buffers used were Mcllvaine (100 raM citric acid-0.2 M $Na_2HP0_{4)}$ pH 2.5-7.5), glycine-HCl (pH 2.5-3.5), Na acetate (pH 3.5-6.0) and NaPi (pH 6.0- 8.0) at 25 °C. The maximum activity of the purified PPO with catechol as the substrate was found at pH 4.0, irrespective of the buffers used for assay (Fig 3.12a). The enzyme was relatively active at low pH. Half the maximal activity was still present at pH 3.5 and 5.5 and 25% at pH 3.0. Similarly, with 4-methyl catechol and L-DOPA as substrates, at pH 3.0, 19% and 17% of activity was observed respectively. The maximum activity with 4-methyl catechol as the substrate was observed at pH 4.0, similar to that of catechol. But at this pH, with L-DOPA as the

substrate, the PPO exhibited only 40% of the activity (Fig 3.12b). With L-DOPA, the enzyme exhibits a maximum activity at pH 5.0. At pH 5.0, with catechol and 4-methyl catechol as substrates, the enzyme exhibits only 50 and 40% of its maximum activity. While catechol and 4-methyl catechol show relatively no activity at higher pH, L-DOPA retains 30% of its reactivity towards field bean PPO at pH 7.5. At this pH, with catechol and 4-methyl catechol, the PPO has 5% and 18% of activity respectively. The single pH optimum exhibited by the three substrates, catechol, 4-methyl catechol and L-DOPA further evidences the presence of a single isoform as demonstrated by native PAGE (Fig 3.4 and 3.5) and capillary electrophoresis (Fig 3.6).



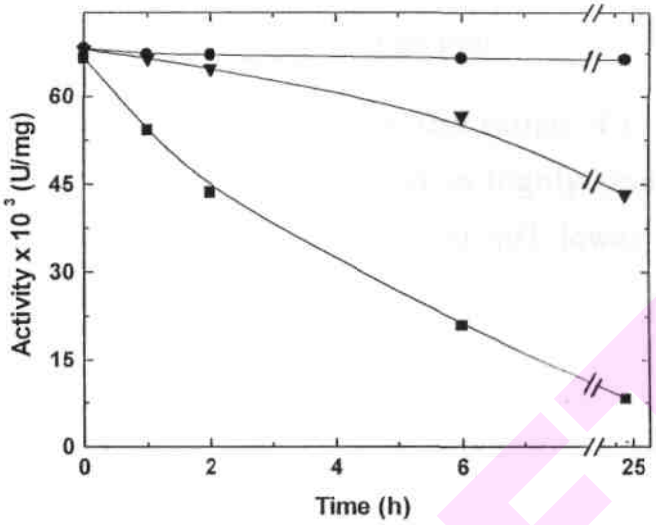


Fig 3.13a. **pH stability of field bean PPO.** PPO in Mcllvaine buffer (•) pH 7.0, (T) pH 4.0 and (■) pH 2.5. Assay was conducted in 50 mM Na acetate buffer, pH 4.0. Substrate used was 50 mM catechol.

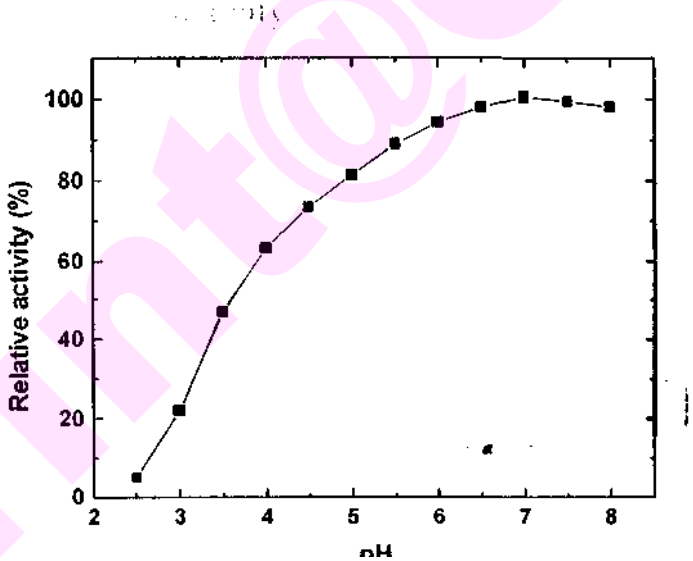


Fig 3.13b. **pH stability of field bean PPO.** Field bean PPO was incubated in different pH Mcllvaine buffers (2.5-7.5) and 100 mM NaPi buffer, pH 8.0 for 24 h,at the end of which the residual activity was measured.

At pH 7.0, the field bean PPO was stable over a period of 24 h retaining 100% of its activity. At pH 4.0, the enzyme is fairly stable retaining 95% of its activity after 2 h and 83% of activity after 6 h. At pH 2.5, the enzyme retains only 53% of its activity after the first 2 h

and 16% after 6 h. PPO at pH 4.0 lost 35% of its activity after 24 h whereas at pH 2.5, 95% of the activity was lost.

Purified field bean PPO is stable in the range of pH 6.0-8.0 over a period of 24 h whereas at pH below 4.0 it is highly unstable. From pH 6.0-4.0, it is moderately stable whereas at pH lower than that, the activity declined drastically (Fig 3.13b)

Temperature optimum and stability

The effect of temperature on purified field bean PPO was studied in 50 mM Na acetate pH 4.0, between 4-75 °C using catechol as the substrate. As shown in Fig 3.14a, the purified enzyme had a temperature optima in the range of 25-35 °C when catechol was used as the substrate. At 15 °C the enzyme activity was 80% of the maximum. At 4 °C and 45 °C, the activity was about 65% of the original activity.

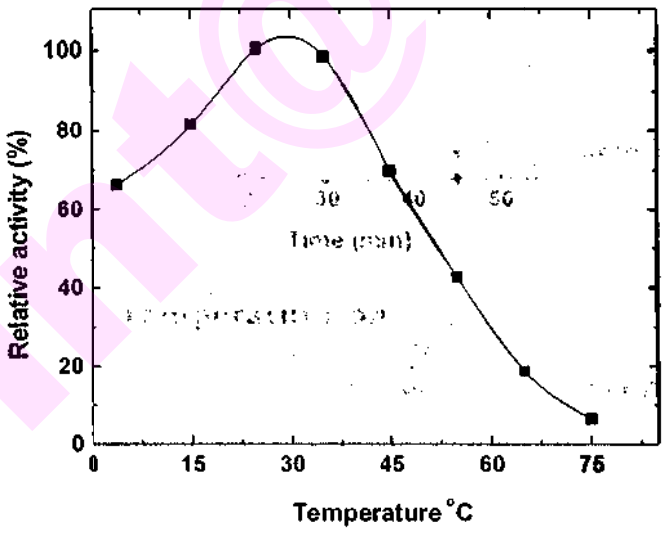


Fig 3.14a. **Effect of temperature on the activity of field bean PPO.**
PPO was assayed at indicated temperatures in 50 mM Na acetate buffer, pH 4.0 containing 50 mM catechol.

The relative stability of the purified enzyme, to temperature inactivation was studied by incubating the enzyme in a water bath at various temperatures (4-75 °C). Aliquots were removed at different time intervals and the residual activity was determined. At 4 °C and 25 °C, PPO retained all its activity over a period of 24 h. Hence the field bean PPO, was stored at 4 °C.

Pure field bean PPO was found to slowly loose its activity when incubated at temperatures above 35 °C. At 45 °G, only 25% of the activity was lost in 45 min whereas at 65 °C, 90% of the total activity was lost in the same time. At 75 °C, the loss of activity is drastic where 85% of the activity was lost in 5 min (Fig 3.14b).

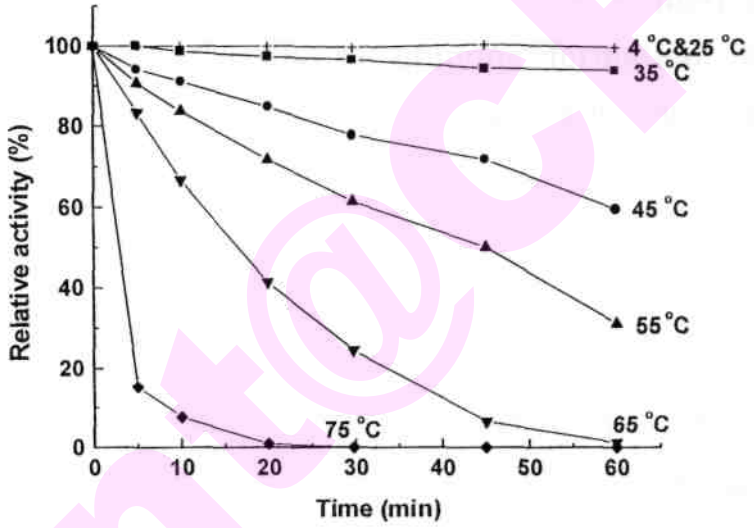


Fig 3.14b. **Effect of temperature on the stability of field bean PPO.**
Field bean PPO was incubated at different temperatures ranging from 4-75 °C. Aliquots were removed at different time intervals and assayed for residual PPO activity using catechol as the substrate.

Substrate Specificity.

PPOs act on a wide range of mono, di and triphenols. PPOs from various sources show a preference to certain phenolic compounds. The specificity of purified field bean PPO towards different phenolic

compounds was determined at the absorption maximum of each individual quinone product (Zhou et al., 1993).

The monophenols used to determine substrate specificity were tyrosine, p-cresol, p-coumaric acid, and ferulic acid. The diphenols, catechol, 4-methyl catechol, L-DOPA, chlorogenic acid, caffeic acid, (+) catechin, protocatechuic acid, protocatechuic aldehyde and the triphenols, pyrogallol, 2,3,4-trihydroxy benzoic acid and gallic acid were used. Reaction with each substrate was measured at different concentrations. Diphenols, catechol (2.5-100 mM), 4-methyl catechol (0.25-20 mM), chlorogenic acid (0.5-50 mM), L-DOPA (0.5-12.5 mM), catechin (0.5-50 mM), caffeic acid (0.5-50 mM), protocatechuic acid (0.5-50 mM) and protocatechuic aldehyde (0.5-50 mM) were used to determine the initial velocity of quinone formation. Of the eight diphenols used, field bean PPO was reactive only towards catechol, 4-methyl catechol and L-DOPA (Table 3.5).

Among the triphenols, pyrogallol was used at a concentration of 2.5-50 mM, 2,3,4-trihydroxy benzoic acid at 2.5-25 mM and gallic acid at 0.5-50 mM concentrations. Both pyrogallol and the 2,3,4-trihydroxy benzoic acid were oxidized by field bean PPO (Table 3.5) whereas gallic acid with o-dihydroxy group did not have any activity towards field bean PPO at the concentrations used. All the monophenols used at concentrations of 0.5-50 mM were not acted upon by PPO suggesting the absence of monophenolase (cresolase) activity.

Relative activities of PPO measured at the absorption maximum of each oxidation product were calculated using catechol for comparison. Maximum activity was achieved with 4-methyl catechol as the substrate followed by catechol, pyrogallol, DOPA and 2,3,4-trihydroxy benzoic acid. The purified enzyme displayed Michaelis-Menten kinetics with all the five substrates which were reactive (Fig 3.15a, only catechol shown).

Table 3.5. **Effect of various substrates on the activity of purified field bean seed PPO.**

Substrate	Wavelength* (nm)	Vmax (U/mg)	Relative activity (%)	K_m (mM)
Catechol	420	67,347	100	10.5
4-Methyl catechol	420	94,285	140	4.0
L-DOPA	480	15,220	22.6	1.18
Chlorogenic acid	325*	ND	0	ND
Catechin	420	ND	0	ND
Caffeic acid	480	ND	0	ND
p-Cresol	420	ND	0	ND
Tyrosine	480	ND	0	ND
Ferulic acid	480	ND	0	ND
p-coumaric acid	480	ND	0	ND
Protocatechuic acid	420	ND	0	ND
Protocatechuic aldehyde	420	ND	0	ND
Pyrogallol	334	16,163		12.5
2,3,4-trihydroxy benzoic acid	420	2,491	3.7	5.2
Gallic acid	420	ND	0	ND
Phloroglucinol	420	ND	0	ND

• Absorption maxima of the corresponding oxidation product.

* Decrease in absorbance at 325 nm was monitored as the index of PPO activity

ND Not Determined

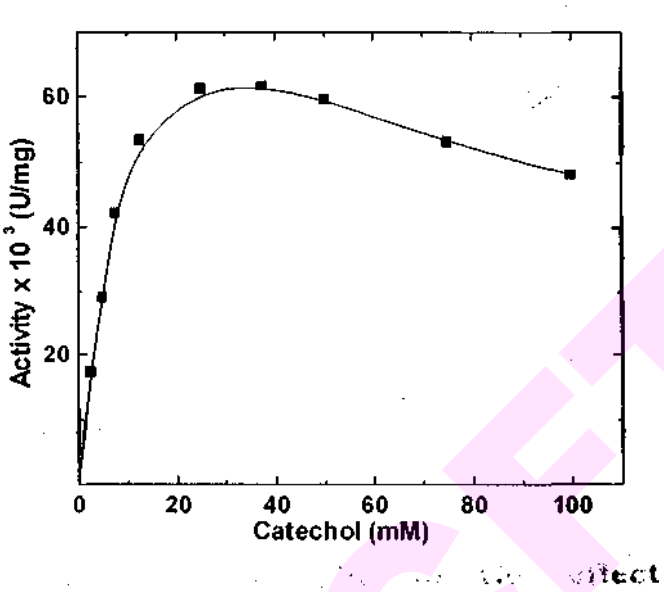


Fig 3.15a. **Michaelis-Menten plot of the effect of catechol concentration on field bean seed PPO.**

Linear regression analysis of V vs [S] (Fig 3.15b only catechol shown) indicated an apparent K_m of 10.5, 4.0, 1.18, 12.5 and 5.2 mM for catechol, 4-methyl catechol, DOPA, pyrogallol, and 2,3,4-trihydroxy benzoic acid respectively. The V_{max} calculated from the Lineweaver-Burk double reciprocal plot of each substrate are given in Table 3.5. L-DOPA was found to have the highest affinity for the enzyme even though the maximum velocity was only 22.6% of that of catechol which was taken as the control. 4-methyl catechol which had the highest activity, 40% greater than that of catechol activity, also showed a stronger binding affinity than catechol. The K_m of 2,3,4-trihydroxy benzoic acid although similar to that of 4-methyl catechol was not oxidized at a high rate.

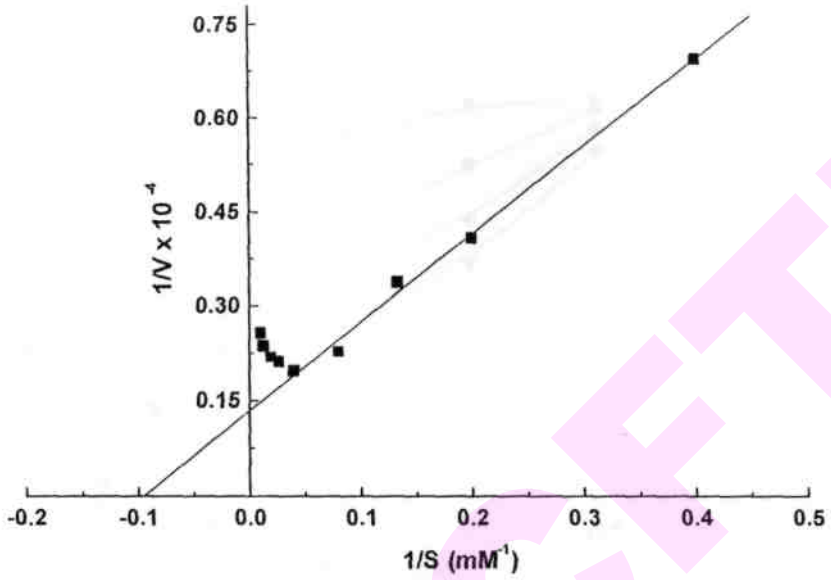


Fig 3.15b. **Double reciprocal plot of the effect of catechol concentration on field bean seed PPO.** Data of Fig 3.15a.

Inhibitor Studies.

Inhibitors of polyphenol oxidase can be grouped according to their mode of action, which may be by a) chelating the Cu-prosthetic group, b) competing with the substrate or c) interacting with the reaction products. Tropolone (2-hydroxy-2,4,6-cycloheptatrien-l-one) is one of the most potent inhibitors of PPO (Espin and Wichers, 1999a) which is structurally analogous to the o-diphenolic substrates of PPO, as well as an effective copper chelator. Cysteine, a sulfur containing amino acid inhibits PPO by either reducing the quinone product formed or by forming colorless adducts with the o-quinones (McEvily et al., 1992; Friedman and Bautista, 1996). Ascorbic acid and potassium metabisulfite inhibit the enzyme reaction primarily by their reducing power i.e., by reducing the o-quinones formed to its phenolic forms, thus avoiding the formation of colored pigments (Galeazzi and Sgarbieri, 1981).

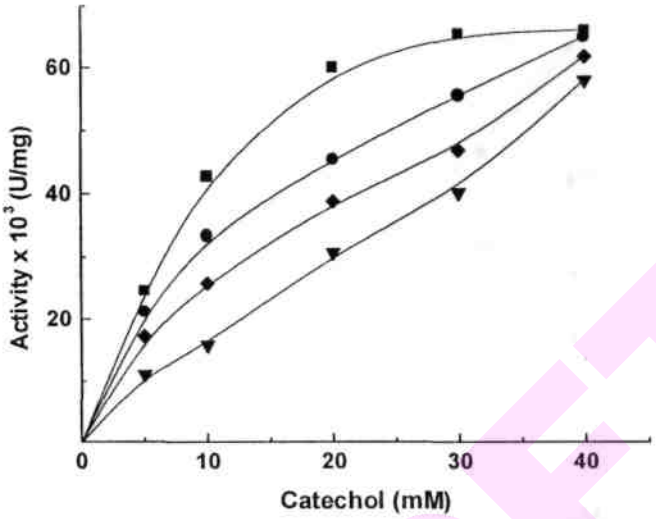


Fig 3.16a. **Effect of tropolone on catechol oxidation by field bean PPO.** Tropolone (■) 0 M, (•) 6.25xl0"^{7} M, (♦) 12.5x10-7 M, (T) 18.75X10"^{7} M.

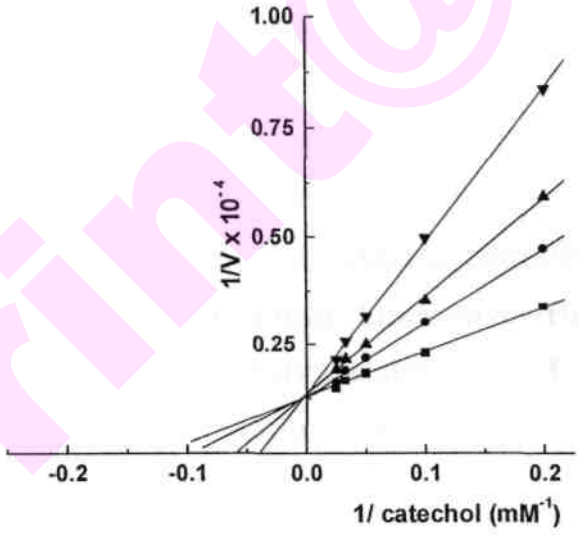


Fig 3.16b. **Double reciprocal plot of tropolone inhibition of field bean PPO. (■)** 0 M, (•) 6.25xl0-^{7} M, (A) 12.5X10-^{7} M, (T) 18.75x10~^{7} M. Data of Fig 3.16a

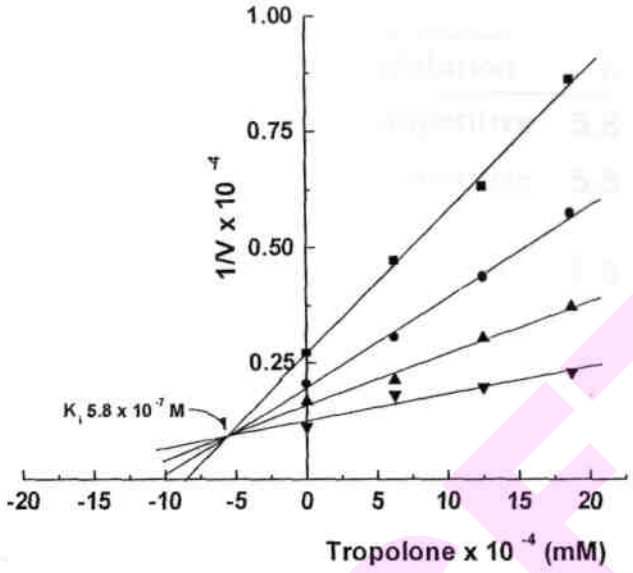


Fig 3.16c. **Dixon plot of tropolone inhibition of field bean PPO.** Catechol (■) 5 mM, (●) 10 mM, (▲) 20 mM, (▼) 30 mM. Data of Fig 3.16a.

The effect of tropolone, ascorbic acid, cysteine and potassium metabisulfite on catechol oxidation of field bean PPO was determined at varying concentrations of catechol and at three different concentrations of the inhibitor. Michaelis-Menten plot of the effect of three different tropolone concentrations on catechol oxidation (Fig 3.16a) is typical of a competitive type of inhibition. Lineweaver-Burk plots of 1/V vs 1/[S] at three inhibitor concentrations determined the type of inhibition (Fig 3.16b). The inhibition constant Ki for these inhibitors was deduced from the Dixon plots (Fig 3.16c, only tropolone shown). Table 3.6 shows the inhibition results with catechol as the substrate. All four inhibitors used in this study inhibited PPO competitively (Table 3.6). Tropolone, the progenitor of a group of compounds called tropolones, is the most potent inhibitor of field bean PPO with an apparent Ki of 5.8×10^{7} M.

Table 3.6. **Effect of various inhibitors on the activity of purified field bean seed PPO.**

Inhibitor	Inhibition	K_i (M)
Tropolone	Competitive	5.8×10^{-7}
Potassium metabisulfite	Competitive	5.5×10^{-6}
Ascorbic acid	Competitive	7.5×10^{-6}
Cysteine–HCl	Competitive	2.0×10^{-2}

The inhibition constants for the reducing agents, ascorbic acid ($7.5 \times 10\text{-}^{6}$) and metabisulfite ($5.5 \times 10\text{-}^{6}$) are similar and 260-360 fold lower than that for cysteine ($2.0 \times 10\text{-}^{2}$). The presence of cysteine-HCl, potassium metabisulfite and ascorbic acid in the assay medium exhibited a lag phase, which increased as the inhibitor concentration increased (Fig 3.17). Similar increase of lag phase with increasing inhibitor concentrations has been observed in banana (Galeazzi and Sgarbieri, 1981) and pineapple fruit (Das et al., 1997) PPOs.

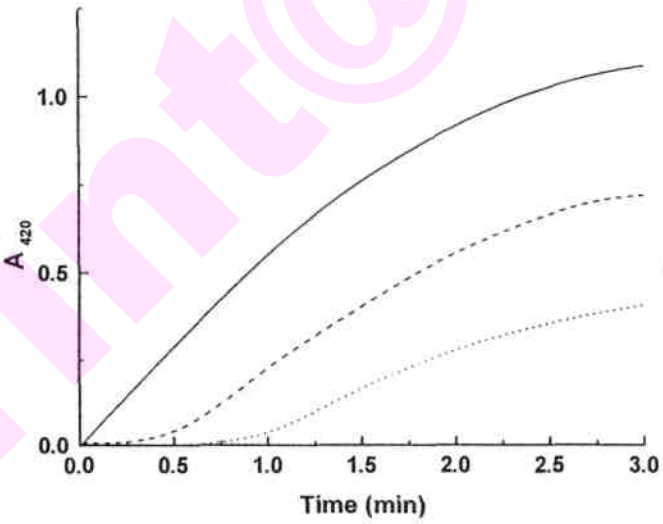


Fig 3.17. **Inhibition of catechol oxidation of field bean PPO by ascorbic acid.** PPO was assayed in 50 mM Na acetate, pH 4.0 containing 50 mM catechol (-------) and ascorbic acid (----------) 6 mM, (.....) 12 mM.

A natural inhibitor of PPO was isolated and purified from *Achras sapota* and identified to be a proanthocyanidin. The details of this study are presented in Chapter 7.

Antibody cross reactivity

Polyclonal antibodies were developed against purified native, field bean PPO, in a New Zealand white rabbit. Crude extract of field bean PPO, purified PPO, SDS (4%) treated PPO, PPO treated with SDS and ß-mercaptoethanol and also PPO denatured by boiling were immobilized on a nitrocellulose membrane and subjected to immunodetection (Section 2.2.19) using the polyclonal antibodies raised against purified field bean PPO. Positive antigen antibody reactions could be detected with all forms of PPO (Fig 3.18). BSA served as the negative control which showed no cross reactivity. Since the antibodies developed against the native enzyme recognized the partially and completely denatured forms of the enzyme, it is suggestive of sequential epitopes of PPO.

Reports on legume PPOs are limited to mung bean *(Vigna radiata)* leaves and seedlings (Shin et al., 1997; Takeuchi et al., 1992) and broad bean leaves (Ganesa et al., 1992). Hence a few legumes were chosen to detect the presence of PPO. Crude extracts of defatted flour of various legume seeds i.e., chick pea *(Cicer arietinum),* mung *bean (Vigna radiata),* pigeon pea *(Cajanas cajan),* soybean *(Glycine max)* and horse gram *{Dolichos biflorus)* were prepared and the PPO activity measured and compared to field bean PPO. The results are shown in Table 3.7. The specific activity of legume seed extracts were 5-20 times lower than that of the field bean. All the legume PPOs showed a strong cross . reactivity towards the field bean PPO antibodies, suggesting common epitopes (Fig 3.19). Compared to legume PPOs, PPOs from vegetables and fruits have assumed a pivotal role in the fruit and vegetable

processing industry. However very little information is available on the three dimensional structure, and its organization into higher structures. Crude extracts of apple *(Pyrus malus),* pear *(Pyrus communis),* banana *(Musa paradisiaca),* mushroom, potato *(Solarium tuberosum)* and brinjal *(Solarium melangena)* were prepared and the activity compared to that of field bean (Table 3.7).

Table 3.7. **Comparison of various plant PPOs.**

Source	Total activity $\times 10^4$(U)	Total protein (mg)	Specific activity (U/mg)
Apple	5.5	1.44	38,194
Pear	6.3	0.78	80,769
Banana	6.7	2.8	23,821
Potato	8.8	46	1,913
Brinjal	9.9	2.8	35,357
Mushroom*	1.4	0.45	30,888
Pigeon pea	4.1	396	104
Chick pea	19.2	595	322
Horse gram	22.4	652	343
Mung bean	22.4	588	381
Soy bean	28.0	832	336
Field bean	121.0	604	2,003

10 g of minced tissue of apple, pear, potato and brinjal, 10 g of the banana acetone powder, 10 g of the defatted bean powders were each extracted in 50 mL of 100 mM Tris HC1, pH 7.0 buffer containing 2% PVPP (w/v) and 1.2% NaCl (w/v). *50 mg of mushroom PPO, obtained from Sigma was dissolved in 1 mL of the above buffer.

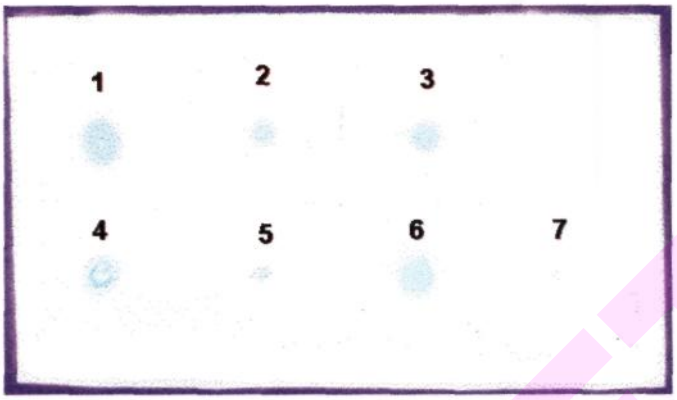


Fig 3.18. **Dot blot analysis of field bean PPO using anti-field bean PPO antibodies.** (1) crude extract (2) PPO (4 ng) in 4% SDS (3) PPO (5 ng) in 4% SDS and 10% p-mercaptoethanol (4) PPO (4 ng) boiled in 4% SDS (5) PPO (5 |ig) boiled in 4% SDS and 10% p-mercaptoethanol (6) PPO (5 ng) (7) BSA (10 ng).

The specific activity of potato was similar to that of field bean PPO whereas the specific activity of pear PPO was -40 times greater than the field bean PPO. The specific activity of the other fruit and vegetable PPOs was greater than that of legume PPOs (Table 3.7). Protein extracted from apple, pear, banana, mushroom, potato and brinjal were immobilized on a nitrocellulose membrane and subjected to immunodetection (Fig 3.20). The strong cross-reactivity of all the PPOs towards the field bean PPO antibody indicate that one or more of the epitopes present on field bean PPO are also present on the PPOs from all the other sources examined.

DISCUSSION

PPO has been studied in many fruits and vegetables including peaches (Luh and Philthakphol, 1972), grape (Wisseman and Lee 1981; Sanchez-Ferrer et al., 1988), apple (Janovitz-Klapp et al., 1989; Murata et al., 1992), oil bean (Chilaka et al., 1993), plantain (Ngalani et al., 1993) potato (Sanchez-Ferrer et al., 1993a & b), pineapple (Das et al., 1997), cabbage (Fujita et al., 1995) and avocado (Kahn, 1977; Espin et al., 1997). However among the leguminous plants, reports are available only on the PPO of broad bean leaves *(Vicia faba* L) (Ganesa et al., 1992), mung bean leaf *(Vigna radiata)* and seedlings *(Vigna mungo)* (Shin et al., 1997; Takeuchi et al., 1992). Preliminary investigations in our laboratory, earlier during the structural characterization of a lectin from the field bean seeds revealed the presence of a single PPO by PAGE, in crude extracts. The purification of PPO from higher plants continues to be a problem compounded by the presence of multiple isoforms. The single isoform of field bean renders it ideal for three dimensional structure analysis, which in plant PPOs has been hindered by its multiplicity. Although PPO was discovered in 1839, the first and only X-ray crystal structure available to date is that of sweet potato

(Ipomoea batatas) determined by Klabunde et al. (1998). The refined structure of sweet potato *(Ipomoea batatas)* at 2.5 A has provided new insights into the mechanism of this enzyme (Eicken et al., 1999).

The weight of evidence indicates the wide occurrence of membrane PPOs, particularly in chloroplasts bound within the chloroplast lamellae and grana (Katz and Mayer, 1969; Parish, 1972). The strength of PPO binding to membranes depends on the tissue and the stage of the development of the plant. In field bean, extraction with buffer alone suffices to release maximum enzyme (Table 3.1). The inclusion of the detergents, Triton X-100, SDS or Triton X-114 in the extraction buffer does not improve the yield of field bean PPO. In most cases of PPO, the drastic conditions used for solubilization causes changes in the enzyme structure and/or conformation and are frequently accompanied by activation (Robb et al., 1965; Mayer et al., 1966; Kenten, 1958) (Chapter 7). The fact that the mature field bean seeds were used as the source, probably allows for the easy solubilization of the enzyme. Apple (Harel et al., 1970) and grape (Kidren et al., 1978) PPO become increasingly soluble during fruit ripening. Green olive PPO is tightly bound to the chloroplast membranes and requires drastic treatments for solubilization. In contrast, the enzyme is completely soluble in the black ripe olive (Ben-Shalom et al., 1977) deeply colored by anthocyanins.

Enzyme purification in plant extracts is hampered by the presence of a large variety and quantity of secondary products that can bind tightly to the enzymes and change their native characteristics (Loomis, 1974). The use of acetone powders, (NH4)2S04 fractionation, salts, insoluble polymers and detergents partially circumvent this problem. The compartmentalization of the endogenous phenolic substrates and PPO results in the separation between enzyme and bulk

of its phenolic substrates *in situ.* However this is destroyed during solubilization with buffers resulting in browning of extracts. The purification method described here involves the use of an insoluble polymer PVPP in the extraction buffer, which can complex endogenous polyphenols and thus prevent the crude extract from browning at 4 °C. PVPP in the extraction buffer appeared to complex most of the polyphenols, as further purification could be carried out in the absence PVPP with no further browning.

$(NH_4)_2S0_4$ treatment was used to partially remove inactive proteins. The purification protocol for field bean PPO involved a combination of ion exchange chromatography, hydrophobic interaction chromatography and gel filtration. The procedures employed to purify PPO include removal of inactive protein by precipitation with protamine sulfate (Bendall and Gregory, 1963; Herzfeld and Esser, 1969) or calcium acetate (Dhar and Bose, 1965; Patil and Zucker, 1965) and fractionation with $(NH_4)_2S0_4$. DEAE-cellulose and hydroxyapatite are widely used along with size exclusion chromatography (Mayer and Harel, 1979) for the purification of PPO. Celite, a relatively specific binder of copper protein, has also been used to purify PPO which is a binuclear copper containing enzyme (Fling et al., 1963; Katan and Galun, 1975). More recently hydrophobic interaction chromatography has been successfully used (Takeuchi et al., 1992; Kader et al., 1997; Partington and Bolwell, 1996; Das et al., 1997) in the purification of PPOs from several sources.

In this study, a combination of these procedures, namely $(NH_4)_2S0_4$ fractionation, ion-exchange chromatography on DEAE-Sephacel, hydrophobic interaction chromatography and size exclusion chromatography have been used to purify the enzyme from field bean seeds to homogeneity. The DEAE-Sephacel matrix used for

ion-exchange chromatography offers the advantage of better flow properties than DEAE-cellulose. In this step of purification, the inactive proteins were bound to DEAE-Sephacel and active PPO collected as the unbound fraction (Fig 3.1), which afforded a three fold purification over the previous step. Phenyl Sepharose CL-4B has been successfully used for purification of PPO from various plant sources (Flurkey and Jen, 1980; Wissemann and Lee, 1980; Janovitz-Klapp et al., 1989). In this study, Phenyl Agarose served as the hydrophobic interaction chromatography matrix with an inactive protein fraction eluting in the void volume (Fig 3.2)

The size exclusion chromatography step on Sephadex G-100 (Fig 3.3) provided a homogenous PPO with a high specific activity of 68,077 U/mg (Table 3.2). The single species on PAGE as observed by both specific enzyme activity staining and protein staining (Fig 3.4 and Fig 3.5) suggest a single isoform. Electropherograms of the capillary electrophoresis at pH 8.3 as well as pH 4.0 (Fig 3.6a & b) with a single peak evidence the presence of a single isoform. Most reports on PPOs mention multiple forms or isoforms (Mayer and Harel, 1979). The heterogeneity or multiplicity could be a native phenomenon or result from the release of membrane bound forms, partial denaturation, fragmentation, proteolysis, activation or tanning reactions (Mayer and Harel, 1979). The isoenzymes of mushroom catechol oxidase differ in their primary structure (Jolley et al., 1969) and therefore are true isoenzymes. There is also inter and intra-species diversity in the number of PPO isoforms and their M_rs.

Field bean PPO is a tetramer of identical subunit M_r 3O±l.5 kDa as revealed by SDS-PAGE (Fig 3.11), not connected by intermolecular disulfides. The size of field bean PPO is similar to that reported for pineapple fruit (Das et al., 1997), oilbean (Chilaka et al., 1993), yam

(Anosike and Ayaebene, 1982) and cocoyam (Anosike and Ojimelukwe, 1982), but was larger than that reported for broad bean leaf PPO (Flurkey, 1989), tomato, potato, carrot and mung bean seedling PPO (Takeuchi et al., 1992). Both the oilbean seed (Chilaka et al., 1993) and pineapple fruit PPO (Das et al., 1997) are tetramers of identical subunit size, similar to field bean PPO. PPOs, although ubiquitous in angiosperms, also display a complex, heterogeneous quatenary structure and the values reported cover a wide range. Flurkey (1989) resolved the broad bean leaf PPO into doublets of M_r 61.5 and 60.0 kDa and 44.5 and 43.0 kDa, all of which have the same amino-terminal sequence. An association-dissociation phenomenon of pineapple PPO was observed in buffers of varying ionic strength (Das et al., 1997). The equilibrium could be shifted towards association by decreasing the ionic strength whereas the dissociation was facilitated by increasing ionic strength. Harel et al. (1973) observed that the predominant form of grape PPO undergoes dissociation upon storage or when exposed to acid pH or urea. A concentration dependent association-dissociation equilibrium of mushroom PPO has been reported by Jolley et al. (1969). Association of the enzyme subunits was induced at high protein concentration and dissociation is facilitated by increased ionic strength and presence of SDS. Field bean PPO was found to form aggregates in 25 mM Tris-HCl at high protein concentrations. Increasing the ionic strength by including 1.2% NaCl (w/v) in all the purification steps prevented the association of PPO.

Aylward and Haisman (1969) reported that the optimum pH for maximum PPO activity in plants varies from about 4 to 7, depending on the extraction methods, substrates used for assay and the localization of the enzyme in the plant cell. The optimum pH of 4.0 of field bean PPO for catechol and 4-methyl catechol is the same as that obtained for apple (Murata et al., 1992) and egg plant (Fujita and Tono, 1988). PPO

purified from blueberry fruit is very active at pH 3.0, which is also the pH of the blueferry fruit *per se* (Kader et al., 1994). We have not determined the pH of the seed.

Different pH optima with different substrates have been demonstrated for plum PPO (Siddiq et al., 1996), where caffeic acid, catechol and 4-methyl catechol had an optima of 5.8, 6.0 and 6.4 respectively. Differences in pH optima with different substrates have also been reported for partially purified strawberry PPO (Wesche-Ebeling and Montgomery, 1990), palmito PPO (Lourenco et al., 1990) and tea-leaf PPO (Gregory and Bendall, 1966). Plum PPO (Siddiq et al., 1996) is also characterized by a rapid decrease in activity in alkaline pH as observed in the case of field bean PPO. Changes in the form of the pH curve during development and maturation or treatment of the isolated enzyme with various agents are documented. These include changes in the pH optimum upon aging, exposure to stress and treatment of this enzyme with denaturing agents such as SDS (Moore and Flurkey, 1990; Espin and Wichers, 1999c) or a short exposure to acid pH (Kenten, 1957). Such changes in field bean PPO accompanied by activation of enzyme are also observed (Results presented and discussed in Chapter 6).

The pH stability of field bean PPO follows a pattern similar to that of PPO from loquat fruit (Ding et al., 1998), cabbage (Fujita et al., 1995) and monroe apple peel (Zhou et al., 1993) where the enzyme is stable near neutral pH and less stable at acidic pH. The field bean PPO though unstable at acidic pH can be activated by acid pH (Chapter 6). PPO from the vascular tissue of lettuce was stable for 20 h at pH 4-8 whereas that from the photosynthetic tissue of lettuce was stable between pH 5-8 (Heimdal et al., 1994). Banana PPO was stable between pH 5-11 upto 48 h (Yangetal., 2000).

Many of PPOs have an optimum temperature around 30 °C. The optimum temperature for the PPO from banana pulp (Yang et al., 2000), parsnip root (Chubey 85 Dorrell, 1972), loquat fruit (Ding et al., 1998) and monroe apple peel was 30 °C. The PPO from the vascular and photosynthetic tissue of lettuce had a temperature optimum of 25 °C-35 °C whereas that of apple (Trejo-Gonzalez and Soto-Valdez, 1991) and grape is in the range of 25 °C-45 °C (Valero et al., 1988).

PPO from field bean appears to be moderately heat stable being able to retain 50% of its activity at 55 °C after 45 min. This is similar to the PPO from cucumber (Miller et al., 1990) and plum (Siddiq et al., 1996). At higher temperatures, the enzyme rapidly loses its activity unlike the banana PPO (Yang et al., 2000) where the enzyme is relatively stable at high temperatures, 80% of the enzyme activity being retained after a heat treatment at 70 °C for 10 min. Relatively high thermal stabilities were also found in the PPOs in kiwi fruit (Park & Luh, 1985), mango (Park et al., 1980), satsuma mandarin (Fujita & Tono, 1979) and head lettuce (Fujita et al., 1991). At 4 °C and 25 °C, PPO retained 100% activity upto 24 h. Purified field bean PPO stored at 4 °C does not loose activity even after 2 months.

Fungal and higher plant enzymes act on a wide range of mono and diphenols. The field bean PPO however acts only on small o-diphenols, suggesting a small and compact substrate binding site with a high affinity for small o-diphenols like catechol and 4-methyl catechol and no affinity to bulky o-diphenols such as caffeic acid, chlorogenic acid, catechin and also diphenolic oligomers (Table 3.5). Black poplar leaf PPO possesses an extended substrate binding site, as the enzyme-substrate affinity is relatively insensitive to the substrate's bulkiness, wherein the K_ms for catechol, 4-methyl catechol, chlorogenic acid and caffeic acid are similar (Tremolieres and Bieth, 1984).

Substrate specificities of plant and fungal PPOs are wide and varied when compared to those from animal tissue where stereospecificity for optical isomers is clear-cut (Mayer and Harel, 1979).

All catechol oxidases require the basic o-dihydroxyphenol structure for oxidase activity with catechol as the simplest possible, but not necessarily the best substrate. 4-Methyl catechol usually shows a high reaction rate (Walker, 1995). Catechol and 4-methyl catechol react with field bean PPO with similar affinities but are oxidized with different Vmax, 4-methyl catechol being oxidized at a much higher rate. The nature of the p-substituent on the diphenol affects the rate of oxidation and corresponds to the substituent constant values, σ in the Hammet equation (Mayer and Harel, 1979). Hence the presence of a methyl group, an efficient electron donor at the para position in 4-methyl catechol accounts for the increased catalytic efficiency. The rate of oxidation of o-diphenols by lettuce catechol oxidase also increased with the increased electron withdrawing power of the para position of the substituent (Mayer, 1962). As the electron withdrawing ability of the *p*-substituent increased, the K_m and the $K_{ca}t$ values of mushroom PPO decreased in the order H>SCN>COCH3>CHO>CN>NO2 (Duckworth and Coleman, 1970). 4-Nitrocatechol, protocatechuic acid and protocatechuic aldehyde with electron abstracting groups reduce the reactivity of PPO substrates. The oxidation rate of PPO is therefore governed by an electrophilic stage and the phenol is oxidized in the keto form (Horowitz et al., 1960). p-Substituted 3,4-dihydroxy phenols are oxidized at higher rates than 2,3-dihydroxy phenols substituted in position 3, eg. 3-methyl catechol, 2,3-dihydroxy benzoic acid cause a decrease in affinity of the enzyme probably by steric hindrance (Horowitz et al., 1960). Due to unavailability of these compounds our study was restricted. Gallic acid and protocatechuic acid which bears the carboxylic group, an electron-withdrawing group are not oxidized by

field bean PPO. Protocatechuic acid, with an electron withdrawing COOH group is not a substrate for poplar leaf PPO (Tremolieres and Beith, 1984) and blueberry PPO (Kader et al., 1997).

The monophenols p-cresol, L-tyrosine, ferulic acid and *p*-coumaric acid were not oxidized suggesting the absence of monophenolase activity in the enzyme preparation. Many preparations of PPO from plants are devoid of monophenolase (cresolase) activity, which is extremely labile, and the loss of this activity can be attributed to changes that occur during the purification procedure (Harel et al., 1964; Harel et al., 1965). However, the monophenolase activity of field bean PPO could be restored by the addition of catalytic quantities of small o-diphenols in the assay medium (results presented and discussed in Chapter 5).

Although most PPOs do not oxidize triphenols, some PPOs do oxidize triphenols such as quinol, resorcinol, pyrogallol and phloroglucinol (Fujita and Tono, 1980; Fujita et al., 1995). Field bean PPO oxidized only pyrogallol at a much lower efficiency than catechol (Table 3.5). The rate of oxidation of 2,3,4-trihydroxy benzoic acid was much lower owing to the electron withdrawing COOH in the vicinity. Although protocatechuic acid (3,4-dihydroxy benzoic acid) is not oxidized, the presence of an additional substituent OH at position 2 renders it as a substrate (Table 3.5). These results suggest that such substituents shield against the electron withdrawing power of COOH group. Gallic acid (3,4,5-trihydroxy benzoic acid) is inactive as a substrate (Table 3.5) but behaves as either an activator or inhibitor of field bean PPO depending on the substrate concentrations (results presented and discussed in Chapter 4).

On the one hand the enzyme-substrate affinity for field bean PPO is high for catechol, 4-methyl catechol, pyrogallol and DOPA and on the

other, inhibition by these substrates is observed at higher concentrations. This phenomenon can be rationalized by assuming that a second substrate molecule binds non-productively to the active site and thereby inhibiting the productively bound one (Trowbridge et al., 1963). Substrate inhibition was also been observed for PPO from potato (Sanchez-Ferrer et al., 1993a), spinach (Sanchez-Ferrer et al., 1989) and wheat (Interesse et al., 1983)

Yasunobu (1959) by comparing substrate specificities of various PPO concluded that although the enzyme oxidized a wide range of phenolics and the rate of oxidation corresponds to the Hammet relationship, individual enzymes 'prefer' a particular substrate or a certain phenolic compounds. Thus it can be inferred that the preferred substrates for field bean are small o-diphenols. The specificity of plant PPOs towards its phenolic substrates has been extensively studied but only a few studies have been devoted to the effect of the second substrate oxygen. The oxygen uptake of field bean PPO was concomitant with the hydroxylation and oxidation reactions of ferulic acid and caffeic acid (Figs 5.5 85 5.14, Chapter 5). In this study, oxygen uptake was used only for the assay of monophenolase and diphenolase activities of field bean PPO. Nicolas et al. (1994) have drawn up an equation from model systems to predict the oxygen uptake and shown that the PPO reaction is an ordered mechanism and oxygen binds first to the enzyme.

The rate of oxidation of phenolic substrates by field bean PPO is high but the affinity is relatively low (Table 3.5). The K_m of plant PPOs is high, generally around 1 mM and is higher than those values reported for fungi and bacteria, -0.1 mM (Mayer and Harel, 1979). However higher affinities of the order of 0.01-0.1 mM in some plant sources *viz.* potato tubers (Alberghina, 1964), cotton (Smith and Krueger, 1962) are

observed. The affinity for oxygen of plant PPOs depends on the phenolic substrate being oxidized (Harel et al., 1964). The affinity of field bean PPO to DOPA is three fold greater (Table 3.5) than 4-methyl catechol, although the oxidation rate of the latter is 6 fold greater. Therefore the molecular size of the side chain has different effects on the binding rate constant. Espin et al. (2000) suggests that the low K_m value of L-DOPA and DOPA methyl ester could be a result of the CO and NH^{3+} groups establishing some critical interaction, increasing the affinity of the enzyme toward them.

Several reagents inhibit PPO activity and inhibition studies have provided valuable insights into the mode of action of this enzyme. Because of the detrimental effects of the browning reaction in fruits and vegetables, caused by PPO, its inhibition and control by efficient and safe methods are of top priority in the food industry. Tropolone inhibits field bean PPO competitively in a classical manner (Fig 3.16a & b), although it is reported to be a slow binding non-classical competitive inhibitor of grape PPO (Valero et al., 1991). A mixed type of inhibition with tropolone was reported for soluble potato PPO (Sanchez-Ferrer et al., 1993a) and mushroom tyrosinase (Kahn and Andrawis, 1985). Tropolone chelates the active site copper of PPO slowly binding to the oxy form of the enzyme (Valero et al., 1991). It is a not an inhibitor for laccase and therefore useful in differentiating PPOs from laccases which also oxidize o-diphenols (Flurkey et al., 1995). Diethyldithiocarbamate, a potent inhibitor of plant PPOs (Anosike and Ayaebene, 1982) also complexes the copper prosthetic group at the active center. Aromatic carboxylic acids and substituted phenols have long been used as inhibitors for PPO. Gallic acid although a substrate for some plant PPOs (Billaud et al., 1996; Passi and Porro, 1981; Arslan et al., 1998), inhibits field bean PPO at low substrate concentration and activates it at high substrate concentration (details discussed in Chapter 4). The

PPO catalyzed reaction (Fig 1.1, Chapter 1) is reversible and as it is an oxidation reaction, requires a reducing agent to reverse it thereby inhibiting it. Inhibition by sulfhydryl compounds and reducing agents such as ascorbic acid and potassium metabisulfite are well documented. The observed inhibition effect of these compounds on the rates of PPO catalyzed reactions is generally attributed to a fast non-enzymatic reduction of the quinones formed back to the colorless o-dihydroxyphenolic substrates. (Iyengar and McEvily, 1992) The inhibitor reaction mechanism differs depending on the reducing agent employed. Inhibition by thiol compounds is attributed to either the stable colorless products formed by an addition reaction with o-quinones (Ikediobe and Obasuyi, 1982) and/or binding to the active center of PPO (Valero et al., 1992). Ascorbate, acting as an antioxidant reduces the initial o-diquinone product prior to it undergoing secondary reactions which lead to browning (Whitaker, 1972) and cysteine and other thiols form colorless thiol-quinone adducts without actually inhibiting the O2 uptake reaction (Dudley and Hotchkiss, 1989)

Antibodies can be useful in identifying the isoenzyme forms and also determining their cross reactivity among different plant species. It is a useful tool to examine how the different isoforms are generated. The cross-reactivity of PPOs from different sources towards polyclonal antibodies raised against field bean PPO gives evidence for the common antigenic determinants present on these PPOs. Flurkey (1986) reported that eight different plant species cross-reacted with anti broad bean PPO antibodies in Ouchterlony double diffusion tests. Lanker et al. (1988) also reported that polyclonal antibody against broad bean PPO recognized PPO in a variety of plant species including those from bushbean, lettuce, mung bean, pea, soybean, spinach, tobacco and tomato. The monoclonal anti-PPO antibody raised against broad bean PPO had more limited use because of its narrow specificity. Antibodies

raised against apple, *Malus pumila* (Murata et al., 1993) cross-reacted with crude extracts of PPO from five different culltivars of apple. Among crude extracts of eggplant, banana, pear, broad bean, spinach and lettuce, only pear PPO cross reacted with the anti- apple PPO antibody when analysed by western blotting. In our study, PPO from all the sources examined was found to be serologically related to the field bean PPO. Marques et al. (1994) raised anti-PPO antibodies and explained the relationships between the multiple forms of apple PPO. They showed that the most important isoform in apple is a 64 kDa monomer, which when folded by an internal disulfide is a 42 kDa protein. This form can be cleaved by proteolysis to a 42 kDa monomer that forms a 27 kDa protein after folding and all are active. The polyclonal anti-PPO serum will be thus useful in further structural studies and characterization of the enzyme being pursued in this laboratory.

Chapter 4
Inhibitory properties of phenolics on field bean *(Dolichos lablab)* polyphenol o×idase

PPOs from various source shows a preference to 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, each individual enzyme tends to prefer a particular substrate or a certain type of phenolic compound. Conformational changes in the active site of PPO by the correct spatial docking of the substrates afford access to the catalytic metal centre resulting in oxidation (Eicken et al., 1998). Although field bean PPO was active towards the diphenols, catechol, 4-methyl catechol, L-DOPA and the triphenols, pyrogallol and 2,3,4-trihydroxy benzoic acid (Table 3.5, Chapter 3), it showed no activity towards the most important and common naturally occurring substrates, (+)catechin, chlorogenic acid and caffeic acid (Table 3.5, Chapter 3). Only minor structural differences among phenolic substrates are necessary to transform substrates into inhibitors and/or activators (Tremolieres and Beith, 1984). Therefore, a study of phenolics as either inhibitors or activators of field bean PPO was pursued, the results of which are presented and discussed in this chapter.

RESULTS

Inhibitory properties of natural substrates

The effect of the natural substrates, caffeic acid, chlorogenic acid and (+)catechin were tested towards oxidation of catechol by field bean PPO. All the three compounds were found to inhibit the oxidation of catechol by PPO. The inhibitory effect of caffeic acid was monitored at concentrations ranging from 0.025-0.075 mM with catechol concentrations of 5-40 mM. The Michaelis-Menten plot of the reaction velocities at different substrate and inhibitor concentrations is presented in Fig 4.1. It is observed that, as the catechol concentrations

increased, the inhibitory effect of caffeic acid decreased, suggestive of a competitive type of inhibition.

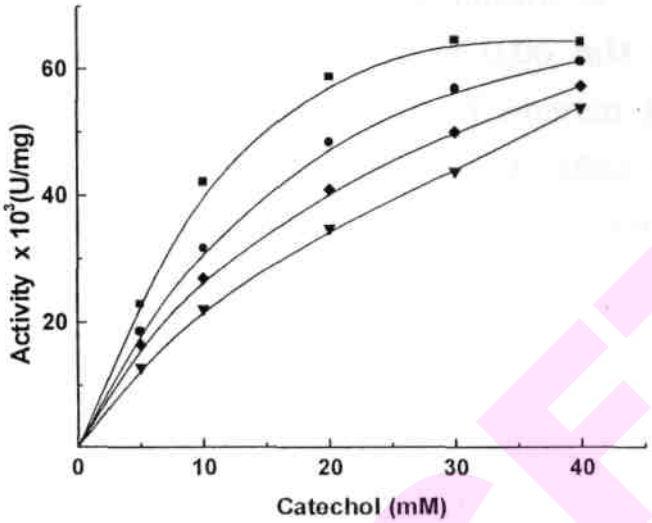


Fig 4.1. **Effect of caffeic acid on catechol oxidation by field bean PPO.** Assay medium contained 50 mM Na acetate buffer, pH 4.0 and 25 µg of field bean PPO pre-incubated in caffeic acid (■) 0 mM, (•) 0.025 mM, (♦) 0.05 mM and (T) 0.075 mM at the indicated concentrations of catechol.

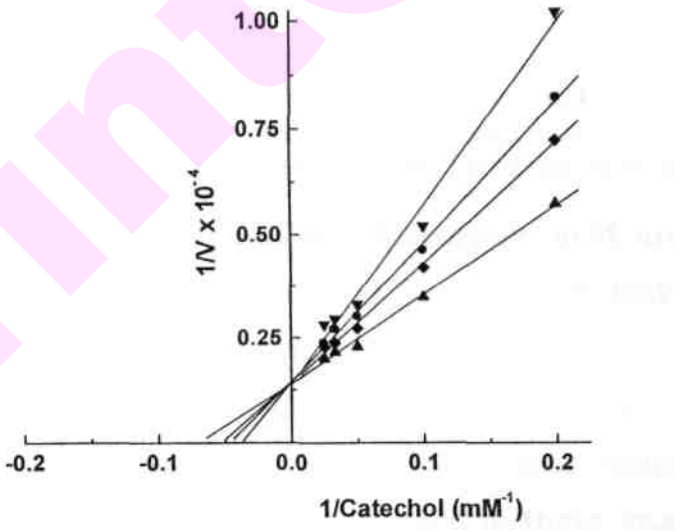


Fig 4.2. **Double reciprocal plot of catechol oxidation at different concentrations of caffeic acid.** Caffeic acid (A) 0 mM, (♦) 0.025 mM, (•) 0.05 mM and (▼) 0.075 mM. Data of Fig 4.1.

The Lineweaver-Burk double-reciprocal plot (Fig 4.2) shows a series of lines that intersect on the Y axis with similar V_{max} and different apparent K_ms indicating the competitive nature of the inhibition by caffeic acid. The inhibition constant Ki of 0.06 mM (Table 4.1) was deduced from the Dixon plot (Dixon, 1942) wherein 1/V was plotted against [I] (Fig 4.3). The inhibition constant of caffeic acid is 170 fold lower than the K_m of catechol (10.5 mM, Chapter 3) indicating the much higher binding affinity of caffeic acid when compared to catechol.

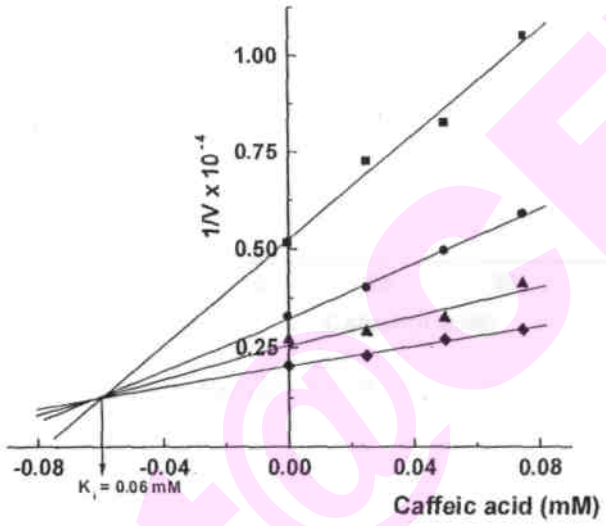


Fig 4.3. **Dixon plot for determining the inhibition constant of caffeic acid.** Caffeic acid at the indicated concentrations and catechol (■) 5 mM, (•) 10 mM, *(A)* 20 mM and (♦) 30 mM. Data taken from Fig 4.2.

The effect of the diphenols, chlorogenic acid and (+)catechin on catechol oxidation was also studied in a similar way. The Michaelis-Menten plots for the effect on chlorogenic acid and catechin on the initial velocity of catechol oxidation are represented in Fig 4.4 & 4.5 respectively. Chlorogenic acid at concentrations ranging from 0.5-1.5 mM and (+)catechin at 1-3 mM were used in these studies. The pattern of the Michaelis-Menten plots obtained were typical of a competitive type of inhibition. The results recorded in Table 4.1 were obtained from Lineweaver-Burk plots of 1/V against 1/[S] (data not shown), at three levels of inhibitor and the inhibition constants for catechin and

chlorogenic acid were confirmed by Dixon plots of 1/V against [I] (not shown). The Ki determined for chlorogenic acid was 0.37 mM which is higher than the Ki of caffeic acid but is lower than the Ki of (+)catechin (1.2 mM). The Ki values followed the sequence catechin>chlorogenic acid>caffeic acid showing that caffeic acid has the highest affinity.

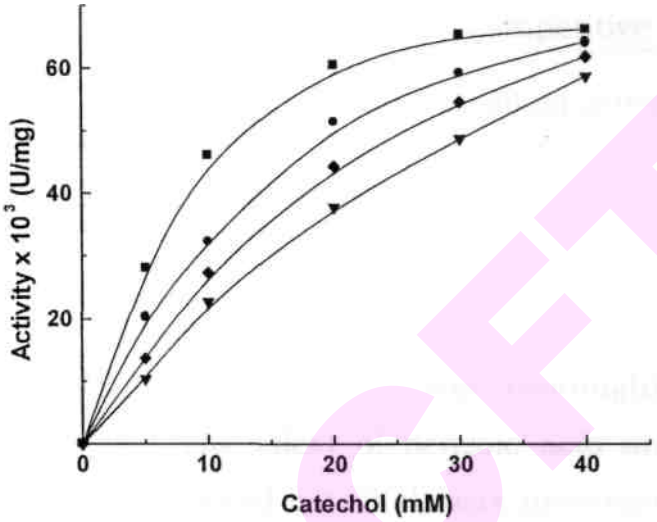


Fig 4.4. **Effect of chlorogenic acid on catechol oxidation by field bean PPO.** Assay medium contained 50 mM Na acetate buffer, pH 4.0 and 25 |ig of field bean PPO pre-incubated with chlorogenic acid (■) 0 mM, (•) 0. 5 mM, (♦) 1 mM and (▼) 1.5 mM, at the indicated concentrations of catechol.

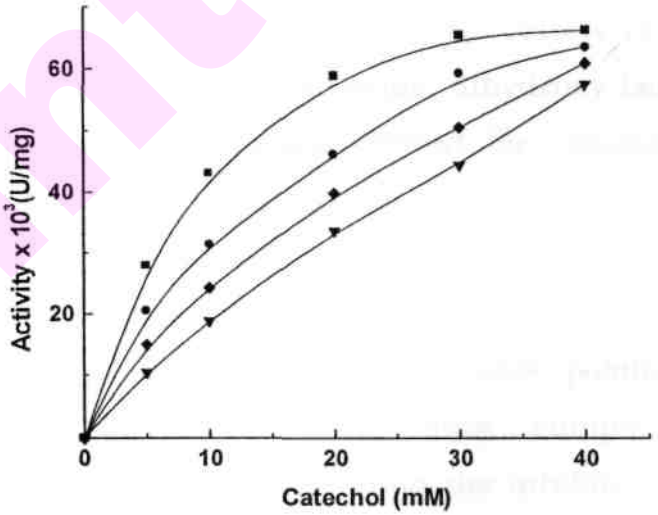


Fig 4.5. **Effect of (+)catechin on catechol oxidation by field bean PPO.** Assay medium contained 50 mM Na acetate buffer, pH 4.0 and 25 (ig of field bean PPO pre-incubated with (+) catechin (■) 0 mM, (•) 1 mM, (♦) 2 mM and (T) 3 mM at the indicated concentrations of catechol.

Table 4.1. **Inhibitory effect of some natural substrates on field bean PPO***

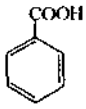
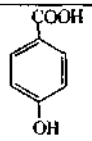
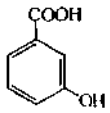
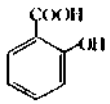
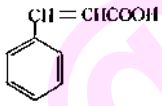
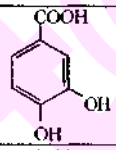
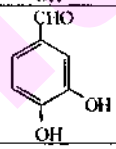
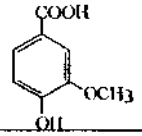
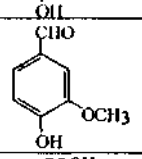
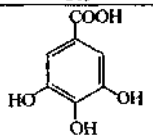
Phenolic compound	**Ki** (mM)	Type of inhibition
caffeic acid	0.06	competitive
chlorogenic acid	0.37	competitive
(+)catechin	1.2	competitive

* PPO was incubated with inhibitors for 3 min. Residual activity was assayed using catechol as the substrate.

Effect of benzoic acid and its substituted compounds on field bean PPO

PPOs from various sources are inhibited by a considerable number of compounds and to date the most thoroughly studied are aromatic carboxylic acids. The effect of benzoic acid and substituted benzoic acids on the oxidation of catechol, was investigated. It should be noted here that the enzyme pre-incubated in these studies was mostly metPPO (E_{met}), known as the resting form (Chapter 5). Benzoic acid was found to be the most effective, among the compounds in the series studied for their inhibitory effect on field bean PPO. On the basis of this potent PPO inhibitory activity, several closely related congeners (Table 4.2) such as hydroxy benzoic acids, dihydroxy benzoic acids and trihydroxy benzoic acids were also tested for comparison. A single substitution on the phenyl ring was found to diminish the inhibitory effect of benzoic acid. Thus, the degree of inhibition of aromatic acids of benzoic acid series decreased in the order of *para*>*meta*>*ortho* when a hydroxyl group was substituted in one of these positions (Table 4.2). The degree of inhibition of these three compounds at 1 mM concentration was considerably less than the inhibition brought about by a ten fold lower concentration of benzoic acid. These studies were carried out at catechol concentrations, five fold greater than the K_m of catechol.

Table 4.2. **Relative inhibition of field bean PPO by phenolic compounds.**

Phenolic compound	Structure	Conc. (mM)	% inhibition
Benzoic acid		0.1 1.0	96.0 98.0
p-Hydroxy benzoic acid		0.1 1.0	68.0 78.9
m-Hydroxy benzoic acid		0.1 1.0	38.4 67.3
o-Hydroxy benzoic acid		0.1 1.0	32.0 59.7
Cinnamic acid		0.1 1.0	88.2 94.1
Protocatechuic acid		0.1 1.0	39.3 69.2
Protocatechuic aldehyde		0.1 1.0	27.5 37.3
Vanillic acid		0.1 1.0	46.6 73.1
Vanillin		0.1 1.0	9.7 32.7
Gallic acid		0.1 1.0 10	1.3% activation 18% activation 84% inhibition

Further hydroxylation of the hydroxy benzoic acid to 3,4-dihydroxy benzoic acid (protocatechuic acid) decreased the inhibitory effect on field bean PPO. The inhibitory effect was ~29% less than p-hydroxy benzoic acid but of the same degree as m-hydroxy benzoic acid. These results indicate that a m- hydroxy substitution is not effective for inhibition. The replacement of the carboxylic group of benzoic acid with acrylate (cinnamic acid) was also found to marginally decrease the inhibition at lower concentration of the inhibitor. At concentrations of 1 mM, the inhibition potency of cinnamic acid was similar to that of benzoic acid. When the carboxylic groups of either protocatechuic acid or vanillic acid were replaced by an aldehyde group to give the corresponding aldehydes, there was a considerable reduction in the inhibitory effect (Table 4.2). A 50% decrease in the inhibitory effect was noted when the carboxylic group of protocatechuic acid was substituted by an aldehyde. Vanillic acid exhibited an inhibition of 73.1% at 1 mM concentration whereas vanillin inhibited to the extent of only 32.7%. These results indicate the requirement of a free carboxylic group, substituted directly on the benzene ring for inhibition. The degree of inhibition by protocatechuic acid and vanillic acid are similar. However, the degree of inhibition brought about by vanillic acid is greater than m-hydroxy benzoic acid but less than p-hydroxy benzoic acid suggesting that a methoxy group in place of a hydroxy group probably enhances their inhibitory effects on field bean PPO. The addition of either one or two phenolic groups to benzoic acid (hydroxyl and dihydroxy benzoic acid) resulted in a decreased inhibition (Table 4.2). Addition of a third phenolic group to obtain 2,3,4-trihydroxy benzoic acid converts into a substrate (Table 3.5, Chapter 3). In contrast, gallic acid (3,4,5-trihydroxy benzoic acid) activates PPO towards catechol oxidation (Table 4.2). Gallic acid, when assessed for its

effect on field bean PPO, at different concentrations had markedly different effect acting both as an inhibitor and as an activator.

Effect of gallic acid on field bean PPO

Gallic acid is not a substrate of field bean PPO (Chapter 3, Table 3.5). At 1 mM concentration, although the other carboxylic acids studied had an inhibitory effect, gallic acid was found to stimulate the catechol oxidation. An 18% increase in the catechol quinone formation was observed (Table 4.2). At a ten fold lower concentration of gallic acid (0.1 mM), there was a negligible increase in the activity. At 10 fold higher concentration (10 mM), gallic acid was found to inhibit the oxidation of catechol by -84%. This dual effect of gallic acid was further investigated. The effect of varying concentrations of gallic acid on the oxidation of a fixed concentration of catechol at pH 4.0 is shown in

-Fig 4.6.

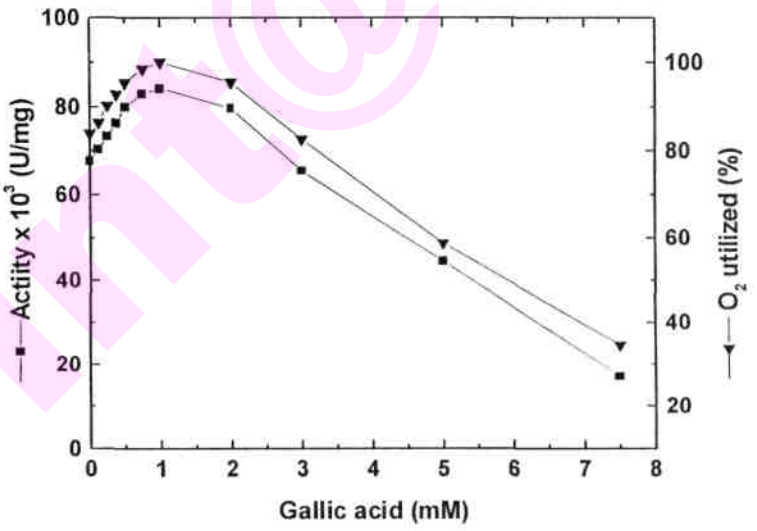


Fig 4.6. **Effect of gallic acid on the rate of catechol oxidation by field bean PPO.** The assay medium consisted of 50 mM Na acetate buffer pH 4.0, 50 mM catechol and 20 *xg* of field bean PPO in the presence of gallic acid at indicated concentrations.

A linear increase in the oxidation of catechol was observed with increasing concentration of gallic acid upto 1 mM. At concentrations >lmM and thereafter, there was a sharp decline in the oxidation. At 7.5 mM of gallic acid, only 25% of the original activity was measurable. Similar results were obtained when this activation and inhibition was monitored by polarographic method of oxygen consumption (Fig 4.6). The effect of fixed concentrations of gallic acid on varying substrate concentrations was analysed. A plot of V versus [S] (Fig 4.7) provides some interesting results. At low substrate concentrations, up to the intersecting point, (35±2 mM), gallic acid (0.5-1.5 mM) inhibits oxidation of catechol by field bean PPO. But at catechol concentrations above the intersection point, gallic acid activates the reaction.

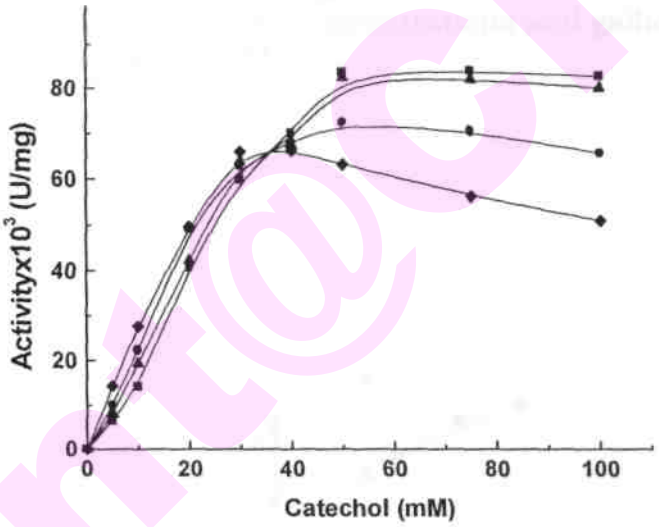


Fig 4.7. **Effect of the gallic acid on catechol oxidation by field bean PPO.** Assay medium contained 50 mM Na acetate, pH 4.0, 25 jag of field bean PPO, catechol at the indicated concentrations and gallic acid (♦) 0 mM, (•) 0.5 mM, *(A)* 1 mM and (■) 1.5 mM.

The respective inhibition and activation constants of gallic acid (Ki and K_a) were determined individually by double reciprocal plots considering the concentrations at which it inhibited or activated. The Lineweaver-Burk plot of 1/V against 1/[S] at the substrate

concentrations where gallic acid inhibits the oxidation of catechol is shown in Fie 4.8.

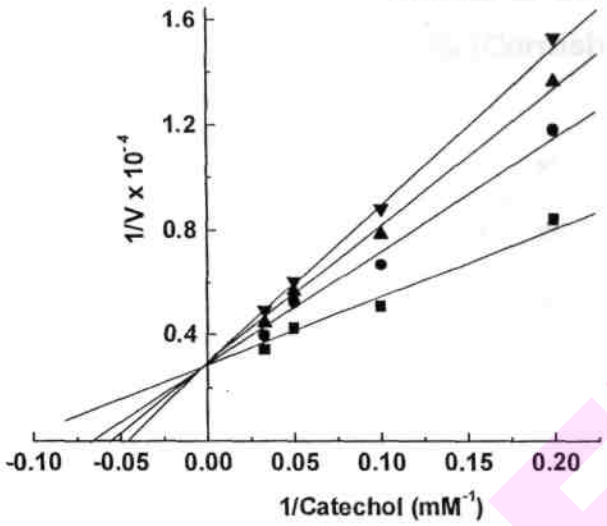


Fig 4.8. **Double reciprocal plot of gallic acid inhibition of catechol oxidation.** Catechol at the indicated concentrations and gallic acid **(■)** 0 mM (•) 0.5 mM, **(A)** 1 mM and **(T)** 1.5 mM.

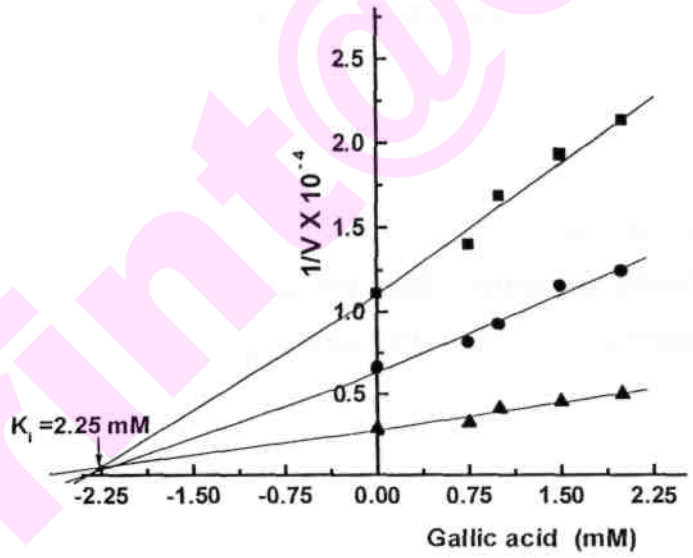


Fig 4.9. **Dixon plot for determining the inhibition constant of gallic acid.** Gallic acid at the indicated concentrations and catechol (■) 5 mM, (•) 10 mM and (A) 20 mM.

The Ki of gallic acid as determined from the Dixon plot of 1/V against [I] (Fig 4.9) was 2.25 mM. The activation constant of gallic acid

was determined by a method analogous to the Dixon plot where, 1/V is plotted against 1/A at two or more values of [S], where 'A' is the activator (Fig 4.10). The abscissa coordinate of the point of intersection of the resulting straight lines gives $-1/K_a$ (Cornish-Bowden, 1995), the apparent K_a being 0.38 mM.

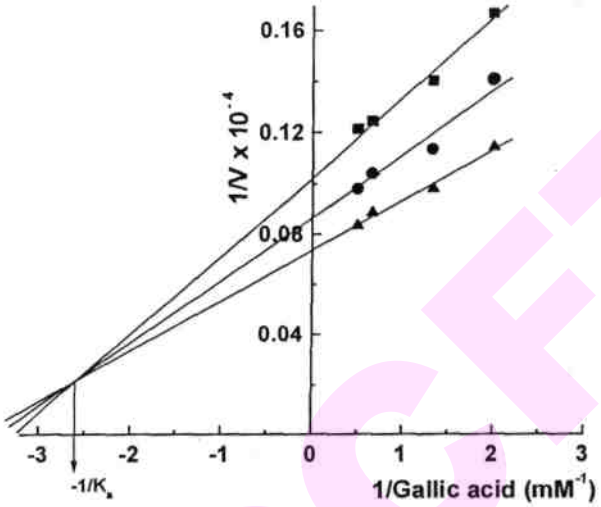


Fig 4.10. **Cornish-Bowden plot to determine the activation constant of gallic acid for catechol oxidation.** Gallic acid at the indicated concentrations and catechol (■) 50 mM, (»)75 mM and (A) 100 mM.

Fig 4.11a shows that catechol oxidation was inhibited with increasing concentrations of gallic acid, wherein the magnitude of inhibition decreased (decrease in the slope) as the substrate concentration increased. At 40 mM catechol, above which substrate inhibition is observed (Fig 3.15a, Chapter 3), increasing concentrations of gallic acid appears to have no effect upon the oxidation rate. At catechol concentrations >50 mM, where substrate inhibition is more pronounced, increasing gallic acid concentration enhances the oxidation rate upto a cross over point of = 1.5 mM. Thereafter, increasing concentrations of gallic acid inhibits catechol oxidation by field bean PPO (Fig 4.11b). The effects of gallic acid observed on catechol oxidation depend solely on the substrate concentration.

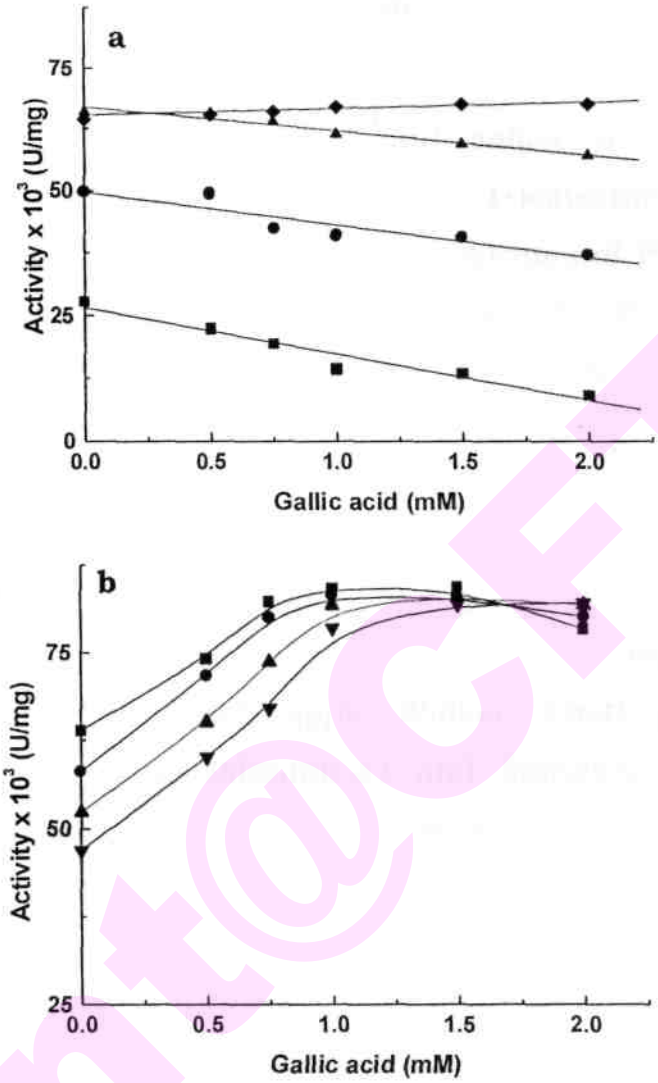


Fig 4.11. **Inhibition and activation effect of gallic acid on catechol oxidation.** Assay medium contained 50 mM Na acetate, pH 4.0, 25 p.g of field bean PPO and gallic acid at the indicated concentrations, (a) catechol **(■)** 10 mM, (•) 20 mM, **(▲)** 30 mM, **(♦)** 40 mM; (b) **(■)** 50 mM, (•) 75 mM, **(▲)** 100 mM and **(▼)** 150 mM.

DISCUSSION

Fruits and vegetables contain a wide variety of phenolic compounds. However, only a relatively small number of them serve as substrates to PPOs. Certain phenolic compounds used as food preservatives are chemically capable of acting as alternative substrates

or competitive inhibitors of PPO *in vitro* (Walker, 1995), hence their effects merit investigation.

(+)Catechin, chlorogenic acid and caffeic acid are common naturally occurring substrates. In apples, (+)catechin, (-)epicatechin and chlorogenic acid were identified as substrates of PPO, chlorogenic acid being the major substrate (Tanfel and Voigt, 1963). In potato, apart from tyrosine, chlorogenic acid and caffeic acid are substrates and form colored products upon enzyme action (Matheis and Belitz, 1977). The substrate specificity of PPOs from different plant sources have been investigated in detail. PPOs from strawberry (Wesche-Ebeling and Montgomery, 1990), grape (Gunata et al., 1987), banana bud (Oba et al., 1992), loquat fruit (Ding et al., 1998), gum arabic (Billaud et al., 1996), pear (Luh et al., 1963), apple (Walker, 1964), peach (Jen and Kahler, 1974) and plum (Moutounet and Mondles, 1976) oxidized catechin, chlorogenic acid and caffeic acid to their quinones. PPO from plantain (Ngalani etal., 1993), iceberg lettuce (Heimdal et al., 1994), plum (Siddiq et al., 1996) and highbush blueberry (Kader et al., 1997) were found to be active towards caffeic acid and chlorogenic acid and not catechin. Monroe apple peel PPO (Zhou et al., 1993) and Portabella mushroom PPO (Zhang etal., 1999) oxidized catechin and chlorogenic acid, whereas raspberry PPO (Gonzalez et al., 1999) was found to be active only towards catechin and not towards caffeic acid and chlorogenic acid.

Field bean PPO however shows no activity to these three o-diphenolic compounds although PPOs require the basic o-dihydroxyphenol structure for utilizing a substrate. The acceptability of a particular substrate depends on the source of the enzyme, nature and position of the substituent group on the aromatic ring, as well as on the length of the side chain. In addition, the spatial orientation of the

essential vicinal dihydroxy groups with respect to the side chain is also important (Espin et al., 1998c). Finkle and Nelson (1963) suggested the use of an o-methyl transferase to methylate and thus block the natural substrates of various fruit PPOs, to inhibit browning. This enzyme converts caffeic acid to ferulic acid and chlorogenic acid to feruloyl quinic acid. By this process, the fruit's PPO was deprived of its substrate (s), which were converted to enzyme inhibitors by the addition of a single methyl group.

Field bean PPO appears to have a small and compact substrate binding site, which does not allow the productive binding of substrates like caffeic acid, chlorogenic acid or (+)catechin (Chapter 3). These phenolics are competitive inhibitors binding strongly to field bean PPO, caffeic acid being the most potent.

The carboxylic side chain with a conjugated double bond of caffeic acid appears to be instrumental in affecting the rate of oxidation by field bean PPO, as the esterfication of the carboxylic group of caffeic acid with quinic acid (chlorogenic acid) lowers the inhibitory efficiency. Esterification of the carboxyl group of benzoic acid and cinnamic acid leads to a considerable decrease of their inhibitory strength on grape catechol oxidase (Gunata et al., 1987), apricots (Soler-Martinez etal., 1965) and cherries (Pifferi etal., 1974). The Ki values reported here (Table 4.1) for caffeic acid are much lower than that reported for cinnamic acid and p-coumaric acid of grape catechol oxidase (Gunata etal., 1987). (+)Catechin with its bulky flavan structure was also not used as a substrate by the field bean PPO. Oxidation of these o-diphenols does not occur probably due to improper positioning of the phenyl ring with its bulky side chain. However, these compounds are competitive inhibitors of field bean PPO when catechol was used as the substrate. Three-dimensional studies on the sweet potato catechol

oxidase indicate that the catalytic copper centre is accomodated in a central four-helix bundle located in a hydrophobic pocket (Klabunde etal., 1998). Access to the catalytic metal centre is primarily controlled by the rotation of the aromatic ring of Phe 261, which lines the hydrophobic pocket (Eicken et al., 1999). In arthropodean He, the aromatic ring of a Phe in the amino-terminal domain shields access to the dimetal centre and this shielding prohibits any binding of substrates (Hazes et al., 1993). This phenyl ring aligns perfectly with the aromatic ring of PTU, an inhibitor of catechol oxidase. Therefore it is possible that the phenolic substrates, such as catechin, in the case of field bean PPO binds, but free rotation of the crucial phenyl ring is prevented by the bulky side chains, which prevents access to the catalytic centre. The binding of PTU to catechol oxidase results in Phe 261 undergoing a conformational change to form hydrophobic interactions with the aromatic ring of the inhibitor (Eicken et al., 1999). Van der Waal's interaction also contributes to the high affinity of PTU for the enzyme. Therefore, depending on their chemical structure, phenolic compounds may act as substrates or as competitive inhibitors of PPOs. Caffeic acid, chlorogenic acid and (+)catechin, not being substrates and being aromatic compounds are good candidates as competitive inhibitors for field bean PPO. The low Ki of caffeic acid, chlorogenic acid and (+)catechin in comparison with the K_m of catechol gives evidence for the high binding affinity to the active site of field bean PPO even though the binding is not a catalytically productive one. These results suggest that the phenyl ring probably align with the so called "gate residue" and maintain Van der Waal's interactions with the residues that line the hydrophobic pocket, resulting in the high affinity for the enzyme.

Aromatic carboxylic acids of the benzoic acid and cinnamic acid series have been extensively investigated. Aromatic carboxylic acids

were competitive inhibitors of PPO due to their structural similarities to its phenolic substrates (Kreuger, 1955), and the type of inhibition was dependent on the substrate (Walker and Wilson, 1975; Gunata et al., 1987). The efficiency of an aromatic carboxylic acid, like the efficiency of a substrate, is governed by the position, nature and orientation of the substituent group (s) on the aromatic ring. Kutner and Wagreich (1953) reported that benzoic acid was found to be the best inhibitor of mushroom catechol oxidase. Its inhibitory effects decreased by esterifying or ionizing the acid group. Pifferi et al. (1974) also reported that the addition of either electron donating or electron attracting groups on the benzene ring of carboxylic acids, either increased or decreased the inhibitory effect on cherry PPO.

The effect of two concentrations (0.1 and 1 mM) of benzoic acid and its derivatives on field bean PPO activity (Table 4.2) indicate that, benzoic acid was the strongest inhibitor of catechol oxidation of field bean PPO. Hydroxylation of the benzene ring decreased the inhibitory capacity, in the order of *para>meta>ortho.* These results are also in agreement with those reported by Pifferi et al. (1974), Gunata et al. (1987) and Kermasha et al. (1993) for inhibiton studies from sweet cherry, grape and mushroom PPO respectively. Billaud et al. (1996) in their studies on PPO from gum arabic, reported that the addition of another phenolic group to the benzoic acid always resulted in a decrease of inhibition but was more marked for substitution in the *ortho* position than in the *para* position. Substitutions in the *ortho, meta* and *para* positions diminishes the degree of inhibition of benzoic acid by creating a steric interference in the interaction between the benzene nucleus with the enzyme (Pifferi et al., 1974).

Protocatechuic acid with an additional hydroxyl group in position *meta* to the carboxylic group of p-hydroxy benzoic acid displays an

inhibition less than p-hydroxy benzoic acid and greater than m-hydroxy benzoic acid (Table 4.2). Protocatechuic acid, with its o-dihydroxy group was found to be used as a substrate by a few PPO. Strawberry (Wesche-Ebeling and Montgomery, 1990) and loquat fruit (Ding et al., 1998) showed reactivity towards protocatechuic acid but the activity towards this substrate was very low when compared to other o-diphenolic substrates. In spite of possessing the o-dihydroxy group, protocatechuic acid is an inhibitor and not a substrate for field bean PPO. The presence of the carboxylic group, a strong electron withdrawing group, converts the substrate to an inhibitor. Batistuti and Lorenco (1985) showed that protocatechuic acid demonstrated a non-competitive inhibition on potato PPO activity when chlorogenic acid was used as substrate, suggesting that the substrate and inhibitor bind at different sites on the enzyme molecule. Kermasha et al. (1993) and Passi and Porro (1981) also observed inhibition by protocatechuic acid on PPO. Replacing the carboxylic group by an aldehyde group (protocatechuic aldehyde and vanillin) further diminished the inhibitory effect, because an aldehyde group has less electron withdrawing capacity than the carboxylic group. Similar effect was also observed by Passi and Porro (1981). In contrast, Kubo and Kinst-Hori (1998) investigating the effects of the flavor compounds of anise oil on mushroom tyrosinase indicate that the aldehyde is a key group in eliciting potent inhibitory action. They established that benzaldehyde was a non-competitive inhibitor whereas p-hydroxy benzaldehyde was a competitive inhibitor of less potency.

Cinnamic acid, where the COOH group of benzoic acid is directly substituted by a CH=CHCOOH group, also was a potent inhibitor of a slightly lower efficiency (Table 4.2). The inhibitory effect was higher than the ring substituted benzoic acids. Cinnamic acid and its analogs were reported to be potent inhibitors of apple PPO (Walker and Wilson, 1975). The separation of the carboxyl group from the benzene nucleus

markedly reduced the inhibition of gum arabic PPO. The Ki for p-coumaric acid and cinnamic acid was ten fold higher than benzoic acid (Billaud etal., 1996). The effect of pH on the inhibition of carboxylic acids is profound, because pH directly affects the dissociation of carboxylic acids. Pifferi etal. (1974) and Janovitz-Klapp et al. (1990) showed that PPO inhibition by carboxylic acids was mainly associated with the protonated form. The studies with field bean PPO were all carried out at pH 4.0, where all the acids are protonated. Billaud etal. (1996) suggest that, provided the pH condition are adequately defined, carboxylic acids could be considered as potential alternatives to sulfites in controlling enzymatic browning.

The effect of gallic acid on field bean PPO was distinct when compared to the other carboxylic acids investigated (Table 4.2) and also to that reported in the literature. Gallic acid has been reported as either a substrate or inhibitor of plant PPOs. PPO from gum arabic (Billaud etal., 1996), mushroom (Passi and Porro, 1981) and Malatya apricot (Arslan et al., 1998) utilized gallic acid as a substrate at a much lower rate than the o-dihydroxyphenols. In contrast, gallic acid was a competitive inhibitor of diphenolase activity of grape PPO (Gunata et al., 1987) and mushroom (Kermasha etal., 1993). Kermasha etal. (1993) reported gallic acid to be a non-competitive inhibitor of mushroom PPO suggesting that gallic acid reduces the affinity of the substrate for the enzyme, yet it does not bind to the active site.

Catechol oxidation of field bean PPO was enhanced by low concentrations of gallic acid but at higher concentrations it inhibited the catechol oxidation (Table 4.2 & Fig 4.6). The dual activation and inhibition phenomena induced by the gallic acid was found to be dependent both on the substrate concentrations and gallic acid concentration. At low concentrations of the substrate (below saturation

level), gallic acid appears to compete with catechol for the active site of field bean PPO. The Ki of gallic acid, 2.25 mM is higher than the other phenolic compounds which are also competitive inhibitors of catechol oxidation (Table 4.1). However, at saturating levels of substrate, higher gallic acid concentration enhances the rate of catechol oxidation. The K_a, 0.38 mM, for gallic acid indicates a high affinity for the enzyme. Gallic acid is a non-essential potent activator of the PPO catalyzed oxidation of catechol.

The phenomenon of activation by phenolic compounds has been observed in several PPOs but it is always the monophenolase activity, which was very often found to be activated. Activation, in most cases was induced by o-diphenols (Pomerantz and Warner, 1967; Duckworth and Coleman, 1970; Hearing and Ekel, 1976). Shannon and Pratt (1985) while studying the activity of apple PPO in relation to various phenolic compounds, found that phloroglucinol and resorcinol increased the rate of oxidation of chlorogenic acid, especially at higher chlorogenic acid concentrations. Tremolieres and Beith (1984) observed a combined activation and inhibition effect of ferulic acid and gallic acid on the PPO catalyzed oxidation of catechol from black poplar. The K_a of gallic acid (0.38 mM) for field bean PPO is similar to the K_a (0.34 mM) of ferulic acid activation of black poplar PPO-catalyzed oxidation of catechol. However, their studies were restricted to a fixed substrate concentration and varying inhibitor concentrations. Tremolieres and Beith (1984) conclude that the activation was mechanistically similar to non-competitive inhibiton or to excess substrate inhibition since a ternary enzyme-substrate-effector complex is postulated (Dixon and Webb, 1964).

The existence of an effector site other than the catalytic site has been proposed by several workers. Walker and Wilson (1975) suggested

the presence of two distinct binding sites on apple PPO "a catalytic site" for the substrate and an adjacent "inhibitor site". The effect of gallic acid on catechol oxidation by field bean PPO appears to involve only binding at the active site. At low substrate concentrations (<35±2.5 mM), gallic acid competitively inhibit catechol oxidation (Fig 4.8 & Fig 4.1 la). At catechol concentrations >35±2.5 mM, substrate inhibition of field bean PPO is demonstrated (Fig 3.15, Chapter 3). At this substrate concentration, gallic acid neither inhibits nor activates the enzyme as the active site is occupied with catechol. At saturating substrate concentrations and higher, catechol exhibits substrate inhibition. At saturating levels of substrate, the active site appears to be inaccessible to gallic acid, however it appears to prevent the nonproductive binding of catechol, which generally causes substrate inhibition (Tremoliers and Beith, 1984). Therefore gallic acid activates field bean PPO primarily, by abolishing the substrate inhibition and not by activation 'per se'.

Benzoic acid and substituted benzoic acid were used as antibrowning agents in processed fruit and vegetable industry. In conclusion, this study shows that the choice of an aromatic acid as an antibrowning agent depends not only on the source of the enzyme but also upon the availability of the substrate, which could render as an inhibitor or as an activator.

Chapter 5
Activation of the monophenolase and diphenolase activities of field bean *(Dolichos lablab)* polyphenol o×idase by diphenols

PPO, a binuclear copper containing enzyme, catalyzes two ostensibly distinct reactions (1) hydroxylation of monophenols to o-diphenols, the only specific reaction catalyzed by this enzyme (cresolase or monophenolase) and (2) oxidation of the self generated o-diphenols to the corresponding o-quinones (catecholase or diphenolase). The second reaction can be achieved not only by PPO but also by peroxidases and laccases (Kalyanaraman et al., 1984). The PPO purified from field bean *(Dolichos lablab)* exhibited no activity towards hydroxylation of monophenols (Chapter 3, Table 3.5). It has been recognized that the addition of catalytic quantities of reducing agents/diphenols as co-substrates, induce the monophenolase activity. In this chapter, the results of the oxidation of monophenols by field bean PPO in the presence of catalytic quantities of diphenols as the co-substrates, ascertaining the existence of a monophenolase activity is presented. The most important characteristic of this activity is that only those diphenols that exhibit high binding affinity for the diphenolase activity (Chapter 3, Table 3.5) act as co-substrates. In addition, these diphenols also activate the diphenolase activity by an entirely different mechanism. The results are presented and discussed with reference to the mechanism proposed earlier by Sanchez-Ferrer et al. (1995).

RESULTS

Monophenolase activity of field bean PPO

PPO purified from field bean *(Dolichos lablab)* seeds showed no activity towards monophenols (Chapter 3, Table 3.5) indicating the absence of hydroxylating activity (monophenolase). The absence of monophenolase activity of several plant PPOs has been attributed to its loss during purification (Matheis, 1987). The monohydroxyphenols, ferulic acid, tyrosine, p-coumaric acid and p-cresol are not hydroxylated by field bean PPO (Chapter 3, Table 3.5).

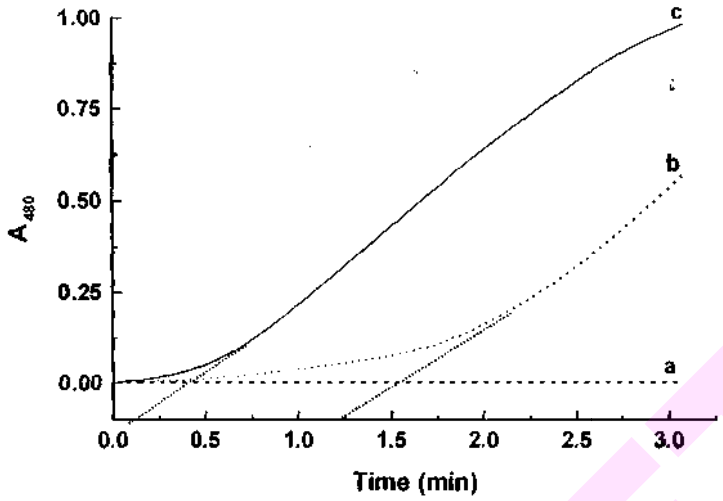


Fig **5.1. Time course of product formation in the oxidation of ferulic acid by field bean PPO.** Progress curve with the reaction medium containing 0.2 mM ferulic acid in 50 mM Na acetate buffer pH 4.5, 20 µg of enzyme and catechol (a) 0 mM (b) 2 mM (c) 20 mM.

Fig 5.1 shows the time course of ferulic acid oxidation under varying experimental conditions. When ferulic acid oxidation was attempted at saturating oxygen concentrations, but in the absence of catechol, there was no measurable activity (Fig 5.1, curve a). In the presence of catalytic concentrations of catechol, ferulic acid was oxidized to its corresponding quinone. Curves b and c (Fig 5.1) show that ferulic acid was hydroxylated when catechol (2 & 20 mM) was included in the assay mixture. However, the reaction rate was characterized by a lag phase.

Purified field bean PPO was subjected to electrophoresis on a 7.5% native-PAGE and stained with catechol and ferulic acid to determine its activity towards mono and diphenols. All the staining solutions contained 0.05% p-phenylenediamine as the complexing agent, which enhances the color formed (Lee, 1991). The gel was electrophoresed at pH 8.3. Lane 2a (Fig 5.2) stained for PPO activity using 0.2 mM ferulic acid as substrate showed no enzyme activity.

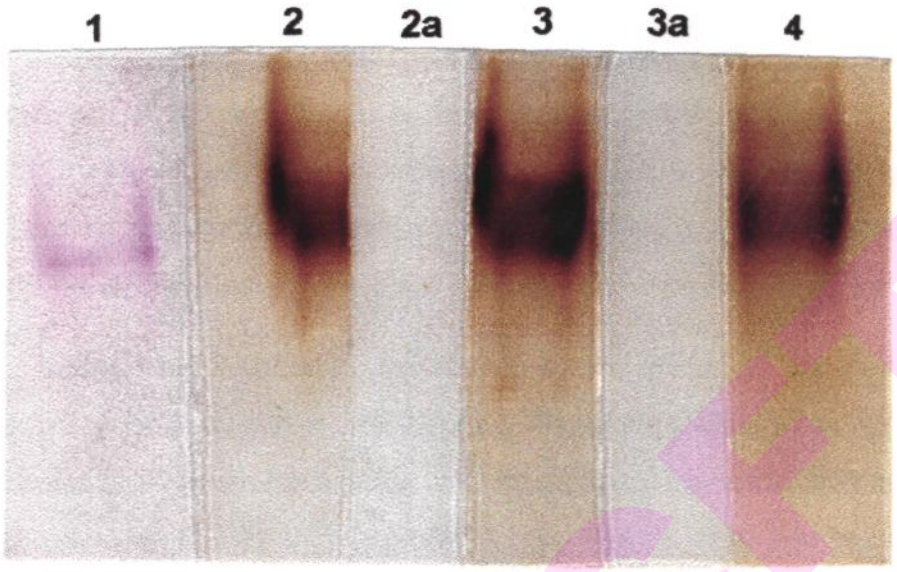


Fig 5.2. **PAGE (7.5% T, 2.7% C) of field bean PPO.** Run in Tris-glycine buffer pH 8.3. The gels were stained for protein (lane 1) and for PPO activity in 50 mM Na acetate pH 4.5 containing 0.05% p-phenylenediamine and (lane 2) 0.2 mM ferulic acid & 10 mM catechol, (lane 2a) 0.2 mM ferulic acid, (lane 3) 0.2 mM caffeic acid & 10 mM catechol, (lane 3a) 0.2 mM caffeic acid (lane 4) 50 mm catechol.

In the parallel lane 2, containing the same amount of protein, when incubated with 10 mM catechol in addition to 0.2 mM ferulic acid, the PPO activity band was clearly visible, the intensity of which was similar to that of the activity band in lane 4, stained with 50 mM catechol alone (Fig 5.2). The final color observed after activity staining is a complexation product of the oxidized quinone product with p-phenylenediamine, and hence no difference in the final color of the bands (Fig 5.2 lanes 2 86 4).

Catechol oxidized by field bean PPO to o-quinone has an absorption maximum at 420 nm (Chapter 3). The absorption maximum of ferulic acid after hydroxylation and oxidation was 480 nm (Fig 5.3). At this wavelength, the contribution to the spectrum by the o-quinone product of catechol oxidation was negligible, 7% (Fig 5.3). This demonstrated that the measured increase in absorbance at 480 nm was due to the formation of quinone from ferulic acid hydroxylation and oxidation and not catechol oxidation.

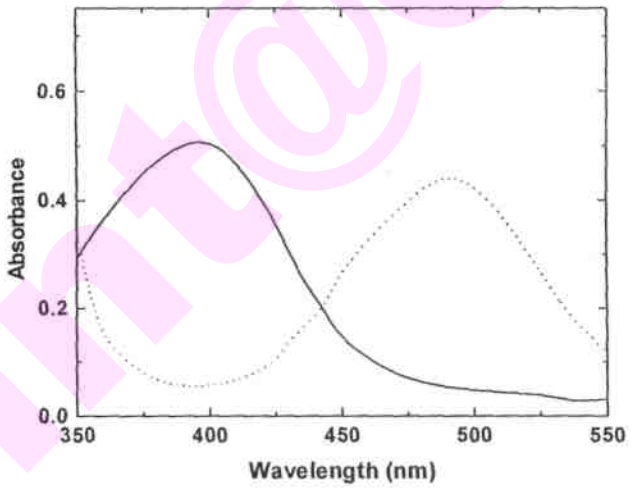


Fig **5.3. Absorbance spectra for the oxidation products of catechol and ferulic acid catalyzed by field bean PPO.** Assay medium contained 50 mM Na acetate buffer, pH 4.5, purified PPO (20 µg) and 50 mM catechol (-------) or 0.2 mM ferulic acid in the presence of 10 mM catechol (.....).

Characterization of the lag phase

The monophenolase activity was characterized by a lag phase, defined as the abscissa obtained by the extrapolation of the linear zone of the oxidation product accumulation curve (steady state rate curve, Fig 5.1b & c). Such a lag phase is common to several plant PPOs. (Lerch, 1981; Sanchez-Ferrer et al., 1988; Sanchez-Ferrer et al., 1993b; Jimenez and Garcia-Carmona, 2000; Fenoll et al., 2000; Ros et al., 1994). The lag phase and the monophenolase steady state rate were affected by the concentrations of both catechol and the substrate ferulic acid. The effect of varying concentrations of catechol on the lag phase of the monophenolase activity at a fixed concentration of ferulic acid is shown in Fig 5.4.

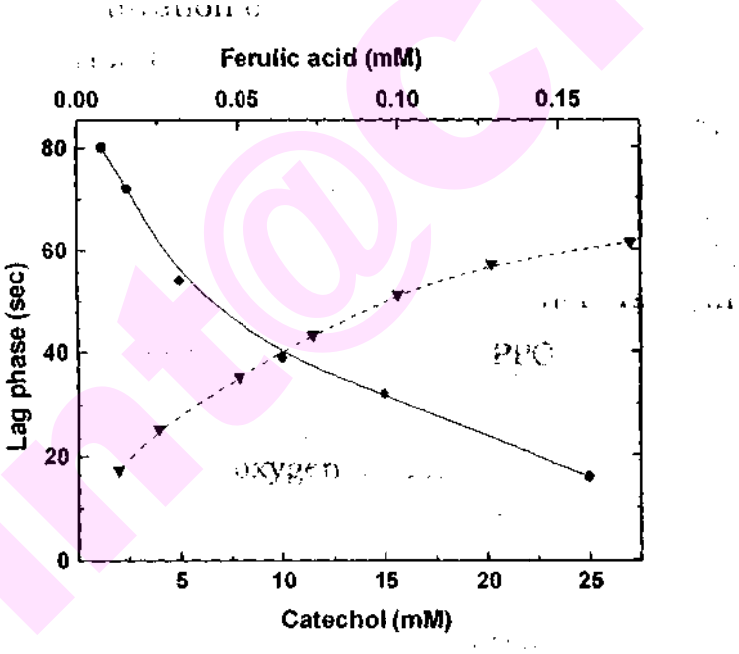


Fig **5.4. Effect of variation of ferulic acid and catechol concentrations on the lag phase of monophenolase activity of field bean PPO.** The reaction medium included 40 µg of purified field PPO in 50 mM Na acetate buffer, pH 4.5 and (•) 0.2 mM ferulic acid and indicated concentrations of catechol (▼) lOmM catechol and indicated concentrations of ferulic acid.

The length of the lag phase decreased with increasing catechol concentrations and almost disappeared at ~25 mM. Increasing the ferulic acid concentration from 0.0125 mM to 0.175 mM resulted in an increase in the lag phase (Fig 5.4) similar to that observed for other plant PPOs (Sanchez-Ferrer et al., 1988; Sanchez-Ferrer et al., 1993; Ros et al., 1994; Jimenez and Garcia-Carmona, 2000; Fenoll et al., 2000). Fig 5.1 shows that unless the enzyme was primed with catalytic quantities of catechol, no monophenolase activity could be measured, hence catechol can be classified as an essential activator.

Effect of ferulic acid concentration on monophenolase activity

To characterize this catechol dependent monophenolase activity towards ferulic acid, the effect of varying catechol concentrations at a fixed ferulic acid concentration on the steady state rate was measured at 480 nm (Fig 5.5). The initial rate showed a hyperbolic response to increasing catechol concentrations, when ferulic acid oxidation was measured at 480 nm. As observed, an increase in catechol concentration resulted in increased enzyme activity and reached a maximum at concentrations ≥20 mM catechol. Further increase in catechol concentration had no effect on the velocity. PPO activity requires the presence of oxygen as a second substrate, therefore the reaction was also monitored by measuring the oxygen consumption. The initial rates of oxygen consumption at a fixed concentration of ferulic acid, but variable catechol concentration showed a similar hyperbolic response (Fig 5.5), the oxygen consumption increasing with increasing concentrations of catechol. The maximum reaction rate measured occurred at a catechol concentration ≥20 mM, irrespective of the method used to measure the reaction rate.

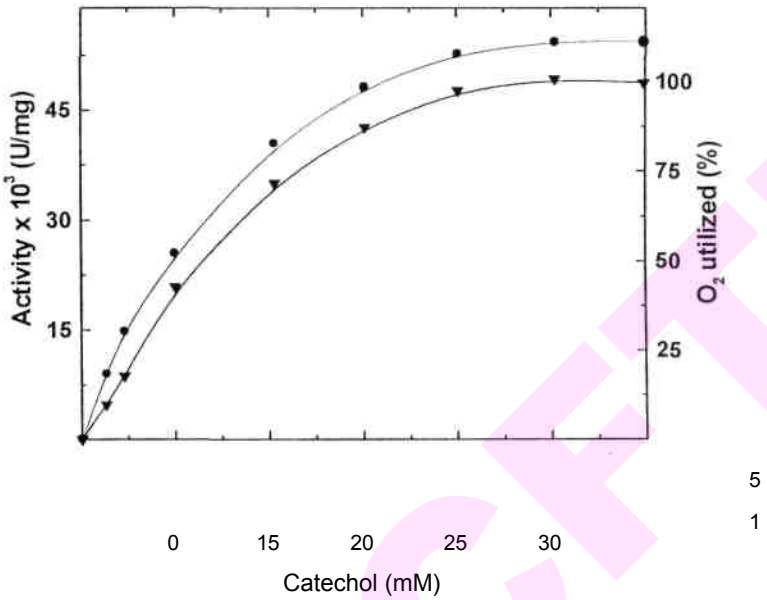


Fig 5.5. **Effect of catechol concentration on the rate of ferulic acid oxidation by field bean PPO.** The assay medium consisted of 50 mM Na acetate buffer pH 4.5, 50 ug of field bean PPO, catechol at indicated concentrations and 0.2 mM ferulic acid, (T) oxygen utilized (•) quinone product formed.

To further study the ferulic acid oxidation by field bean PPO, the kinetic parameters V_max and apparent K_m in the presence of catechol as an activator were estimated at pH 4.5 using Na acetate buffer. The enzyme showed Michaelis-Menten kinetics (only quinone product measurement shown), irrespective, of whether the oxidation product was measured or whether the oxygen consumption rate was measured (Fig 5.6). Reciprocal plots for the kinetic data of ferulic acid oxidation resulted in linear relationships for all catechol concentrations (Fig 5.7). The kinetic parameter K_m for ferulic acid evaluated from these data in the presence of catechol was 0.09 mM. The enzyme activation constant K_a, was evaluated from triplicate measurements of the steady state rate V, for each initial catechol concentration [Ao]. The K_a for catechol was calculated from the linear plot (1/V vs 1/A) according to the method of Cornish-Bowden (Cornish-Bowden, 1995), as shown in Fig 5.8. The apparent K_a for catechol was 5 mM, lower than the K_m of catechol for the diphenolase activity (Chapter 3, Table 3.5).

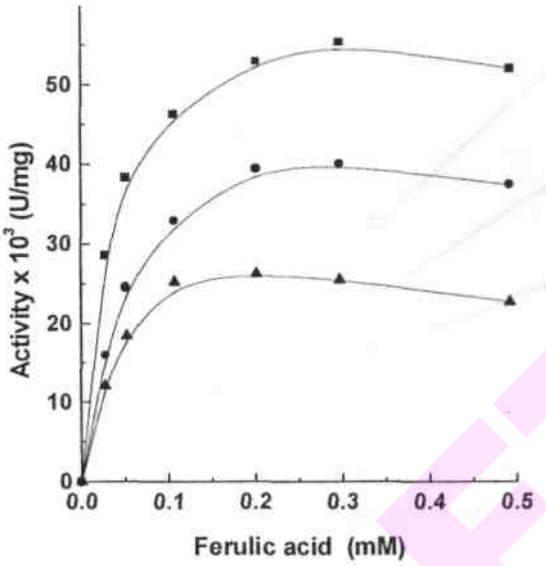


Fig **5.6. Effect of the variation of catechol concentrations on monophenolase activity at different concentrations of ferulic acid.**
Assay medium contained 50 mM Na acetate, pH 4.5, 50 µg of field bean PPO, ferulic acid at the indicated concentrations and catechol (A) 2 mM, (•) 5 mM and (■) 10 mM.

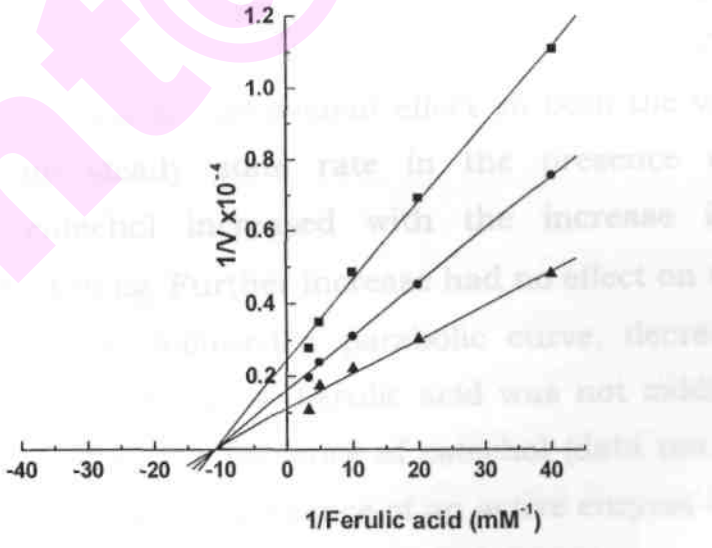


Fig **5.7. Double reciprocal plot of ferulic acid oxidation at different concentrations of catechol.** Ferulic acid at the indicated concentrations and catechol (■) 2 mM, (•) 5 mM and (A) 10 r∩M. Data of Fig 5.6.

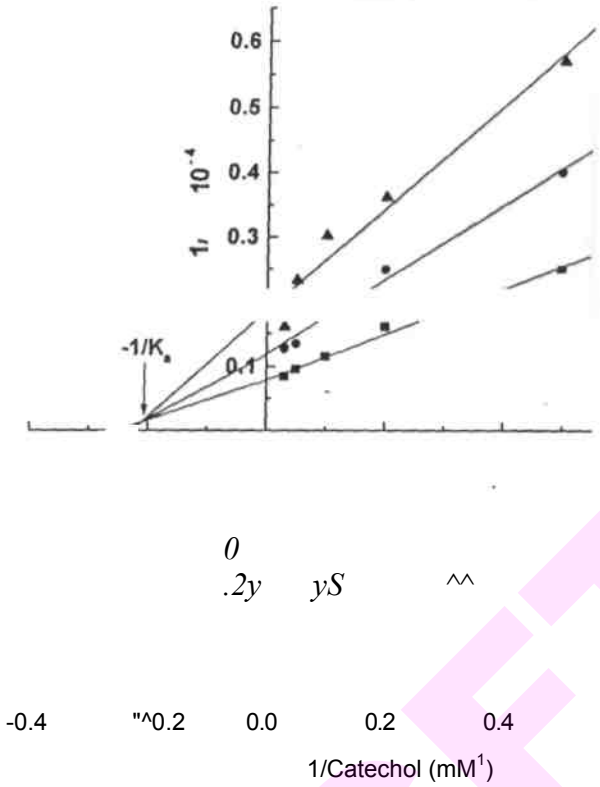


Fig 5.8. **Cornish-Bowden plot of ferulic acid oxidation at different concentrations of catechol to determine the K» of catechol.** Catechol at the indicated concentrations and ferulic acid **(A)** 0.05 mM, (•) 0.1 mM and **(■)** 0.2 mM.

Effect of enzyme concentration on monophenolase activity

The monophenolase activity of field bean PPO in the presence of catechol was also dependent on the enzyme concentration. Variation in the enzyme concentration has an evident effect on both the velocity and the lag phase. The steady state rate in the presence of a fixed concentration of catechol increased with the increase in enzyme concentration up to 100 µg. Further increase had no effect on the activity (Fig 5.9). The lag phase followed a parabolic curve, decreasing with increasing enzyme concentrations. Ferulic acid was not oxidized in the absence of enzyme and in the presence of catechol (data not shown). It can thus be concluded that the presence of an active enzyme is essential for ferulic acid hydroxylation and further oxidation.

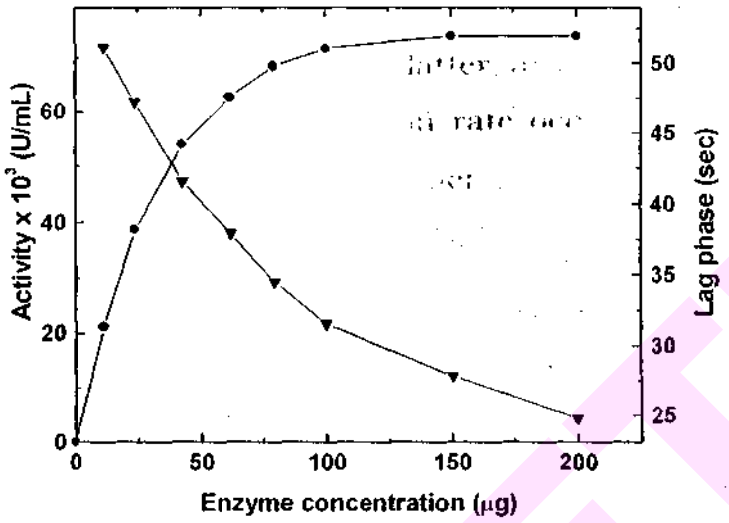


Fig 5.9. **Effect of enzyme concentration on the monophenolase activity of field bean PPO and the lag phase.** The reaction medium included 0.2 mM ferulic acid, 10 mM catechol and field bean PPO concentrations as indicated, in 50 mM Na acetate buffer, pH 4.5 (•) PPO activity, (T) lag phase.

Tyrosine as a substrate for monophenolase

When this catechol dependent monophenolase activity of field bean PPO was extended to tyrosine, activation similar to that in Fig 5.1, with a lag phase was observed. Tyrosine was not hydroxylated to DOPA by field bean PPO (Chapter 3, Table 3.5). The product of tyrosine oxidation, dopaquinone also had an absorption maximum of 480 nm similar to that of ferulic acid.

To characterize the catechol dependent monophenolase activity towards tyrosine, the effect of varying catechol concentrations at a fixed tyrosine concentration on the steady state rate was measured at 480 nm (Fig 5.10). An increase in catechol concentration resulted in increased enzyme activity and reached a maximum at concentrations ~2 mM catechol. Further increase in catechol concentration had no effect on the reaction rate. Initial values of oxygen consumption at a fixed concentration of tyrosine, but variable catechol concentration are shown

in Fig 5.10. The oxygen consumption rates increased with higher levels of catechol upto >2 mM concentration of the latter, at which point the curve started to level off. The maximum reaction rate occurred at a catechol concentration ≥ 2 mM, irrespective of whether the quinone formation or oxygen consumption was measured (Fig 5.10).

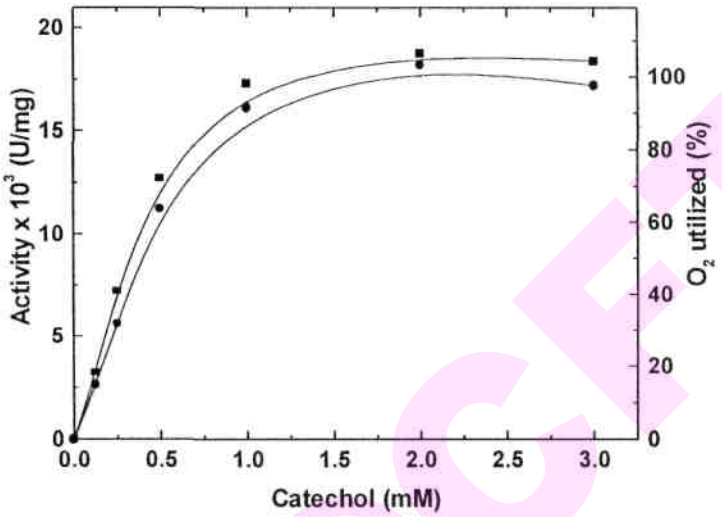


Fig 5.10. **Effect of catechol concentration on the rate of tyrosine oxidation by field bean PPO.** The assay medium consisted of 50 mM Na acetate buffer pH 4.5, 50 µg of field bean PPO, catechol at indicated concentrations and 10 mM tyrosine, (•) oxygen utilized (■) quinone product formed.

The effect of tyrosine concentrations on the catechol dependent hydroxylation reaction rate was enumerated. The enzyme showed Michaelis-Menten type kinetics similar to that observed for ferulic acid (data not shown). Reciprocal plots for the kinetic data of tyrosine oxidation resulted in linear relationships (Fig 5.11) similar to the plots for ferulic acid, for all catechol concentrations. The kinetic parameter K_m for tyrosine, evaluated from this data was 3.13 mM. In the presence of catalytic quantities of catechol, tyrosine was oxidized to dopaquinone, with a Vmax of 19,841 U/mg (Table 5.1). The enzyme activation constant K_a, was evaluated from measurements of the steady state rate V, for each initial catechol concentration [Ao]. The $K_a$was calculated from the linear

plot (1/V vs 1/A) at different tyrosine concentrations (Fig 5.12). K_a for catechol was 0.58 mM, ten fold lower than that for ferulic acid.

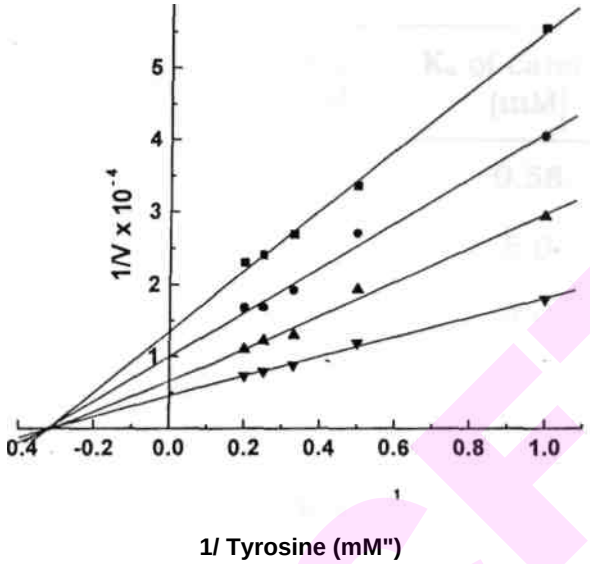


Fig 5.11. **Double reciprocal plot of tyrosine oxidation at different concentrations of catechol.** Tyrosine at the indicated concentrations and catechol (■) 0.25 mM, (•) 0.5 mM, (A) 1 mM and (▼) 1.5 mM.

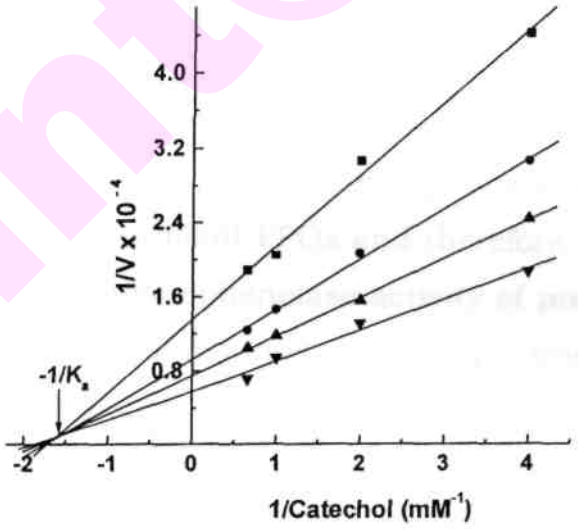


Fig 5.12. **Cornish-Bowden plot of tyrosine oxidation at different concentrations of catechol to determine the Ka of catechol.** Catechol at the indicated concentrations and tyrosine **(■)** 1 mM, (•) 3 mM, **(A)** 4 mM and **(r)** 5 mM.

Table. 5.1. **Observed kinetic parameters for the monophenolase and diphenolase activities of field bean PPO.**

Substrate	V max (U/mg)	K_m (mM)	K_a of catechol (mM)	pH optimum*
Tyrosine	19,841	3.13	0.58	4.5
Ferulic acid	56,687	0.09	5.0	4.5
Caffeic acid	32,440	0.08	4.6	4.5

The assay medium contained 50 mM Na acetate buffer, pH 4.5, 20 mM catechol as co-substrate and 50 µg of field bean PPO. K_ms are apparent.

*McIlvaine buffers (pH 2.5-7.5) used to determine the pH optima

pH optima for monophenolase

The pH optima of both ferulic acid and tyrosine in the presence of catechol as an activator were determined using Mcllvaine buffer (pH 2.5-7.5). Both the substrates (tyrosine and ferulic acid), in the presence of catechol activator had an optimum pH of 4.5 (Table 5.1).

Monophenolase activation by other diphenols

Several diphenols and substituted diphenols have been reported to be excellent substrates of plant PPOs and therefore were investigated for activation towards the monophenolase activity of purified field bean PPO (Table 5.2). Each of the compounds, 2-10 differs from catechol only with respect to one substitution on the phenyl ring. The results show that among the diphenols, other than catechol, 4-methyl catechol, L-DOPA, the triphenols, pyrogallol and 2,3,4-trihydroxy benzoic acid stimulate the monophenolase activity. These diphenols are also efficiently oxidized by field bean PPO to their respective quinones (Table 3.5 of Chapter 3). In the absence of these compounds, the monophenolase activity towards both ferulic acid and tyrosine was absent.

Table 5.2. **Effect of co-substrates on monophenolase and diphenolase activity of field bean PPO.**

	Co-substrate	Monophenolase Vmax (U/mg)		Diphenolase Vmax (U/mg)	
		Ferulic acid (0.2 mM)	Tyrosine (10 mM)	Caffeic acid (0.2 mM)	Catechin (5mM)
1	Catechol	56,687	19,841	32,440	6,021
2	4-Methyl catechol	54,248	19,281	31,340	5,924
3	L-DOPA	52,340	19,841	30,130	5,897
4	Pyrogallol	32,450	10,980	17,894	0
5	2,3,4-Trihydroxy benzoic acid	6,876	1,537	3,452	0
6	Protocatechuic acid	0	0	0	0
7	Caffeic acid	0	0	0	0
8	Catechin	0	0	0	0
9	Chlorogenic acid	0	0	0	0
10	Gallic acid	0	0	0	0

Assay medium contained 50 mM Na acetate buffer, pH 4.5, substrate concentrations as indicated and 50 *xg* of PPO. The co-substrates were used at a final concentration of 20 mM.

The stimulation of the monophenolase activity by these compounds was also characterized by a lag phase similar to that shown in Fig 5.1. Among the substituted hydroxy benzoic acids, 2,3,4-trihydroxy benzoic acid stimulated this activity, whereas protocatechuic acid (3,4-dihydroxy benzoic acid) and gallic acid (3,4,5-trihydroxybenzoic acid) did not have the same effect. The latter two are also not substrates for field bean PPO but rather inhibit the activity

(Chapter 4), in contrast to 2,3,4-trihydroxybenzoic acid which is oxidized by field bean PPO to its quinone (Table 3.5 of Chapter 3).

Diphenolase activation by **diphenols**

The diphenols, caffeic acid (3,4-dihydroxycinnamic acid) and catechin did not activate the monophenolase activity of field bean (Table 5.2). These two diphenols are not oxidized by field bean PPO (curve a, Fig 5.13) and also are potent competitive inhibitors of catechol oxidation (Chapter 4, Table 4.1). However, if catalytic amounts of catechol are added to the reaction medium, these compounds serve as substrates and are rapidly oxidized to their corresponding quinones (curves b 85 c, Fig 5.13). The steady state occurred immediately with no lag phase unlike the monophenolase activity. The quinone product of caffeic acid also has an absorption maximum of 480 nm similar to that of the ferulic acid oxidation product and is distinct from the product of catechol oxidation whose absorption maximum is at 420 nm.

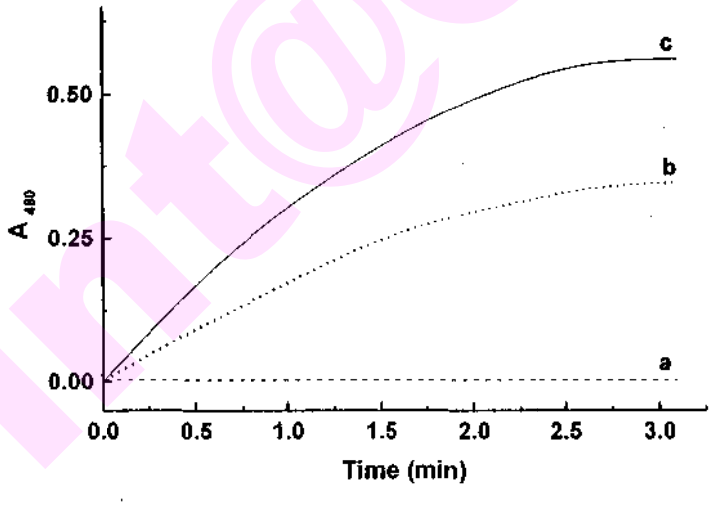


Fig 5.13. **Time course of product formation in the oxidation of caffeic acid by field bean PPO.** Progress curve with the reaction medium containing 0.2 mM caffeic acid in 50 mM Na acetate buffer pH 4.5, and 20 µg of enzyme in the initial presence of catechol (a) 0 mM (b) 2 mM or (c) 10 mM.

Activity of field bean PPO towards caffeic acid in the presence of catechol was also detected by native-PAGE, followed by activity staining.

PPO (Fig 5.2, lane 3) was stained by immersing the gel in 0.2 mM caffeic acid in the presence 10 mM catechol and the activity was detected as a dark brown band against a transparent background. Lane 3a, stained with 0.2 mM caffeic acid in the absence of catechol, showed no such band. The staining solutions contained 0.05% p-phenylenediamine which was included to intensify the dark brown color of the quinone product by complexing it.

This diphenolase activity in the presence of catechol was also measured both by polarographic oxygen utilization technique in addition to changes in the absorption spectrum at 480 nm for caffeic acid. The initial rates of oxygen consumption and quinone formation, at fixed concentrations of caffeic acid but variable concentrations of catechol are shown in Fig 5.14. Oxygen consumption and quinone formation increased concomitantly with increasing levels of catechol upto ~20 mM. No further increase in activity was observed at concentrations >20 mM. A similar result was observed with (+)catechin (results not shown).

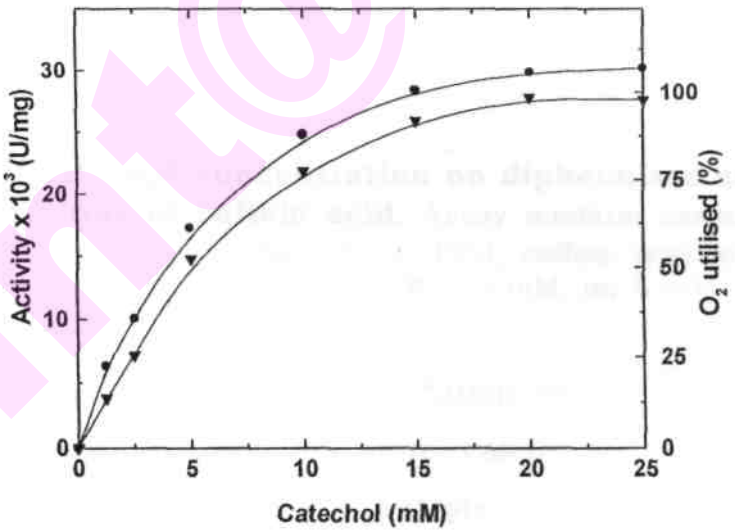


Fig 5.14. **Effect of catechol concentration on the rate of caffeic acid oxidation by field bean PPO.** The assay medium consisted of 50 mM Na acetate buffer pH 4.5, 50 µg of field bean PPO, catechol at indicated concentrations and 0.2 mM caffeic acid, (T) oxygen utilized (•) quinone product formed.

The pH optimum for this catechol dependent caffeic acid oxidation was determined using Mcllvaine buffers (pH 2.5-7.5). The maximal activity occurred at pH 4.5, similar to the pH optimum of catechol dependent monophenolase activity toward ferulic acid and tyrosine (Table 5.1). The dependence of the oxidation rate of field bean PPO on catechol concentration and varying caffeic acid concentrations was therefore examined at pH 4.5. Varying the initial concentration of caffeic acid at a fixed concentration of catechol, the enzyme showed Michaelis-Menten kinetics (Fig 5.15).

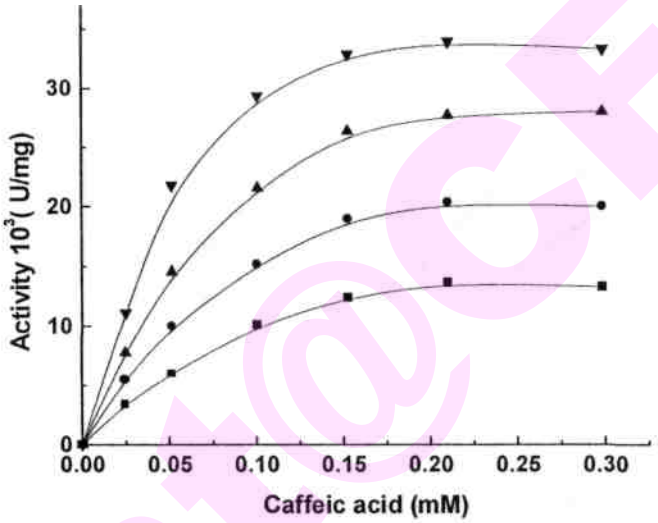


Fig 5.15. **Effect of catechol concentration on diphenolase activity at variable concentrations of caffeic acid.** Assay medium consisted of 50 mM Na acetate pH 4.5, 50 µg of field bean PPO, caffeic acid at indicated concentrations and catechol concentrations (■) 2.5 mM. (•) 5 mM, (A) 10 mM and (r) 25 mM.

Reciprocal plots for caffeic acid oxidation showed no important differences when kinetic parameters were calculated from the data obtained with variable catechol concentration from 5 to 25 mM (Fig 5.16). These data were averaged. The apparent K_m for caffeic acid in the presence of catechol was 0.08 mM with a $V_{ma}\chi$ of 32,440 U/mg. The activation constant K_a for catechol evaluated from the Cornish-Bowden plots of these data was 4.6 mM (Fig 5.17), very close to the catechol

activation constant for the monophenolase activation. It is evident from these results that catechol, a diphenol is an essential activator for caffeic acid oxidation by field bean PPO. In addition to catechol, among the several diphenols and substituted diphenols examined, 4-methyl catechol and L-DOPA stimulated the diphenolase mediated oxidation of caffeic acid and catechin (Table 5.2). Pyrogallol and 2,3,4-trihydroxybenzoic acid could stimulate the PPO activity toward caffeic acid oxidation and not catechin. The maximal reaction rate of the diphenol dependent caffeic acid oxidation is the same irrespective of the diphenol used to activate the reaction (Table 5.1 & 5.2).

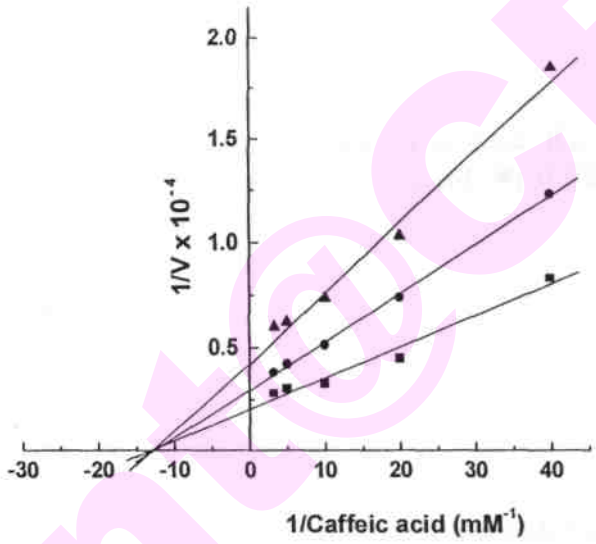


Fig 5.16. **Double reciprocal plot of caffeic acid oxidation at different concentrations of catechol.** Caffeic acid at the indicated concentrations and catechol (A) 5 mM, (•) 10 mM and (■) 25 mM. Data of Fig 5.15.

The kinetic data of all the o-diphenols which served as co-substrates (cofactor) for the tyrosine and ferulic acid hydroxylation and caffeic acid oxidation indicated interesting requirements for optimal cofactor activity (Table 5.1 86 5.2). While L-DOPA has the lowest K_m of all the cofactors, the relative efficiency of stimulation by 4-methyl catechol, catechol and L-DOPA are similar. Pyrogallol with an additional OH substituent is less efficient, and 2,3,4-trihydroxy benzoic acid with a strong electron

withdrawing COOH group elicits an even less significant stimulation of the monophenolase and diphenolase activation (Table 5.2).

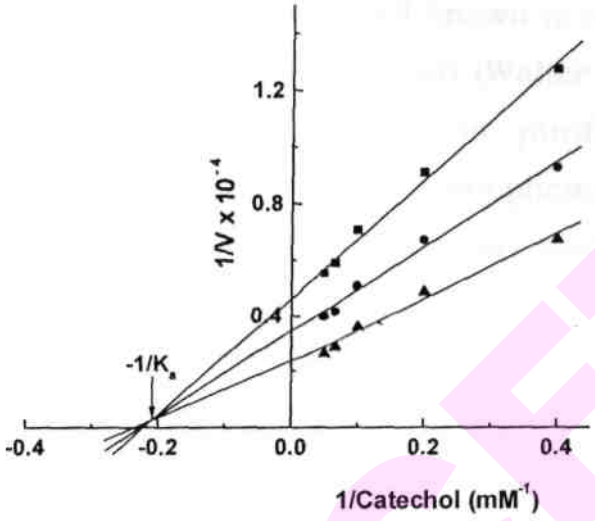


Fig 5.17. **Cornish-Bowden plot of caffeic acid oxidation at different concentrations of catechol to determine the K_a of catechol.** Catechol at the indicated concentrations and caffeic acid (■) 0.05 mM (•) 0. 10 mM and (A) 0.25 mM. Data of Fig 5.15.

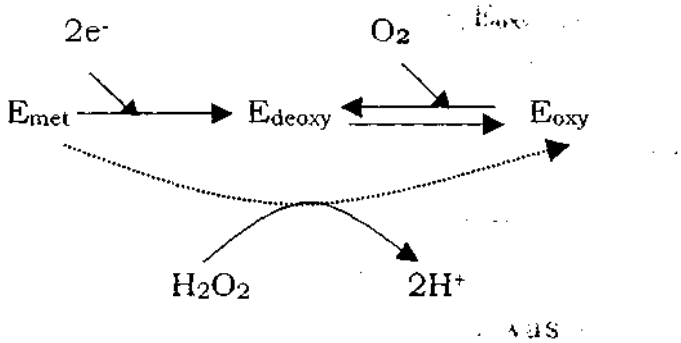
DISCUSSION

Plant PPOs are nuclear encoded chloroplast proteins that generally occur in plastids, although their occurrence in other cell compartments has been reported (Mayer and Harel, 1979). In higher plants, the enzyme is mostly membrane bound, but solubilizes as the fruit matures (Murata et al., 1993). A definitive way to confirm that an active PPO is present in plant material is by determining the monophenolase activity (Sanchez-Ferrer et al., 1988). The monophenolase activity is generally defined as the first step in the melanization pathway, and consists of the o-hydroxylation of the monophenol to the o-diphenol. The fact that the hydroxylating activity is expressed in conjunction with the oxidation of o-diphenol to o-quinone (second step in the melanization pathway) has íed some authors to define monophenolase activity as the complete conversion of monophenol to o-quinone (Naish-Byfield and Riley, 1992).

The soluble field bean PPO did not exhibit any monophenolase activity towards the monophenols ferulic acid, tyrosine and p-cresol (Table 3.5 of Chapter 3). This phenomenon is also well known in other PPOs resulting from structural changes during purification (Walter and Purcell, 1980). The absence of monophenolase activity in purified plant PPOs is generally attributed to; (1) the lability of monophenolase activity during the harsh purification process and (2) the assay methods used to follow this activity (Sanchez-Ferrer et al., 1988; Sanchez-Ferrer et al., 1993a; Sanchez-Ferrer et al., 1993b). Temperature induced phase partitioning in Triton X-l 14 and other mild extraction methods have been used to purify PPOs that avoid the loss of monophenolase activity (Sanchez-Ferrer et al, 1989; Werck-Reichhart et al., 1991; Espin et al., 1995; Espin et al., 1997; Sojo et al., 1998a; Sojo et al., 1998b; Perez-Gilabert and Garcia-Carmona, 2000). Chemical and spectroscopic studies indicate that the binuclear copper active site of tyrosinase can be prepared in any of the three forms: met, oxy and deoxy which are interrelated (Solomon, 1981). Only the oxy form acts on monophenols, whereas the met form can be converted to the oxy form by H_2O_2, that acts on monophenols (Jimenez and Garcia-Carmona, 2000). It has long been recognized that the monophenolase activity of some plant PPOs requires a reducing agent for its initiation (Pomerantz and Warner, 1967; Duckworth and Coleman, 1970). The close association of the two activities (monophenolase and diphenolase) is borne out by the reciprocal competitive inhibition patterns of monophenolic and diphenolic substrates (Mason, 1956).

Ferulic acid and tyrosine were not oxidized by field bean PPO (Fig 5.1, curve a). In the presence of catalytic quantities of catechol (Fig 5.1, curve b & c) and other diphenols (Table 5.1 & 5.2) they were oxidized to the o-quinone. This induced monophenolase activity of field bean PPO is characterized by a lag phase, that is common to PPOs from various sources when the monophenolase activity was measured (Lerch, 1981;

Sanchez-Ferrer et al., 1988; Sanchez-Ferrer et al., 1989; Espin et al., 1995b; Espin et al., 1997b; Sojo et al. 1998a). The lag phase and the steady state rate of field bean PPO were affected by the substrate concentration, concentration of the diphenol and the enzyme concentration (Fig 5.4, 5.5, 5.6 & 5.9). The lag phase of field bean is shortened by the increase in catechol concentration (Fig 5.4) and increased with increasing ferulic acid concentrations. The time required to reach the steady state also depends on the enzyme source (Valero etal., 1988) and the concentration of the monophenol. The lag phase increases when the monophenol concentration is increased (Osaki, 1963, Vaughan and Butt, 1972). The lag phase diminishes when the enzyme concentration was increased (Pomerantz and Warner, 1967; Duckworth and Coleman, 1970). Addition of catalytic quantities of diphenols (Pomerantz and Warner, 1967; Garcia-Carmona etal., 1979), reducing agents such as ascorbic acid, NADH, dimethyltetrahydropteridine (Osaki, 1963; Vaughan and Butt, 1972) or transition metal ions (Fe^{2+}) (Palumbo et al., 1985) were found to reduce the lag phase. The characteristic existence of a lag phase in monophenolases has been explained in a review by Sanchez-Ferrer et al. (1995). Cooksey et al. (1997) explain the kinetics of the lag phase of mushroom tyrosinase by an autocatalytic mechanism dependent on the generation of a dihydric phenol which acts as an activator. However, the autoactivation of tyrosinase failed with N,N-dimethyltyramine, a monohydric phenol analog. This reaction required the addition of a dihydric phenol. The diphenol activator in autoactivation is formed indirectly by the disproportionation of the o-quinone, but in the case of N,N-dialkyl substrates, this reaction does not occur, necessitating the addition of o-diphenols (Cooksey et al., 1997). The binuclear copper active site prepared in various forms; E_{met}, Edeoxy and E_{oxy} (Solomon, 1981) are interconvertible as shown below.



The oxygenated form (E_{oxy}) is the form that binds and acts on monophenols giving rise to an ordered sequential mechanism (Wilcox etal., 1985). The $E_m$et (resting form) cannot act upon monophenols, but can be converted to E_{OX}y form (Jolley et al., 1974; Jimenez and Garcia-Carmona, 2000) and can bind diphenols (Matheis, 1987). The redox potential of the active site copper atoms results in a significant proportion of isolated PPO having a bicupric active site, unable to bind molecular oxygen and thus being incapable of monophenol oxidation. This so-called Emet requires a cofactor able to reduce the active site copper atoms to give Edeoxy which is then able to bind oxygen and take part in phenol oxidation (Lerch, 1981). The monophenolase reaction requires that a minimum quantity of enzyme be present as Eoxy (Ros-Martinez et al., 1993). During diphenolase activity, the o-diphenol binds to both E_{0X}y and $E_m$et producing a random sequential mechanism (Wilcox et al., 1985). In the monophenolase cycle, binding of monophenol to the Eoxy form enables the reaction to proceed. On the other hand, binding of E_{me}t scavenges a portion of the PPO from the catalytic turnover as a dead end complex in the steady state of monophenolase activity because $E_m$et-monophenol complex is nonfunctional. The enzyme slowly re-enters the catalytic cycle by means of diphenols, which serve as $2e^-$ donors. This puts an end to the lag phase. Thus the lag phase can be defined as the time required by the enzyme to accumulate through several steps, a certain concentration of o-diphenol characteristic of the

steady state, which is required to drain E_{oxy} form from $E_m$et into the catalytic cycle.

Ros et al. (1994) observed that, contrary to the characteristic lag phase increase with an increase in monophenol concentration, the length of the lag phase of mushroom tyrosinase showed no significant variation when 4-tertiary butyl phenol concentration was varied. They concluded that the high stability of the reaction product 4-t-butyl-o-benzoquinone results in the non-regeneration of the diphenol in the medium, which is required to convert $E_m$et to E_{OX}y.

Purified field bean PPO is unable to catalyze the oxidation of the monophenols, tyrosine and ferulic acid suggesting that the purified PPO is in the $E_m$et (resting) form. However, catalytic quantities of catechol, 4-methyl catechol, L-DOPA, pyrogallol and 2,3,4-trihydroxy benzoic acid serve as the $2e^-$ donors, convert E_{me}t to Edeoxy, that is capable of binding oxygen reversibly to form E_{0X}y. The E_{0X}y form thus formed binds the monophenols, ferulic acid and tyrosine, hydroxylates them and further oxidizes them to the quinones (Fig 5.1, curve b). The K_m values for ferulic acid and tyrosine are several fold lower than the K_m of the diphenols tested (Table 5.1) indicating the higher binding affinity of the field bean PPO for monophenols. Increasing catechol concentrations produce a greater transformation of the Emet field bean PPO to E_{oxy} leading to an increase in the rate of ferulic acid oxidation (Fig 5.5). The oxygen consumption also increases concomitantly leading to an increased turnover of the active form. However, increasing monophenol concentrations in the reaction medium leads to an increase in the lag phase (Fig 5.4) implying that the turnover to the active form is insufficient and therefore the time required to attain steady state is longer. Increase in enzyme concentration involves an increase in the E_{oxy} concentration which produces higher amounts of diphenols which in

turn accelerate the passage of E_{met} to $E_{0}xy$, thereby shortening the observed lag phase.

Among all the diphenols and substituted diphenols tested, only those that were reactive towards the diphenolase activity of the field bean PPO catalyze this unusual activation phenomenon. Catechol, 4-methyl catechol, L-DOPA, pyrogallol and 2,3,4-trihydroxybenzoic acid exhibit a high binding affinity to field bean PPO (Chapter 3, Table 3.5). Therefore, it is most likely that only those compounds that bind to the enzyme productively can serve as electron donors at the active site for the conversion of the binuclear copper from E_{met} to $E_{0X}y$. Tyrosine hydroxylation by melanosomal tyrosinase is dependent on the presence of DOPA in catalytic quantities. DOPA in addition to its known activity as a $2e^-$ donor for the reaction also appears to act as a positive allosteric effector for tyrosine hydroxylation (Hearing and Ekel, 1976). Protocatechuic acid, caffeic acid, chlorogenic acid and gallic acid, compounds that show no substrate binding affinity to field bean PPO (Chapter 3, Table 3.5) do not catalyze this activation. The observation that the Ka for catechol is less than the K_m of catechol suggests its function as a cofactor (2e-donor). The mechanism of the inter conversions of the E_{met} to E_{oxy} varies. Mclntyre and Vaughan (1975) demonstrated that the diphenol caffeic acid acts as an electron donor at the active site of spinach-beet phenolase, whereas other reducing agents such as ascorbic acid and NADH function mainly to recycle cofactor amounts of caffeic acid, from the quinone product, rather than donate electrons. Ascorbic acid, hydroxylamine and dithionite (Krueger, 1950) also produce oxytyrosinase via this mechanism. In the presence of excess peroxide, Emet is directly converted to $E_{0X}y$ (Jolley et al., 1974; Jimenez and Garcia-Carmona, 2000).

Field bean PPO showed no activity towards the diphenol, caffeic acid (Table 5.2 and Fig 5.13 curve a). Caffeic acid is shown to be a potent competitive inhibitor for catechol oxidation (Chapter 4, Table 4.1) indicating that it has a high affinity for the active site of field bean enzyme. The inhibition constant of caffeic acid (Ki=0.06 mM, Chapter 4) and the binding constant K_m=0.08 mM, in the presence of catechol are very similar and much lower than the K_m of catechol suggesting that the enzyme has a greater binding affinity for caffeic acid than catechol.

Jacobsohn and Jacobsohn (1984) reported that 2-hydroxy estradiol (catechol estrogen) would react in the presence of tyrosinase only if catechol is present. Kinetic analysis of their data indicates that catechol functions as an activator of tyrosinase. Similarly, only with catechol present in the incubation media, caffeic acid was oxidized rapidly (Fig 5.13, curves b & c). Both the oxygen consumption and o-quinone formation measurements show the absence of the lag phase, earlier observed with monophenols (Fig 5.1 curve b). Kinetic studies (Wilcox et al., 1985) indicate that the oxidation of diphenols to o-quinones by tyrosinase has less geometric and electronic requirements than does monophenol oxidation. Diphenol co-ordinates to the reduced (Edeoxy), oxidized ($E_m$ct) and oxygenated forms of the binuclear copper active site and is oxidized by the latter two forms (Garcia-Carmona et al., 1988). Although catechol activation of the monophenolase suggests that a major proportion of the purified PPO is in the $E_m$et form, yet it did not bind caffeic acid or catechin productively. The absence of a lag phase (Fig 5.13) indicates that this activation does not require the conversion of Emet to Eoxy. The inability of caffeic acid to stimulate monophenolase activity indicates that it is not a $2e^-$ donor for the conversion of Emet to Eoxy (Table 5.2). Compounds possessing aromatic rings such as benzoic acid and substituted benzoic acid including caffeic acid also show inhibition of field bean PPO and act competitively (Chapter 4, Table 4.1).

The apparent K_a found for the activation was smaller by one half than the Km of catechol as substrate, 10.5 mM (Chapter 3, Table 3.5). No clear view emerges regarding how catechol and other small diphenols activate the enzyme towards caffeic acid, which otherwise is a competitive inhibitor. The lower K_a suggests that catechol functions as a diphenolase activator, perhaps by binding to an activator site, altering the conformation to allow for a productive interaction between the caffeic acid and the residues of the active site involved in the catalytic mechanism.

Depending on the catechol concentration, gallic acid behaves both as an activator and a competitive inhibitor of catechol oxidation by field bean PPO, (Chapter 4). These observations together with the fact that only those diphenols that are substrates for field bean PPO activate caffeic acid and catechin oxidation implicate that the activator site is similar in conformation to the substrate binding site. The active site of field bean PPO appears to be compact and it does not accommodate substrates with bulky substituents (Chapter 3). The CH=CHCOOH substituent of caffeic acid perhaps hinders the presentation of the substrate to the active site residues of the enzyme. The diphenol-induced conformational change of field bean PPO permits the positioning of caffeic acid in the correct orientation to facilitate its oxidation by field bean PPO. The catechol activation constants (K_as) for monophenolase (ferulic acid oxidation) and diphenolase (caffeic acid oxidation, Table 5.1) are almost similar. However, the mechanism by which it stimulates these two distinct activities differs, the former characterized by a lag phase and the latter attaining the steady state immediately.

Observing the stimulation of maximal velocities by the o-diphenols (Table 5.1 8B 5.2), the effect of co-substrate (cofactor) structure can be seen to have markedly different effects. Those with the highest V_{max}

values are o-diphenols whereas, the addition of a third hydroxy substituent markedly lowers the stimulation. Besides this, it appears that a prerequisite for stimulation of PPO is a binding affinity. Hearing et al. (1978) showed that the precise steric organization of all the major groups present on L-DOPA are necessary for maximal binding to or maximal stimulation of the activity of malignant and normal melanocyte tyrosinase.

The observation that L-DOPA had two K_m values for PPO from hamster melanoma, supports the allosteric model hypothesis, wherein L-DOPA binds at different sites on the enzyme when functioning as a co-substrate or as a sole substrate (Pomerantz and Warner, 1967). The allosteric model was also supported by Hearing and Ekel (1976), who found that L-DOPA at low concentrations was not a competitive inhibitor of tyro sine hydroxylation but rather a non-competitive activator of tyrosine binding. It was proposed that a conformational change elicited by L-DOPA might align the catalytic sites for tyrosine and L-DOPA leading to an increased efficiency in the coupling of tyrosine hydroxylation with DOPA oxidation. The initial lack of adequate DOPA concentration in the golgi apparatus and endoplasmic reticulum in melanocytes, even though tyrosinase is detected, probably explains why these organelles lack melanin formation

The lack of monophenolase activity observed in field bean PPO is probably due to the loss of the endogenous natural diphenols that occurs during the drastic methods used to purify the enzyme, resulting only in the Eπiet (resting) form. The results presented are in agreement with the mechanism described (Sanchez-Ferrer et al., 1988) that o-diphenols are needed in the medium before the enzymatic reaction starts. In parallel, diphenols also activate the diphenolase by a different mechanism. The presence of high concentrations of 'endogenous diphenols in the intact

fruit or vegetable, which can activate both the monophenolase and diphenolase reactions probably explains the instantaneous and severe browning reactions that occur upon mechanical damage during harvesting, handling, storage and processing of fruits and vegetables.

Chapter 6
Induced activation of the field bean *(Dolichos lablab)* polyphenol oxidase, by SDS and pH

Since it was first discovered by Mallette and Dawson (1949), multiple forms of PPO have been described in the literature. An unusual and intriguing characteristic of PPOs is their ability to exist in a latent (inactive or less active) state. PPO can be released from latency or activated by a variety of treatments or agents such as acid or base shock (Kenten, 1957), urea (Swain et al., 1966), polyamines (Jimenez-Atienzer et al., 1991), anionic detergent, such as SDS (Kenten, 1958; Golbeck and Cammarata, 1981; Flurkey, 1986; Sanchez-Ferrer et al., 1993c), proteases (Golbeck and Cammarata, 1981; King and Flurkey, 1987; Soderhall and Soderhall, 1989) and fatty acids (Hutcheson and Buchanan, 1980; Golbeck and Cammarata, 1981). Field bean PPO purified from a neutral pH extraction buffer contains a single active form with pH optima of 4.0 (Chapter 3, Fig 3.12). During the preliminary investigation, it was observed that field bean PPO activity of acidic (pH 2.5) buffer extracts, when assayed at pH 6.0 was 1.6 fold higher than at pH 4.0. The fact that plant PPOs can be activated *in vitro* by exposure to acidic or basic pH suggested the presence of a latent PPO. The results *on* the activation of field bean PPO by acid pH and by the anionic detergent SDS are presented and discussed in this chapter.

RESULTS

PPO purified from neutral pH buffer extracts of defatted field bean flour indicated the presence of a single active form with a pH optimum of 4.0 (Fig 3.12, Chapter 3). During the standardization procedures for optimal PPO extraction, in the pH 2.5 buffer extracts, it was observed that catechol oxidation was greatest at pH 6.0 (Fig 6.1) whereas that of the neutral pH extract was pH 4.0. However, if the pH of the extract was adjusted to 7.0, the optimal pH was 4.0. This shift of optimum pH could be reversed (Fig 6.1). To examine this displacement of the pH optimum towards higher values and the fact that plant PPOs are activated by acid pH (Kenten, 1957; Swain et al., 1966), this effect was investigated in detail.

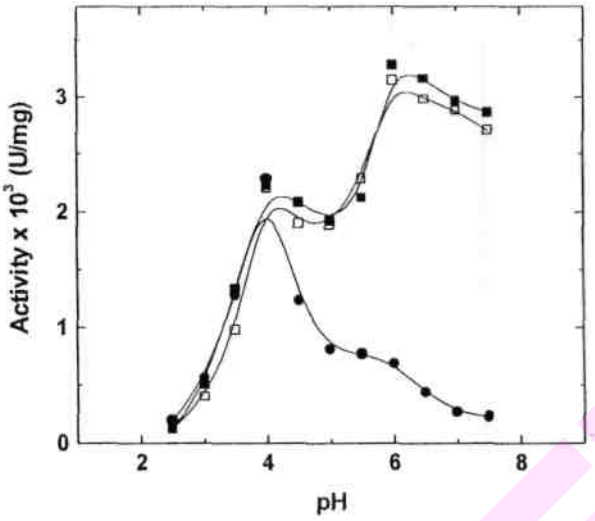


Fig 6.1. **Effect of pH on the activity of crude field bean PPO. (■)** PPO solubilized in pH 2.5 Mcllvaine buffer, (•) pH of solubilized PPO adjusted to pH 7.0 and (¤) pH of PPO readjusted back to pH 2.5.

Purified PPO (Table 3.2, Chapter 3) was incubated in buffers of pH 7.0, 4.0 and 2.5 for 30 min at 25 °C and the PPO activity measured in assay buffers of varying pH using catechol as the substrate. The purified PPO incubated at neutral pH (native PPO) was active at acidic pH (3.0-4.5) with a maximum at 4.0, but relatively inactive at pH >5.O (Fig 6.2). In contrast, PPO exposed to acidic pH (2.5 and 4.0) was active between 5.0-7.0. The maximum activity, 2.2×10^5 U/mg at pH 6.0 was 3.3 fold greater than the activity of native PPO ($O.67\times10^5$ U/mg) stored at pH 7.0.

The measurement of the catecholase activity as a function of pH indicated a pH optimum of 6.0 (Fig 6.2) above, which the activity decreased. At pH 4.0, the activated form exhibited only -30% of the maximal activity. These results indicate that field bean PPO is activated *in vitro* by exposure to acid pH and are in agreement with those obtained with crude extracts at pH 2.5. The product accumulation curve (not shown) attained its steady state value immediately at all pH values with no lag phase.

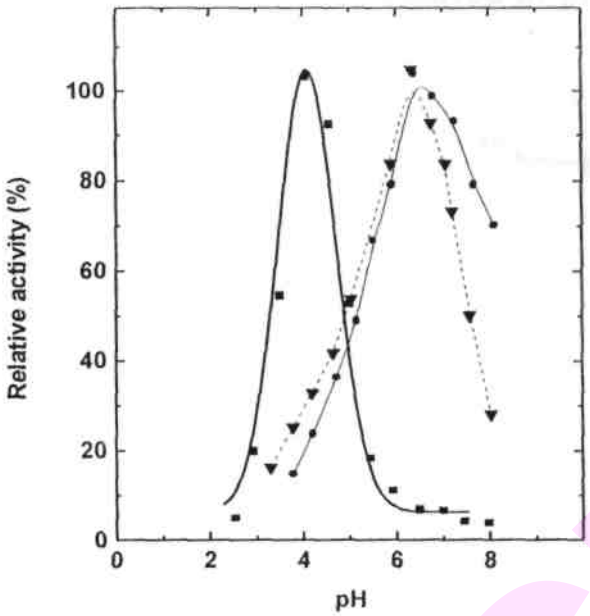


Fig **6.2. Effect of pH on the activity of field bean PPO.** (■) purified PPO (native) in 25 mM Tris-HCl, pH 7.0 containing 1.2% NaCl (w/v), (T) acid activated PPO and (•) SDS activated PPO. Assays were conducted in Mcllvaine buffer, pH 2.5-7.5 and 100 mM Tris-HCl, pH 8.0, using 50 mM catechol as substrate.

Effect of pH and time on activation process

The activation phenomenon as a function of time was investigated. The native PPO was incubated in buffers of pH 2.5, 4.0 and 7.0 and at regular time intervals aliquots were assayed for PPO activity at pH 6.0. The results indicate that the activation at pH 2.5 was faster than that at pH 4.5 (Fig 6.3) reaching a maximum in 30 min. However, at pH 4.5 (Fig 6.3) although the maximal velocity attained was similar (2.2×10^5 U/mg), the time taken to reach this maximum was between 1-6 h, indicating a much slower rate of activation. PPO activated at pH 2.5, although activated rapidly, was unstable with a sharp decline in the reaction rate, loosing 97% of its activity in 24 h. In contrast, the slowly activated enzyme, at pH 4.0 was stable even after 24 h (Fig 6.3). The activity of the native PPO (pH 7.0) assayed at pH 6.0 is less than 10% of the activated forms and remains unaltered. The

native PPO stored at pH 7.0 and assayed at pH 4.0 is also stable at pH 7.0 (Fig 3.13a, Chapter 3)

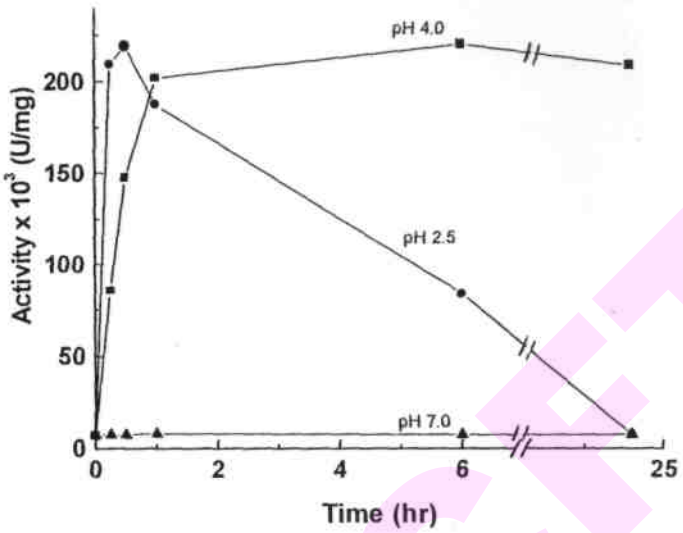


Fig 6.3. **Activation of field bean PPO at varying pH.** Native PPO was incubated in Mcllvaine buffers, pH 7.0, 4.0 and 2.5. Aliquots were assayed in 50 mM Na acetate, pH 6.0 containing 50 mM catechol.

These results suggest that field bean PPO exists in two forms, the native form with a pH optimum of 4.0 and the activated form with a pH optimum of 6.0. Native PAGE was used to ascertain whether the two pH optima observed are associated with two different proteins or a single protein. The electrophoretic mobility of the native PPO and the activated PPO are similar as revealed both by protein (Fig 6.4, lanes 1 & 2) and activity staining (lanes la and 2a).

Electrophoretic analysis of PPO isoforms from a variety of angiosperms has revealed that the enzymes remain active in the presence of 0.1% SDS. Hence SDS was included in the staining solution (Angleton and Flurkey, 1984). Additional protein bands or enzyme bands were not visible confirming the fact that this shift in the pH optimum following exposure to acid pH of field bean PPO is associated with a single protein.

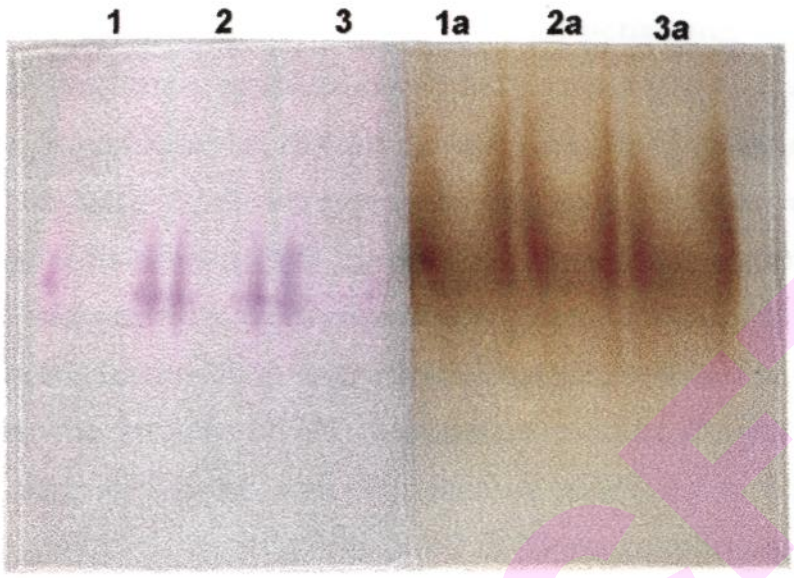


Fig 6.4. **Native PAGE (7.5% T, 2.7% C) of native and activated field bean PPO.** The gel was stained for protein with Coomassie brilliant blue (lanes 1, 2 & 3) and for PPO activity (lanes la, 2a & 3a) in 50 mM Na acetate containing 50 mM catechol, 1.5 mM SDS and 0.05% p-phenylenediamine. (lanes 1 8B la) native PPO, (lanes 2 & 2a), PPO incubated in Mcllvaine buffer pH 2.5 for 30 min and (lanes 3 & 3a) PPO incubated in Mcllvaine buffer pH 6.0 buffer containing 0.5 mM SDS.

SDS activation of field bean PPO

SDS, an anionic detergent as an activating agent, has been used by several researchers because few enzymes are known to be activated by it, in contrast to the many enzymes that are inactivated. Activation studies with SDS were carried out to determine whether any other forms of field bean PPO, in addition to the acid activated form exist. For this reason, the determination of the optimum pH and the kinetic characterization was carried out in the presence and absence of SDS. When the activity of PPO was determined in the presence of SDS, in a pH range of 2.5-8.5, the pH optimum was 6.0 (Fig 6.2), with the activity relatively high between pH 5.5-7.5. These results differ from the activity determined in the absence of SDS, wherein the optimum is pH 4.0, and the activity falling significantly on either side. In contrast, these results are very similar to the activating effect of PPO exposed to acid pH (Fig 6.2). The maximal activity measured in the presence of SDS was 2.6×10^5 U/mg, quite similar to the acid activated form and is 3.8 fold higher than the native form (Table 6.1).

Table 6.1.**Kinetic properties of the native and activated field bean PPO.**

Properties	Native[1]	Acid activated[2]	SDS activated[3]
pH optimum	4.0	6.0	6.0
K_m (mM)	10.5	11.6	13.3
Vmax(U/mg)	67,347	2,22,220	2,55,100
Ki of tropolone (x 10"[7] mM)	5.8	2.0	1.8
Vmax/Km (min[1])	6,476	19,156	19,180
Substrate inhibition	>40 mM	>40 mM	>40 mM

!Data taken from Chapter 3; [2]PPO incubated in Mcllvaine buffer, pH 4.0 for 6 h; [3]PPO incubated in Mcllvaine buffer, pH 6.0 containing 0.5 mM SDS for lh.

Effect of SDS concentration on activation of PPO

To study this activating effect of SDS on field bean PPO, the native enzyme was incubated in 25 mM Tris-HCl buffer, pH 7.0 containing 1.2% NaCl (w/v), in varying concentrations of SDS for 30 min, and the catechol oxidation measured at pH 6.0. The results shown in Fig 6.5 indicate that the purified field bean PPO was activated in a sigmoidal manner with increasing SDS concentrations upto a maximum of 1.5 mM. Insignificant activation occurred until 0.5 mM, followed by a sharp increase in the activity from 0.5-1.5 mM, with half the maximal activation occurring at 0.79 mM. Escribano et al. (1997) reported that the activation process induced by SDS is saturable whereas Moore and Flurkey (1990) reported that activation was more successful and effective using SDS, at concentrations below the critical micellar concentration (CMC) of SDS. The CMC was estimated to be 3.5 mM in 0.1 M NaPi buffer, pH 6.0 (Moore and Flurkey, 1990). Therefore, purified field bean PPO was activated much below the CMC for SDS. The enzyme lost activity at concentrations greater than 1.5 mM.

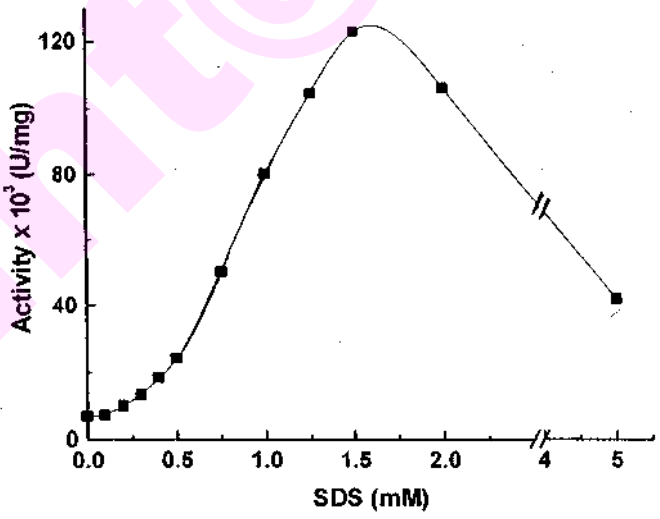


Fig **6.5. Effect of SDS concentration on the activity of field bean PPO.** PPO was incubated in 25 mM Tris-HCl, pH 7.0 containing 1.2% NaCl (w/v) and indicated concentrations of SDS for 30 min and then assayed using catechol as the substrate at pH 6.0.

Electrophoretic mobility of SDS activated PPO

The electrophoretic mobility of PPO activated by SDS was examined by native PAGE. A single enzymatically active protein band (Fig 6.4, lanes 3 & 3a) was observed. The staining intensity and relative mobility of this band was similar to that of the acid activated PPO (Fig 6.4, lanes 2 & 2a) suggesting that the two forms are similar and are associated with a single protein. The stability of the activated form in the presence of SDS was investigated. The results (Fig 6.6) showed that in the presence of 1.5 mM SDS, although the PPO activation was maximal, it gradually lost activity probably due to inactivation. In contrast, the activation at 0.5 mM was slower, requiring 6 h to attain the maximum reaction rate, but was more stable. At very low SDS concentration (0.1 mM) although the activation is <5%, the enzyme is stable for 24 h.

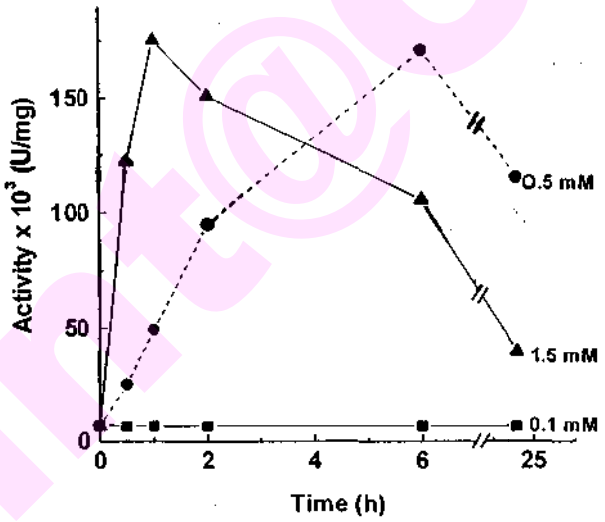


Fig 6.6. **Stability of SDS activated field bean PPO.** PPO was incubated in the presence of different concentrations of SDS in 25 mM Tris-HCl pH 7.0 containing 1.2% NaCl (w/v). PPO was assayed at regular time intervals in 50 mM Na acetate, pH 6.0 containing 50 mM catechol.

Effect of pH on SDS activation

The dependence of SDS activation on the pH of the medium has been documented for plant PPOs. Fig 6.7 shows the results obtained after incubating the enzyme at pHs between 4 and 7 in the presence of 0.5 mM SDS. pH has a profound effect on SDS activation with maximal activity observed when incubated at pH 6.0 in the presence of SDS for 1 h (Fig 6.7). Further incubation leads to a decrease in the activity. At lower pH, in the presence of SDS, activation is profound initially, but rapid inactivation occurs after 1 h. In all these studies PPO was assayed at the optimum pH 6.0, of the activated form. The highest value in the SDS activation process was also observed in pH 6.0 buffer (Fig 6.7). It was therefore decided to further study the activation by SDS (0.5 mM) at pH 6.0.

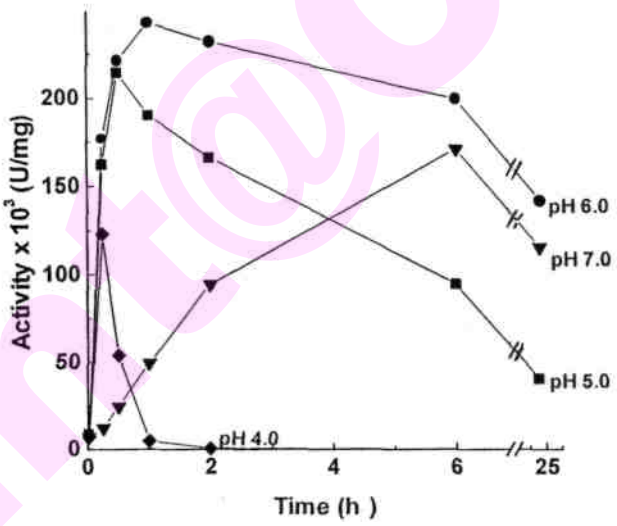


Fig **6.7. Effect of pH on the activity of the SDS activated field bean PPO.** PPO was incubated in McIlvaine buffers pH 4.0-7.0 containing 0.5 mM SDS. Aliquots were removed at regular time intervals and assayed using catechol as the substrate at pH 6.0

Kinetic characteristics of acid pH and SDS activated forms of field bean PPO

Enzymatic characteristics associated with SDS activation and acid pH activation were examined and compared to the data of the native purified form (Table 3.2, Chapter 3). These results demonstrated that by acid shocking and in the presence of SDS the low pH optimum of field bean PPO was abolished (Table 6.1 and Fig 6.2). The effect of catechol concentration on the initial velocity of the pH and SDS activated forms show a regular Michaelis-Menten kinetics (Fig 6.8), the initial velocity increasing linearly with increasing catechol concentrations upto -40 mM. Further increase in catechol concentration leads to substrate inhibition (Fig 6.8) as observed for native PPO (Chapter 3, Fig 3.15a). The kinetic constants calculated by double reciprocal plots of these data (Table 6.1) indicate no apparent change in the binding affinity of catechol. The K_m values for the activated forms are similar to the native or non-activated form. However, the values of V_{max} are increased -3.5 fold. The kinetic parameters of both the acid activated and SDS activated forms are similar (Table 6.1) and this suggests that both these methods of activation render active PPO with similar catalytic and affinity properties towards catechol. The change in the kinetic parameter values produced by the activation process either by exposure to acid pH or SDS was even greater when the catalytic efficiency $V_{mU}x/K_m$ was compared (Table 6.1). The V_max/K_m increased up to three times for both the acid activated and SDS activated forms, being due to an increase in the Vmax values in both the cases. The similarity in the increase in the catalytic power of field bean PPO, activated by two different agents suggests that the mechanism of activation could be similar.

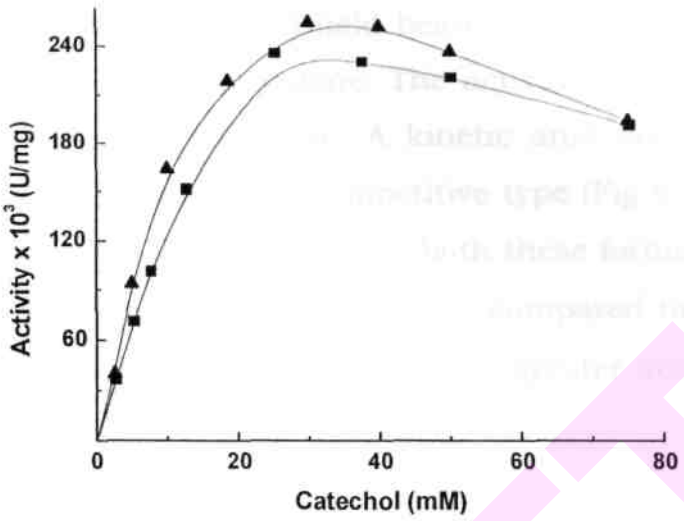


Fig 6.8. **Michaelis-Menten plot of the effect of catechol concentration on activated field bean PPO. (■)** Field bean PPO was incubated in Mcllvaine buffer, pH 4.0 for 6 h and (A) Mcllvaine buffer, pH 6.0 containing 0.5 mM SDS for 1 h, prior to the assay.

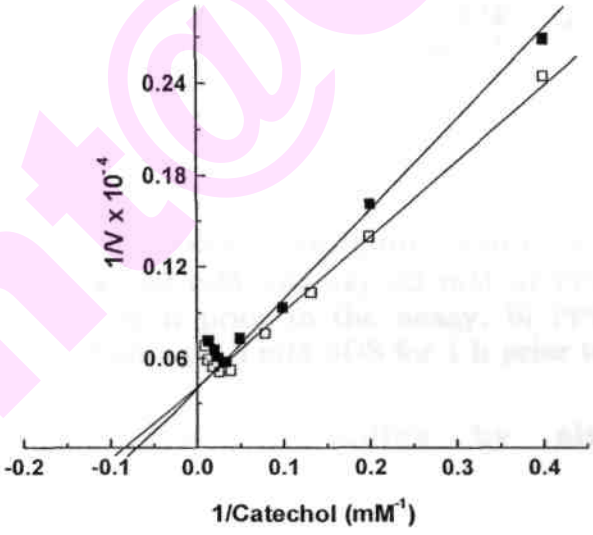


Fig 6.9. **Double reciprocal plot of the effect of catechol concentration on acid activated PPO.** Data of Fig 6.8. (■) PPO exposed to pH 4.0 for 6 h and (□) PPO incubated in 0.5 mM SDS at pH 6.0 for 1 h.

Inhibition by tropolone

Inhibition by tropolone, a potent competitive inhibitor of plant PPOs and also field bean PPO (Chapter 3, Fig 3.16a, b 85 c) was carried

out for these two activated forms of field bean PPO. In the presence of SDS, PPO was inactivated by tropolone. The activated forms are more susceptible to inhibition by tropolone. A kinetic analysis of tropolone inhibition showed that it was of the competitive type (Fig 6.10) both for the SDS and pH activated PPO. The KiS for both these forms are similar (Fig 6.10, Table 6.1). The lower Kj of ~2 x 10^{-7} compared to that of the native form with a Ki of 5.8 x 10^{-7} suggests the greater accessibility to the active site.

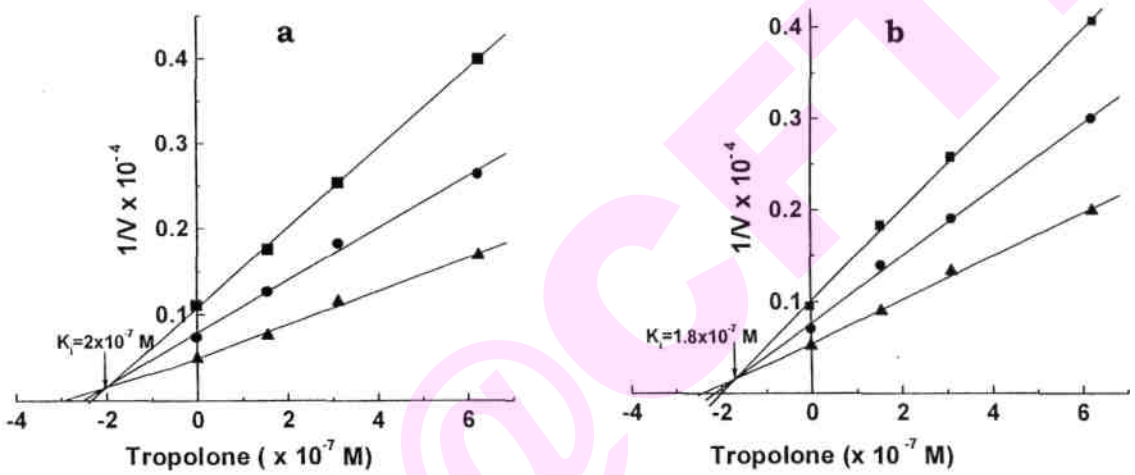


Fig 6.10. **Dixon plot of tropolone inhibition of activated field bean PPO.** Catechol (■) 5 mM, (•) 10 mM and (A) 20 mM a) PPO incubated in Mcllvaine buffer, pH 4.0 for 6 h prior to the assay, b) PPO incubated in Mcllvaine buffer, pH 6.0 containing 0.5 mM SDS for 1 h prior to the assay.

Determination of hydrodynamic radius by size exclusion chromatography

Swain et al. (1966) suggested that activation in broad bean leaf PPO could be due to a conformational change of the enzyme. The conformational status of a protein can be ascertained by measuring the hydrodynamic radius. Size exclusion chromatography was used to determine the Stokes radius (R_s) of the pH activated and the SDS activated forms.

A set of proteins whose M_r and Stokes radii are known (Corbett and Roche, 1984; Uversky, 1993) were used to construct the calibration curve of log R_s vs migration rate ($1000/V_e$). The calibration curve obtained from the Progel™-TSK G2000 SWXL (7.8 mm idx30 cm) column can be described by the equation 1000/Ve=(84.671 + 1.681) R_s+1.4378±0.039 (Fig 6.11). In the absence of SDS and without exposure to acid pH, field bean PPO eluted from the column at a retention time of 6.9 min, which corresponds to a Stokes radius of 41.8 (Fig 6.12).

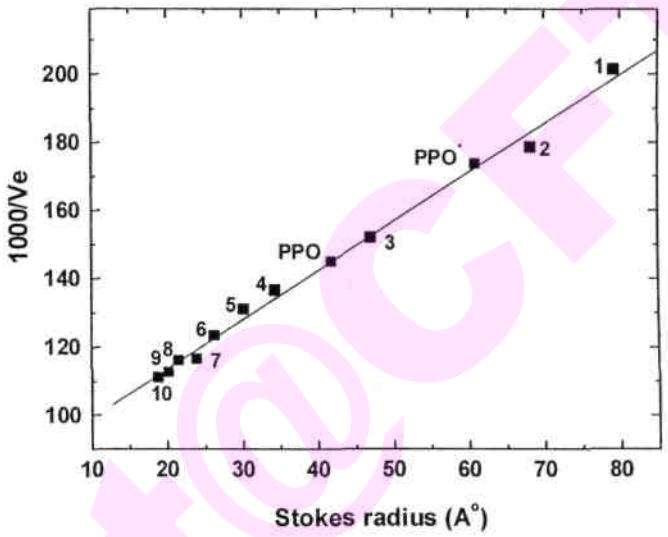


Fig 6.11. **Dependence of migration rate ($1000/V_e$) of calibration proteins on their Stokes radius (R_s).** PPO, native field bean PPO, PPO*, activated field bean PPO. The column was calibrated using (1) thyroglobulin, 660 kDa, (2) ferritin, 450 kDa, (3) aldolase, 158 kDa, (4) BSA, 66.3 kDa, (5) ovalbumin, 43.5 kDa, (6) ß-lactoglobulin, 36.8 kDa, (7) chymotrypsinogen, 25.7 kDa, (8) carbonic anhydrase, 25.0 kDa, (9) myoglobin, 16.9 kDa and (10) ribonuclease, 13.7 kDa.

Activation by either SDS or exposure to acid resulted in a shift of the R_s towards much higher R_s values of 60.9 (Fig.6.11 & 6.12). This increased radii in the presence of SDS or by exposure to acid indicate that native PPO assumes a more compact conformation than the activated forms. The increase in the Stokes radius that acid pH induces could be interpreted as representing the unfolding of the enzyme

molecule rather than association because, prolonged incubation at pH 4.0 (>24 h) resulted in the same radii (result not shown). Fig 6.12 shows that although a conformational change occurs by acid activation, the change is incomplete with about <15% remaining in the native form. In the presence of SDS, >95% has the altered conformation, with an increased Stokes radius.

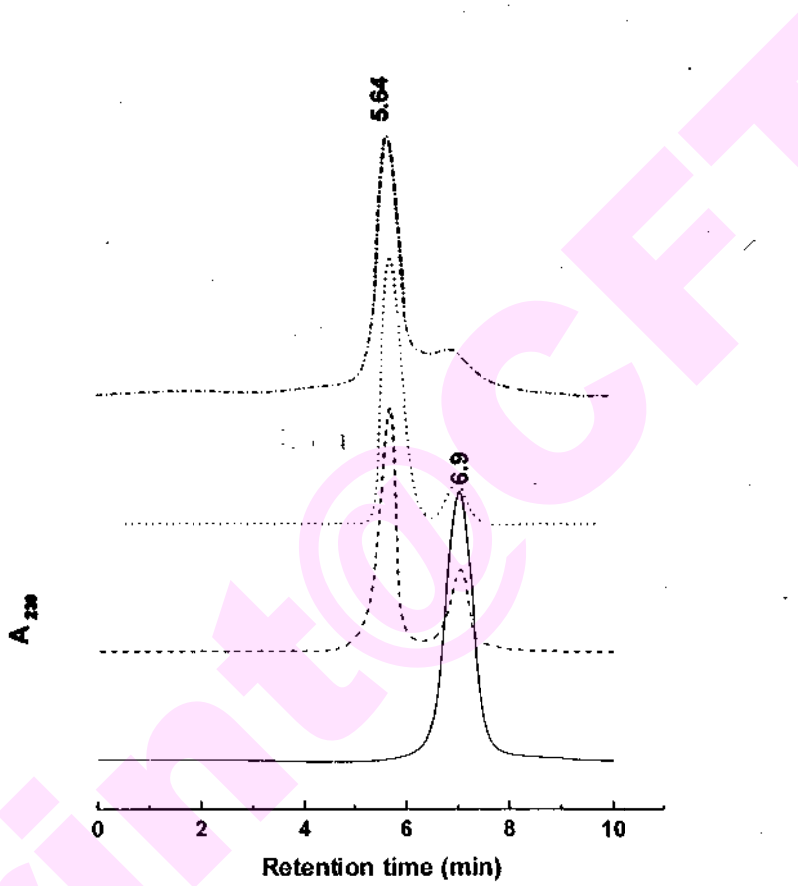


Fig 6.12. **HPLC-gel filtration profile of native and activated field bean PPO.** Column used: Progel™-TSK G2000 SWXL (7.8 mm idx30 cm). The eluent used was 0.1 M NaPi pH 7.0 containing 0.1 M sodium sulfate, at a flow rate of 1 mL/min. (---------) native field bean PPO, (----------)PPO incubated in Mcllvaine buffer, pH 4.0 for 30 min, (.....) PPO incubated in Mcllvaine buffer, pH 2.5 for 30 min, (-------) PPO incubated in Mcllvaine buffer, pH 6.0 containing 0.5 mM SDS for 30 min. The same amount of protein (20 (j.g) was injected.

Intrinsic fluorescence spectra

The fluorescence spectra of native field bean PPO and the acid and SDS activated forms of PPO were measured in a Shimadzu (Model Rf 5000) recording spectrofluorimeter. The intrinsic tryptophan and tyrosine residues were excited at 280 nm and emission spectra recorded at a wavelength ranging from 300-400 nm. The conformational status of PPOs was analysed at pH 4.0 and in the presence of SDS at pH 6.0 as shown in Fig 6.13.

The fluorescence emission spectra were distinguishable for the native PPO and acid activated form when the activation was carried out for 30 min. Although no shift in the emission maximum was observed (Fig 6.13), the fluorescence intensity decreased by about 36% after 6 h in the presence of SDS (Fig 6.13b) as compared to 16% after 30 min incubation (Fig 6.13a). Incubation at pH 4.0 decreased the intensity to 20% of the native PPO after 6 h. The fluorescence data are consistent with a view that the two activated forms assume similar conformations but different from that of the native form.

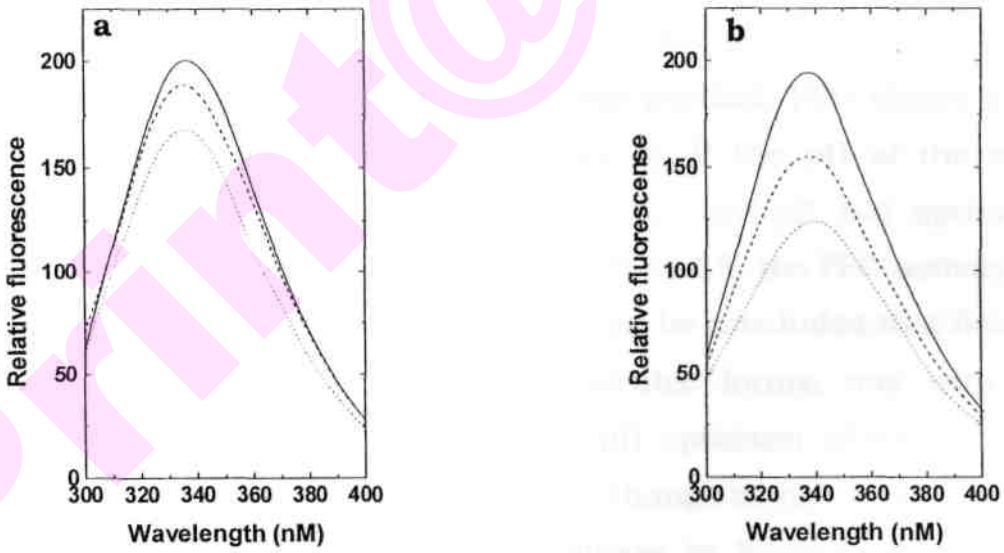


Fig 6.13. **Fluorescence emission spectra of field bean PPO.** (----------) native PPO, (-------) PPO in Mcllvaine buffer, pH 4.0 (.....) PPO in Mcllvaine buffer, pH 6.0 containing 0.5 mM SDS; a) After 30 min b) After 6 h.

DISCUSSION

In plants, PPO is located in the chloroplast thylakoid membrane and presents unusual intriguing characteristics since it can exist in an inactive or latent state (Mayer and Harel, 1979) and can also undergo reversible inactivation during the oxidation of o-diphenols to o-quinones (Mason, 1965). PPO can be activated or released from latency by various treatments or agents. In some cases, the activation was caused by denaturing agents of some kind such as acid, alkali, detergent or organic solvent treatment (Kenten, 1958; Flurkey, 1986; Espin and Wichers, 1999b). In other cases, activation was also induced by proteolytic enzymes (Golbeck and Cammarata, 1981; King and Flurkey, 1987), fatty acids (Sugumaran and Nellaiappan, 1991) or pathogen attack (Golbeck and Cammarata, 1981; Soler-Rivas et al., 1997). In many cases, the enzyme is activated upon release from the thylakoid membrane, but there is no indication that solubilization and activation are part of the normal function of the enzyme in the chloroplast. Indeed, the only physiological activator known is the process of aging (Golbeck and Cammarata, 1981). PPO solubilized at low pH (2.5) showed two pH optima of 6.0 and 4.0 (Fig 6.1) with the activity at pH 6.0, predominant. However, when solubilized at pH 7.0 and purified, PPO shows a single pH optimum of 4.0 (Fig 3.12, Chapter 3). If the pH of the enzyme solubilized at low pH is adjusted to 7.0, the pH 6.0 optimum is abolished (Fig 6.1). Upon adjusting the pH to 2.5, the PPO activity at pH 6.0 is maximum. From these results, it can be concluded that field bean PPO exists in at least two interconvertible forms, one with a pH optimum of 6.0 and the other with a pH optimum of 4.0. These two forms are interconvertible by a sudden change in pH. A pH dependent interconversion of two forms of tyrosinase in human skin has been characterized (Tripathi et al., 1988). Experiments carried out by Kenten (1957) showed that the crude extracts of broad bean *(Viciafaba)* leaves

contained a latent PPO, which could be released by brief exposure to acid or alkaline conditions.

PPO from field bean seeds was extracted as a soluble enzyme and purified to apparent homogeneity (Table 3.2, Chapter 3). pH is a determinant factor in the expression of field bean PPO activity and in the case of latent PPO, activation by acid or basic shock has been described (Kenten, 1957). Fig 6.1 demonstrates that two forms of PPO can be found in the field bean seed if it is solubilized at pH 2.5. The single form of PPO (Fig 3.12, Chapter 3) with a pH 4.0 optimum, purified by solubilization at pH 7.0, can be converted by a sudden exposure to acid pH of 2.5 or 4.0, to the form with a pH optimum of 6.0 (Fig 6.1 8s 6.2). Prolonged exposure to pH 2.5 leads to a loss in the activity (Fig 6.3). Kenten (1957) showed that the maximum activity of broad bean leaf PPO was released by exposure to acid pH and was greater than that obtained by exposure to alkaline pH. Lerner et al. (1972) found that, prolonged exposure of grape berry PPO to acid pH provoked irreversible activation of the enzyme and Lerner and Mayer (1975) further showed that the process was accompanied by a conformational change. A lag phase dependent on the pH of the assay medium of a latent grape PPO (Valero and Garcia-Carmona, 1992) was reported, suggesting the hysteretic nature of the enzyme. A kinetic model has been proposed to account for this hysteresis (Ricard et al., 1994). The basis of the model is that, upon ionization or protonation of a group located outside the active site, the protein undergoes a slow conformational transition. Glu 236, present in the active centre of ibCO, is proposed to assist in deprotonating the substrate (Klabunde et al., 1998). Field bean PPO shows no lag phase when assayed between 2.5-8.5 (results not shown) and therefore it is not hysteretic in nature and effect of exposure to acid pH does not follow the kinetic model of Ricard et al. (1994). Enzymatic characteristics associated with the two

interconvertible forms of field bean PPO show that although the binding affinity of two forms for catechol are similar, there is a -3.5 fold increase in the V_{max} of the acid and SDS activated forms (Table 6.1). The increase in the catalytic efficiency V_{max}/K_m is due to the V_{max} values. Such increase in catalytic power are similar to those described for potato and table beet leaf PPO (Sanchez-Ferrer et al., 1993c, Escribano et al., 1997). Marques et al. (1994) reported that the activation of PPO does not modify substrate affinity, which is also reflected by the similar K_m of the activated forms of field bean PPO (Table 6.1).

Plant PPO in its latent state can also be induced or activated by SDS (Moore and Flurkey, 1990). PPO could be activated by SDS to a different form, with a pH optimum of 6.0 (Fig 6.2) similar to that of the acid exposed form. The exposure of field bean PPO to SDS concentrations below its CMC abolished the low pH optimum of the native PPO from 4.0 to 6.0. Activation by two different denaturing agents produces the same effect on the pH optimum (Fig 6.2). Purified field bean PPO was activated by SDS in a sigmoidal manner below the CMC for the detergent (Fig 6.5). Half the maximal activation occurred at 0.79 mM, slightly lower than that reported for broad bean PPO (Moore and Flurkey, 1990). Maximal activation of partially purified broad bean PPO (Swain et al., 1966) and purified *Xenopus* tyrosinase (Wittenberg and Triplett, 1985), occurred below 3.5 mM SDS, its CMC. The sigmoidal nature of SDS activation of field bean PPO with increasing SDS concentrations (Fig 6.5) is suggestive of a cooperative interaction between SDS binding and activation. A similar sigmoidal nature of SDS activation has been reported for *Xenopus* tyrosinase (Wittenberg and Triplett, 1985) and broad bean leaf PPO (Moore and Flurkey, 1990). Kenten (1958) also showed that a variety of methyl sulfates activated PPO in a sigmoidal manner and that the concentration of alkyl sulfate needed to bring about a given activation decreased with increasing

chain length. The concentration of SDS required to bring about maximum activation was 1.5 mM (Fig 6.5), which is one half the CMC of SDS. Wittenberg and Triplet* (1985) showed that the amount of SDS required for activation was related to the maximum number of monomers in the solution. Isotherms for SDS binding to *Xenopus laevis* tyrosinase were correlated with SDS activation/stabilization, suggesting that activation was a result of SDS binding to the enzyme (Wittenberg and Triplett, 1985). Field bean PPO is activated by high SDS concentrations, which generally provoke loss of catalytic efficiency (Moore and Flurkey, 1990). Concentrations greater than 1.5 mM SDS inactivate PPO by denaturation.

Enzymatic characteristics associated either by exposure to acid pH or with SDS activation are similar (Table 6.1), both abolishing the low pH optimum of purified PPO. The similarities in the optimum pH, increased catalytic efficiency, similar substrate inhibition and increased inhibition by tropolone suggest that the activation processes are related by a common mechanism and the activated forms are similar in conformation at the active site of the enzyme. Differences found in the optimum pH of mushroom tyrosinase activated by SDS and acid pH was related to the different conformation of the active site of the enzyme (Espin and Wichers, 1999c). The two conformational forms must be similar enough to catalyze the same reaction but dissimilar enough to have different catalytic or binding rate constants. Kinetic characteristics (Table 6.1) of the activated field bean PPO forms, obtained by either pH activation or SDS are similar enough to exhibit similar conformations.

SDS activation of PPO has been suggested as a process involving an induced conformational change in the protein that could give rise to active forms with an altered catalytic efficiency (Sugumaran and Nellaiappan, 1991; Moore and Flurkey, 1990). Kenten (1975) and Lerner

et al. (1972) suggest that activation by changes in the pH of the medium involved a change of the Stokes radius of the PPO, a conformational change. HPLC size exclusion chromatography of the acid pH activated form and the SDS activated form (Fig 6.11) shows a decrease in the retention times when compared to the native form. The similar elution volumes of the acid activated form and SDS activated form evidence that these two forms are conformationally similar but different from that of the native form. The significant and similar increase in the Stokes radius of these activated forms, when compared to the native form (Fig 6.12) implies the involvement of a conformational change, which occurs due to exposure to acid pH or the presence of SDS. Moore and Flurkey (1990) noted a small increase in the size of broad bean PPO in the presence of SDS from 43 kDa to 48 kDa and attributed this to the result of SDS binding and increase in size. The same transformation of field bean PPO from 41.8 A to 60.9 A can be induced by either incubation at pH 2.5, 4.0 or SDS, all of which exhibit increased enzyme activity. Lerner and Mayer (1975) interpret the increase from 26 to 40 A in the Stokes radius of grape catechol oxidase during its reversible activation at pH 5.0 as representing an unfolding of the enzyme rather than association of several enzyme molecules because it was possible to induce the same enzyme transformation by different treatments, which irreversibly increased enzyme activity. Field bean PPO is a homotetramer of subunit M_r~30+1.5 kDa. Preliminary studies showed that the enzyme could aggregate in low ionic strength buffer with loss in catecholase activity. However, addition of 1.2% NaCl resulted in dissociation with total regain of activity (results not shown). The increase in the activity and size of field bean PPO (Fig 6.12) due to SDS or acid pH is therefore not due to a quaternary change as the enzyme activity is enhanced. Swain et al. (1966) suggest that the activation process involves a limited conformational change such as swelling of

the protein rather than dissociation or aggregation. Swain et al. (1966) found little difference in the sedimentation coefficient of PPO in the presence (4.4 S) vs in the absence (4.3 S) of SDS. Thus in the few reports available, SDS does not appear to affect quaternary changes or subunit dissociation of the enzyme. The electrophoretic mobility of these activated forms (Fig 6.4) is similar indicating no radical change in the charge. Valero and Garcia Carmona (1992) detected a single species for thylakoid bound grape PPO in both the latent state and upon activation with trypsin.

Intrinsic fluorescence can be used to probe perturbations in protein structure and conformation. That SDS and exposure to acid pH cause a conformational change in field bean PPO is further evidenced from the decrease in the fluorescence intensity (Fig 6.13a & b). Reynolds and Tanford (1970) report that alterations in protein structure begin to occur at monomer concentrations of 0.1 mM SDS. Decrease in intrinsic fluorescence along with the change in Stokes radius, strongly suggest that a conformational change has occurred by pH and SDS activation of field bean PPO. The treatments apparently cause a conformational change near the active site, resulting in a considerable acceleration of the reaction rate and increased susceptibility to inhibition (Table 6.1). Tropolone, a potent inhibitor of PPOs and has been reported to compete for the copper active site of the enzyme (Kahn and Andrawis, 1985). The nearly 3-fold increased affinity of tropolone for the pH and SDS activated forms suggest that the conformational change is indeed at the active site, that renders it more accessible to the inhibitor. It thus emerges that the exposure of field bean PPO to acid pH or to SDS at levels that bring about activation, or conformational change, leads to an opening of the active site. The slightly higher reactivity of the SDS activated form when compared to the acid activated form (Table 6.1) could be due to the higher levels of the

conformationally more active form (Fig 6.12). The oxidation rate of PPO exposed to pH 2.5 (30 min) is greater than that of pH 4.0 (Fig 6.3) and is also reflected in the higher levels of conformationally altered form (Fig 6.12). The existence of a regulatory domain or region, where pH controls enzyme activity has been suggested for apple PPO (Marques et al., 1995). This region could be shifted by the addition of SDS or by protease digestion. The most unusual feature of acid activation of field bean PPO is the rapidity of the conformational changes (Fig 6.12) and its rapid reversibility (Fig 6.1). A slow reversibility of acid activation of *Vicia faba* (Swain et al., 1966) and an irreversible activation of grape catechol oxidase are documented (Lerner and Mayer, 1975). Intrinsic fluorescence of tyrosine and tryptophan residues of purified broad bean leaf PPO increased in a complex fashion as SDS concentration increased (Moore and Flurkey, 1990). The intrinsic fluorescence curve showed an increasing trend upto 0.5 mM which suggested that conformational changes occurred during SDS binding.

Due to an implication of PPO in the detrimental effects of enzymatic browning leading to quality loss, the kinetic characterization of this enzyme and the study of its ability to exist in an inactive or latent state in both soluble and membrane bound forms is a prerequisite when designing antibrowning agents. A study of PPO latency during fruit ripening and storage is essential to develop better control measures, as activation of PPO is believed to occur during aging and senescence.

Chapter 7
Purification and characterization of a natural inhibitor of field bean (*Dolichos lablab)* polyphenol oxidase

Browning of raw fruits, vegetables and beverages is a major problem in the food industry and is a major cause of quality loss in post harvest handling, storage and processing. Enzymatic browning mediated by PPO, causes deleterious effects on appearance, nutritional and organoleptic properties resulting in decreased market value. Because of the detrimental effects of browning, its control is of high priority for the food processing industry. Many compounds inhibit PPO activity. However, only a limited number are considered acceptable on the grounds of safety and/or expense for use in food processing. The most widely used anti-browning agents are probably the sulfites (Mayer and Harel, 1991; Walker, 1995; Sapers, 1993). Sulfites have come under scrutiny because of their possible harmful side effects (Sapers, 1993). The Generally Recognized As Safe (GRAS) status of the sulfites has been revoked when used in fruits and vegetables that are sold or served raw (Walker and Ferrar, 1995). In the light of this, the research on PPO inhibitors has been focused on non-toxic inhibitors for use as food additives. The most exciting of this has been inhibitors from natural sources.

Prabha and Patwardhan (1986a) of this institute observed that the aqueous extracts of unripe sapota peel *(Achras zapota)* and pulp prevented PPO mediated catechol oxidation suggesting the presence of endogenous PPO inhibitors. The non-dialyzable nature and precipitation of the inhibitor by TCA led them to conclude that the inhibitor was proteinaceous in nature. A protein inhibitor is an ideal macromolecule for structural characterization and lends itself to genetic manipulation. Therefore, a detailed study on the purification and characterization of the inhibitor present in unripe sapota *(Achras zapota)* was undertaken.

RESULTS

The crude extract of raw sapota was found to inhibit field bean PPO using catechol as the substrate. The activity of field bean PPO in the absence of crude sapota extract was 68,000 U/mg whereas in the presence of 0.01 mL of the water extract of raw sapota, the measured activity was 56,430 U/mg. Inhibitory units were considered as the difference in the activity of the enzyme in the presence and absence of the inhibitor.

The crude extract was dialyzed extensively against distilled water (3x1000 mL) using a membrane with a M_r cut off of 12.5 kDa. The inhibitory activity was recovered in the retentate (Table 7.1). These results reflect upon the non-dialyzable nature of the inhibitor with a $M_r \geq 12.5$kDa.

Table 7.1. **Inhibitory activity of the crude sapota extract before and after dialysis.**

Crude Sapota extract	Total inhibitory activityx10^6(IU)
Before dialysis	16.3
After dialysis	16.29

The UV-Vis spectrum of the crude extract had two absorption maxima, one at 220 nm and the other at 280 nm (Fig 7.1). This, together with the earlier findings (Prabha and Patwardhan, 1986a), led to the assumption that the PPO inhibitor could be a protein. The dialyzed sapota extract was freeze dried and stored at 4 °C for all further studies.

Test for protein

1. Trichloroacetic acid (TCA) precipitation: Ten milligrams of the freeze dried sapota extract was dissolved in 1 mL of 10% TCA (w/v) in 10 mM NaPi, pH 7.0 and allowed to stand at 4 °C for 10 min. The precipitate was removed by centrifugation at 10,000 *g* at 4 °C. Greater than 99% of the inhibitory activity was recovered in the supernatant indicative of the non-proteinaceous nature of the inhibitor (Table 7.2).

2.Trypsin digestion: To further confirm these results, 10 mg of the extract was dissolved in 1 mL of 10 mM NaPi pH 7.0, and was digested with trypsin (1% w/w) at 37 °C for 6 h. The reaction was stopped by lowering the pH to 2.0. No inhibitory activity was lost after digestion with trypsin suggesting that the inhibitor was not a protein (Table 7.2).

3.Heat treatment: Proteins are generally very labile to heat. An aqueous solution of the extract (1% w/v) was incubated at 100 °C for 30 min and then residual inhibitory activity assayed. The inhibitor was stable to heat, with >99% of the activity recovered (Table 7.2).

Table 7.2. **Inhibitory activity of the crude sapota extract following protein denaturation.**

Treatment	Total inhibitory activity (IU)
Control	10, 530
TCA supernatant	10, 490
Trypsin digest	10, 512
Heat treated	10, 510

Ten milligrams of lyophilized sapota extract was subjected to different methods of protein denaturation and checked for inhibitory activity

TCA is a well known agent used for precipitating proteins (Bennet, 1967), and trypsin is the most widely used proteolytic enzyme

(Darbre, 1986) and most proteins are denatured at high temperatures. No loss in inhibitory activity, after exposure to these treatments suggest that the inhibitor is not a protein.

Test for carbohydrate

Phenol-sulfuric acid test for sugars (Dubois et al, 1956), was used to investigate the nature of the inhibitor. The phenol-sulfuric acid test resulted in the formation of a deep purple color. Simple sugars, oligosaccharides, polysaccharides and their derivatives, including the methyl ethers with free or potentially free reducing groups, generally react to give an orange yellow color with an absorption maximum of 480 nm. Therefore the inhibitor was not a carbohydrate.

Chemical identification of the inhibitor

Literature indicates that a certain group of compounds called proanthocyanidins (PA) although colorless, give colored compounds on treating with hot mineral acids (Bate-Smith, 1953; Haslam, 1982). These PA, also known as leucoanthocyanidins or condensed tannins are widely distributed in the plant kingdom (Haslam, 1982).

In addition, Lakshminarayana and Mathew (1967) reported that the sapota fruit, especially at the early stages of the development contained large amounts of PAs. All these studies led us to conduct specific tests for PAs to chemically identify the inhibitor.

1. *Butanolic HCl treatment:* The butanolic-HCl hydrolysis test is very specific for PAs. The method of depolymerization with butanolic-HCl is based on the transformation of PA into anthocyanidins in hot mineral acid solutions (Porter et al, 1986). A crimson red color was obtained when an aqueous solution of the inhibitor (1% w/v) was heated with butanolic-HCl. The UV-Vis absorption spectrum of the solution was recorded and the maximum absorption was observed at 550 nm

(Fig 7.1). This UV-Vis spectrum of the solution was typical to that of anthocyanidins reported earlier (Swain and Hillis, 1959). The hydrolyzate however showed no inhibitory activity towards field bean PPO. The positive result suggested that the endogenous inhibitor in sapota could be a PA (condensed tannin).

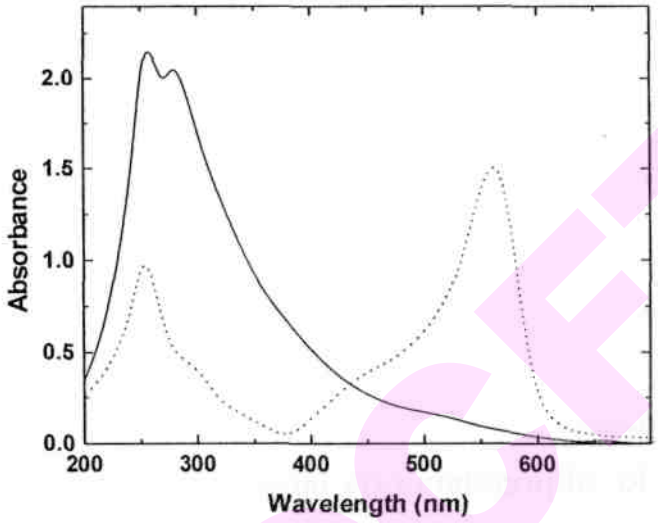


Fig 7.1. **UV-Vis spectrum of crude sapota extract.** Crude extract(----------), crude extract hydrolyzed in 5% butanolic-HCl for 2 h ( )

2. Extraction of condensed tannins: To further confirm this, the condensed tannins were extracted by a modified procedure of Jones et al. (1976) and checked for their inhibitory properties. One hundred milligrams of the freeze-dried sapota extract was dissolved in 5 mL of 70% acetone saturated with NaCl and extracted. The upper acetone layer was removed and washed with the 'aqueous phase of extraction'. To the acetone (2.6 mL) fraction, an equal volume of water was added and extracted twice with 4 mL each of hexane. The hexane layer, which contained pigments and lipids, was discarded. The PA content of this layer was determined as described in Section 2.2.27. About 4.3 mg of tannin was recovered from 100 mg of the original freeze-dried powder. This acetone layer also accounted for ~95% of the inhibitory activity present in the crude extract (Table 7.3).

Table 7.3. Extraction of condensed tannins from crude sapota extract.

Extract	Total inhibitory activity (IU)	Tannin (mg)
Crude	10, 4300	4.8
Acetone phase	10, 4200	4.3
Aqueous phase	ND	0.4
Ripe fruit extract	ND	ND

Hundred milligrams of lyophilized sapota extract was extracted with 70% aqueous acetone. Ripe sapota (5 g) was extracted in 10 mL of 10 mM NaPi, pH 7.0 and checked for inhibitory activity and tannin content. ND: Not detected

3. Two-dimensional chromatography: Two-dimensional chromatography has been used as an efficient tool to detect and identify PAs by several workers (Govindarajan and Mathew, 1963; Lakshminarayana and Mathew, 1967). Two-dimensional chromatography of the crude sapota extract was performed using a Whatman no. 1 filter paper with n-butanol:acetic acid:water (v/v 6:1:2) as the solvent in the first direction and 2% acetic acid in the second direction (Govindarajan and Mathew, 1973; Jones et al., 1976). The PA was detected with 5% vanillin in 12 N HC1 (Hathway, 1958). The crude extract moved as a long trailing spot in the second direction, orange red in color, characteristic of PA (Fig 7.5a).

4. Protein precipitation: PA in sufficient concentrations precipitates proteins (Bate-Smith, 1973) and can be used as a confirmatory test. An aqueous solution of the extract (1% w/v) was added to 2 mL of BSA (0.5% w/v). The solution turned turbid as a result of BSA being precipitated. All these tests confirmed that the inhibitor present in unripe sapota was PA. Further, purification and characterization of the PA was carried out.

Purification of PA

The purification of PA involved two steps of column chromatography.

Sephadex G-50 chromatography: One hundred mg of the lyophilized crude extract dissolved in 1 mL of 10 mM NaPi, pH 7.0, was loaded to a Sephadex G-50 gel filtration column (100 × 2 cm) previously equilibrated in the same buffer. The absorbance of the eluent was monitored at 280 nm. The elution pattern of the Sephadex G-50 chromatography is shown in Fig 7.2.

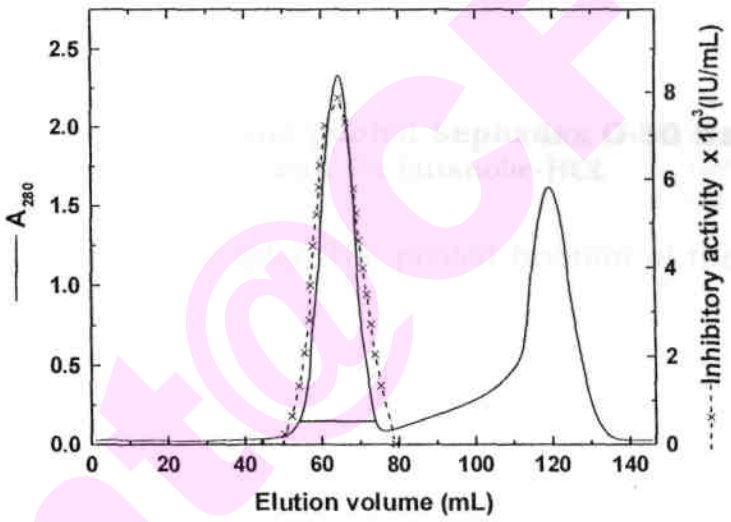


Fig 7.2. **Sephadex G-50 chromatography of sapota PA.** Freeze dried crude sapota extract (100 mg) was dissolved in 1 mL of 10 mM NaPi, pH 7.0 and chromatographed on a Sephadex G-50 column (100 × 2 cm) equilibrated in 10 mM NaPi, pH 7.0. The fraction size was 3.0 mL with a flow rate 18 mL/h. Fractions were pooled as shown (----------).

Although two individual peak fractions with a 280 nm absorbance eluted, only the initial peak showed inhibitory activity towards field bean PPO. These fractions displaying inhibitory activity also showed a positive reaction for PA. The second fraction neither exhibited inhibitory activity nor showed the presence of PA. The UV-Vis spectrum of the pooled inhibitor fraction is shown in Fig 7.3. Hydrolysis of the inhibitor

resulted in the appearance of anthocyanidins with a characteristic absorption maximum at 550 nm and concomitant decrease in 280 nm absorption.

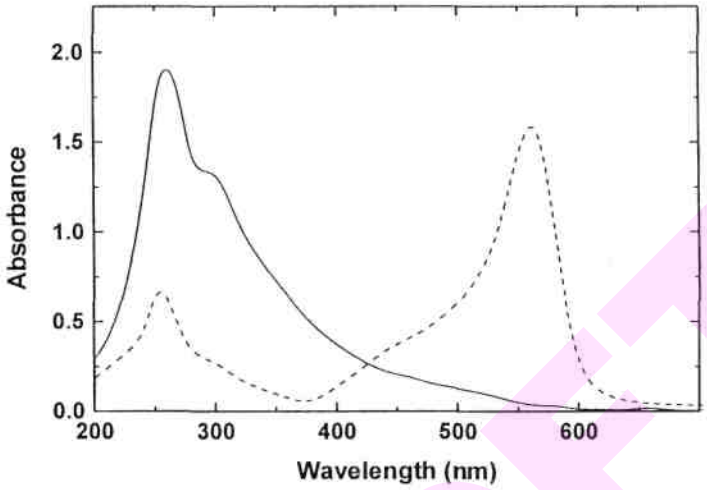


Fig **7.3. UV-Vis spectrum of the pooled Sephadex G-50 fraction.** (----------) PA fraction, (-----) PA hydrolyzed with 5% butanolic-HCl.

Sephadex LH-20 chromatography: The pooled fraction of the above step was dialyzed against water and concentrated by lyophilization. An equal volume of methanol was added to the concentrate and loaded to a Sephadex LH-20 column (20 × 1 cm) previously equilibrated in 50% aqueous methanol. The column was washed with 40% aqueous methanol to remove unbound materials. The bound PA was eluted with 70% aqueous acetone as described earlier (Jones, 1976). The PA eluted as a single symmetrical peak when assayed for inhibitory activity (Fig 7.4).

The active fractions were pooled and acetone removed by dialysis before concentration by lyophilization. The results of the purification are summarized in Table 7.4. The recovery of the PA was ~I4.5% after a 5.5 fold purification with a specific activity of 1,26,133 IU/mg of tannin.

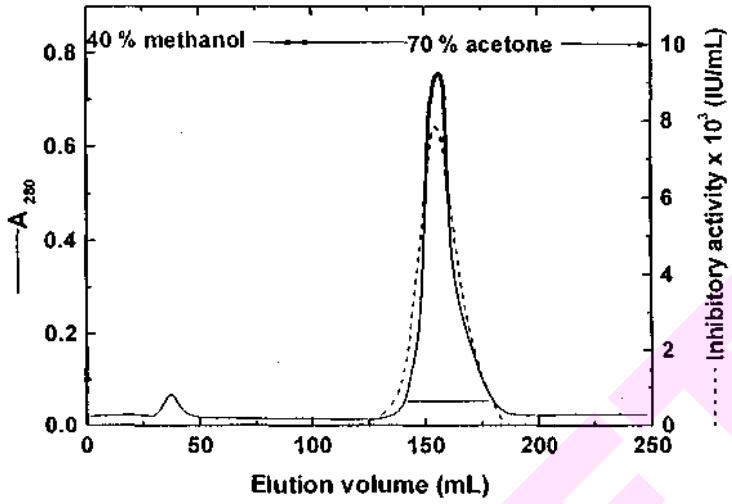


Fig 7.4. **Sephadex LH-20 chromatography of sapota PA.** The pooled fraction of Sephadex G-50 was concentrated, dissolved in 50% methanol and chromatographed on a Sephadex LH-20 column (20 × 1 cm), which was equilibrated in 50% methanol. The column was washed with 40% methanol. The bound inhibitor was eluted with 70% acetone. Fraction size was 2 ml with a flow rate 12 mL/h. The active fractions were pooled as shown (----------).

Table 7.4. **Purification table of PA from sapota extract***

Purification step	Total inhibitory units (IU)	Tannin (mg)	Specific activity (IU/mg)	Fold purification	Yield (%)
Crude	1,02,400	4.5	22,755	-	100
Sephadex G-50	20, 850	0.18	1,15,833	5.1	20.4
Sephadex LH-20	18,920	0.15	1,26,133	5.5	14.5

* These are the results of a typical purification using 100 mg of freeze-dried sapota extract. These values were reproduced in three separate purifications.

Criteria of purity

The purity of the PAs was assessed by 2-D chromatography and HPLC gel filtration.

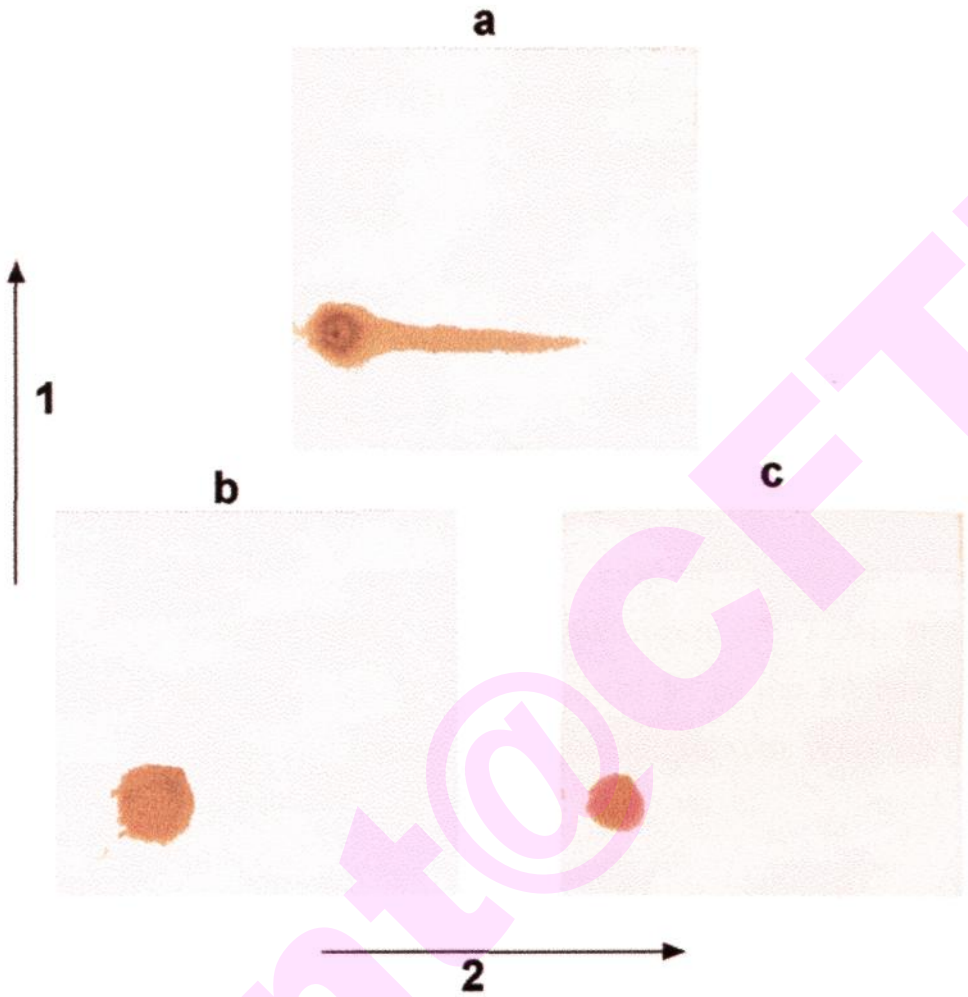


Fig 7.5. **Two-dimensional paper chromatograms of sapota PA.** Chromatography was conducted by the downward development on Whatman no. 1 filter paper using n-butanol:acetic acid:water (6:1:2) in the 1st direction and 2% aqueous acetic acid in the 2nd direction. The chromatograms were developed with vanillin-HCl (5% vanillin (w/v) in 12 N HC1). (a) crude sapota extract, (b) Sephadex G-50 fraction and (c) Sephadex LH-20 fraction.

Two-dimensional chromatography: The purified PA was subjected to 2-D chromatography on Whatman no. 1 filter paper. The PA was located using 5% vanillin in HC1. The purified PA appeared as a single orange red spot at the origin (Fig 7.5c), indicating the absence of any phenolic contaminants, which were present in the crude extract (Fig 7.5a).

HPLC gel filtration: HPLC gel filtration of the purified PA was carried out using a Zorbax G-50 gel filtration column. Both Sephadex G-50 and the LH-20 fractions eluted as single symmetrical peaks (Fig 7.6, profile of only Sephadex LH-20 fraction shown) indicative of a single species. The approximate M_r of the compound was also estimated on the same column. The standards used for calibration of the column were ribonuclease (13.7 kDa), chymotrypsinogen (25 kDa), ovalbumin (43 kDa) and BSA (66.3 kDa). The apparent M_r of the PA was estimated to be ~3 kDa, from the calibration curve of globular proteins (Fig 7.7).

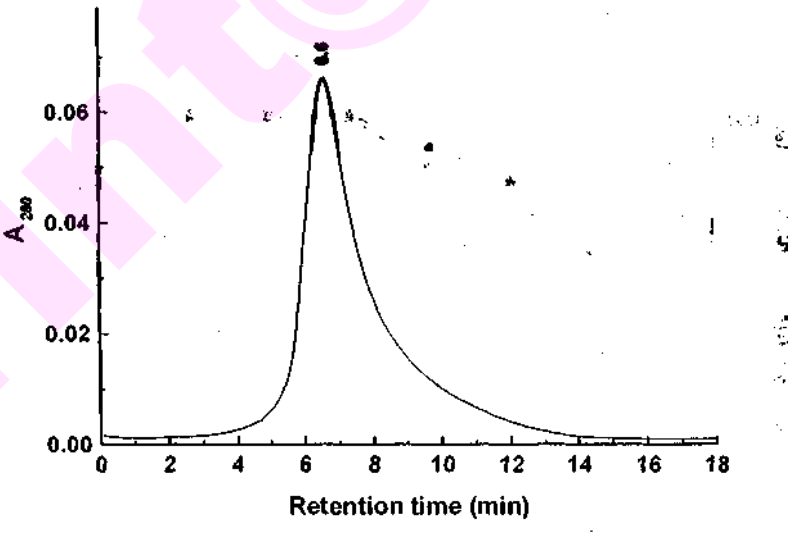


Fig **7.6. HPLC gel filtration profile of purified PA.** Column used was Zorbax GF-250 (250 × 9.4 mm) and eluted with 10 mM NaPi pH 7.0 at a flow rate of 2 mL/min. Detector was set at 280 nm.

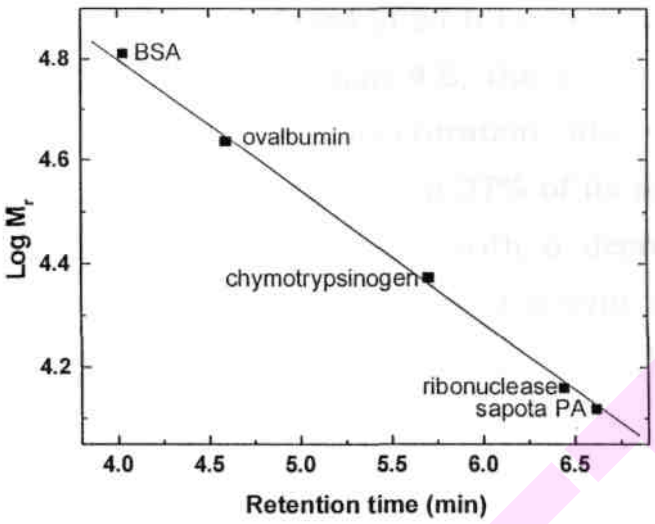


Fig 7.7. **Estimation of M_r of sapota PA.** Fig shows the plot of retention time vs log M_r of standard proteins and the inhibitor.

pH stability of PA

The stability of the inhibitor at different pH was determined by incubating the PA in 100 mM buffers of different pH, glycine-HCl (pH 2.5-3.5), Na acetate (pH 3.5-6.0), NaPi (pH 6.0- 8.0) and Tris-HCl (pH 8.5) for 24 h. The residual inhibitory activity was assayed. The results are presented in Fig 7.8.

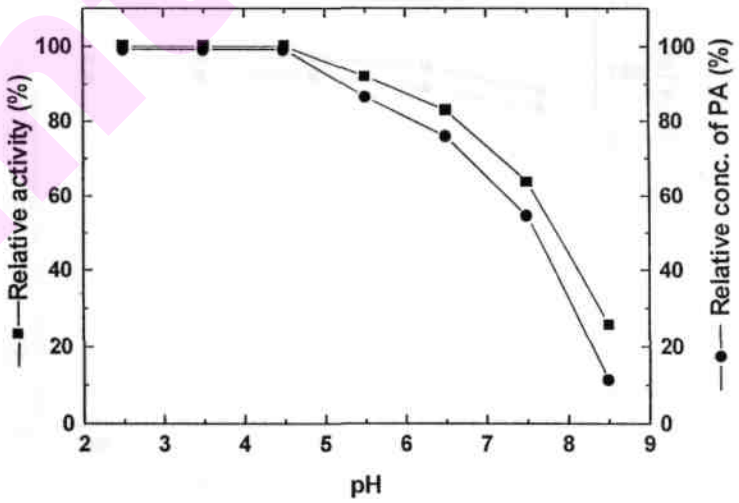
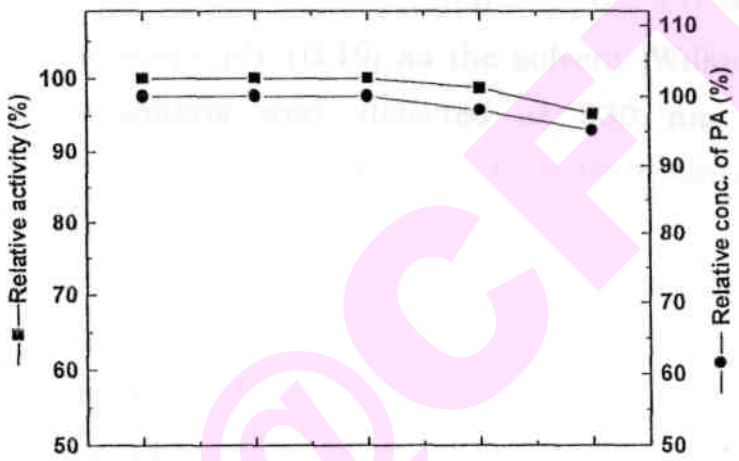


Fig 7.8. **Effect of pH on the stability of PA.** The PA was incubated in different pH buffers, glycine-HCl (pH 2.5-3.5), Na acetate (pH 3.5-6.0), NaPi (pH 6.0-8.0) and Tris-HCl (8.5) for 24 h at 25 °C. The residual inhibitory activity and PA content were determined.

The inhibitor was stable over a period of 24 h between pH 2.5-4.5, with no loss in activity. At pH greater than 4.5, the activity was found to decrease progressively. The PA concentration also was found to decrease concomitantly. At pH 6.5, PA lost 37% of its activity, whereas at pH 8.5, 75% of the activity was lost with a decrease in the PA content. These results suggest that the loss of activity is probably due to degradation of the PA.



Temperature stability of PA

An aqueous solution of the purified PA was incubated in a water bath at temperatures ranging from 25-100 °C preset to the appropriate temperatures. Aliquots removed at 15 min, 30 min and 1 h intervals were checked for the residual inhibitory activity. Fig 7.9 shows the relative inhibitory activity as well as the PA content after 1 h incubation. The inhibitor was found to be extremely heat stable. Even after 1 h at 100 °C, the inhibitor retained >95% of the activity (Fig 7.9). There was no significant decrease in the PA content, indicating it to be intact.

Fig 7.9. **Effect of temperature on the stability of PA.** The purified PA was incubated at different temperatures for 1 h and the residual inhibitory activity and PA content assayed.

Identification of monomers of PA

The colorless polymeric PA when treated with hot mineral acid are hydrolyzed to the corresponding monomeric anthocyanidins, which are colored compounds. The purified PA was hydrolyzed as described earlier (section 2.2.29). The hydrolyzed sample was subjected to ascending chromatography on Whatman no. 3 filter paper in Forestal solvent (water:acetic acidxonc. HC1 (10:30:3 v/v), Bate-Smith, 1954), to separate the different anthocyanidins. The anthocyanidins were identified by their color and relative mobility. Ascending chromatography of the purified, acid hydrolyzed samples gave three separate spots with R_f values of 0.34, 0.58 and 0.66 (Fig 7.10). The R_f values of these three spots were found to correspond to the anthocyanidins, delphinidin, cyanidin and pelargonidin respectively when compared to the Rf values of the same compounds obtained by Bate-Smith (1954) and Lakshminarayana and Mathew (1967).

The monomeric anthocyanidins were also identified by RP-HPLC. RP-HPLC of the hydrolyzed condensed tannins was carried out on a Phenomenex Cis column (250 x 4.6 mm) using water:acetic acid: methanol (71:10:19) as the solvent (Wilkinson et al., 1977). The anthocyanidins were detected at 530 nm and since standards were not available, were identified from the retention times of the same compounds obtained by Wilkinson et al. (1977). Three well resolved anthocyanidins were detected in the chromatogram (Fig 7.11). The major anthocyanidin was delphinidin (61%). The structure of anthocyanidins is presented in Fig 7.12. Delphinidin (Fig 7.12b) with two hydroxyl groups is the most hydrophilic of the three and therefore elutes first followed by cyanidin which accounts for 34% of the anthocyanidin. Pelargonidin was found to an extent of 5%.

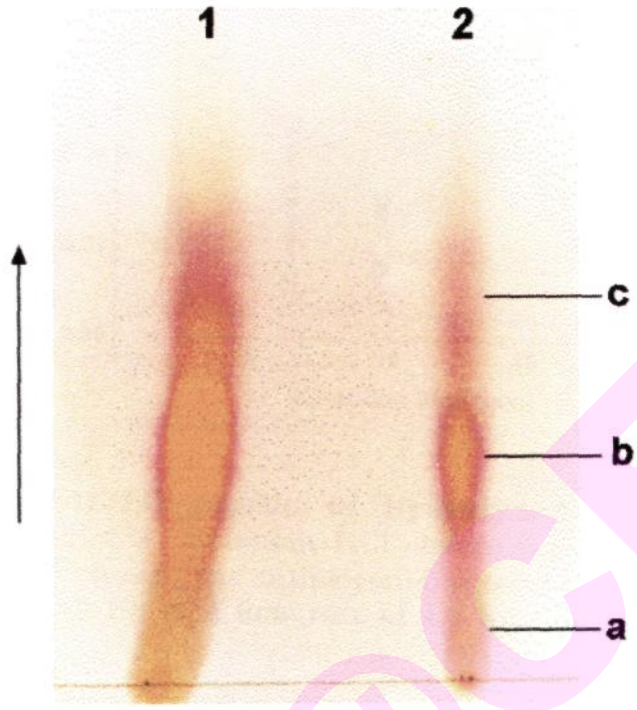


Fig 7.10. **Chromatogram of the hydrolyzed anthocyanidins.** The sapota PA (1) 4 ng and (2) 2 ^g, was hydrolyzed in 5% butanolic-HCl and subjected to ascending chromatography on Whatman no. 3 filter paper in Forestal solvent (watenacetic acidrconc. HC1, 10:30:3 v/v) R_f a= 0.34, b= 0.58, c= 0.66.

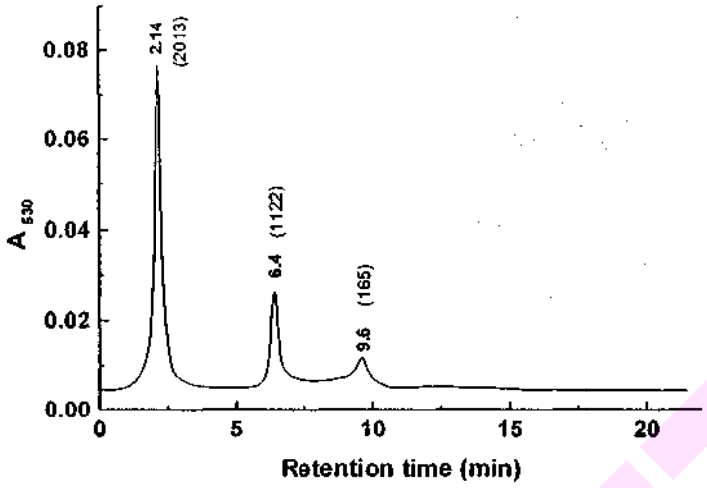
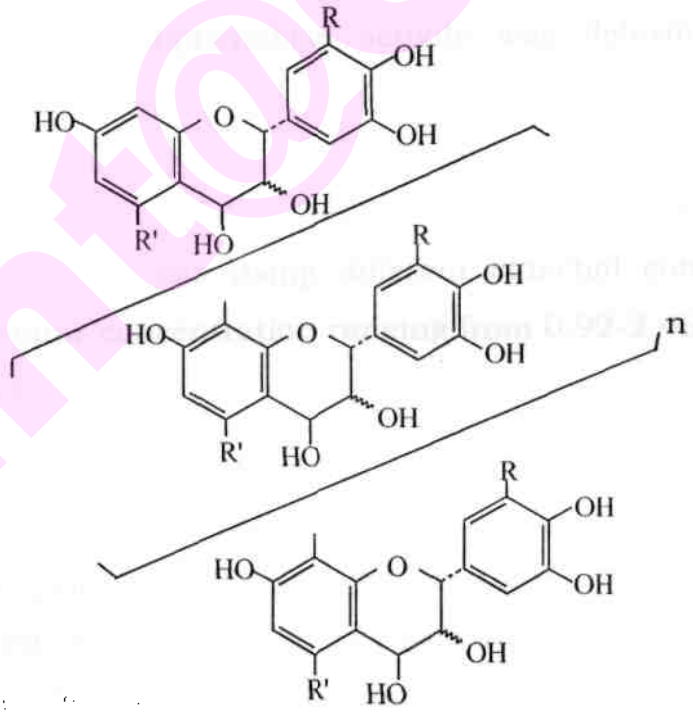
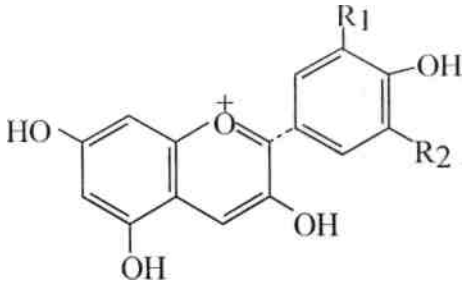


Fig 7.11. **RP-HPLC profile of hydrolyzed anthocyanidins.** Elution profile of the 5% butanolic-HCl hydrolyzate on a Phenomenex Cis (250x4.6 mm) column. The anthocyanidins were eluted with water: acetic acid: methanol (71:10:19) at a flow rate of 1.5 mL/min. Peak areas are shown in parenthesis.



R/R'= H/OH

Fig 7.12a. **General structure of PA.**



Ri=R_2=OH Delphinidin

Ri=OH, R_2=H Cyanidin

Ri=R_2=H Pelargonidin

Fig 7.12b. **Structure of anthocyanidin monomer.**

Inhibitory properties of PA

Purified field bean PPO (Table 3.2, Chapter3) was used to study the inhibitory effect of sapota PA. The purified PA was found to inhibit the diphenolase as well as monophenolase activity of the field bean PPO. The inhibition of diphenolase activity was determined using catechol as the substrate at PA concentrations ranging from 0.92-2.46xlO^8 M (concentrations of the inhibitor were calculated based on the Mr of 13 kDa determined by HPLC). The Michaelis-Menten plot of the initial reaction velocities using different catechol concentrations (5-50 mM) at inhibitor concentration ranging from 0.92-2.46 x 10~^{8} M is plotted in Fig 7.13.

In the presence of the inhibitor, the reaction velocity was markedly reduced at all the substrate concentrations studied. Lineweaver-Burk plots of 1/V vs 1/[S] at three PA concentrations showed an increase in the slope and 1/V axis intercept pivoting counter clock-wise, about the point of intersection with the control curve (Fig 7.14).

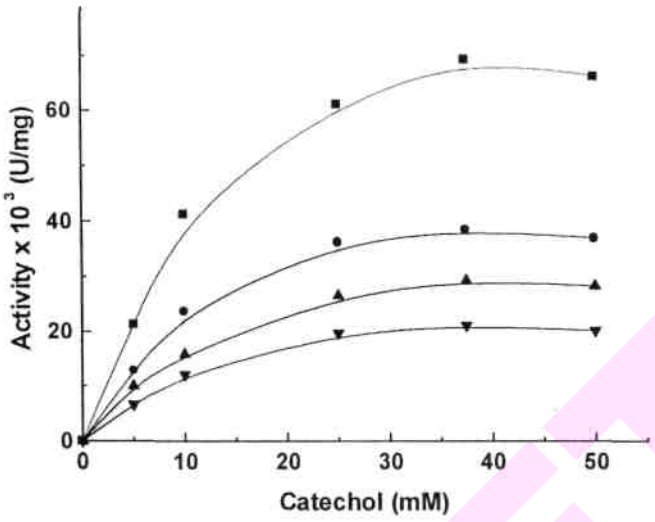


Fig 7.13. **Michaelis-Menten plot showing the effect of catechol concentration on field bean PPO activity in the presence of various PA concentrations.** Reaction was carried out in 50 mM Na acetate buffer, pH 4.0 with catechol at indicated concentrations and with inhibitor at (■) 0 M, (•) 0.92 x 10-s M, (A) 1.84 x 10"^{8} M and (r) 2.46 x 10~^{8} M

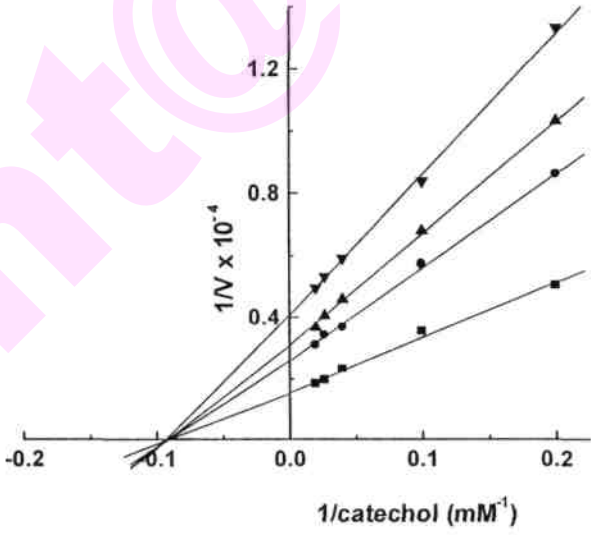


Fig 7.14. **Double reciprocal plot of field bean PPO activity showing the effect of substrate concentration on initial velocity in the presence of various PA concentrations.** Data from Fig 7.13. Assay in the absence (■) and presence of inhibitor (•) 0.92 x 10-^{8} M, **(A)** 1.84 x 10-^{8} M and **(▼)** 2.46 x 10-^{8} M.

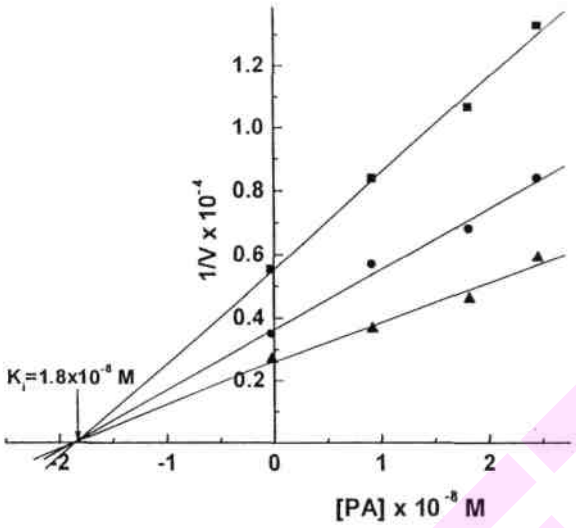


Fig 7.15. **Dixon plot for determining the inhibition constant of the PA with field bean PPO.** Data from Fig 7.13. Catechol (■) 5 mM, (•) 10 mM and (A) 25 mM.

This is representative of pure non-competitive inhibition. The inhibition constant Ki for PA was deduced from a Dixon plot of the same data (Fig 7.15). PA was a potent non-competitive inhibitor with an apparent Ki of 1.8xl0~^{8} M, which is 3 times lower than the Ki of tropolone, the most potent of the inhibitors examined (Chapter 3, Table 3.6).

Inhibition of monophenolase activity of the field bean was studied using ferulic acid as the substrate in the presence of catechol activator. Purified field bean PPO does not act on monophenols. However, in the presence of catalytic quantities of a diphenol like catechol, ferulic acid, a monophenol is oxidized to its corresponding quinone (Chapter 5, Table 3.5). The effect of PA on this monophenolase activity was studied. The induced monophenolase activity in the presence of PA exhibits a characteristic lag phase. This lag phase increased as the PA concentration increased (Fig 7.16). A similar increase in the lag phase with increasing concentrations of the inhibitor was observed for field bean PPO with ascorbic acid, potassium metabisulfite and cysteine-HCl (Chapter 3, Fig 3.17).

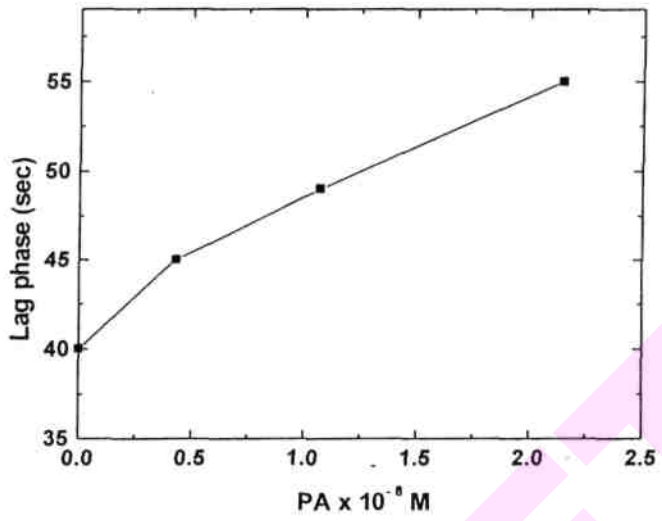


Fig 7.16. **Effect of PA concentration on the lag phase of monophenolase activity.** Assay medium contained 50 mM Na acetate (pH 4.5), ferulic acid 0.2 mM, catechol 10 mM and 40 µg of field bean PPO.

The effect of PA on the initial velocity of ferulic acid oxidation presented in Fig 7.17 show that PA inhibit the reaction. V vs [S] curve shows that as the concentration of the PA increases, the degree of the inhibition increases. However, even at saturating ferulic acid concentration, the $V_{ma}\chi$ obtained is less than the minus inhibitor curve suggesting that the inhibitor affects only the oxidation rate. Double reciprocal plots (Fig 7.18) of these data show a characteristic non-competitive type of inhibition.

The inhibition constant of PA for monophenolase activity was deduced from the Dixon plot (Fig 7.19). The Ki for this activity is 1.45×10^{8} M. The binding constants for both the activities are similar indicating that both the monophenolase and diphenolase activities are probably carried out at a single site on the enzyme.

Extracts of the ripe sapota fruit did not exhibit the inhibition of catechol oxidation by field bean PPO (Table 7.3). In addition, this ripe extract was devoid of PA, estimated by the butanolic-HCl test. In contrast, the ripe fruit exhibited PPO activity.

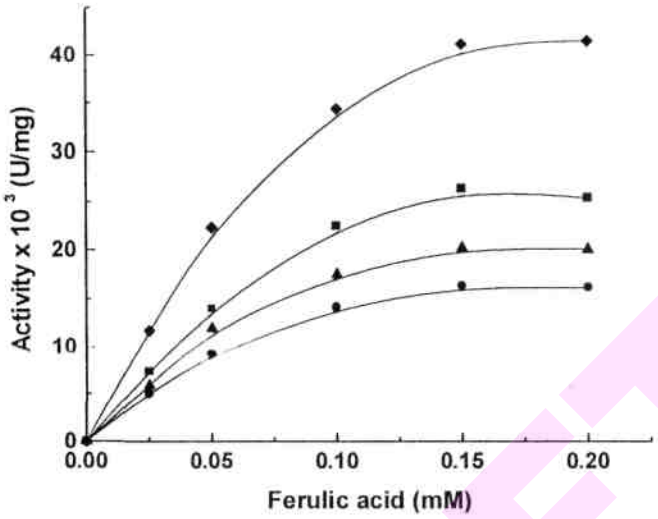


Fig 7.17. **Michaelis-Menten plot showing the effect of ferulic acid concentration on the initial velocity of field bean PPO (monophenolase) in the presence of varying PA concentration.**
Reaction was carried out in 50 r∩M Na acetate buffer, pH 4.5 in the presence of 10 mM catechol. PA (♦) 0 M, (■) 0.43 × 10-8 M, (A) 1.07 × 10-8 M and (•) 2.15 × 10-8 M.

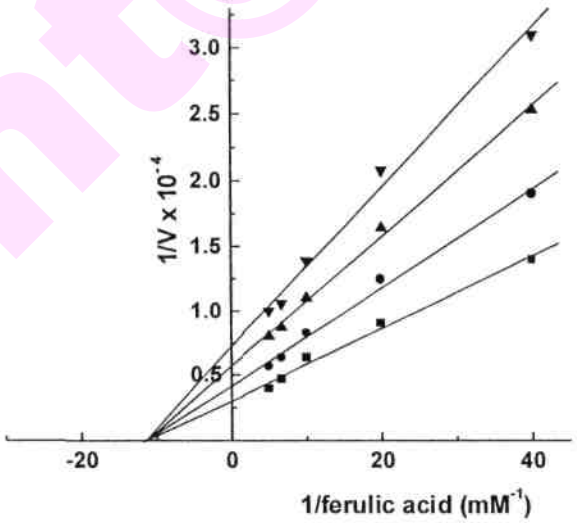


Fig 7.18. **Double reciprocal plot of data of Fig 7.17.** Ferulic acid at the indicated concentrations and PA at (■) 0 M, (•) 0.43 × l(H M, (A) 1.07 × 10^{-8} M and(τ) **2.15** × l(H M.

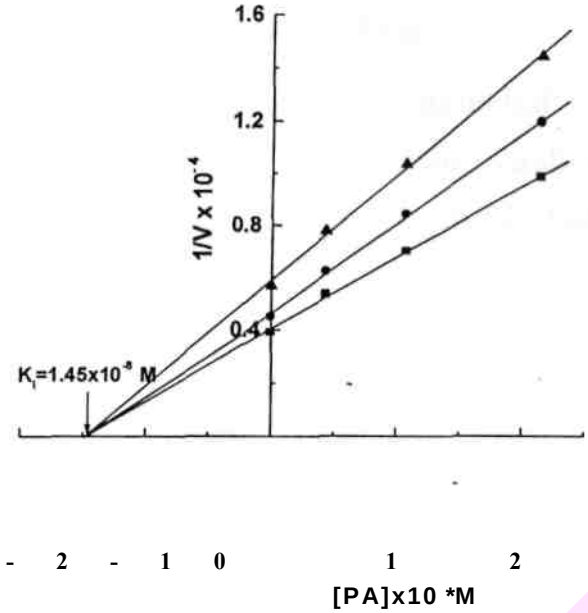


Fig 7.19. **Dixon plot for determining the inhibition constant of PA.**
Data from Fig 7.17, ferulic acid (A), 0.025 mM, (•) 0.05 mM and **(■)** 0.1 mM.

DISCUSSION

PPO is found in all fruits and vegetables (Vamos-Vigyazo, 1981). The PPO activity in potatoes, apples, mushrooms, bananas, peaches, lettuce, fruit juices and wines is high, as these foods are sensitive to oxidative browning. Browning is exacerbated by tissue damage caused by food processing techniques *viz.* cutting, peeling, comminuting, pureeing, pitting, pulping or freezing. Antibrowning agents are compounds that act primarily on PPO or the intermediates of pigment formation. Many reagents inhibit PPO activity (Chapter 3) and studies with them have provided valuable insights into the mode of action of this enzyme (Luo and Barbosa-Canovas, 1995; Osuga and Whitaker, 1995). However, only a limited number of PPO inhibitors are considered acceptable on grounds of safety, effects on the sensory attributes and/or expense for the use to control browning during food processing. Because of recent changes in scientific knowledge and life style and an awareness of the relationship between food and health, the consumers'

demand for more natural foods and safer, fewer added chemicals in processed food products has been increasing.

Several PPO inhibitors have been detected or isolated from natural sources including honey, pineapple, cashew nut and figs (Walker, 1995). 4-Hexyl resorcinol isolated from figs has now been given GRAS status and is used to inhibit shrimp melanosis (Iyengar and McEvily, 1992). Oszmianski and Lee (1990) found a polypeptide noncompetitive PPO inhibitor in honey. Tan and Kubo (1990) reported that the roots of *Zea mays* secreted a 6.3 kDa protein which inhibited catechin oxidase activity. Screening of plant extracts has led to the identification of pseudostellarins, a group of cyclopeptides, which inhibit PPO (Morita et al, 1994).

Prabha and Patwardhan, (1986b) suggest the presence of endogenous inhibitors in unripe sapota *(Achras zapota)* which disappear on ripening. From their preliminary investigation, they concluded that the high M_r inhibitor of PPO present in raw sapota extract was a protein and was not investigated further. Interest in proteins as novel enzyme inhibitor led to this detailed study. The inhibitor was non-dialyzable (Table 7.1) confirming the high M_r nature of the inhibitor. However, TCA precipitation and protease digestion indicate that the inhibitor is non-proteinaceous (Table 7.2). The inhibitor, by several specific tests, was identified to belong to a group of compounds called PA, also known as leucoanthocyanidins or condensed tannins. Two groups of tannins occur in plants, the "hydrolyzable tannins" and the "condensed tannins", which are the polymers derived from various flavonoids. Fruit bearing plants are a rich source of oligomeric condensed tannins (Haslam, 1982).

The butanolic-HCl hydrolysis, 2-D chromatography and the ability to precipitate proteins confirmed that the high M_r compound was

a PA. The observation that acid depolymerization of PAs in heated alcohol yielded anthocyanidin pigments with an absorption maximum at 550 nm, led Swain and Hillis (1959) to develop a spectrophotometric assay for PA. The hydrolyzed product of sapota PA exhibited a UV-Vis absorption spectrum with a maximum at 550 nm (Fig 7.1 and 7.3). Anthocyanidins produced are not stoichiometrical to the original PA, but after 2 h approaches a steady state value.

The 70% aqueous acetone phase which contained all the PA, also showed inhibitory activity of >95% when compared to the aqueous phase (Table 7.3). Aqueous acetone (usually 70%) has been regarded as the most efficient solvent for extracting PA from plant material (Dalzell and Kerven, 1998; Terrill et al, 1990; Cork and Krockenberg, 1991; Jackson et al, 1996). Tannins are considered to be phenolic compounds of sufficiently high M_r (>500 Da), capable of forming reasonably strong complexes with proteins and other polymers under suitable conditions of concentration and pH (Goldstein and Swain, 1963; Somers, 1967). The high M_r PA isolated from sapota, precipitated albumin. Porter and Woodruff (1984) observed that polymeric PAs of sufficiently high average M_r of 2500 Da or more are efficient in precipitating hemoglobin.

Sapota PA was effectively purified by two steps of column chromatography, consisting of gel filtration on Sephadex G-50 and adsorption chromatography on Sephadex LH-20. Gel filtration on Sephadex G-25 has been successfully used for the resolution of phenolic constituents from grape pigment (Somers, 1967) and Sephadex G-50 for condensed tannins of pasture legume species (Jones et al, 1976). Sephadex LH-20 chromatography has been used to fractionate condensed tannins from temperate forages (McNabb et al, 1998) and procyanidins from apple pomace (Foo and Lu, 1998). The first Sephadex G-50 chromatography was effective in separating most of the impurities

(Fig 7.5b). Further purification on Sephadex LH-20 however did not increase the specific activity of the inhibitor. The fractions obtained after Sephadex G-50 as well as Sephadex LH-20 chromatography were homogenous (Fig 7.5b 8s 7.5c) suggesting that the additional chromatographic step was futile.

The Sephadex G-50 and Sephadex LH-20 gave single spots at the origin of the 2-D chromatogram indicating the lack of any mobile organic contaminants as seen in the crude (Fig 7.5a). The purity of the PA fractions was also determined by HPLC gel filtration on a Zorbax G-50 column where a single symmetric peak was observed. The apparent M_r determined using standard globular proteins was -13 kDa. The M_r of 13 kDa has been attributed, assuming that the shape of the compound is globular. The M_r of PAs varied from 5.8-28 kDa when the Mr distribution in some legume pasture species was studied (Jones et al, 1976). PA from cocoa has a fairly low M_r (1.2-1.8 kDa, Quesnel, 1968) whereas those from sainfoin are of high M_r (17-28 kDa, Bate-Smith, 1973).

The only means of detecting PAs in plants is their ability to yield anthocyanidins on heating in acid solution. Only oligomers and polymers of anthocyanidins are regarded as tannins. Ascending chromatography of hydrolyzed PA, in Forestal solvent is used to identify individual anthocyanidins in the polymer (Bate-Smith, 1954; Lakshminarayana and Mathew, 1967). Three distinct spots, colored orange, bright pink and faint pink were identified to be delphinidin, cyanidin and pelargonidin (Fig 7.10), respectively, based on the findings of Lakshminarayana and Mathew (1967). The corresponding Rf values obtained, 0.34, 0.58 and 0.66 was less than that obtained by Lakshminarayana and Mathew (1967), but slightly higher than that obtained by Bate-Smith (1954).

Raw sapota extract was found to contain large amounts of PAs (Lakshminarayana and Mathew, 1967), especially at the early stages of development, which reduced during maturation and ripening. In a systematic estimation of the polyphenolic content of raw sapota fruit, Lakshminarayana and Mathew (1967) had established that the condensed tannin in sapota mainly comprises of delphinidin, cyanidin and pelargonidin. The three monomeric anthocyanidins formed after hydrolysis was confirmed by RP-HPLC. Comparing the peak areas, delphinidin was the highest in concentration (61%) and pelargonidin the least (5%), which is similar to the result obtained by Lakshminarayana and Mathew (1967).

The predominance of leucodelphinidin and leucocyanidin in sapota polyphenols is in agreement with the general pattern of PA distribution in nature although between the two, leucocyanidin is more abundant in nature. However, in fruits, it has been noticed that leucodelphinidin is the more common contributor for astringency (Joslyn and Goldstein, 1964).

The sapota PA was unstable at alkaline pH, whereas it was highy stable at pH ≤4.5. The sapota PA was also found to be extremely heat stable like the Bowman-Birk inhibitors of trypsin and chymotrypsin (Godbole et al, 1994; Mulimani and Paramjyothi, 1994). McNabb et al. (1998) observed that inhibition of trypsin and chymotrypsin by lotus PA was affected by pH wherein at pH 6.0, the inhibition was greater than pH 7.0, but completely lost at pH 8.0.

Sapota PA was found to inhibit both the monophenolase and diphenolase activities of field bean PPO. The inhibition kinetics analysed by the Line weaver-Burk plots (Fig 7.15 and 7.19) indicate the sapota PA to be a potent non-competitive inhibitor, which means that the inhibitor binds to a site, other than the active site of the enzyme.

The Ki for the sapota PA, 1.8 x 10"^{8}M for diphenolase and 1.45 x 10'^{8} M for monophenolase is lower than the Ki observed for many of the common inhibitors used (Vamos-Vigyazo, 1981). The phenolic inhibitors studied by Walker and Wilson (1975) had Kis ranging from 0.06-3.3 x 10-^{3} M. Since the sapota PA is effective at such low concentrations, it should serve as an effective inhibitor of enzymatic browning which occurs during processing of fresh fruits and vegetables. Although sapota PA is astringent, the very low Ki suggests that it can be used as the inhibitor, at concentrations much lower than that at which it precipitates protein. Kaempferol, a flavonol from saffron flower was found to inhibit only the catecholase activity of mushroom tyrosinase in a competitive manner (Kubo and Kinst Hori, 1999) with a Ki of 0.23x10^{-3} M. Anisaldehyde, a phenolic compound isolated from anise oil, exhibited a non-competitive type of inhibition towards L-DOPA oxidation of mushroom tyrosinase whereas it did not inhibit the monophenolase activity (Kubo and Kinst-Hori, 1998).

The ripe sapota fruit extract neither exhibits an inhibition on field bean PPO nor precipitates albumin. The loss of inhibitory activity may be attributed to the absence of PA in the mature fruit (Table 7.3). Lakshminarayana and Mathew (1967) also observed the disappearance of PA on maturation and ripening of the sapota fruit. Loss of astringency is a major change that occurs during the ripening of many edible fruits (Guadagni and Nimmo, 1953). It is generally agreed that the astringent property is due to the presence of tannins, and many of the astringent fruits show a reduction in tannin and astringency on ripening (Barnell and Barnell, 1945).

In conclusion, the PA of sapota is a potent non-competitive inhibitor of PPO, the enzyme responsible for the browning effects on fruit and vegetable during post harvest, storage and processing. Since

the PA is an inhibitor of both monophenolase and diphenolase activities of field bean PPO and is from a natural source, it should serve as a useful antibrowning agent to the food processing industry.

Chapter 8
Summary and Conclusions

Enzymatic browning, with few exceptions (prunes, black raisins, dates, tea, coffee, cocoa etc.) is considered as product degradation which lowers fruit quality both visually and with regard to taste and nutritional characteristics. The main enzyme involved in this process is polyphenol oxidase (PPO), which uses molecular oxygen to catalyze the o-hydroxylation of monophenols to o-diphenols (monophenolase activity) and their further oxidation to coloured and highly reactive o-quinones (diphenolase activity). The prevention or inhibition of enzymatic browning is a major concern of the food industry all over the world. Understanding the details of the browning process and insights into molecular and structural aspects of this intriguing enzyme is a pre-requisite to evolve effective preventive measures for enzymatic browning.

The present investigation entitled **"Purification and structural characterization of a polyphenol oxidase from field bean *(Dolichos lablab)"*** has been undertaken to purify and characterize the single isoform of PPO present in field bean. This investigation also includes the isolation and characterization of a potent natural inhibitor of field bean PPO. The following are the salient features of the present investigation.

- The PPO of field bean seeds did not require the presence of detergents in the extraction buffer, implying that the enzyme is soluble rather than membrane bound.
- The single isoform of PPO from field bean was purified by buffer extraction, ammonium sulfate precipitation, DEAE-Sephacel anion exchange chromatography and hydrophobic interaction chromatography on Phenyl Agarose followed by gel filtration on SephadexG-100.

- The field bean PPO was purified with a yield of 34%. The specific activity 68,077 U/mg of the purified enzyme was 31 fold higher than that of the crude extract (Table 3.2).

- The homogeneity of PPO was determined by native PAGE (Figs 3.4 8& 3.5), capillary electrophoresis (Fig 3.6) at acid and alkaline pH and by HPLC analytical gel filtration chromatography (Fig 3.9a). The homogeneity was also confirmed by the release of a single amino-terminal amino acid (Fig 3.7a).

- The M_r of the field bean PPO as revealed by analytical gel filtration on HPLC and gel filtration on Sephadex G-100 was 120±3 kDa (Figs 3.9b 85 3.10). Field bean PPO is a homotetramer of subunit M_r 30±1.5 kDa as determined by SDS PAGE (Fig 3.11) and release of the same amino-terminal amino acid sequence by the native and denatured PPO (Fig 3.7a & b).

- The optimum pH of the purified enzyme was determined to be pH 4.0 using catechol and 4-methyl catechol as the substrates and pH 5.0 using L-DOPA as the substrate (Fig 3.12). The enzyme was stable in the pH range 6-8 (Fig 3.13).

- The optimum temperature for field bean PPO activity was 25-35 °C (Fig 3.14).

- Field bean PPO has a compact substrate-binding site as it utilized only small di- and tri-phenols as substrates and was unreactive towards substrates with bulky substituents. 4-Methyl catechol exhibited the highest V_max whereas L-DOPA had the highest affinity for the enzyme (Table 3.5).

- High substrate concentration inhibited field bean PPO activity. PPO shows no activity towards the monophenols, p-cresol, L-tyrosine, ferulic acid and p-coumaric acid (Table 3.5).

- Tropolone, ascorbic acid, cysteine-HCl and potassium metabisulfite are competitive inhibitors of field bean PPO, of which tropolone is the most potent with a Ki of 5.8 x lO^7 M (Table 3.6).

- Polyclonal antibodies developed against pure field bean PPO cross-reacted with PPO isolated from several other legumes, fruits and vegetables (Figs 3.19 & 3.20) indicating the presence of common antigenic determinants.

- Caffeic acid, chlorogenic acid and catechin which are common natural substrates for PPO were found to be potent competitive inhibitors of catechol oxidation catalyzed by field bean PPO (Figs 4.1, 4.4, 4.5 & Table 4.1).

- Benzoic acid is the most potent inhibitor among a group of substituted aromatic carboxylic acids studied for their inhibitory effect on field bean PPO (Table 4.2), with ring substitutions of benzoic acid decreasing the inhibitory effect.

- At low concentrations of the substrate catechol (<35±2.5 mM), gallic acid inhibits field bean PPO. However, at high substrate concentrations where there is inhibition by excess substrate, gallic acid activates field bean PPO, by abolishing the substrate inhibition (Fig 4.7).

- Monophenols, ferulic acid and tyrosine, which are not utilized as substrates by field bean PPO, can be oxidized in the presence of catalytic quantities of substrate o-diphenols (Fig 5.1).

- This monophenolase activity is characterized by a lag phase, which is dependent on substrate and co-substrate (o-diphenol) concentration (Fig 5.4).
- The activation constant K_a of catechol for monophenolase activation is lower than the K_m (10.5 mM) of catechol as substrate (Table 5.1)
- Diphenols, caffeic acid and catechin, which are otherwise inhibitors of field bean PPO (Table 4.1), are oxidized immediately by the enzyme in the presence of catalytic amounts of o-diphenols (Fig 5.13) with no lag phase.
- o-Diphenols serve as co-substrates to convert the $E_{mc}t$ (resting form) to Eoxy form that binds monophenols.
- Though the activation constant K_a of catechol for both monophenol and diphenol activation is similar, the mechanism of activation is different.
- Field bean PPO can be activated by exposure to acid pH and by the anionic detergent SDS (Table 6.1).
- The activated forms of PPO exhibit an optimum pH of 6.0, higher than the native form (Fig 6.2). This activation by exposure to acidic pH is reversible (Fig 6.1).
- The Vmox of the activated enzyme is -3.5 times higher than that of the native enzyme, whereas the substrate affinity remains unaltered (Table 6.1).
- The activated forms of PPO are more susceptible to inhibition by tropolone (Table 6.1).
- The Stokes radius of the PPO increases upon activation by either exposure to acid pH or SDS (Fig 6.11).

- Intrinsic fluorescence measurements of the activated forms decrease, indicating conformational changes in the activated form (Fig 6.13).
- The high M_r PPO inhibitor isolated from unripe sapota extract, with absorbance maxima at 220 and 280 nm is not a protein, but identified to be a PA.
- The PA inhibitor was purified by buffer extraction followed by gel filtration chromatography on Sephadex G-50 and adsorption chromatography on Sephadex LH-20.
- The Mr of the PA inhibitor was estimated to be -13 kDa from analytical HPLC gel filtration (Fig 7.7).
- The inhibitor was highly heat stable (Fig 7.9) whereas it was not stable at alkaline pH (Fig 7.8).
- The monomeric anthocyanidin components of sapota PA were identified after acid hydrolysis. Delphinidin was the major anthocyanidin followed by cyanidin and pelargonidin (Fig 7.11).
- Sapota PA inhibits both the monophenolase and diphenolase activity of field bean PPO non-competitively (Figs 7.14 & 7.18) with a Ki of 1.45 x 10^{-8} M and 1.8 x 10^{-8} M respectively and therefore can be used as an antibrowning agent in the food processing industry.

The results presented in the thesis describe the purification of PPO from field bean *(Dolichos lablab)*. The study characterizes the molecular and kinetic properties as well as the induced activation of the enzyme. The results are discussed with respect to the structure and function of PPO in plants. The isolation and characterization of a potent natural inhibitor of field bean PPO, which can be used as an alternate antibrowning agent is presented.

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