Molecular characterization and expression of an oxidase from field bean (Dolichos lablab)

A Thesis Submitted to the
Department of Biochemistry
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degree of Doctor of Philosophy

by

Santosh R. Kanade

Under the supervision of

Dr. Lalitha R. Gowda

Department of Protein Chemistry and Technology
Central Food Technological Research Institute
Mysore –570 020, India

July, 2007

DECLARATION

I hereby declare that this thesis entitled "**Molecular characterization and expression of an 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 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 of March, 2002 - July, 2007.

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

Mysore
July, 2007. **SANTOSH KANADE**

Guide

Head of the Department

Dr. Lalitha R. Gowda
Department of Protein Chemistry and Technology

CERTIFICATE

I hereby certify that this thesis entitled **"Molecular characterization and expression of an oxidase from field bean (*Dolichos lablab*),"** submitted by **Mr. Santosh Kanade** to the University of Mysore, Mysore, for the degree of **Doctor of Philosophy in Biochemistry** is the result of research work carried out by him 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.

Mysore
Date:

Dr.Lalitha R. Gowda

……to my beloved parents

brothers and sisters

ABSTRACT

Polyphenol oxidases (PPO) are type III enzymes with a dinuclear copper centre which initiates enzymatic browning. PPOs are mixed function oxidases which catalyze both the hydroxylation of monophenols to diphenols (monophenolase/cresolase) and also oxidation of *o*-diphenols to *o*-quinones. The PPO has been purified to apparent homogeneity. The purified enzyme had a specific activity of 35553 U/mg with a yield of 20 %.The PPO was found to be a hetero dimer of 29000 Da and 31000 Da with a native molecular mass of 120000 Da. The amino-terminal sequence of the subunits are identical. The kinetic studies revealed that tertiary butyl catechol is the best substrate followed by 4-methyl catechol and catechol. The purified protein was a glycoprotein, the neutral sugar composition being 8.0 % and pI of 9.3. Both the subunits of PPO cross react with PPO antibodies. The spatial and temporal expression of PPO during germination and seed development were evaluated.

A series of phenolic compounds experimentally evaluated for their binding affinity and inhibition constants were computationally docked to the active site of catechol oxidase. Analyses of the complexes provide structural explanations for correlating subtle changes in the position and nature of the substitutions on diphenols to their functional properties as substrates and inhibitors. Higher reaction rates and binding are reckoned by additional interactions of the substrates with key residues that line the hydrophobic cavity. The docking results suggest that inhibition of oxidation stems from an interaction between the aromatic carboxylic acid group and the apical His^{109} one of the four co-ordinates of the trigonal pyramidal co-ordination polyhedron of CuA. The spatial orientation of the hydroxyl in relation to the carboxylic group either allows a perfect fit in the substrate cavity leading to inhibition or due to a steric clash flips the molecule vertically facilitating oxidation. This is the first study, which explains at the molecular level the determinants of substrate/inhibitor specificity of a catechol oxidase.

The enzyme is activated manifold either in the presence of the anionic detergent SDS below its critical micellar concentration or on exposure to acid pH. The activation is accompanied by a marked shift in the pH optimum enhanced k_{cat}, an increased sensitivity to the competitive inhibitor tropolone, altered susceptibility to proteolytic degradation and decreased thermal

stability. The activation is characterized by a unique and large increase in the Stokes radius. The activation is due to a localized conformational change that is anchored around the active site. The BLAST search of internal peptide sequences indicated a high homology (>90%) to the galactose specific lectins of legumes. The cDNA sequence of 786 bp was obtained using degenerate primers corresponding to the amino-terminal sequence and internal peptide sequence and submitted to Gene Bank (Accession No.EF204527) This sequence is highly homologous to galactose specific legume lectins. Consequently the protein was purified by two independent methods and multifunctional property was characterized.

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Mysore ***Santosh Kanade***
July, 2007

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LIST OF ABBREVIATIONS

2,3,4-THB	2, 3, 4-Trihydroxybenzaldehyde
2,3,4-THBA	2, 3, 4-Trihydroxybenzoic acid
2, 3-DHBA	2, 3-Dihydroxy benzoic acid
2, 4-DHBA	2, 4-Dihydroxy benzoic acid
2, 5-DHB	2, 5-Dihydroxy benzoic acid
MOPS	3-(N-morpholino)propanesulfonic acid
3, 4, 5-THB	3, 4, 5-Trihydroxybenzalldehyde
3, 4, 5-THBA	3, 4, 5-Trihydroxybenzoic acid
DOPAC	3, 4-Dihydroxy phenyl acetic acid
3, 4-DHB	3, 4-Dihydroxybenzaldehyde
3, 4-DHBA	3, 4-Dihydroxybenzoic acid
CAPS	3-[Cyclohexylamino]-1-propanesulfonic acid
MBTH	3-Methyl benzothizolinone
4-MC	4-methylcatechol
BCIP	5-Bromo-4-chloro-3-indolylphosphate
AU	Absorbance unit
E_a	Activation energy
APS	Ammonium persulfate
Å	Angstrom unit
ATZ	Anilinothiazolinone
BA	Benzoic acid
K_B	Boltzmann constant
BSA	Bovine serum albumin
CCl_4	Carbon tetrachloride
CAT	Catechol
cm	Centimeter
CD	Circular dichroism
cDNA	Complementary DNA
CBB	Coomassie brilliant blue
Cu	Copper
Da	Daltons
DAF	Days after flowering

°C	Degree centigrade
dNTPs	Deoxynuleotide mixture
DNA	Deoxyribonucleic acid
Gal	D-galactose
DEAE	Diethylaminoethyl
DEPC	Diethylpyrocarbonate
DMSO	Dimethylsulfoxide
DTT	DL-Dithiothreitol
DPN	Dopamine
V_e	Elution volume
EtBr	Ethidiumbromide,
EDTA	Ethylene diamine tetra acetic acid
EXAFS	Extended X-ray absorption fine structure
Gal-Nac	Galactosamine
Glc	Glucose
GME	Glycine methyl ester
g	Grams
GuHCl	Guanidine hydrochloride
Hc	Hemocyanin
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
h	Hour
HCl	Hydrochloric acid
[I]	Inhibitor concentration
K_i	Inhibitory constant
id	Internal diameter
ibCO	*Ipomoea batatas* catechol oxidase
pI	Isoelectric point
DOPA	L-3,4-Dihydroxyphenylalanine
L	Liter
LiCl	Lithium Chloride
LB	Luria Broth
$MgCl_2$	Magnesium chloride
Man	Mannose
MALDI-TOF	Matrix Assisted Laser Desorption Ionization-Time of flight

*m*HBA	m-Hydroxybenzoic acid
K_m	Michaelis-Menten constant
µg	Microgram
µL	Microliter
µm	Micrometer
mL	Milliliter
mM	Millimolar
mtorr	Millitorr
min	Minute
M	Molar concentration
ε	Molar extinction coefficient
Mr	Molecular weight
EDAC	N-(3-dimethylaminopropyl) N'-ethylcarbodiimide hydrochloride
TEMED	N,N,N'N'-Tetramethyl 1, 2-diaminoethane
nm	Nanometer
NBT	Nitroblue tetrazolium
Tricine	N-Tris (hydroxymethyl) methyl glycine
NMR	Nuclear magnetic resonance
*o*HBA	*o*-Hydroxybenzoic acid
PAA	Phenylacetic acid
PITC	Phenylisothiocyanate
PPA	Phenylpropionic acid
PTU	Phenylthiourea
*p*HBA	*p*-Hydroxybenzoic acid
3(p-HPP)	*p*-Hydroxyphenyl propionic acid
pmole	Picomole
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PVDF	Polyvinilidine diflouride membrane
RP	Reverse phase
RNA	Ribonucleic acid
NaCl	Sodium chloride
SDS	Sodium dodecyl sulfate
NaOH	Sodium hydroxide

NaPi	Sodium phosphate
R_s	Stokes' radius
[S]	Substrate concentration
TBC	Tertiary butyl catechol
TPCK	Tosyl-phenyalanine chloromethylketone
TCA	Trichloroacetic acid
TEA	Triethylamine
TFA	Trifluoroacetic acid
Tris	Tris (hydroxymethyl) amino methane
TAE	Tris acetate ethylene diamine tetra acetic acid
TE	Tris ethylene diamine tetra acetic acid
TBS	Tris-buffer saline
UV-VIS	Ultraviolet-Visible
vs	Versus
V_o	Void volume
v/v	Volume by volume
λ	Wavelength
w/v	Weight by volume
w/w	Weight by weight

Chapter I

Introduction

OXIDASES

Oxidoreductases are a class of enzymes that catalyze oxidation-reduction reactions. The trivial names for this group are dehydrogenases, oxidases and oxygenases. The systemic name for an enzyme of this group is donor: acceptor-oxidoreductase. Among the six groups of enzymes, oxidoreductase is the most abundant. Oxygenases catalyze the direct incorporation of oxygen into a substrate molecule. Oxygenases are divided into two groups, namely, monooxygenases and dioxygenases. Monooxygenases are an important group of enzymes, which use only one of the two atoms of molecular oxygen during the catalytic reaction and require two substrate molecules to reduce molecular oxygen. The main substrate accepts one of the two atoms and a co-substrate provides hydrogen atoms to reduce the other oxygen atom to water. The reaction catalyzed by monooxygenases is represented below.

Figure 1.1 The general reaction of an oxidoreductase.

AH is the main substrate and BH_2, the co-substrate. Since the main substrate is hydroxylated, monooxygenases are also called hydroxylases. They are also at times called mixed-function oxidases or mixed function oxygenases, to indicate the oxidation of two different substrates simultaneously. The term oxidase is used only in cases where O_2 is the acceptor.

Oxidases and antioxidative enzymes such as superoxide dismutase, catalase and glutathione peroxidases have recently gained widespread importance as phytopharmacuticals that enhance the natural antioxidant defense. These enzymes as cosmetic ingredients help to maintain low levels of oxidants by scavenging intermediate oxygen radicals. These enzymes are used in their highly concentrated or chemically pure form in small quantities yet offer enhanced benefits. All oxidases are metalloproteins.

Copper-containing oxidases catalyze the oxidation of a wide variety of substrates ranging from small molecules such as methane, to large peptides,

with concomitant reduction of oxygen to hydrogen peroxide or water. Initially, all copper containing proteins were classified based on their spectroscopic features, which led to distinguishing of type-1, type-2 and type-3 active sites. Recent developments in crystallographic and spectroscopic techniques has enabled the discovery of other types of copper-containing active sites. A current classification distinguishes seven different types of active sites in the oxidized state of copper containing proteins; they are briefly described below (Koval et al., 2006).

Type-1 active site: The copper proteins with the type-1 active site are commonly known as "blue copper proteins" due to their intense blue color in the oxidized state, which causes strong absorption at 600 nm. The type-1 active site is also found in some multicopper oxidases, such as ascorbate oxidase.

Type-2 active site: The copper proteins containing the type-2 active site are also known as "normal" copper proteins, a name historically based on their EPR features which are similar to common Cu II complexes, containing a N, O chromophore with a tetragonal geometry.

Type-3 active site: This class is represented by three proteins, namely hemocyanin, tyrosinase and catechol oxidase. The active site contains a dicopper core, in which both the copper ions are surrounded by three nitrogen donor atoms from histidine residues. A characteristic feature of the proteins with this active site is their ability to reversibly bind dioxygen at ambient conditions. Hemocyanin is responsible for dioxygen transport in certain mollusks and arthropods, whereas tyrosinase and catechol oxidase utilize it to perform the oxidation of phenolic substrates to quinones. The copper (II) ions in the oxy state of these proteins are strongly antiferromagnetically coupled, leading to an EPR-silent behavior.

Type-4 active site: The copper site in these proteins is usually composed of a type-2 and a type-3 active site.

The CuA active site: This type of active site is also known as a mixed-valence copper site. Both copper ions have a tetrahedral geometry and are bridged by two thiolate groups of two cysteinyl residues. Each copper ion is also coordinated by a nitrogen atom from a histidine residue.

The CuB active site: This active site was detected close to an iron center in cytochrome c oxidase. In this site, a mononuclear Cu ion is coordinated by three nitrogen atoms from three histidine residues in a trigonal pyramidal geometry. Two metal ions are strongly antiferromagnetically coupled in the oxidized state.

The CuZ active site: The CuZ active site consists of four copper ions, arranged in a distorted tetrahedron and coordinated by seven histidine residues and one hydroxide anion. This site was detected in nitrous oxide reductase and is involved in the reduction of N_2O to N_2.

Polyphenol oxidase

Polyphenol oxidases or tyrosinases (PPO) (1, 2 benzene:oxygen oxidoreductase, EC 1.10.3.1), are type III enzymes with a dinuclear copper centre which initiates enzymatic browning. PPO is also known as phenol oxidase, monophenol oxidase, cresolase, catechol oxidase etc. PPOs are mixed function oxidases which catalyze both the hydroxylation of monophenols to diphenols (monophenolase/cresolase) and also oxidation of *o*-diphenols to *o*-quinones (diphenolase/catecholase, Figure 1. 2).

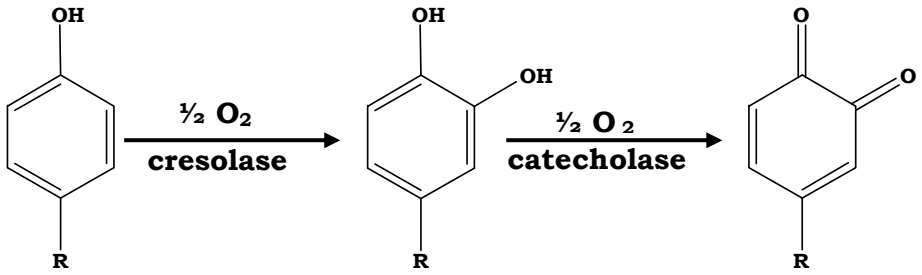

Figure 1.2 Reactions catalyzed by polyphenol oxidase.

Reactions catalyzed by PPO

The insertion of an oxygen in a position ortho to an existing hydroxyl group in an aromatic ring often referred to as cresolase activity is usually followed by oxidation of the diphenol to the corresponding quinone. The oxidation of the *o*-diphenol to the corresponding *o*-quinone is often referred to as catecholase activity. Molecular oxygen participates in both the reactions.

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 (Figure 1. 3).

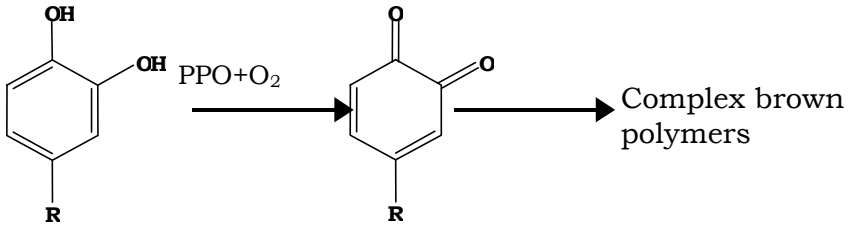

Figure 1.3 End products of PPO catalyzed reaction.

Enzyme nomenclature differentiates between monophenol oxidase (tyrosinase, EC 1.14.18.1) and catechol oxidase or o-diphenol:oxygen oxidoreductase (EC 1.10.3.1). **In this review the general term PPO will be used. Where the two activities need to be compared and differentiated the terms tyrosinase and catechol oxidase are used**.

Occurrence and location of PPO

The list of species in which PPO has been described and partly characterized is growing steadily. 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 et al., 1991), in some fungi especially those that produce brown filaments (Osuga et al., 1994), 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 (Holst and Yopp, 1976), bryophytes (van Poucke, 1967; Babbel, 1974), moss (Ritcher et al., 2005), pteridophytes (Malesset-Bras, 1962), gymnosperms (Cambie and Bocks, 1966) and practically every order of angiosperms where it has been looked for.

Determination of PPO activity

A number of quantitative assay methods are available to assess PPO activity and are essential in order to characterize the enzyme and understand its physiological role. Oxygen uptake measured manometrically or polarographically via a Warburg apparatus are still the definitive assay for tyrosinase activity (Mayer, 2006). The radioassay discontinous method, which measures the incorporation of tritium from tritiated tyrosine to ^{3}HOH (Kahn and Pomeratz, 1980) is a highly sensitive method for assaying tyrosinase or monophenolase activity. The rate of removal of tritium from a monophenol is considered a better approach for monitoring monophenolase activity (Whitaker, 1995). The most commonly used routine assay for catecholase activity is the spectrophotometric monitoring of the quinone formation from *o*-diphenolic compound at wavelengths ranging from 400-500 nm. Both the spectrophotometric and oxygen consumption polarographically with an oxygen sensitive electrode are rapid, practical and sensitive methods (Yoruk and Marshall, 2003).

More sophisticated methods involve the quantitative measurement of enzymic reaction products by HPLC, equipped with an electrochemical detector. Generally tyrosine is used to assay the monophenolase activity (Osaki, 1963; Friedman and Bautista, 1995). 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, Paul and Gowda, 2000), 4-methyl catechol (Siddiq et al., 1993; Wesche-Ebeling and Montgomery, 1990; Froderman and Flurkey, 1997), tertiary butyl catechol (Sanchez-Ferrer et al., 1993b; Sojo et al., 1998), and L-DOPA (Halim and Montgomery, 1978; Dawley and Flurkey, 1993; van Leeuwen and Wichers, 1999).

A continuous spectrophotometric method proposed by Espin et al., (1995a) is based on the coupling reaction between 3-methyl-2-benzothiazolinone hydrazone (MBTH) and *o*-quinone product of the PPO mediated reaction. MBTH, which is a potent nucleophile, traps the enzyme generated *o*-quinones, to render a soluble and more stable MBTH-quinone adduct with high molar absorptivity. The stability of the MBTH-quinone

adducts and the rapid assay provides one of the most reliable methods for determining both the monophenolase and diphenolase activities of PPO. Different phenolic substrates are used in conjunction with MBTH. The 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 were stable and did not show solubility problems unlike the other substrates (Espin et al., 1995a; Espin et al., 1995b; Espin et al., 1997a; Espin et al., 1997b; Espin et al., 2000). A dark purple propyl-quinone adduct for increased sensitivity has been used in PPO solution assay (Briganti et al., 2003).

Only a few reports focus on PPO activity detection in polyacrylamide gel electrophoresis (PAGE) slab gels. Electrophoretic methods can be widely used because enzymes remain active under non-denaturation conditions, even in the presence of SDS. An analytical method allowing the detection of PPO activity on PAGE is rapid, sensitive and specific and is based on a coupling reaction between enzymic reaction product with chromophores, which forms an adduct and gives specific color (Rescigno et al., 1997; Dicko et al., 2002). Very recently Cheng et al., (2007) developed a simple and rapid method to identify PPO in fruit and vegetable extracts. Catechol as an immobilized PPO-substrate on 3 mm chromatographic paper was layered on a gel. Instead of a Western blot, the ready-to-use catechol-paper was used on the gel without additional liquid reagents for instant visualization.

Multiplicity of PPO

PPO is present in a variety of plants, some unusual or exotic. The major progress in research has been on the multiplicity of genes coding for PPO, their description and the characterization of the expression pattern of some of these genes (Newman et al., 1993; Steffens et al., 1994). Robinson and his co-workers indicate the presence of only a single PPO gene in grape vine (Dry and Robinson, 1994). In hybrid poplar two PPO genes are differentially expressed (Wang and Constabel, 2003). Differential, tissue specific, cultivar expression of six genes coding for PPO in potatoes (Thygesen et al., 1995; Marri et al., 2003) and for seven genes in different tissues of tomatos (Thipyapong et al., 1997) has been reported. Apple PPO

is encoded by a multiple gene family, whose expression is up-regulated by wounding of the tissue (Boss et al., 1994; Kim et al., 2001). This PPO was cloned and expressed in *E. coli*. The PPO was characterized by a transit peptide and processing led to a mature PPO of Mr. 56000 Da. A gene coding for PPO is expressed only at the post-anthesis stage (Kim et al., 2001). The PPO of apricot belongs to a multigene family and is highly expressed in young immature-green fruit and turned of early in the ripening process (Chevalier et al., 1999).

The PPO from the aerial roots of an orchid Aranda and located in the plastids was found to occur in four isoforms, which were partially characterized, including the amino terminal sequences of the isoforms (Ho, 1999). Two distinct PPOs are present in leaves and seeds of coffee (Mazzafera and Robinson, 2000) in the parasitic plant *Cuscuta* (Dodder) (Bar Nun and Mayer, 1999), and in chinese cabbage (Nagai and Suzuki, 2001). PPO occurs as two forms in *Annona muricata* (Bora et al., 2004). PPO has been isolated from oregano (Dogan et al., 2005a), persimmon (Ozen et al., 2004), artichoke (Dogan et al., 2005b), marula (*Sclerocarya birrea*) (Mduli, 2005), loquat (*Eriobotrya japonica*) (Selle´s-Marchart et al., 2006) and *Uapaca kikiana* fruit, a plant belonging to *Euphoriaceae* (Muchuweti et al., 2006). At least three PPOs exist in the red clover and are differentially expressed in different parts of the plant (Sullivan et al., 2004). The red clover PPO is apparently the only expressed PPO that is functional (Sullivan et al., 2004). Although it is generally agreed that PPO is plastid located, the site at which it is present in potato tubers is not entirely clear. Using immuno-gold localization PPO was located in starch grains and the cytoplasm (Partington et al., 1999).

The subcellular location of PPO is used to ascribe a physiological function to the enzyme (Mayer and Harel, 1979). 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. PPO is found in a variety of subcellular fractions such as peroxisomes, mitochondria and microsomes (Mayer and Harel, 1979; Martinez-Cayuela et al., 1989). The weight of evidence indicates the wide occurrence of membrane bound PPO, is 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 have reached the same conclusion (Ruis 1972; Tolbert, 1973; Kato et al., 1976). Furthermore, histochemical work employing 3, 4-dihydroxyphenylalanine (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 et al., 1987). In potato tubers, nearly all of the sub cellular fractions were found to contain PPO, in amounts proportional to the protein content (Craft, 1966). Localization of PPO in 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. Similarly, 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).

Considerable evidence that the PPO is a nuclear-coded protein (Lax et al., 1984), located in the plastids has been provided (Vaughn and Duke, 1984; Vaughn et al., 1988; Shernman et al., 1991; Murata et al., 1997; Escribano et al., 2002). Rathjen and Robinson, (1992) suggested that PPO in grape berries could accumulate into an aberrant form, with a Mr weight of 60000 Da, and not the expected one of 40000 Da. Sommer et al., (1994) investigated the pathway by which plant PPO reaches the chloroplast. They studied in detail the synthesis, targeting and processing of PPO. Using an in *vitro* system and pea chloroplasts they showed that tomato PPO, coded by

cDNA, was processed in pea chloroplasts in two steps during its import. The precursor PPO with Mr 67000 Da was imported into the stroma of the chloroplasts by an ATP-dependent step. It was then processed into a 62000 Da form by a stroma peptidase. The processing is carried out by a stromal peptidase, which was purified and characterized (Koussevitzky et al., 1998). The synthesis of PPO and its transport to its site in chloroplasts, where plant PPOs are thought to be located, is a complex process. The finding on the possible ability of PPO to act as a protease has been reported (Sokolenko et al., 1995; Kuwabara, 1995; Kuwabara et al., 1997; Kuwabara and Katoh, 1990). PPO is synthesized on cytoplasmic ribosomes and is inactive until integrated into the plastid (Yalovskg et al., 1992; Mayer et al., 1995).

pH optima and stability

The changes in ionization of prototropic groups in the active site of an enzyme at different pH reckon changes in conformation of the active site, binding of substrates, and/or catalysis of the reaction (Segel, 1976; Whitaker, 1994). The catalytic activity of enzymes is influenced by irreversible denaturation of the protein and/or reduction, and instability of substrate as a function of pH. The kinetic behavior of PPO was reported to alter depending on the pH of the assay buffer due to pH-induced conformational changes in the enzymes (Janovitz-Klapp et al., 1990; Valero and Garcia–Carmona, 1992, 1998).

The pH optimum of PPOs varies widely with plant sources but generally is in the range of 4.0-8.0. Cherry and strawberry PPOs show a narrow pH optimum with a maximum at about pH 4.5 with 4-methylcatechol as substrate (Wesche-Ebeling and Montgomery, 1990; Fragnier et al., 1995). Several fruit PPOs including almond, apricot, peach and plum generally have maximum activities around pH 5.0 (Fraignier et al., 1995a). PPO from highbush blueberry fruit had an optimum of 4.0 (Kader et al., 1997), morula fruit 4.5 (Mdluli and Owusu-Apenten, 2003), loquat fruit, 4.5 (Ding et al., 1998) broad bean, 5.0 (Jimenez and Garcia-Carmona, 1999), field bean 4.0 (Paul and Gowda, 2000), egg plant 5.0 (Perez-Gilabert and Carmona, 2000) palmito, 5.2 (Robert et al., 1995) plantain, 6.5 (Ngalani et al., 1993), potato, 6.5 (Sanchez Ferrer et al., 1993a), cocoa bean, 6.8 (Lee et al., 1991) and cap

flesh of Portabella mushroom, 7.0 (Zhang et al., 1999). PPO from apple, eggplant, pear and olives had a broad 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 pH of 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). PPO isoenzymes from mung bean leaf had an optimum of 5.5-6.5 (Shin et al., 1997). The isoenzymes of banana bud had an optima of 6.8 and 5.5 (Oba et al., 1992). PPO from subtropical fruit such as pineapple and longan are most active near neutral pH (Das et al., 1997; Jiang, 1999).

The pH optimum of PPO varies from vegetable plants; lettuce PPO shows a broad pH optima of 5.0 to 8.0 (Heimdal et al., 1994) whereas spinach PPO is optimally active at pH 8.0 with no activity below pH 6.0 (Sheprovitszy and Brudvig, 1996). Some PPOs exhibit two pH optima such as potato which shows maximal activity at pH 4.5-5.0 and 6.0-6.5 (Sanchez-Ferrer et al., 1993b). This was attributed to two ionization states of the enzyme substrate complexes although only a single isoform was visible under electrophoresis (Sanchez-Ferrer, 1999). The two isoform of sweet potato catechol oxidase (ibCO) showed a pH optima of 5.4 and 6.7 for oxidation of chlorogenic acid (Nozue et al.,1998).

Differences in pH optima with different substrates have been reported for PPO from different sources. The pH optima of crude plum PPO (Siddiq et al., 1996) was 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 an 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 for maximum activity of PPO from tea leaf varies depending upon the original material, extraction methods and substrates (Yoruk and Marshall, 2003).

Molecular weight of PPO

The molecular weights of PPO vary significantly from source to source. This variability is due to the phenomenon of multiplicity observed in plant PPOs (Mayer, 2006). Sherman et al., (1991) reported that the Mrs of plant PPOs ranged from 33000 to 200000 Da. The PPO from bacteria *Streptomyces glaucescens* and *Streptomyces antibioticus* have Mrs of 30900 Da and 30700 Da respectively while the PPO from fungus *Neurospora crassa* has a Mr of 46000 Da. Zhang et al., (1999) found that tyrosinase from the cap flesh of Portabella mushrooms had a Mr of 41000 Da, which compares with that proposed by Wichers et al., (1996) for mushroom tyrosinase. PPO purified from cabbage (Fujita et al., 1995) had a Mr of 39000 Da, loquat fruit 55000 Da (Ding et al., 1998), oil bean 110 000 Da (Chilaka et al., 1993), potato 129000 Da (Partington and Bolwell, 1996) and pineapple 104000 Da (Das et al., 1997). Sachde et al., (1989) reported a relatively low Mr of 17000 Da for date PPO. The coffee leaf and endosperm PPO has a Mr of 46000 and 50000 Da (Mazzafera and Robinson, 2000), loquat fruit 592000 and 612000 Da (Seles Marchant et al., 2006), *Ocimum basilicum* PPO 54000 Da (Dogan et al., 2005), Creosote bush (*Larrea tridentate*) PPO isoforms have Mr of 43000 Da (Cho Mantto et al., 2003), garland chrysanthemum (*Chrysanthemum coronarium* L) 45000 Da, bean sprout (*Glycine max*) 54000 Da (Nagi and Suzuki, 2003), cucumber PPO has 53000 Da (Gandia-Herrero et al., 2003), *Hevea brasiliensis* 32000 and 34000 Da (Wititsuwannakal et al., 2002). Many of the higher plant PPOs are multi subunit proteins. Oil bean PPO is a tetramer of 28000 Da (Chilaka et al., 1993) and pineapple PPO a tetramer of 25000 Da (Das et al., 1997). PPOs from cabbage (Fujita et al., 1995), sweet potato (Eicken et al., 1999) and loquat fruit (Ding et al., 1998) are single subunit proteins whereas PPO from the dwarf variety of banana (Galeazzi and Sgarbieri, 1993) was found to be a dimer of 30000 Da.

As seen above the molecular weight of plant PPO are very diverse and variable. Part of this variability is due to partial proteolysis of the enzyme during its isolation and multiplicity in the genes coding for PPO. The native (42000 Da) and proteolysed (27000 Da) forms of apple PPO detected under partially denaturing conditions were found to have Mr of 62000 Da and 42,000 Da respectively (Macques et al., 1995).

Substrate specificity

Phenolic compounds are the primary substrates of PPO. All *o*-diphenol oxidases require the basic *o*-dihydroxyphenol structure for oxidase activity, so that catechol is the archetype but not necessarily the best substrate. 4-methyl catechol is usually oxidized faster (Walker, 1995). Several diphenolic compounds serve as substrates. The types and native concentrations of natural phenols vary widely for different plant sources. The catechins, cinnamic acid esters, L-DOPA and tyrosine are the most important natural substrates of PPO in fruits and vegetables (Baruah and Swain, 1959; Hermann, 1974; Walker, 1975; Rocha and Morais, 2001). Some fruit PPOs use other phenolic substrates. The major endogenous substrate in bananas and latex of *Hevea brasiliensis* (Vamos-Vigyazo, 1981: Yang et al., 2001; Wititsuwannakul et al., 2002) is dopamine. Grape catechol oxidase acts on *p*-coumaryl and caffeoyl-tartaric acids while dates contain unusual substrates including a range of caffeoyl-shikimic (dactylferic) acids, which are analogous to the ubiquitous isomers of chlorogenic acid (Walker, 1995; Erat et al., 2006). The Kolkhida tea leaves preferentially oxidize *p*-coumaric acids (Pruidze et al., 2003). Among the cinnamic acid esters, chlorogenic acid (3-*o*-caffeoyl-D-quinic acid) is the most widespread natural PPO substrate. Chlorogenic acid was used as a substrate to assay PPO from several sources, including apple, ferula sps (Janovitz-Klapp et al., 1989; Erat et al., 2006; Rapeanu et al., 2006), lettuce (Fujita et al., 1991), ocimum (Dogan et al., 2005), sweet potato (Lourenco et al., 1992), potato (Sanchez Ferrer et al., 1996), highbush blueberry fruit (Kader et al., 1997) and plums (Siddiq et al., 1996). 4-Methyl catechol was used as a substrate to assay sweet potato, field bean (Lourenco et al., 1992; Paul and Gowda, 2000), apple (Janovitz-Klapp et al., 1990), grape (Sanchez Ferrer et al., 1992), plantain (Ngalani et al., 1993) and plum PPOs (Siddiq et al., 1996). PPO oxidizes flavonoids (Jimenez-Atienzar et al., 2005). The caffeic acid (3, 4-dihydroxy cinnamic) acid moiety of chlorogenic acid is reported to be hydroxylated by PPO to *p*-coumaric acid (Sato, 1962, Erat et al., 2006; Nkya et al., 2003; Lima and Bora, 2003). The extent of naturally occurring phenolic substrates contribute to degree of enzymatic browning of fruits and vegetables. 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 nature and position of the substituent groups has profound effects on the rate of substrate oxidation (Passi and Porro, 1981; Janovitz-Klapp et al., 1990) and studies of these problems can shed light on the nature of the interaction between the substrate and the active site of PPO. The *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 increases the PPO catalyzed reaction (Solomon et al., 1996; Espin et al., 1998; Espin et al., 2000). The greater the electron donating nature, better the suitability of the substrate. Substitutions in position 3 (3-methyl catechol, 2, 3-dihydroxy benzoic acid) cause 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 black poplar PPO (Duckworth and Coleman, 1970). Chloro and fluro phenols are also hydroxylated by tyrosinases (Battaini et al., 2002).

Yasunobu, (1959) concluded from a comparison of the substrate specificity of various catechol oxidases that although, the enzymes could oxidize a wide range of phenolics, each 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; Tanfel and Voigt, 1963; Jen and Kahler, 1974). PPO from oil bean (Chilaka et al., 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 the 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; Mdluli and Owusu-Apenten, 2003; Selles-Marchart et al., 2006) and field bean PPO (Paul and Gowda, 2000) did not exhibit any activity towards monophenols or *p*-diphenols. 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.

PPOs from strawberry fruit (Wesche-Ebeling and Montgomery, 1990), cocoa bean (Lee et al., 1991) highbush blueberry fruit (Kader et al., 1997), vascular lettuce tissue (Heimdal et al., 1994) and dwarf variety of banana (Galeazzi and Sgarbieri, 1981) did not have monophenolase activity. Raspberry fruit PPO (Gonzalez, 1999), eggplant PPO (Perez-Gilabert and Garcia-Carmona, 2000) and broad bean thylakoid bound PPO (Sanchez Ferrer et al., 1990) were active towards *p*-cresol. Strawberry (Espin et al., 1997b), Verdoncella apple (Espin et al., 1995b), potato tuber (Sanchez Ferrer et al., 1993b), ferula (Erat et al., 2006) and quince fruit (Orenes-Pinero et al., 2005) PPOs have cresolase activity. The monophenolase activity is activated in the presence of catalytic amounts of diphenols as co-substrates (Gowda and Paul, 2002; Sanjust et al., 2003; Orenes-Pinero, et al., 2005).

The affinity of plant PPOs for phenolic substrates is relatively low. The K_m is high, usually in the mM range (Janovitz-Klapp et al., 1989; Lee et al., 1991; Lourenco et al., 1992; Zhou et al., 1993; Siddiq et al., 1996; Shin et al., 1997; Espin et al., 1997b; Das et al., 1997; Ridgway and Tucker, 1999; Nunez-Delicado et al., 2005; Rapeanu et al., 2006). The affinity of PPO for oxygen depends on the phenolic substrate being oxidized (Duckworth and Coleman, 1970; Harel et al., 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; Frieden et al., 1965; Bull and Carter, 1973; Lerner and Mayer, 1976). The values reported are in the range of 0.1-0.5 mM (Tocher and Meeuse, 1966; Bull and Carter, 1973; Rivas and Whitaker, 1973; Lerner and Mayer, 1976).

The specificity for optical isomerism which is clear-cut in the

mammalian enzyme (Pomerantz, 1963; Lerner, 1953) is less evident in catechol oxidase of higher plants (Palmer, 1963). The stereospecificity of monophenolase and diphenolase activity of mushroom tyrosinase with several enantiomorphs (D-, L- and DL-tyrosine, methyltyrosine, DOPA, methyl-DOPA and isoprenaline) of monophenols and o-diphenols was assayed by Espin et al., (1998a). The lower K_m values of L-isomers than the D-isomers, revealed stereospecificity (Espin et al., 1998b). The phenolic compounds containing electron withdrawing groups were shown to be poor substrates for tyrosinase as compared to the electron-donating groups (Espin et al., 1998a).

Substrate specificity of PPO is not only dependent on species but cultivar and tissue also. Among grapes the activity of DeChaunac grape PPO is highest toward caffeic acid (Lee et al., 1983). In contrast the activity of koshu grape PPO is greatest with chlorogenic acid followed by caffeic acid (Nakamura, et al., 1983), whereas concord grape PPO oxidized catechol more rapidly than caffeic acid (Cash et al., 1976).

Inhibitors of PPO

Enormous economic impact of PPO-induced, deleterious browning reactions in fruit and vegetables, as well as in seafood necessitates its control in order to maintain the quality and extend product shelf life. More than 98 % of PPOs in plants are present in a latent form, the remaining active form has a potential for enzymatic browning during maturity or damage at the time of post harvest handling and processing which causes severe economic loss to food industries. Millions of money in crop loss occurs yearly due to enzymatic browning (Martinez and Whitaker, 1995; Whitaker, 1996 and Kim et al., 2000). Several approaches are experimentally demonstrated to diminish or prevent browning of injured tissues. The most commonly applied inhibitor of the discoloration process currently is sulfite, which, however, is meeting increasing resistance. A number of inhibitors from both natural and synthetic sources that inhibit monophenolase, diphenolase, or both of these activities have been identified (Seo et al., 2003). Inhibitors of PPO are often grouped according to their mode of action (McEvily et. al., 1992).

a) Reducing agents (ascorbic acid and analogs, sulfites)
b) Copper chelating agents (EDTA, DIECA, sodium azide)
c) Competing agents (cyclodextrin, chitosan)
d) Acidulants (ascorbic acid, malic acid, phosphoric acid)
e) Active site inhibitors (substrate analogs, halides)
f) Enzyme treatments (proteases, *o*-methyltransferase)

These compounds diminish or inhibit the browning reaction rate by means of eliminating from the reaction an active reaction element(s), that may be either enzyme, substrate, copper or a reaction intermediate (*o*-quinones) (Vamos-Vigyazo, 1981). In addition the search for naturally occurring inhibitors has led to the discovery of number of active compounds.

Reducing agents

Reducing agents are broadly used in the food industry. They inhibit browning by preventing accumulation of the *o*-quinones or form products that are colorless (Kim et al., 2000). Sulfur dioxide, sulfites (sodium sulfite, sodium bisulfite and sodium metabisulfite) were used in the fruit and vegetable industry as antibrowning agents as they were effective and inexpensive. Ashie et al., (1996) showed that sulfite interacts with quinones preventing the formation of brown pigments. Owing to safety concerns FDA (Martinez and Whitaker, 1995) banned their use in fresh fruit and vegetables although they are still allowed for shrimp to delay black spot formation during processing and storage (Kim et al., 2000). Ascorbic acid and its isomer erythrobic acid are commonly used as reductants for antibrowning in fruit juices and canned vegetables and fruits. However browning occurs after ascorbic acid is exhausted (Osuga, 1994; Ashie, 1996). Citric acid inhibits PPO through its site directed specificity toward histidine residues on the PPO (Golan-Goldhirsh et al., 1992). Therefore a combination of ascorbic acid and citric acid, more effectively prevents the browning (Sappers, 1993).

Copper chelating agents

These agents are believed to either bind to the active site of PPO or reduce the level of copper availability. The many copper chelating agents that inhibit PPO include diethyldithiocarbamate, azide,

mercaptobenzothiozloe (Walker, 1975), cyanide, thiourea (Mathew and Parpia, 1971) and carbon monoxide (Albisu et al., 1989).

Acidulants

The pH optimum of PPO activity varies with the source of the enzyme and the particular substrate. In most of the cases the pH optimum is in the range of 4.0-8.0 for PPO. By lowering the pH of the media below 2.0, the enzyme is effectively inhibited. Hence the role of acidulants is to maintain the pH well below that necessary for optimal activity.

The most widely used acid in the food industry for prevention of browning is citric acid. It has a dual effect 1) reduces the pH and 2) chelates the copper at the active site (McEvily and Iyengar, 1992). The other alternatives are ascorbic acid, malic acid and phosphoric acid (Yoruk and Marshall, 2003).

Polyphenols

These are a group of compounds widely distributed in nature. The recently reported chalcones and related compounds such as glabridin, isoliguiritigenin (Nerya, et al., 2004) showed that the number and position of hydroxyl groups were important to the degree of inhibition (Khatib et al., 2005). The oxidiazoles (Khan, et al., 2005), flavonols (Kubo et al., 2000), procyanidins (Paul, 2000; Le Bourvellec et al., 2004) are potent inhibitors of diphenolase. An important group of compounds, which inhibit PPO, are gallic acid derivatives widely used in the food industry (Mayer, 2006).

Substrate analogs

Various carboxylic acids and their derivatives are known to be powerful inhibitors of PPO (Seo et al., 2003). Lim et al., (1999) reported that *p*-coumaric acid inhibits oxidation of diphenols. Salicylic acid and its derivatives are found to inhibit oxidation of diphenols by competing for the active site. The inhibitory strength increased in the order; 4-methyl salicylic acid > 5-methyl salicylic acid > 4-methoxy salicylic acid > salicylic acid > 5-methoxy salicylic acid (Zhang et. al., 2006). In addition many aromatic acids inhibit diphenolase activity very effectively. The mono, di and tri hydroxyl derivatives of aromatic carboxylic acids have inhibitory potency with

different scale. The number and position of side groups has a major effect on the inhibitory potency. Benzoic acid has long been known as a PPO inhibitor (Kunner et al., 1953; Kruger, 1955; Gunata et al., 1987; Menon et al., 1990; Ferrer and Walker, 1996). Substitution around the aromatic nucleus has shown varied effects on the degree of PPO inhibition. 2, 3-Dihydroxy benzoic acid displayed no inhibition whereas 2, 4-dihydroxy benzoic acid was a strong inhibitor of apple PPO (Ferrer and Walker, 1996). Hydroxylation and methylation of the aromatic rings decreased the inhibitory effect of fruit PPOs (Menon et al., 1990; Pifferi et al., 1974; Walkwe and Wilson, 1975; Duckworth and Coleman, 1970). The type and degree of inhibition by aromatic carboxylic acids on PPO activity are dependent on the structure of both the substrate and inhibitor (Duckworth and Coleman, 1970; Tremoliers and Beith, 1984). Kermasha et al., (1993) in their study concluded that the inhibition of PPO by aromatic carboxylic acids and their derivatives was a complex phenomenon.

A large number of aldehydes and other derivatives such as trans-cinnamaldehyde (Lee et al., 2000), 2-hydroxy-4-methoxybenzaldehyde (Kubo and Kinst-Hori, 1999), anisaldehyde (Kubo and Kinst-Hori 1998), cuminaldehyde and cumic acid (Kubo et al., 1988) are potent inhibitors of PPO. The aldehyde group reacts with biologically important sulfhydryl, amino, and hydroxy groups, and thereby inhibits the PPO. The inhibitory activities of various aldehydes and closely related compounds such as cinnamic acid, anisic acid, cumic acid, and benzoic acid proved, that the inhibitory potency was dependant on the number and position of side groups (Seo et al., 2003).

Chemicals

A number of chemicals such as hydrogen peroxide, hydroxylamine, and aromatic carboxylic acids have been reported for their anti-browning activity. Hydrogen peroxide inactivates PPO in a biphasic manner (Seo et al., 2003). Hydroxylamine inhibits *o*-dihydroxy phenolase activity and lowers the extent of pigment production (Kahn and Andrawis, 1986). Dimethyl sulfide (DMS) was found to be a slow binding competitive inhibitor of PPO (Perez-Gilabert and Garcia-Carmona, 2001). DMS has a physiological role within

plant tissues and its high concentration inhibits endogenous PPO, and prevents premature phenolic oxidation in plants (Sayavecra et al., 1986).

The more recent approach is to reduce browning by controlling PPO *in vivo* by means of antisense RNA technology (Martinez and Whitaker, 1995). The mRNA encoded by the antisense gene hybridizes with mRNA encoded by endogenous gene and prevents the translation. The antisense down regulation of PPO has been reported in potato and apple (Bachem et al., 1994; Coetzer et al., 2001; Murata et al., 2000).

Presently, the use of 4-hexylresorcinol is considered to be safe in the food industry (Iyernger et al., 1991) and for browning control in fresh and dried fruit slices (Frankos et al., 1991). However, as safety is of prime concern for an inhibitor to be used in the food industry, there is a constant search for better inhibitors from natural sources as they are largely free of any harmful side effects. Many of the inhibitors described are flavor condiments and are listed as food flavor ingredients in *Fenaroli's Handbook of Flavor Ingredients* (Burdoch, 1995), which makes their use favorable in food industries.

Structure of PPO

Primary structure

The amino acid sequence of a large number of PPOs, of plants and other organisms derived from cDNA and comparative information has been reviewed (van Gelder, 1997; Wichers et al., 2003; Marusek et al., 2006; Halaouliin et al., 2006). Among plants the PPO sequences of sweet potato *(Ipomea batatas*), tomato, (*Lycopersicon esculretum*; Newman et al., 1993), potato (*Solanum tuberosum;* Hunt et al., 1993), apple, (Boss et al., 1995) grape (*Vitis vinifera*; Dry and Robinson, 1994), broad beans (*Vicia faba*; Cary et al., 1992) are known. As is the case with all nuclear encoded plastid enzymes higher plant PPOs possess plastid transit peptides that posttranslationally direct the proteins to the chloroplast (Sommer et al., 1994). The transit peptide is of 80-90 residues and located at the amino-terminus. The transit peptide is characterized by three domains. A small cysteine rich region in the amino-terminal domain is conserved (van Gelder, 1997).

The most prominent features in all PPO sequences are the two Cu binding regions called the CuA and CuB site. Multiple sequence alignment of PPOs shows that all PPOs possess six histidine residues that ligate the two Cu ions of the active site. The first three encompass the CuA site and the next three the CuB. A conserved thioether bridge in plant PPOs is involved and is located two residues prior to the second histidine of CuB. This bridge is not present in arthropod hemocyanins. A phenylalanine corresponding to Phe^{261} of all plant PPOs is conserved with the exception of pineapple PPO and bread wheat PPO (Marusek et al., 2006). Garcia-Borron and Solano, (2002) in their sequence alignment noted a pair of tyrosines conserved in PPOs ranging from humans to bacteria, but did not realize their significance. Marusek et al., (2006) in their sequence alignment of a larger number of PPOs showed that one of the tyrosines can be substituted by a phenylalanine leading to a "tyrosine motif" down stream of the CuB site. An aspartate residue located four residues after the third histidine is conserved in all plant PPOs important for structural integrity. From their crystal structure of ibCO Klabunde et al., (1998) implicate a role for Glu^{236} in catalysis. Multiple sequence alignment of plant catechol oxidases shows this residue is conserved (Kanade et al., 2006). This residue is glutamine in human tyrosinase and leucine in *Neurospora crassa* tyrosinase (Klabunde et al., 1998). The amino acid sequence identity between tyrosinase and catechol oxidase is 26 % (Klabunde et al., 1998).

Three dimensional structure

The crystal structure of the enzyme from sweet potato (ibCO) in the resting dicupric Cu(II)-Cu(II) state, the reduced dicupric Cu(I)-Cu(I) and in complex with a potent competitive inhibitor phenylthiourea has been resolved at 2.5 Å. The monomeric ibCO is an ellipsoid with dimensions of 55 × 45 × 45 Å (Klabunde et al., 1998). The secondary structure is primarily an α-helix with the catalytic dinuclear copper site in the core of a four helix bundle (Figure 1.4). This four helix bundle is located in a hydrophobic pocket close to the surface. Each of the Cu atoms are ligated to three histidine residue from the α-helix bundle. In addition His^{109} liganded to CuA is in an unusual covalent thioether linkage with Cys^{92}. In the oxy state the

distance between the two Cu atoms is 2.9 Å, which increases to 4.4 Å on reduction. The Cu-O distance is 1.89 Å from EXAFS data (Rompel et al., 1998).

The thiourea linkage between His and Cys is reported for the tyrosinase of *Neurospora crassa* (Lerch, 1982) and for molluscan hemocyanin (Gielens et al., 1997; Cuff et al., 1998). This thioether link is absent in human and arthropod tyrosinase.

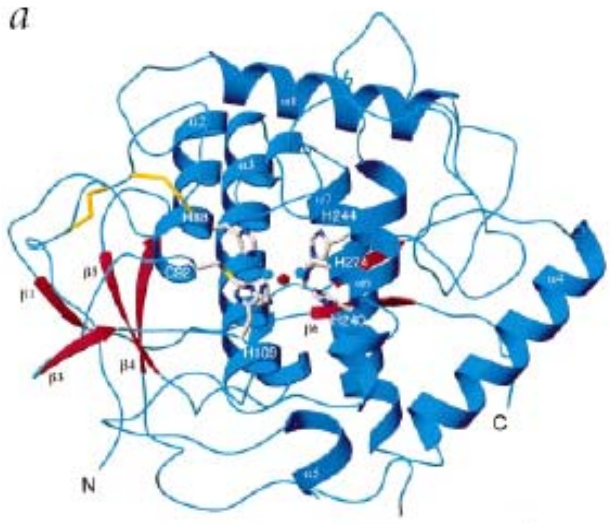

Figure 1.4 Ribbon drawing of sweet potato (*Ipomea batatas*) catechol oxidase (PDB id 1BT1)

The hydrophobic pocket of the dicupric catalytic center is lined by the side chains of Ile^{241}, Phe^{261}, His^{244} and Ala^{264}. Phe^{261} is termed as the "gate residue" as access to the catalytic metal center is controlled by the aromatic ring of this residue (Eicken et al., 1999; Gerdemann et al., 2002). The binding of the inhibitor is with its phenyl ring perfectly stacked against His^{244} and the sulfur of PTU replacing the hydroxibridge and rendering it a powerful inhibitor.

The dicopper centers of hemocyanin, the oxygen transport protein of *Panulirus interruptus* and *Limulus polyphemus* are similar (Gaykema et al., 1984; Volbeda et al., 1989; Hazes et al., 1993). A Phe residue in the amino-terminal domain is fully conserved in all hemocyanins. It shields substrate

access to the dicopper center thereby making the hemocyanin function as an oxygen carrier and is postulated to be the key residue in allosteric regulation mechanism of hemocyanin (Hazes et al., 1993). This Phe is termed as a "place holder" for potential substrates (Decker and Tuczek, 2000). The crystal structure of tyrosinase complexed with a caddie protein ORF378 at 1.2-1.8 Å resolution shows it is ellipsoid and the catalytic dinuclear copper center lodged in a helical bundle is similar to ibCO (Matoba et al., 2006). Gerdemann et al., (2002) used comparative modeling to propose that the carboxy-terminal fragment of ibCO prior to cleavage adopts a tertiary structure similar to the carboxy-terminal domain of hemocyanin of *Octopus doflei*. They propose that this domain shields the copper catalysis site from substrate access prior to cleavage. Marusek et al., (2006) in a comparative analysis of PPOs from plant and fungal species opine that several structural features are conserved in the amino-terminal domains (YIFXY) located after the CuB site and that the tyrosine motif is a landmark connecting the carboxyl-and amino-terminal domains. Their sequence alignment and secondary structure predictions indicate that the tertiary structure of the carboxy-terminal domains of PPO resemble hemocyanin and are likely to adopt a β-sandwich structure.

Koval et al., (2006) have developed a biomimetric model of the active site of catechol oxidase after the reported X-ray structure of ibCO. Studies on substrate binding to a model complex, structure-activity relationship, kinetic studies of substrate oxidation and substrate interaction with oxo-dicopper adducts are reviewed.

Catalytic mechanism of polyphenol oxidase

The overall mechanism of monophenolase and diphenolase proposed by Lerch (1995) was based on the geometric and electronic structure of the copper active site, which structurally resembled that in hemocyanin (Himmerlwright et al., 1980). This mechanism reckoned that only monophenols bind with oxy-PPO forming a ternary PPO-oxygen-monophenol complex. PPO is converted to deoxy-PPO after the *o*-quinone is released and ready for the next cycle. In the diphenol oxidation pathway the diphenols not only react with oxy-PPO but also met-PPO (Lerch, 1995). The kinetic data

indicated the reaction is through an ordered sequential mechanism (Wilcox et al., 1985) for monophenolase and random sequential mechanism for binding *o*-diphenols and oxygen. The ordered sequential bi-bi mechanism with oxygen as the first substrate to be bound to PPO is generally assumed (Janovitz-Klapp et al., 1990, Whitaker, 1994). Kinetic aspects and structural aspects of the reaction mechanism for PPO has been reviewed by Sanchez-Ferrer et al., (1995). The characteristic lag period observed for the monophenolase activity of several plant PPOs is required to generate catalytic quantities of *o*-diphenol necessary to form oxy-PPO from met-PPO (Rodriguiz-Lopez et al., 1992; Sanchez-Ferrer et al., 1995; Gowda and Paul, 2002).

Klabunde et al., (1998) proposed a mechanism for the catalytic process of ibCO based on biochemical and spectroscopic (Eicken et al., 1998) as well as structural data. The reaction mechanism is shown in Figure 1.5. The catalytic cycle begins with the met-ibCO, the resting form. The dicopper (II) center of the met form reacts with one equivalent of catechol, leading to the formation of quinone and to the reduced deoxy dicopper (I) state. Stoichiometric amounts of the quinone product form immediately after the addition of catechol, even in the absence of dioxygen (Klabunde et al., 1998; Eicken, et al., 1999). A monodentate binding of the substrate to the CuB center has been proposed. Oxygen binds to the dicopper (I) active site replacing the solvent molecule bonded to CuA in the reduced enzyme form. The binding of the catechol substrate to the deoxy state prior to dioxygen binding was ruled out, as no substrate binding occurred when the enzyme was reduced with dithiothreitol. UV-Vis spectroscopy and Raman data suggested that dioxygen binds in the bridging side-on μ-η^2: η^2 binding mode with a copper–copper separation of 3.8 A°(Rompel et al., 1999). The rotation of the side chain of Phe^{261} in ibCO opens the dicopper center to permit the binding of catechol. This model shows that simultaneous binding of catechol and dioxygen is possible. The aromatic ring of the catechol substrate and the phenyl ring of phenylthiourea position the coordinated hydroxylate group of the substrate close to the coordinated amide nitrogen of the inhibitor and maintains the favorable van der Waals interactions observed in the inhibitor complex. The CuA site retains the tetragonal pyramidal geometry with

dioxygen, His[88] and His[118] in the equatorial positions, His[109] in an axial position and a vacant sixth coordination site. In this proposed ternary CO-O_2^{2-} catechol complex, two electrons can be transferred from the substrate to the peroxide, followed by the cleavage of the O–O bond, loss of water and the formation of the quinone product, together with the restoration of the met state, completing the catalytic cycle (Klabunde et al., 1998).

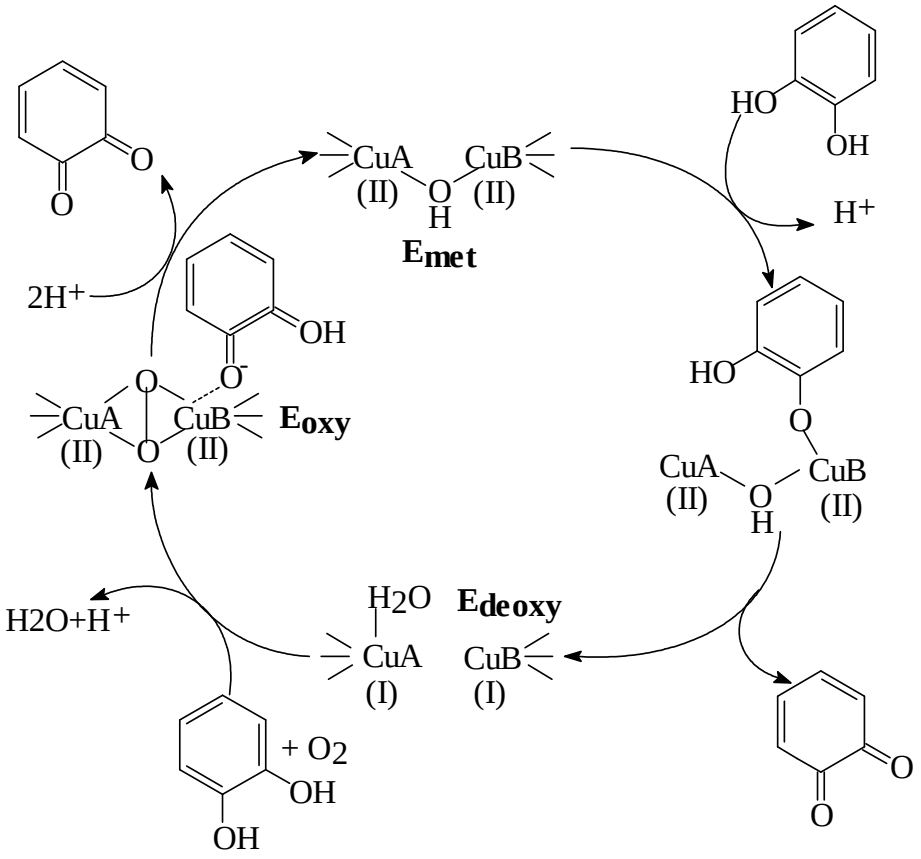

Figure 1.5 Catalytic cycle of catechol oxidase from *Ipomoea batatas*, as proposed on the basis of structural, spectroscopic and biochemical data. Two molecules of catechol (or derivatives thereof) are oxidized, coupled with the reduction of molecular oxygen to water. The ternary CO-O_2^{2-}catechol complex was modeled, guided by the binding mode observed for the inhibitor phenylthiourea.

A very similar catalytic mechanism was proposed by Solomon et al., (1996) for the catecholase activity of the structurally related type-3 protein tyrosinase. The main difference between the two mechanistic proposals involves the binding mode of the substrate to the dicopper (II) core, whereas a monodentate asymmetric coordination of the substrate was proposed by Klabunde et al., (1998), a simultaneous coordination of the substrate to both

copper centers in the bidentate bridging fashion was suggested by Solomon et al., (1996). The growing numbers of theoretical and experimental studies suggest that the active site of an enzyme, which is deeply buried in the low dielectric of a protein, as observed in catechol oxidase, should not change its charge during the catalytic cycle (Koval et al., 2006).

Tyrosinases catalyze two oxidation reactions as shown in Figure 1.6. In cycle 1 tyrosinase accomplishes the oxidation of monophenols by oxygen as it passes through four enzyme states (E_{deoxy}, E_{oxy}, E_{oxy}-M and E_{met}-D). In cycle two *o*-diphenols are oxidized as the enzyme passes through five enzyme states (E_{deoxy}, E_{oxy}, E_{oxy}-M, E_{met} and E_{met}-D). The two cycles lead to the formation of *o*-quinones which spontaneously react with each other to form oligomers (Sanchez-Ferrer et al., 1995; Fenoll, et al., 2001; Seo et al., 2003).

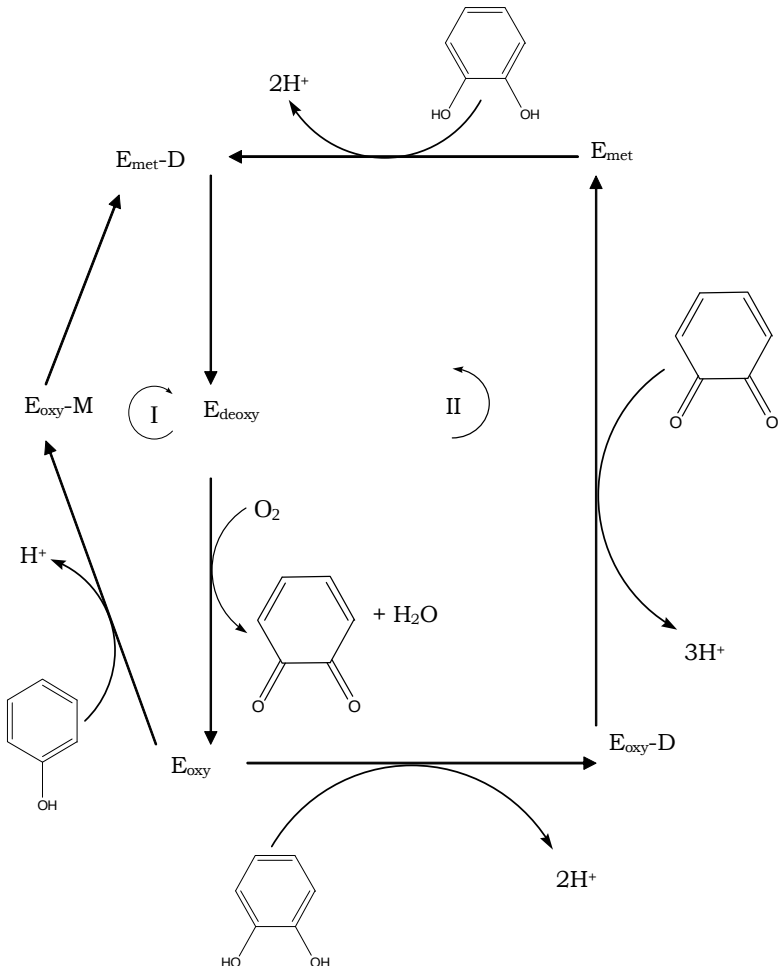

Figure 1.6. The catalytic cycle for the I) Hydroxylation of monophenols II) Dehydrogenation of *o*-diphenols to quinones by tyrosinase (M corresponds to monophenols and D corresponds to diphenols).

Latency of PPOs

An unusual and intriguing characteristic of PPO is its ability to exist in either a latent and/or an active form (Manson, 1965 and Whitaker, 1995). PPO is released from latency or activated by acid and base shock (Kenten, 1957; Lerner et al., 1972), an anionic detergent such as SDS (Kenten, 1958; Flurkey, 1986; Moore and Flurkey 1990; Sugumaran and Nelliappan 1991; Jimenez and Garcia-Carmona 1996; Espin and Wichers 1999a; Laveda et al., 2000; Lopez-Serrano et al., 2002; Jiang et al., 2003; Gandia-Herrero et al., 2005a and2005b), urea (Swain et al., 1966; Lerner et al., 1972 and Okot-Kotber et al., 2002), proteases (King and Flurkey, 1987 and Espin et al., 1999), fatty acids (Golbeck and Cammarata, 1981), polyamines (Chazarra et al., 1997; Jimenez-Atiener et al., 1991), lipases (Hearing, 1973), organic solvents (Menon and Haberman 1970), light (Mikkesen et al., 1975), alcohols (Asada et al., 1993; Espin and Wichers, 1999b) and pathogen attack (Soler-Rivas et al., 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). 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; Kanade et al., 2006). 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). The degree of latency varies widely with species and plant tissues.

The latency and activation of a 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 leaf 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, 1999a), iceberg lettuce, (Chazzara et al., 1997) table beet (Escribano et al., 1997) and William pear (Gauilard 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, (1999a) 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. Overall latent PPO was activated by numerous factors. A simple pictorial presentation of acid pH and SDS activation is represented in Figure 1.7. Activation of latent PPO by endogenous proteases is by proteolytic digestion of the latent protein (King and Flurkey, 1987; Dry and Robinson, 1994; Laveda et al., 2001) and a protease inhibitor would prevent further PPO activation. In *vivo* carboxy-

terminal processing within the chloroplast appears to be a pre requisite for activation of grape PPO (Dry and Robinson, 1994).

PPO activation with the anionic detergent SDS occurs at low concentrations and higher concentrations inhibit the enzyme (Sanchez-Ferrer, 1993a). SDS concentrations greater than the critical micellar concentration inhibit broad bean leaf PPO (Moore and Flurkey, 1990). A similar trend is noted with PPO from mushroom (Espin and Wichers, 1999a), peach (Laveda et al., 2000) and table beet (Escriban et al., 1997). The degree of SDS activation is dependent on the substrate used (Sanchez-Ferrer, 1993a). The SDS activation caused a shift in the pH optimum from low to higher (Moore and Flurkey, 1990; Jimenez and Garcia-Carmona, 1996; Escribano et al., 1997; Laveda et al., 2000). The SDS-activation is pH dependent and occurs above pH 4.0 due to the existence of a pH sensitive SDS binding center (Jimenez and Garcia-Carmona, 1996). Despite a plethora of studies on latent and active PPO the ratio of these two forms in the natural state is unclear because it is not known whether the isolated enzyme is in its natural form or latent. The endogenous activators, and physiological implications are clearly not known. The degree of latency also is obscure and would differ depending on the plant source, stages of development and the natural activators (Yoruk and Marshall, 2003).

Physiological functions of PPO

The function of PPO is best understood in humans where this enzyme is responsible for the synthesis of melanin which determines the mammalian skin, eye, and hair coloration. It is found in epidermal melanocytes, as well as the pigment epithelia of the retina, iris and ciliary body of the eye (Wang and Hebert, 2006). Absence of melanin biosynthesis, results in oculocutaneous albinism in humans (Witkop, 1984) and an over production of melanin by melanocytes may lead to accumulation of 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).

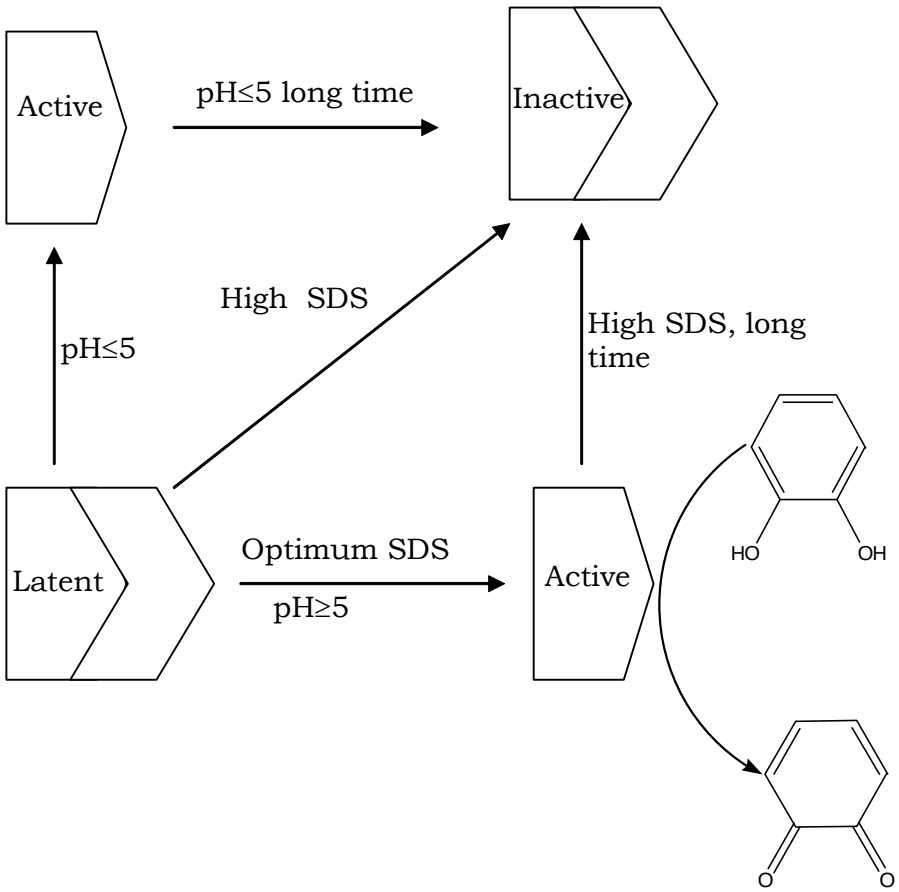

Figure 1.7 Activation of latent PPO by pH and SDS.

The physiological functions of PPO in higher plants are not entirely clear (Mayer, 2006). One of the oldest suggested physiological role of PPO in plants is in the synthesis of diphenols. Strack, (2003) and Gandia-Herrero, et al., (2005c) have reported the involvement of PPO in biosynthesis of betalins. Nakayama, (2002) reported the role of PPO in the biosynthesis of aurone a flavanoid which provides bright yellow color to flowers.

PPOs are antinutritive enzymes that decreases the nutritive value of the wounded plant by cross linking proteins or catalyzing the oxidation of phenolic secondary metabolites to reactive and polymerizing quinones (Kessler and Baldwin, 2002). Thipyapong et al., (2004) reported the role of PPO against the resistance of the plants to the pathogen *Pseudomonas syringae,* by antisense PPO cDNA which makes the plant more susceptible to infection. The role of PPO in plant defense mechanism is clear from another case where PPO over expression was accompanied by enhanced resistance to

the same pathogen (Li and Stefens, 2002). Melo et al., (2006) showed that coffee tree PPO has a defensive role against pathogens and insects to a leaf rust disease. It is assumed that PPO has a general beneficial effect to resistance against herbivores and fungal pathogens (Mayer, 2004).

Lectins

Lectins are a class of proteins of non-immune origin that possess at least one non-catalytic domain that specifically and reversibly binds to mono or oligosaccharide (Lis and Sharon, 1986; Peumans and Van Damme, 1995; Vijayan and Chandra, 1999). A typical lectin is multivalent hence it is able to agglutinate cells. Lectins are extensively distributed in nature and several hundred of these molecules have been isolated so far from plants, viruses, bacteria, invertebrates and vertebrates, including mammals. Because of their binding specificity, they have the capability to serve as recognition molecules within a cell, between cells or between organisms. Last few decades lectins have been investigated in detail owing to the variety of their interesting biological properties including antitumor (Dicko, et al., 2005), anti insect (Singh et al., 2006), antifungal (Ciopraga et al., 1999; Ye et al., 2001), antibacterial (Hatakeyama et al., 2004), anti-HIV (Balzarini et al., 1992; Barrientos et al., 2005; Wong et al., 2006) and mitogenic (Wong and Ng, 2005) activities.

It is assumed that lectins play a fundamental biological role in plants because they are found in many different species and in many different organs and tissues. Typical lectins from different plant sources exhibit a considerable degree of structural similarity. Amino acid sequence homologies exist among the lectins, despite differences in their carbohydrate-binding specificities (Carrington et al., 1972; Foriers et al., 1977; Rouge et al., 1987).

Lectin in plant defense

The role of lectins in plant defense mechanisms has been reviewed (Chrispeels and Raikhel, 1991; Peumans and Van Damme, 1995; Gatehouse et al., 1995). Various plant lectins have shown entomotoxic effects when fed to insects from *Coleoptera*, *Homoptera*, and *Lepidoptera* orders. It is known that the some lectins bind to the brush-border membrane of the insect's intestinal epithelial cells. The chitin-binding lectins bind to the peritrophic

membrane (Chrispeels and Raikhel, 1991; Peumans and Van Damme, 1995). Other possible toxic effect includes binding of lectins to glycosylated digestive enzymes. It has been shown that lectins displaying similar if not identical monosaccharide specificity may have very different effects on closely related insects (Gatehouse et al., 1995; Rahbe et al., 1995).

Multifunctional proteins

Multifunctional proteins are defined by two characteristic properties. Structurally, they consist of a single type of polypeptide chain, but they have multiple catalytic or binding functions. The idea of one gene and one function has become debatable because an increasing number of proteins are found to have two or more different functions (Jeffery, 1999). They represent the phenomenon of one protein–many functions. These multifunctional proteins or chimeras are defined by two or more characteristic properties (Kirschner and Bisswanger, 1976, Ramasarma 1994). Structurally they consist of a single protein, but exhibit multiple catalytic or binding functions. The number of such proteins reported is increasing and complicates protein functional interpretation and yet provides a fascinating window into the complex interactions among the various components that make up a modern cell.

There are several reports on multifunctional proteins. Mung bean lectin shows both α-galactosidase and β-galactosidase activities (Suseelan et al., 1997). The black gram lectin is bifunctional (Suseelan et al., 2004). The galactose specific lectins of *Vigna radiata*, soybean, *Phaseuolus vulgaris* and mung bean exhibit an associated enzymic function, (Hankins and Shannon, 1978; Hankins et al., 1979; Campillo and Shannon, 1982). Horsegram DBL-lipoxygenase is a galactose specific lectin exhibiting lipoxygenase activity (Roopashree et al., 2006). The mannosidase from *Phaseolus vulgaris* possesses lectin properties (Paus and Steen, 1978). Therefore, it is not unreasonable to assume that most if not all galactose specific legume lectins have enzymatic function.

Aim and scope of the present investigation

Although the first PPO, a mushroom tyrosinase was discovered a century and half ago, the first three-dimensional structure of a plant PPO became available only recently (Klabunde et al., 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 glucose specific 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 Native PAGE. 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.

The use of these enzymes in their highly pure state as phytopharmacueticals is limited due to their low thermostability. In addition control of enzymatic browning is a prerequisite to prevent quality loss during post harvest handling and processing. Over many years sulfites were used, to prevent browning effectively and economically. However, because of the adverse health effects imparted by the chemical inhibitors and increased awareness in the life styles related to food and health, there is an increased demand for more natural foods and safer added chemicals in processed foods. Hence alternate methods to prevent browning need to be investigated through an understanding of the structure and mechanism of action of the oxidase at the molecular level.

PPO has been isolated from a variety of sources. With the advent of recombinant DNA technology, cDNA derived amino acid sequence from tomato, potato, broad bean leaf and bacteria have become available recently. However the first and only three-dimensional structure of a PPO available to date, is that from *Ipomea batatas.* The PPO of field bean seeds has been isolated and exists as a single isoform of molecular weight ~120,000 Da. This PPO existing as a single form is ideally suited for understanding and correlating at the molecular level, the relationships between its structure-function and stability. Therefore in the present investigation a study at the molecular level by determination of primary structure and gene sequence

was pursued. The sequence analysis, kinetics and mechanism of activation/inactivation were studied. The main objectives of the present investigation are:

- Characterization of the polyphenol oxidase sequence from field bean.
- Studies on the kinetics, stability and mechanism of activation /inactivation
- Studies on the expression of the polyphenol oxidase in (*Dolichos lablab)* and its characterization.

It is expected that these studies will lead to a better understanding of the structure-function relationship of PPOs. The molecular level studies could eventually provide a platform for the design of tailored inhibitors to prevent enzymatic browning.

Chapter II

Materials and methods

2. MATERIALS AND METHODS

2. 1. MATERIALS

2. 1. 1. Chemicals

Catechol, diethyl pyrocarbonate (DEPC), ethidiumbromide, formamide, formaldehyde, deoxynucleotide mixture (dNTPs), 4-methyl catechol (4MC), L-3,4-dihydroxyphenylalanine (DOPA), gallic acid, caffeic acid, vanillic acid, *p*-phenylenediamine, polyvinylpolypyrrolidone (PVPP), tropolone, DEAE-sepharose, Phenyl sepharose, pyrogallol, 3(p-hydroxyphenyl) propionoic acid, bovine serum albumin (BSA), tris (hydroxy methyl) amino methane (Trizma base), acrylamide, N,N′-methylenebis-acrylamide (BIS), coomassie brilliant blue R-250, bromophenol blue, tris saturated phenol, triethylamine (TEA), N,N,N′,N′-tetramethyl 1,2-diaminoethane (TEMED), pentafluoro propionic acid (PFPA), TPCK-trypsin, glutaraldehyde, sodium deoxycholate, glycine methyl ester hydrochloride (GME), N-(3-dimethylaminopropyl) N'-ethylcarbodiimide hydrochloride (EDAC), urea, iodoacetamide, 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS), β-mercaptoethanol, galactose, N-acetyl galactosamine, α-D-galactosamine hydrochloride, analytical gel filtration molecular weight markers, agarose gel extraction kit, mushroom tyrosinase and primers were obtained from Sigma Chemical Co., St. Louis, MO, USA.

Water saturated phenol, DNA Taq polymerase, goat anti-rabbit IgG alkaline phosphatase conjugate, 5-bromo-4-chloro-3-indolylphosphate (BCIP) / nitroblue tetrazolium (NBT), substrate for alkaline phosphatase, and SDS-PAGE molecular weight marker were purchased from Bangalore Genei Pvt. Ltd., Bangalore, India.

Restriction enzymes and T_4 DNA ligase were procured from New England Biolabs, Inc. Beverly MA USA

M-MLV Reverse transcriptase was obtained from USB Corporation Cleveland, Ohio USA.

DNA ladder and 6 × DNA loading dye were purchased from Fermentas Inc. Canada.

pRSET C Vector was obtained from Invitrogen Life Technologies USA.

Tertiary butyl catechol (TBC), 4-phenyl butyric acid and 3-phenyl propionic acids were purchased from E. Merck, Gmbh. Germany.

Sequanal grade sodium dodecyl sulfate (SDS) was obtained from Pierce Chemical Co, USA.

2,3,4-Trihydroxy benzoic acid, 3,4-dihydroxy cinnamic acid, 2,4-dihydroxy benzoic acid and 3,4,5-trihydroxy benzaldehyde were obtained from Aldrich Chemical Co. WI, USA.

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

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

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

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

Dopamine hydrochloride, agar, agarose, bacto-yeast extract, bacto-tryptone, ammonium per sulfate, glycine, calcium chloride, magnesium chloride, manganese chloride, glucose and sodium acetate were 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.

High performance liquid chromatographic (HPLC) grade solvents were obtained form Spectrochem Pvt. Ltd., India.

Sephadex G-150 and Blue dextran-2000 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden.

All other chemicals used were of analytical grade.

2. 1. 2. HPLC columns: Progel™-TSK G2000 SW_{XL} (7.8 mm id × 30 cm) was obtained from Supelco, Sigma-Aldrich (India), Bangalore, India. Waters

Symmetry Shield C_{18} column (4.6 x 150 mm, 5 μm) was from Waters Corporation MA, USA. Discovery ® Biowidepore C8-5 Supelco (4.6 mm × 25 cm, 5 μm) was from Supelco, Supelco Park, Bellefonte, PA, USA.

2. 2. METHODS

2. 2. 1. Field bean powder

Field bean seeds were purchased from the local market. The seeds were dehulled and powdered to a mesh size of 60-80. Powder was defatted with carbon tetrachloride in the ratio of 1:5 (w/v) by gently mixing at room temperature for 12 h. The suspension was filtered using Whatman No.1 filter paper. The defatted powder was air-dried at room temperature and stored at 4 °C.

2. 2. 2. Collection of plant tissues

2. 2. 2a. Germination of seeds and preparation of extracts: Field bean seeds (5 g) aliquots were imbibed in distilled water for 24 h at 25±2 °C. The seeds were rinsed with distilled water. The seeds were allowed to germinate on a moist filter paper in the dark. After the desired periods of germination the seeds were rinsed with distilled water and cotyledons dissected free of the seed coat and axes and frozen at –20 °C until used. Germination time was reckoned from the beginning of imbibition. Enzyme was extracted for 16 h at 4 °C with 1:5 ratio of seeds to buffer (w/v) in 100 mM Tris-HCl buffer, pH 7.0 containing 2 % (w/v) PVPP and 1.2 % NaCl (w/v). The extract was centrifuged at 15000 rpm for 45 min at 4 °C. The supernatant served as the crude extract.

2. 2. 2b. Seed Development: Collection of tissue: The flowers were tagged on the day of opening. The pods were harvested 5, 10, 15, 20 and 40 days after flowering (DAF). The vegetative pods along with the seeds were used upto 10 DAF. Seeds were separated from pods of 15, 20 and 40 DAF. Leaves were removed from the mature plant. The seeds and leaves were frozen at –20 °C until used. Extract was prepared as explained above.

2. 2. 3. Enzyme extraction from dried field bean flour

The dried defatted field bean seed powder (20 g) was extracted for 16 h at 4 °C with 100 mL of 100 mM Tris-HCl buffer, pH 7.0 containing 2 % (w/v) PVPP and 1.2 % NaCl (w/v). The extract was centrifuged at 15000 rpm for 45 min at 4 °C. The supernatant served as the crude extract. To the supernatant solid $(NH_4)_2SO_4$ (22.6 g/100 mL) was added to obtain 40 % saturation. The precipitated protein was removed by centrifuging at 15000 rpm for 45 min at 4 °C and discarded. Solid $(NH_4)_2SO_4$ (25.8 g/100 mL) was added slowly to the supernatant at 4 °C to obtain 80 % saturation and allowed to stand at 4 °C overnight and centrifuged at 15000 rpm for 45 min at 4 °C. The precipitate thus obtained was redissolved in 0.1 M Tris-HCl buffer pH 8.2 containing 1.2 % NaCl.

2. 2. 4. Protein purification matrices

2. 2. 4a. Preparation of DEAE-sepharose: DEAE-sepharose was equilibrated in 10 mM Tris-HCl buffer, pH 8.2 containing 1.2 % NaCl (w/v) for 24 h. The DEAE-Sepharose was packed in a glass column with dimensions 3.5 × 12 cm under gravity. The column was washed with the equilibrating buffer until the pH of the eluent was 8.2.

In our experiments, field bean PPO $(NH_4)_2SO_4$ (40-80 %) fraction was allowed to flow through the column as most of the contaminants bound to the column. The unbound protein eluting in the wash was collected and used for the next step. Following elution the column was regenerated as described by the manufacturer.

2. 2. 4b. Preparation of phenyl sepharose column: Phenyl sepharose purchased from Sigma Chemical Co. was supplied as a suspension in 0.5 M NaCl containing 0.02 % thimerosal. The phenyl sepharose was washed and equilibrated in 25 mM Tris-HCl buffer, pH 7.0 containing 1 M $(NH_4)_2SO_4$ and 1.2 % NaCl (w/v). The medium was packed in a glass column of dimensions, 3.5 × 11.5 cm at a flow rate of 40 mL/h. PPO purification was performed according to Paul and Gowda, (2000).

2. 2. 4c. Preparation of Sephadex G-150: Fifteen grams of Sephadex G-150 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. Sephadex G-150 has a particle size of 40-120 μm, which gives a bed volume of 15-20 mL per gram of dry gel. The exclusion limit of Sephadex G-150 is 5000 Da-300000 Da for globular proteins. After swelling of the gel, the slurry was packed into a glass column (100 × 2 cm) at a flow rate of 18 mL/h. Size exclusion chromatography on sephadex G-150 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. 4d. Preparation of galactose sepharose: Galactose sepharose was prepared according to the protocol of Siva Kumar and Rajagopal, (1986). Sepharose 6B (25 mL) was washed over a sintered glass with distilled water (10 × 100 mL) and suspended in 0.5 M Na_2CO_3 buffer pH 11.0 and protected from light. Divinyl sulfone (2.5 mL) was added with continuous stirring and allowed to mix end on for 10 min. The gel was washed over sintered glass to remove unreacted divinyl sulfone. The gel was resuspended in 50 mL of galactose (20 % w/v) in 0.5 M Na_2CO_3 buffer, pH 10.0 and allowed to bind for 72 h at 4 °C. The sepharose was washed thoroughly with distilled water followed by 0.5 M Na_2CO_3 buffer pH 8.5. To the resuspended gel 2.5 mL of β-mercaptoethanol was added with continuous stirring and incubated at 25±2 °C for 3 h. Finally the gel was washed thoroughly with distilled water to remove excess β-mercaptoethanol followed by two washes with TBS buffer. The prepared galactose sepharose was stored in TBS at 4 °C.

2. 2. 5. Enzyme assay

2. 2. 5a. PPO assay: PPO was assayed according to the spectrophotometric method of Cosetang and Lee (1987) at 27±1 °C using a Shimadzu UV-Visible spectrophotometer Model 1601 at 400 nm with TBC. The assay mixture consisted of 0.9 mL of 0.05 M sodium acetate buffer, pH 4.5, 0.1 mL of 0.04 M TBC and 10-100 μg of enzyme. The quinone formed was measured at 400 nm (ε_{400} = 1150 $M^{-1}cm^{-1}$). One unit of enzyme activity is defined as the amount of enzyme that produce one μmol of *tert*-butylquinone per min under the assay conditions.

2. 2. 5b. Catechol oxidase assay (ibCO): Sweet potato catechol oxidase assay was performed using catechol as a substrate in 0.1 M NaPi buffer, pH 6.8 containing 1 mM MBTH. The quinone-MBTH adduct was monitored at 500 nm (ε_{400} = 32500 $M^{-1}cm^{-1}$). One unit of enzyme activity is defined as the amount of enzyme that produced one μmol of quinone-MBTH adduct per min under the assay condition.

2. 2. 5c. α-Galactosidase activity: The activity of α-galactosidase was assayed using *p*-nitrophenyl α-D galactopyranoside (PNPG). The assay mixture contained 20-100 μg protein in 1 mL of 3 mM PNPG in 0.1 M sodium acetate buffer, pH 4.7. The mixture was incubated at 37 °C for 10 min. The reaction was stopped by adding 1 mL of 5 % Na_2CO_3. The released *p*-nitrophenol (PNP) was measured at 405 nm. The molar extinction coefficient of PNP used was 18300 at 405 nm. One unit of enzyme activity is defined as the amount of enzyme which releases 1μmole of PNP per min per mL at 37 °C.

2. 2. 5d. Hemagglutinating activity: Hemagglutinating activity was measured using 2 % tryptinized human erythrocytes suspension in phosphate buffer (PBS). Two fold serial dilutions of 200 μL of lectin solution (10-50 μg) in PBS pH 7.4 was incubated with 200 μL of 2 % tryptinized erythrocyte suspension in a micro titer plate for 60 min at 37 °C. One haemagglutination unit (HAU) is defined as the lowest concentration of lectin giving visible erythrocyte agglutination. The sugar inhibition assays were carried using stock solutions of the sugars dissolved in 0.9 % NaCl. To each well 0.1-10.0 mM of sugar solution and 4 HAU of lectin in a total volume of 200 μL was added and incubated for 1 h at 25±2 °C. Later 200 μL of a 2 % suspension of tryptinized human erythrocytes was added and incubated for 1 h at 37 °C. The lowest concentration of sugar which inhibits agglutination was taken as the minimum inhibitory concentration (MIC) of the sugar.

2. 2. 6. 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. 7. Carbohydrate estimation

Total neutral sugar was determined colorimetrically by the phenol/sulfuric acid method (Dubios et al., 1956) using glucose as standard.

2. 2. 8. Substrate specificity of PPO

Tertiary butyl catechol (TBC), catechol, 4-methyl catechol (4MC), DOPA, dopamine, 3,4-dihydroxy phenyl acetic acid, 2,3,4-trihydroxy benzoic acid, 2,3,4-trihydroxy benzaldehyde, gallic acid, and pyrogallol 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. The values for K_m and V_{max} were obtained by evaluation of Lineweaver-Burk plots (Lineweaver and Burk, 1934) of the kinetic measurements.

2. 2. 9. Effect of inhibitors on PPO activity

Inhibitors were evaluated for their effectiveness of inhibiting PPO activity using TBC as the substrate. The enzyme (10-100 μg) was pre-incubated with inhibitor in 50 mM sodium acetate buffer pH 4.5, for 3 min in the cuvette. The reaction was started by adding the substrate to the reaction mixture. The increase in absorbance at 400 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_i was determined from the Dixon plot (Dixon, 1942) where 1/V vs [I] was plotted at different substrate concentrations.

2. 2. 10. Effect of pH on PPO activity

PPO activity as a function of pH was determined using TBC, DOPA, dopamine, and DOPAC as substrates. The buffers used were McIlvaine (0.1 M citric acid-0.2 M Na_2HPO_4, pH 2.5-7.5) and Tris-HCl (pH 8.0 and 8.5).

2. 2. 11. Effect of pH on α-galactosidase activity

The effect of pH on α-galactosidase activity was studied using 3 mM PNPG as substrate at 37 °C for 10 min. The concentration of all the buffers used was 0.1 M. The buffers used were glycine-HCl for pH 3.0, sodium

acetate for pH 4.0-5.0, NaPi for pH 6.0-6.5 and Tris-HCl for pH 7.0. All the assays were carried out in triplicate.

2. 2. 12. Polyacrylamide gel electrophoresis (PAGE)

Vertical slab gel electrophoresis was carried out on a Broviga mini slab gel electrophoresis unit, at 25±2 °C.

2. 2. 12a. SDS-Polyacrylamide gel electrophoresis (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. 30 % Acrylamide: Acrylamide (29.2 g) and bisacrylamide (0.8 g) were dissolved in water (100 mL), filtered and stored in a dark brown bottle at 4 °C.

B. 4× Separating gel buffer (1.5 M, pH 8.8): Tris (18.15 g), was dissolved in water, the pH of the solution was adjusted to 8.8 with HCl (6 N), the volume made up to 100 mL and stored at 4 °C.

C. 4× Stacking gel buffer: (0.5 M, pH 6.8): Tris (6 g) was dissolved in water. The pH of the solution was adjusted to 6.8 with HCl (6 N), volume made up to 100 mL with water and stored at 4 °C.

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

E. 10 % Ammonium persulfate: was freshly prepared by dissolving 50 mg in 0.5 mL of distilled water.

F. 10× Tank buffer: (0.25 M Tris, 1.92 M Glycine): Tris (3.0 g), glycine (14.41 g) were dissolved in 100 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 25±2 °C.

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

I. 2× Sample buffer: It was prepared in solution C diluted 1:4, containing SDS (4 % w/v), β mercaptoethanol (10 % v/v), glycerol (20 % v/v) and bromophenol blue (0.1 % w/v).

Preparation of separating and stacking gel: The contents of separating gel (Table 2.1) were mixed, degassed and poured between the assembled glass

plates with edges sealed with agar (2 % w/v). The gel was layered with 0.5 mL of distilled water and allowed to polymerize at 25±2 °C for 30 min. The contents of stacking gel were mixed and poured above the polymerized separating 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) 1 × in sample buffer. The samples were heated in a boiling water bath for 5 min. Cooled samples were loaded into the wells immersed in 1× solution tank buffer (25 mM Tris, 192 mM glycine and 0.1 % SDS) and run at constant voltage (60 V) for 3-4 h or until the tracking dye, reached the anode tank buffer. Medium range protein Mr markers, phosphorylase b (97400 Da), BSA (66300 Da), ovalbumin (43000 Da), carbonic anhydrase (29000 Da), soybean trypsin inhibitor (20100 Da) and lysozyme (14300 Da) were used. The markers were supplied as a solution having each protein at a concentration of 2.5 mg/mL. The markers were diluted 1:1 with solution I and boiled prior to use.

Staining: The gels were stained for protein with reagent G for 6 h at 25±2 °C. and destained in reagent H.

2. 2. 12b. Native PAGE (Non-denaturing)

Polyacrylamide gel electrophoresis under non-denaturing condition 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 shown in Table 2.2.

Sample buffer was prepared as explained earlier minus SDS and β-mercaptoethanol. For Native-PAGE containing SDS, sample buffer was prepared as mentioned earlier minus β-mercaptoethanol. Tank buffer (solution F) was prepared minus SDS. About 5-20 μg of protein was mixed with an equal volume of sample buffer and loaded to the gel. After

electrophoresis at constant voltage (100 V) proteins were visualized using Coomassie brilliant blue R-250.

Table 2. 1 Preparation of separating gel and stacking gel.

Solution	Separating gel (mL)		Stacking gel (mL)
	(12.5% T, 2.7% C)	(10% T, 2.7% C)	(5% T, 2.7% C)
Solution A	3.33	2.66	0.83
Solution B	2.00	2.00	-
Solution C	-	-	1.25
Distilled water	2.55	3.22	3.03
Solution D	0.08	0.06	0.05
TEMED	0.01	0.01	0.01
Solution E	0.03	0.03	0.03
Total	8.00	8.00	5.00

Table 2. 2 Preparation of Native-PAGE gels

Solution	Separating gel (mL)	Separating gel with SDS (mL)
	(7.5% T, 2.7% C)	(7.5% T, 2.7% C)
Solution A	2.00	2.00
Solution B	2.00	2.00
Distilled water	3.96	3.88
Solution D	-	0.08
TEMED	0.01	0.01
Solution E	0.03	0.03
Total	8.00	8.00

2. 2. 12c. PPO activity staining: PPO was visualized by two methods, Following Native-PAGE. 1) The gel was incubated in assay buffer for 5 min. Catechol was added to achieve a final concentration of 0.05 M followed by 2 mM MBTH in 0.05 M sodium acetate buffer pH 4.0. The appearance of pink bands against a transparent background indicated the presence of PPO.

2) After incubation in assay buffer (sodium acetate pH 4.5, 0.1 M) TBC was added to obtain a final concentration of 40 mM followed by 5 mM of *p*-phenylenediamine in 0.05 M sodium acetate buffer pH 4.5. The appearance of purple-blue bands against a clear background indicated the presence of

PPO.

2. 2. 13. Erythrocytes haemagglutinin overlay assay

The erythrocyte overlay for assay of haemagglutination was performed as described by Souza et al., (2005). The PPO-haemagglutinin was separated by native PAGE (7.5 %T, 2.7 % C) and the protein was transferred to a nitrocellulose membrane using a semi dry system operating at 0.8 mA/cm^2 for 2 h. The membrane was incubated in TBS containing 1 % Triton X-100 for 1 h at 25±2 ºC. The membrane was washed three times with TBS followed by incubation in TBS containing 1 % BSA (TBS-BSA) for 1 h at 25±2 ºC. The membrane was finally incubated with human erythrocytes suspension (2 % in TBS-BSA) for 1 h at 25±2 ºC with gentle shaking. The membrane was fixed for 10 min in 3 % formalin in TBS. The lectin band was visible by the bound erythrocytes.

To detect PPO, the membrane with the bound erythrocytes was washed once with TBS to remove formalin and subsequently incubated with TBS for 30 min at 25±2 ºC. The membrane was stained for PPO activity using catechol and MBTH as described above.

2. 2. 14. Glycoprotein staining

This stain was used to identify the presence of glycoproteins. The periodic acid-Schiff's staining was carried out following the method of Robert et al., (1969) and Kapitany and Zebrowski, (1973). After electrophoresis the gel was immersed in 12.5 % TCA (w/v) for 30 min, then rinsed lightly with distilled water for 15 sec, and incubated in 1 % periodic acid in 3 % acetic acid for 30 min. The gel was washed with distilled water (6 × 50 mL) 10 min each. The washed gel was immersed in Schiff"s reagent and then allowed to develop color in the dark at 4 °C. The dark pink color appeared in about 50 min. Freshly prepared 0.5 % sodium metabisulfite (50 mL) was added to the gel and washed. Finally the gel was incubated in water overnight and stored in 3 % acetic acid. Glycoproteins appeared as pink color bands in the stained gel. Ovalbumin was used as the positive control.

2. 2. 15. Isoelectric focusing

Reagents

Anode solution: Orthophosphoric acid, 34.0 mL of (88-93 %) H_3PO_4 was diluted to 500 mL with distilled water.

Cathode solution: Sodium hydroxide solution; 4 g of NaOH was dissolved in 100 mL of distilled water.

Fixing solution: 10 % tricholroacetic acid (w/v).

Equilibration solution: Aqueous solution of 25 % methanol, 5 % acetic acid (v/v).

Staining solution: 0.1 % (w/v) Coomassie Brilliant Blue G-250 in an aqueous solution of 25 % methanol and 5 % acetic acid (v/v).

Destaining solution: 25 % methanol, 5 % acetic acid (v/v).

Pre-cast ampholine PAG gel of dimensions 245 × 110 × 1 mm, pH range 3.5-9.5, 5 % T, 3 % C, was used. The PAG gel was placed on a pre cooled (10 °C) Multiphor plate, avoiding trapping of air bubbles. With the help of gel loading strips, the samples (10-15 µg) and pI markers were loaded. The anode and cathode buffer strips were pre-equilibrated in anode and cathode solutions separately. The wet buffer strips were carefully placed at the anode and cathode ends of the gel. The electrodes were arranged to come in contact with the buffer strips. The proteins were allowed to focus for 90 min at 1500V, for 1 h. At the end of the run the gel was fixed in fixing solution for 1 h. Then the gel was washed in destaining solution and then immersed in staining solution, preheated to 60 °C for 30 min. The gel was destained using several change of destaining solution.

2. 2. 16. Molecular weight determination by Matrix Assisted Laser Desorption Ionization-Time of flight (MALDI-TOF)

The exact molecular mass of the PPO was obtained by MALDI-TOF; (Bruker Daltanics, Germany model Ultra Flex TOF-TOF, equipped with the linear reflector). The instrument was calibrated over the high mass range of 10000-150000 Da.

2. 2. 17. Metal ion analysis

The copper and manganese content of exhaustively dialyzed and lyophilized protein sample was measured by atomic absorption spectrometry. Protein (10 mg) was refluxed in 2 mL of concentrated HNO_3 for 2 h and cooled. The volume was adjusted to 10 mL using high purity water exhibiting an electrical resistance of 18-mega-ohm cm^{-1} (Millipore Corp.). The copper (Inorganic Ventures Inc.) and manganese (E. Merck Germany), atomic absorption standards were diluted appropriately. An AA6701F Atomic

absorption flame spectrophotometer (Shimadzu Inc. Japan) was employed using the following parameters: 324.8 nm wave length for copper and 279.5 nm wave length for manganese with a 0.2 nm slit width and air–acetylene flame with a 10 cm slot burner. The absorption was recorded. Quantity of Cu^{2+} and Mn^{2+} present was determined from the calibration curve of peak height versus the standard concentration. Experimental values were corrected for background levels of copper and manganese in the controls without protein.

2. 2. 18. HPLC: Size exclusion chromatography

Stokes' radii measurements were carried out on a TSK gel G2000 SW_{XL} (7.8 mm × 30 cm, 5 μM) column, using a Waters HPLC system, equipped with a 1525 binary pump and Waters 2996-photodiode-array detector. The column was pre-equilibrated with 0.1 M NaPi buffer, pH 7.0 containing 0.1 M Na_2SO_4 at a flow rate of 0.5 mL/min. A set of proteins (thyroglobulin 660 000 Da, 79.9 Å; BSA 66000 Da, 33.9 Å; carbonic anhydrase 29000 Da, 21.2 Å; cytochrome C 14300 Da, 17.0 Å) whose Mr and Stokes' radii are known were used to construct the calibration curve of log Rs *versus* migration rate.

2. 2. 19. Automated gas phase protein sequencing

The amino-terminal sequence was determined by Edman degradation using an Applied Biosystems 477A automated gas phase protein sequencer. This sequenator carries out Edman-degradation by supplying gaseous reagents for the coupling and cleavage reactions. The flow diagram for the sequence of events is shown in Figure 2.1. The protein or peptide was spotted on a glass fiber disc previously coated with polybrene and washed for three cycles. Alternatively protein electro blotted (Section 2.20) was directly used for the pulsed gas phase sequencing. The coupling reaction is carried out with phenyl isothiocynate (R1) in the presence of gaseous methyl piperidine (R2). Excess of reagents and by products are washed with *n*-heptane (S1) and ethyl acetate (S2). The cleavage reaction is carried out with the gaseous TFA to form an aniline-thiazolinone (ATZ) derivative. Both the coupling and cleavage reactions are performed in a temperature controlled reaction chamber. The free ATZ-amino acid extracted from conversion flask by n-butyl chloride (S3) is converted to the more stable PTH-amino acid by

reaction with 25 % TFA (R4). The PTH-amino acid dissolved in acetonitrile (S4) is automatically injected into the HPLC. The PTH-amino acids are separated by RP-HPLC and detected at 269 nm. The PTH-amino acid in each cycle is identified, quantified and recovery percentage was calculated using the Seq 2.2 programme. The results are displayed and recorded. Figure 2.2 represents the separation of the standard PTH-amino acid mixture. β-lactoglobulin was used for the performance check of the instrument.

2. 2. 20. Electroblotting of proteins

Preparation of PVDF membrane: The PVDF membrane cut to the required size (slightly larger than the gel) was soaked in methanol for 5 min before use. (Matsudaira, 1987; Speicher, 1989).

Blotting: Following electrophoresis, the gel was immediately rinsed in transfer buffer (10 mM CAPS, pH 11.0 containing 10 % methanol (v/v) and 0.1 % SDS (w/v) for 15 min. Semi-dry electroblotting was carried out using a semi-dry blotting apparatus (Towbin et al., 1979). The transfer was carried out for 2 h using a current of 0.8 mA/cm^2 of the blotting paper. The membranes were stained with coomassie blue for protein sequencing or probed with antibodies as described later (Section 2.2.21).

Destaining: The PVDF membrane was destained in 50 % methanol. For amino-terminal sequence the corresponding bands were excised, washed with 100 % methanol and dried

2. 2. 21. Dot blot analysis

PPO was immobilized on a nitrocellulose membrane by repeated application employing a current of hot dry air to accelerate the drying until the required amount of protein was immobilized. The blot was then subjected to immuno detection as described (Section 2.2.22).

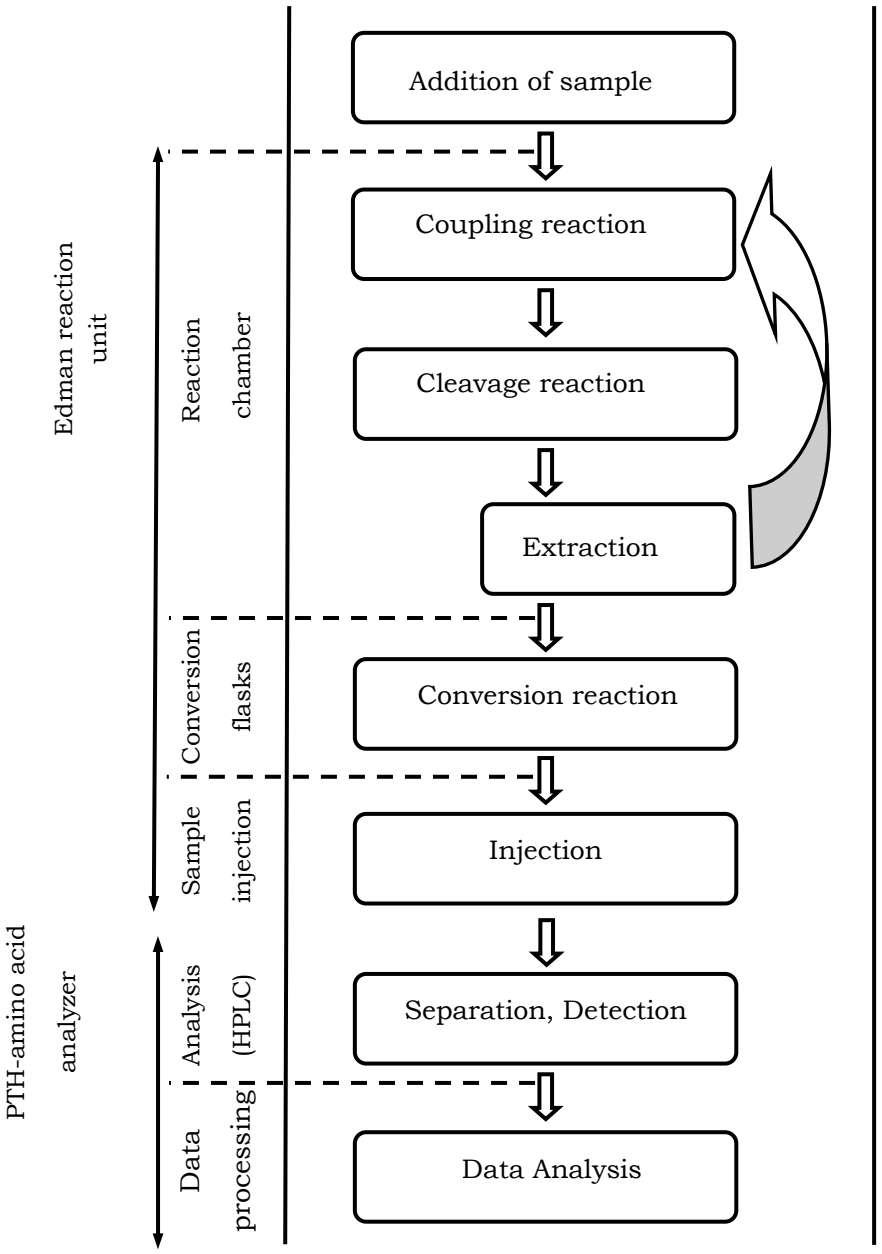

Figure 2.1. Flow diagram of the reactions that occur during gas phase sequencing of protein or peptide on ABI-477A (Applied Biosystem) sequenator.

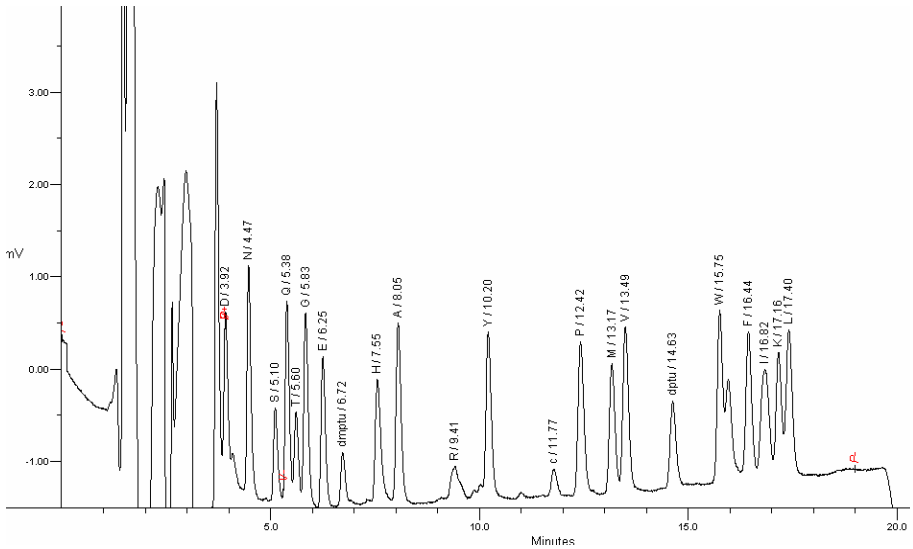

Figure 2.2. RP-HPLC separation of PTH-amino acids standards on the automated protein sequenator

2. 2. 22. Immunodetection of PPO

Following immobilization or electro-transfer, the membrane was washed with immunoblot buffer (5 % skimmed milk powder in PBS, pH 7.0) four times (30 min each wash). The membrane was incubated overnight at 4 °C in immunoblot buffer containing antibodies raised against PPO (1:1000 dilution). After repeated washes (4 × 30 min) in the immunoblot buffer, the membrane was incubated with the secondary antibody, alkaline phosphatase conjugated goat anti-rabbit immunoglobulins, for 2 h at 25±2 °C. After four washes (4 ×10 min) in immunoblot buffer and a final wash with substrate buffer (0.1 M Tris, 0.5 M NaCl, 5 mM $MgCl_2$, pH 9.5), alkaline phosphatase activity was detected with a mixture of BCIP and NBT in substrate buffer. Alternatively when a HRP conjugate was used the HRP activity was detected using benzamidine.

2. 2. 23. SDS and acid pH activation of PPO

PPO was pre-incubated at various concentrations of SDS prepared in 25 mM Tris-HCl (pH 7.0) containing 1.2 % (w/v) NaCl for 30 min, following which activity was assayed at pH 6.0 (Section 2.2.5a). Relative activity was plotted against SDS concentration. Acid-pH activation of PPO was performed by pre-incubating PPO in McIlvaine buffer (0.1 M citric acid and 0.2 M

disodium phosphate, pH 2.5 or 4.0). PPO activity was measured at pH 6.0 periodically as described (Section 2.2.5a).

2. 2. 24. Urea activation of PPO

PPO was incubated at varying concentrations of urea in 25 mM Tris-HCl, pH 6.0 containing 1.2 % NaCl. Following which the PPO activity was assayed at pH 6.0 as described in Section 2.2.5a.

2. 2. 25. Circular dichroism studies

Circular dichroism measurements were carried out using a Jasco J-810 automatic recording spectropolarimeter fitted with a xenon lamp and calibrated with +d-10-camphor sulfonic acid. Dry nitrogen was purged continuously before and during the experiment. The measurements were made at 25 °C. The path length of the cell used was 1 mm in the far UV region and 10 mm in near UV region. The scan speed was 20 nm/min and spectra were taken as an average of three scans. The results were expressed as the mean residue ellipticity $[\theta]_{MRW}$, obtained from the relation $[\theta] = 100 \times \theta_{obs} / (lc)$, where θ_{obs} is the observed ellipticity in degrees. The mean molar ellipticity $[\theta]_{MRW}$ was calculated using a value of 115 for mean residue mass of PPO, c is the concentration in grams per liter and l is the length of the light path in cms. The values obtained were normalized by subtracting the baseline recorded for the buffer under similar conditions.

2. 2. 26. Fluorescence studies

2. 2. 26a. Intrinsic fluorescence: Fluorescence measurements of PPO were carried out at 27 °C using a Shimadzu (Model RF 5000) recording spectrofluorimeter in a 3 mL cuvette with a path length of 1 cm. Protein was excited at 280 and 295 nm and emission was recorded between 300 and 400 nm. Appropriate blanks were used for baseline correction of fluorescence intensity.

2. 2. 26b. Fluorescence quenching by acrylamide: The quenching of enzyme fluorescence with the progressive addition of a dynamic quencher like acrylamide was measured for native and SDS activated PPO in increasing concentrations of acrylamide. The fluorescence intensity was recorded at 334 nm with excitation at 295 nm. The fractional quenching

(F_0/F) was plotted against acrylamide concentration, where F_0 and F represent the fluorescence in the absence and presence of acrylamide respectively. Fluorescence intensities were corrected for dilution effects. The absorption of acrylamide at 295 nm was corrected using the equation.

$$F_{corr}=F_{abs}\ 10^{A/2}$$ (Lehrer and Leavis, 1978)

A is the increase in the absorbance by the addition of acrylamide.

Fluorescence quenching data were analyzed using the general form of Stern-Volmer equation

$$F_0/F = I + K_{sv}\ (Q)$$

Where Q is quencher concentration, K_{sv} is dynamic quenching constant.

To monitor conformational changes, fluorescence quenching data were also analyzed with modified Stern-Volmer equation (Lehrer, 1971)

$$F_0/\triangle F = 1/f_a\ K_{sv}\ (Q) + 1/f_a$$

Where $\triangle F = F_0 - F$

f_a is maximum fractional accessible protein fluorescence.

2. 2. 27. Thermal inactivation studies

The loss of enzyme activity as a function of temperature was studied for the native and activated forms of PPO. Native PPO and the activated forms were incubated for 15 min in their respective buffers at different temperatures ranging from 25 to 90 °C. After cooling to 4 °C, the residual PPO activity was measured at 25±1 °C as described (Section 2.2.5a). The midpoint of thermal inactivation, T_m, at which the activity was diminished by 50 %, was calculated from the plot of percent residual activity *versus* temperature.

Kinetics of thermal inactivation of native, SDS-activated and acid-pH activated PPO were studied, using a constant temperature bath, at desired temperatures. Aliquots of enzyme, removed at periodic intervals were subjected to assay after cooling in an ice bath. The residual PPO activity was measured as a percentage of initial activity. From the semi logarithmic plot of residual activity as a function of time, the inactivation rate constants (k_r) were calculated. The apparent half-lives were estimated and energy of activation (E_a) calculated from the slopes of the Arrhenius plots. Thermodynamic functions were calculated according to the following relationships:

$$\Delta H^* = E_a - RT$$

$$\Delta G^* = -RT \ln (k_r)\, h / K_B T$$

$$\Delta S^* = (\Delta H^* - \Delta G^*) / T$$

(h is Plancks constant, K_B is Boltzmann constant, R is universal gas constant and k_r is rate constant.)

2. 2. 28. Determination of activation energy (E_a)

Activation energy was determined by evaluating the K_m and V_{max} of PPO, at different temperatures using TBC as the substrate. The assay was carried out using a Shimadzu UV-Visible spectrophotometer 1240 mini model equipped with a TCC-240 temperature controlled cell holder. A plot of log (V_{max}/K_m) against 1/T was used to calculate E_a from the following relationship.

$$Ea = -2.303 \times slope \times R$$

Ea =Activation energy,

$R = 1.987$ cal/mol · K

2. 2. 29. Limited proteolysis

Native and activated forms of PPO were subjected to limited proteolysis using TPCK–trypsin (2:100, w/w) at pH 8.2 for 60 min at 37 °C. The proteolysis was arrested by incubating the reaction mixture for 20 min in a boiling-water bath and then freeze-dried. The tryptic digest was analyzed by RP-HPLC using a Waters Symmetry Shield C_{18} column (4.6 mm×150 mm; 5 μm) on a Waters HPLC system equipped with a 1525 binary pump and Waters 2996 photodiode array detector in a water/acetonitrile gradient containing 0.1 % PFPA. The peptides were monitored at 230 nm.

2. 2. 30. Cross-linking of PPO using glutaraldehyde

Native and activated forms of PPO were subjected to cross linking to study the quaternary structural changes during activation. PPO was cross-linked according to the method of Akhtar and Bhakuni (2003). Glutaraldehyde (25 % w/v) was added to aliquots of the both native and activated PPO (8 μg) to a final concentration of 10 % and incubated at 27 °C for 5 min. Quenching of cross-linking was achieved by addition of glycine to a final concentration of 97 mM. After 20 min incubation, 3 μL of 10 % aqueous sodium deoxycholate was added. The pH of the reaction was

lowered to ~ 2.5 by the addition of orthophosphoric acid (85 %), which resulted in precipitation of the cross-linked protein. After centrifugation (10000 rpm, 4 °C, 20 min) the precipitate was re-dissolved in 0.1 M Tris-HCl, pH 8.0 containing 1 % SDS and 50 mM dithiothreitol and then heated to 90-100 °C. Samples were analyzed by SDS-PAGE (10 % T, 2.7 % C) as described (Section 2.2.12a).

2. 2. 31. Chemical modification of carboxyl residues

The importance of the carboxylate group of glutamate in the activity of PPO was investigated by reaction with EDAC. The carboxyl residue of PPO was modified according to the method of Hoare and Koshland (1967). PPO was coupled to glycine methyl ester using N- (3-dimethyl amino propyl) N'-ethylcarbidiimide hydrochloride. To 5 mL (1.3 mg/mL) of PPO solution, 350 mg of glycine methyl ester hydrochloride and 85 mg of the carbodiimide were added. The pH was adjusted to 4.75 and the reaction was allowed to proceed for 10 min. The mixture was desalted and concentrated using a 10000 Da cut off centrifugal filter device. PPO and ibCO activity were determined as described earlier (Section 2.2.5a).

Kinetics of carboxyl residue modification: Carboxyl residue modifications were performed at 25 °C with the field bean PPO and ibCO in 0.05 M sodium acetate buffer (pH 4.8) with 0.275 M GME and 0–0.3 M EDAC. EDAC and GME were dissolved in water immediately before use and inactivation was initiated by the addition of EDAC. A control experiment of enzymes and the nucleophile GME in buffer was run simultaneously which corresponded to 100 % activity for both field bean PPO and ibCO. Aliquots were removed for determination of residual activity at 10 min intervals and the pseudo first-order rate constants for inactivation were determined by the plot of log % residual activity against time. The inactivation kinetics was fitted to the equation: log (% residual activity) = $-k_i$ t, where k_i is the pseudo first-order inactivation rate constant for a given concentration of EDAC and *t* is time of inactivation. The inactivation order (*n*) was calculated from the equation: log k_i = n log [inactivator] + log k_i, where k_i is the second-order inactivation constant. The stoichiometry of the inactivation reaction was determined by a plot of log k_i versus log [EDAC]. The slope of the curve represents the stoichiometry.

2. 2. 32. Enzymatic cleavage of PPO for protein sequencing

2. 2. 32a. α-TPCK-trypsin cleavage: The PPO was cleaved at R-X and K-X bond by digestion with TPCK-trypsin at a ratio of 2 % (w/w). PPO (5 mg) was dissolved in 50 µL of 0.4 M NH_4HCO_3 containing 8 M urea (pH 7.5-8.5). Five µL of 45 mM dithiothreitol was added and the reaction mixture was incubated at 50 °C for 15 min. After cooling to 25±2 °C, 5 µL of 0.1 M iodoacetamide was added and further incubated at 25±2 °C for 15 min. This was followed by the addition 140 µL of water. TPCK-trypsin was added from a stock solution of 10 mg/mL in 1.0 mM HCl, incubated at 37 °C for 24 h. The digest was concentrated to dryness and re-dissolved in 0.5 mL of 0.1 % TFA (Matsudaira, 1989). The peptides were separated by RP-HPLC as described below.

2. 2. 32b. Purification of peptides for amino-terminal sequencing: The peptides obtained after enzymatic cleavage were purified by RP-HPLC using the following solvent system.

Solvent A: 0.1 % TFA in water
Solvent B: 0.05 % TFA in 70 % acetonitrile and 30 % water (v/v).
(Mahoney and Hermodson, 1980 and Hermodson and Mahoney, 1983)

The water used in the preparation of the above solvents was Milli Q water having a conductance of 18.2 mhos. The aqueous solvents were degassed for 15 min and 2 min respectively using an oil vacuum pump before addition of TFA.

The freeze-dried peptide mixtures were dissolved in a minimal amount of 0.1 % TFA (Solvent A), and loaded on a Waters Symmetry Shield C-18 column (4.6 × 150 mm, 5 µm) pre-equilibrated with solvent A. The column was washed for 10 min in solvent A to remove unbound peptides. The bound peptides were eluted using a linear gradient of solvent B (0-70 %) at a flow rate of 0.7 mL/min. The elution of peptides was monitored at 230 nm and 280 nm. The peak fractions were collected individually over several runs. A final wash with 100 % solvent B for 10 min, removed all bound peptides following which the column was re-equilibrated in solvent A for 15 min prior to the next separation. The peak fractions collected over several runs were

concentrated and subjected to gas phase amino-terminal sequencing as described earlier (Section 2.2.19).

2. 2. 33. Molecular docking

The Accelrys software package (INSIGHT-II modules; Accelrys inc, (http://www.accelrys.com) was used to visualize, analyze and manipulate various structures. Location of binding sites were carried out independently by an automated docking algorithm, Autodock 3.0 (Morris et al., 1998). The possible binding conformations and orientations were also analyzed by clustering methods, embedded in Autodock. Following docking, a post-docking energy minimization was carried out using the DISCOVER suite, by allowing full freedom to all protein and ligand atoms. Automated docking was performed with Autodock 3.0. The crystal structure of ibCO complexed with an inhibitor phenylthiourea (PDB ID: 1BUG) was used as the template in all cases. All water molecules and the inhibitor atoms were removed, the copper atoms were retained in the active site through out the process. Hydrogens were added using builder module of Insight II and the atomic partial charge were computed using CVFF forcefield. Each ligand was individually docked into this grid using the Lamarckian genetic algorithm and its interactions monitored using detailed energy estimates. One hundred cycles of docking with about 250000 energy evaluations in each cycle were carried out, which sampled all possibilities of conformations of the ligand in each cycle. Clustering was performed based on the similarities in binding modes and strengths in these cycles.

Phenylthiourea (PTU) was docked into the template structure as a positive control experiment by starting the docking run with different initial positions of the inhibitor. The docking mode obtained from this validation exercise was
highly super imposable with that of the crystal structure.

2. 2. 33. Reagents for molecular biology techniques

*2.2.*33a: Basic *precautions*

I) Use of sterile techniques throughout.

II) Use gloves to avoid contamination with RNases.

Glassware: All cleaned glassware filled with 1 N NaOH and kept overnight were individually rinsed with DEPC water. Residual DEPC was removed by autoclaving for 15 min at 121 °C, 15 lb pressure.

Nondisposable Plastic ware: All plasticware were incubated overnight in 1 N NaOH and rinsed with DEPC-water.

2. 2. 33b. Preparation of reagents: All laboratory chemical reagents used were of molecular biology or AR grade or higher grade wherever possible. All stock solutions and media were sterilized by autoclaving at 121 °C for 15 min at 15 lb pressure. Reagents and buffers not suitable for autoclaving were sterilized by filter sterilization using Millipore disposable sterile filters.

RNase free water: MilliQ water was treated with 0.1 % DEPC and stirred vigorously to bring DEPC into solution. This solution was allowed to stand overnight and then autoclaved for 15 min at 121 °C, 15 lb pressure to remove traces of DEPC. All solutions were prepared in this RNase free water and then autoclaved.

The following reagents were prepared according to Sambrook and Russel (2001), and sterilized at 121 °C, for 15 min at 15 lb pressure unless otherwise indicated.

A. 50× TAE: Tris-base (242 g), 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA dissolved in 1 L distilled water.

B. TE: Tris-HCl (10 mM), pH 7.5 and 1 mM EDTA.

C. 1 M Tris: Tris (12.1 g) was dissolved in 100 mL of DEPC treated water and pH adjusted to 8.0 with 6 N HCl.

D. 8 M LiCl: LiCl (33.9 g) was dissolved in 100 mL RNase free water.

E. EDTA Solution (250 mM): Dipotassium salt of EDTA (9.3 g) was dissolved in 100 mL RNase free water.

F. Sodium acetate: (3 M pH 4.6, pH 5.5 and pH 5.2) Sodium acetate trihydrate (40.81 g) was dissolved in water and adjusted to pH 4.6 or 5.5 as required with glacial acetic acid. Volume was made up to 100 mL and sterilized as described.

G. SDS (10 %): SDS (10 g) dissolved in 100 mL of RNase free water, was heated to 60 °C to completely dissolve SDS. Cooled and pH adjusted to 7.2 with 6N HCl. Stored at room temperature.

H. LB medium: Sodium chloride (10 g), tryptone (10 g), yeast extract (5 g) dissolved in deionized water and pH adjusted to 7.0 with 5 N NaOH. Final volume made up to 1 L with deionized water and sterilized.

I. 2× LB Media: Sodium chloride (2 g), tryptone (2 g), yeast extract (1 g) dissolved in water, and pH adjusted to 7.0 with 5 N NaOH. Final volume was made up to 200 mL with deionized water and sterilized. Filter sterilized glucose solution was added to a final concentration of 0.2 %.

J. LB agar: Tryptone (10 g) and yeast extract (5 g) sodium chloride (10g) were dissolved in water, and pH adjusted to 7.0 with 5 N NaOH. Final volume was made up to 1 L with deionized water then added agar (20 g), sterilized and poured into sterile petri dishes (25 mL/10 cm diameter plate).

K. Preparation of antibiotic: A stock solution of the sodium salt of ampicillin (100 mg /mL) was prepared in sterile water, filter sterilized and stored in aliquots at –20 °C.

L. Plates with appropriate antibiotics: LB-agar medium was sterilized and cooled to 55 °C. Ampicillin or kanamycin was added to a final concentration of 100 µg/mL prior to plating.

M. MOPS buffer (10X): MOPS 20.93 g (200 mM), 2.05 g of sodium acetate, 1.86 g EDTA (10 mM) were dissolved in RNase free water, pH adjusted to 7.0 with 2 N NaOH and filter sterilized. Stored in dark brown bottle at room temperature.

N. Acid salt buffer: (100 mM $CaCl_2$, 70 mM $MnCl_2$, 40 mM sodium acetate) the pH of the sodium acetate solution was adjusted to 5.5 with acetic acid, the salts were added and final volume made to 100 mL and filter sterilized.

0. Preparation of SOB: Bacto-tryptone (20.0 g), bacto-yeast extract (5.0 g), sodium chloride (0.6 g), potassium chloride (0.19 g) dissolved in 1 L water. Magnesium sulphate and magnesium chloride (10.0 mM), added from 1.0 M stock. The magnesium salts were autoclaved individually before addition of SOB medium.

P. SOC: Filter sterilized glucose (1 M) solution was added to the SOB media to obtain a final concentration of 20 mM.

2. 2. 34. RNA isolation procedure

Principle: RNA from homogenized tissue was extracted in buffer. Phenol at acidic pH was used to partition RNA into the aqueous layer. Chloroform

removes residual phenol and other contaminants. LiCl selectively precipitates RNA. Ethanol precipitation further purifies RNA. RNA collected as a pellet was washed with 70 % ethanol (500 µL × 2) and resuspended in RNase free water.

Extraction buffer: 1 M Tris (2.5 mL), 8 M LiCl (0.625 mL), 250 mM EDTA (1.0 mL), 10 % SDS (5.0 mL), β-mercaptoethanol (0.5 mL) made to 50 mL with RNase free water.

One gram of finely powdered tissue frozen in liquid N_2 was suspended in 10 mL buffer (5 mL extraction buffer + 5 mL water saturated phenol (pH 4.0-6.0), vortexed thoroughly and centrifuged at 8000 rpm for 10 min at 4 °C. The aqueous phase was transferred to a new tube. An equal volume of water-saturated phenol (pH 4.0-6.0) was added, mixed well and centrifuged at 8000 rpm for 10 min at 4 °C. The aqueous phase was transferred to a new tube to which an equal volume of chloroform was added mixed and centrifuged at 8000 rpm for 10 min at 4 °C. To the aqueous phase 1/3 volume of 8 M LiCl was added and allowed to precipitate at 4 °C overnight (16-18 h). The precipitate was collected by centrifugation at 8000 rpm for 10 min at 4 °C. The pellet was washed with 70 % ethanol (500 µL × 2 times) air dried, and then resuspended in 500 µL of RNase free water. RNA was precipitated by adding 40 µL of 3 M sodium acetate pH 5.2 and 1 mL of 70 % ethanol. This solution was kept at –20 °C for 2 h and centrifuged at 8000 rpm for 10 min at 4 °C. The pellet was washed with 70 % ethanol and dried at 25±2 °C for 2-4 min. RNA was dissolved in RNase free water. Traces of genomic DNA were removed by DNase treatment. RNA was reprecipitated by adding 1/10 volume of 3 M sodium acetate pH 5.2 and 2.5 volumes of 95 % ethanol and stored at –80 °C for long-term storage.

2. 2. 35. Isolation of genomic DNA

Genomic DNA was isolated from defatted field bean seed powder using a commercial kit (Sigma Chemical Co., St. Louis, MO, USA) following the manufacturer's instructions. DNA purity and quantity was determined as described below.

2. 2. 36. Quantification of DNA/RNA

The yield of RNA and DNA were determined spectrophotometrically at 260 nm, where 1 AU (A_{260}) equals 40 μg of single stranded RNA/mL or 50 μg of double stranded DNA/mL. The purity was estimated from the relative absorbance at 260 and 280 nm. An A_{260} / A_{280} ratio of 2.0 and 1.8 for RNA and DNA respectively are acceptable.

2. 2. 37. Denaturing agarose gel electrophoresis of RNA

Denaturing agarose gel electrophoresis was used to evaluate the isolated RNA. To migrate at their true molecular weights the secondary structure of RNA must be denatured. The percentage of agarose used affects resolution and transfer. High agarose concentration improves resolution but decreases the rate and efficiency of RNA transfer to membranes.

Preparation of gel: The required amount of agarose was heated in 72 mL of water until completely dissolved and then cooled to 60 °C. Then 10 mL of 10 X MOPS running buffer and 18 mL of 37 % formaldehyde (12.3 M) was added. The gel was assembled in the tank and 1× MOPS running buffer was added to cover the gel. The purified RNA was mixed with 1.0 μL EtBr (10 mg/mL), 4.0 μL formaldehyde and 10 μL formamide and incubated at 85 °C for 10 min and chilled on ice. To this 2 μL of 10× sample buffer was added (sample buffer 50 % glycerol diluted in RNase free water containing 10 mM EDTA pH 8.0, 0.25 % (w/v) , bromophenol blue and 0.25 % (w/v), xylene cyanol FF) and loaded. Electrophoresis was carried out in 1× MOPS buffer at 100V.

2. 2. 38. Native agarose gel electrophoresis of RNA

The agarose (1 %) was melted in 1× TAE buffer, cooled to 55 °C and EtBr was added to achieve a final concentration of 0.5 μg/mL. The gel was poured in to the gel trough and allowed to solidify. RNA was mixed with loading dye to a final concentration of 1×. The samples were directly loaded without heating and eletrophoresed.

2. 2. 39. DNA agarose gel electrophoresis

The extracted DNA was evaluated by agarose gel electrophoresis. Depending on the percentage of the gel, agarose was weighed and added to required volume of 1× TAE, cooked in a microwave oven and cooled to 55 °C. EtBr was added to a final concentration of 0.5 μg/mL, mixed well and poured into the gel trough and allowed to solidify.

Samples were mixed with sample buffer and loaded in the well. The gel was electrophoresed for 45-60 min in 1X TAE buffer at 100V. DNA was visualized under a UV-transilluminator.

2. 2. 40. Primer design

A set of degenerate primers were designed based on the amino terminal sequence of PPO. The gene sequences were obtained from NCBI and other protein data banks. The DNA and protein sequences were aligned using CLUSTALW (http://www.ebi.ac.uk/clustalw/). The sense and antisense degenerate primers were designed for the conserved and invariant regions of PPO and lectin sequences.

The parameters considered during primer design were; The primer length was 18-30 nucleotides and a G/C content of 30-50 %. T_m was calculated using the formula T_m = 2 ºC × (A+T) + 4 ºC × (G+C). Optimal annealing temperature was calculated as 5 ºC below the estimated melting temperature. Complementary sequences within a primer sequence were avoided to reduce hairpin formation. Primers with A or T at 3' end were avoided. The common code for degenerate oligos used are as follows A/C: M, A/G: R, C/G: S, C/G/T: B, A/T: W, C/T: Y, G/T: K, A/C/G/T: N, A/C/T: H, A/C/G: V, A/G/T: D.

2. 2. 41. cDNA synthesis

Total RNA was isolated from mid mature seeds according to the described lithium chloride method. Murine-MLV reverse transcriptase (USB corporation, USA) was used to generate cDNA from 8.0 μg of total RNA and 20 μM of oligo(dT) using standard reaction conditions, following the manufacturer's protocol.

2. 2. 42. PCR and cDNA cloning

The cDNA ends were synthesized by rapid amplification. The PCR reaction was performed by using 40 cycles of 30 s 94 ºC, 40 s at 51 ºC, and 1 min at 72 ºC and final extension step 72 ºC for 10 min. Reactions were carried out in 20 µL containing 30 pmol of each primer, 0.2 mM dNTPs and1.0 unit of *Taq* polymerase (Banagolre-Genei) in the corresponding buffer. The blunt end products were generated using high fidelity DNA polymerase.

Putative amplified gene products were cloned into *PvuII* site of p RSET C (Invitrogen). Inserted DNA was sequenced with T7 and SP6 vector primers by the chain termination Big Dye Chemistry (Applied Biosystems, Foster City CA, 310 Genetic analyzer).

Chapter III

Field bean polyphenol oxidase: purification, characterization and temporal expression

In this laboratory, during affinity purification of a glucose-mannose lectin from field bean (*Dolichos lablab*) seeds, severe browning of the extracts was observed (Gowda et al., 1994). Preliminary investigations revealed the presence of a single PPO, in the 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. PPO from field bean seeds was therefore purified and partially characterized (Paul, 2000). In this chapter extending the previous studies of Paul (2000), further biochemical characterization of PPO and its spatial and temporal expression are presented.

RESULTS

Extraction and purification

The defatted field bean seed powder was extracted overnight at 4 °C with 0.1 M Tris-HCl buffer, pH 7.0 (1:5 w/v) containing 1.2 % NaCl (w/v) and 2 % PVPP (w/v). The crude extract was filtered through cheesecloth and filtrate was centrifuged at 15000 × g for 45 min at 4 °C. The supernatant was collected and used for further purification steps.

Ammonium sulfate fractionation

The crude extract was saturated to 40 % $(NH_4)_2SO_4$ (22.6 g/100 mL) by addition of finely powdered solid $(NH_4)_2SO_4$ at 4 °C. The solution was allowed to stand for 2 h at 4 °C for complete precipitation. The precipitate obtained after centrifugation at 15000 × g for 45 min at 4 °C was discarded and supernatant was subjected to 80 % $(NH_4)_2SO_4$ (25.8 g/100 mL) precipitation. The solution was allowed to stand overnight at 4 °C for complete precipitation of proteins. The precipitate was collected by centrifugation at 15000 × g for 45 min at 4 °C. 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 (5 × 500 mL). The specific activity of PPO increased ~2 fold with a yield of 73 % (Table 3.1).

Anion exchange chromatography

The dialyzed fraction was loaded onto a DEAE-sepharose column 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 20 mL/h. PPO did not bind to DEAE-Sepharose at this pH and eluted as a single peak, in the column wash. The fractions exhibiting PPO activity were pooled as shown in the Figure 3.1. The pooled fraction had a specific activity of 7416 U/mg (Table 3.1). A purification of 6.0 fold with a 60 % yield over the previous step was achieved.

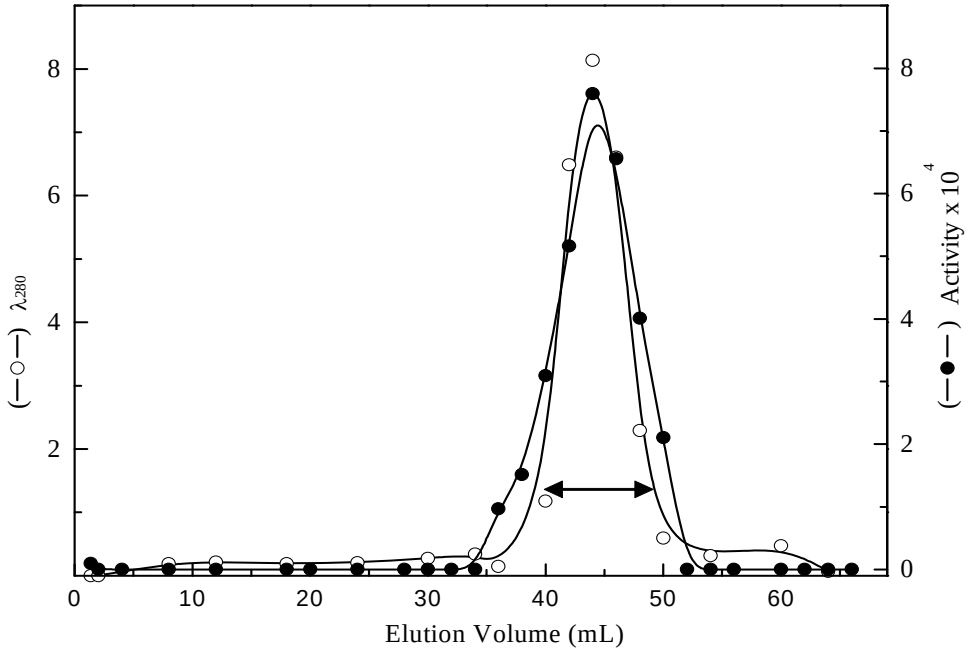

Figure 3.1. DEAE-sepharose chromatography elution profile of field bean seed PPO. The dialyzed fraction of $(NH_4)_2SO_4$ precipitation was loaded on a DEAE-sepharose column equilibrated with 10 mM Tris-HCl containing 1.2 % NaCl, pH 8.2. Elution was carried out in the same buffer at a flow rate of 20 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 hydrophobic interaction chromatography on phenyl sepharose. The pH of the pooled fractions obtained from the previous step was adjusted to 7.0. Solid $(NH_4)_2SO_4$ was added to a final concentration of 1 M and loaded onto a phenyl sepharose column (2.5 × 30 cm), previously equilibrated with 25 mM Tris-HCl buffer, pH 7.0 containing 1 M $(NH_4)_2SO_4$ and 1.2 % NaCl (w/v). The column was washed with the same buffer. In this step, inactive proteins eluted in the

washing. Bound PPO was eluted using the same buffer minus $(NH_4)_2SO_4$. The elution profile of field bean PPO from phenyl sepharose is shown in Figure 3.2.

PPO eluted as a single symmetrical peak with a low ionic strength elution buffer, containing no $(NH_4)_2SO_4$, with a 12.0 fold increase in specific activity (Table 3.1). The protein fractions containing PPO were appropriately combined and precipitated with 80 % $(NH_4)_2SO_4$.

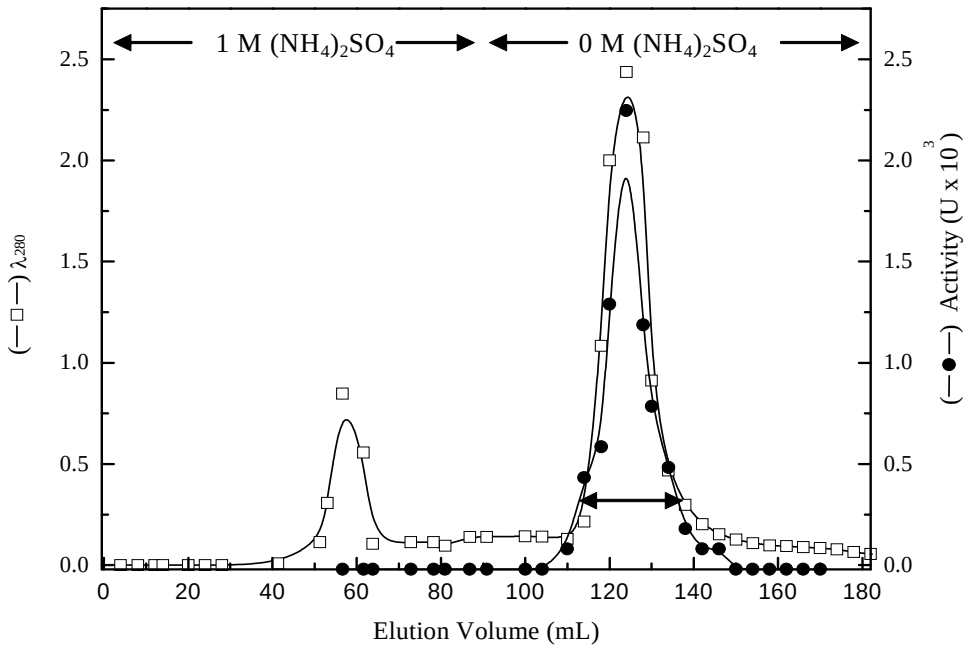

Figure 3.2. Phenyl sepharose chromatography elution profile of field bean seed PPO. The active fraction obtained from DEAE-sepharose column was loaded to a phenyl-sepharose column equilibrated with 25 mM Tris-HCl, pH 7.0 containing 1.2 % NaCl and 1 M $(NH_4)_2SO_4$. PPO was eluted with same buffer without $(NH_4)_2SO_4$ at flow rate of 25 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-150 column (2 × 120 cm) equilibrated in 100 mM Tris-HCl buffer pH 7.0 containing 1.2 % NaCl (w/v). Elution was carried out at a flow rate of 10 mL/h. PPO eluted as a single peak on the descending shoulder of the major protein peak (Figure 3.3). The final recovery of PPO was ~20 %, after a 29.0 fold purification, with a specific activity of 3.55×10^4 U/mg (Table 3.1).

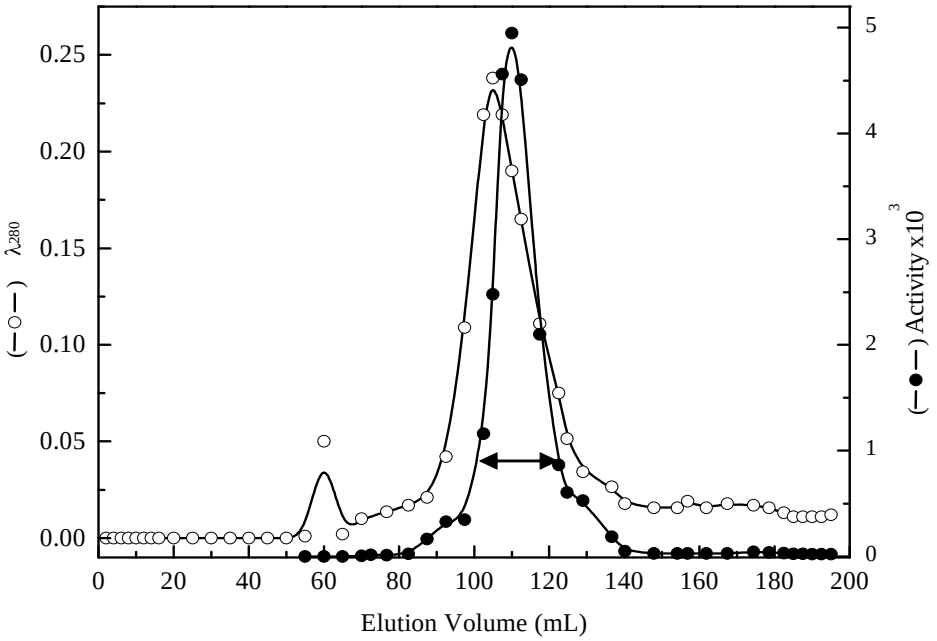

Figure 3.3. Sephadex G-150 chromatography elution profile of field bean seed PPO. The active fractions of phenyl-sepharose chromatography were concentrated and loaded onto Sephadex G-150 column. The column was equilibrated with 0.1 M Tris-HCl containing 1.2 % NaCl pH 7.0. Fractions of 2 mL were collected at a flow rate of 10 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 3.1. The purified PPO was stored at 4 °C and used for further studies.

Criteria of homogeneity

The homogeneity of the purified protein was assessed by native PAGE, HPLC gel filtration and amino-terminal sequencing. The purified enzyme was subjected to native PAGE (7.5 % T, 2.7 % C) in Tris–glycine buffer pH 8.3. PPO was located by protein staining and enzyme activity staining. The purified PPO revealed a single band both by protein staining using Coomassie brilliant blue R-250 (Figure 3.4A) and by specific enzyme staining with catechol and MBTH (Figure 3.4B). The quinone formed with catechol is pink in color. With TBC and *p*-phenylenediamine a blue colored band is visualized. The tertiary butyl quinone forms a blue colored adduct with *p*-phenylenediamine indicating the presence of a single isoform. These results indicate the homogenous nature of the purified PPO.

Table 3.1. Purification of field bean seed PPO*

Step	Total activity ($U \times 10^5$)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Fold Purification
Crude	13.4	1071	1251	100	—
40-80 % $(NH_4)_2SO_4$	9.8	429	2284	73	1.9
DEAE-sepharose Chromatography	8.0	108	7416	60	6.0
Phenyl sepharose Chromatography	4.0	27	14962	30	12.0
Sephadex G-150 Size exclusion chromatography	2.7	7.6	35553	20	29.0

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

The purity of PPO was also determined by analytical HPLC size exclusion chromatography using a Progel™-TSK G2000 SW_{XL} column. Figure 3.5 shows the typical elution profile of field bean PPO. The single symmetrical peak eluting at 13.37 min observed indicates the homogenous nature of the PPO.

Molecular weight

The apparent M_r of field bean PPO was determined by analytical gel filtration on a Progel™-TSK G2000 SW_{XL} HPLC column, SDS-PAGE (Laemmli, 1970) and by MALDI-TOF. The M_r of the purified enzyme estimated by analytical HPLC was 125000±3000 Da from a plot of log M_r versus V_e/V_o (data not shown).

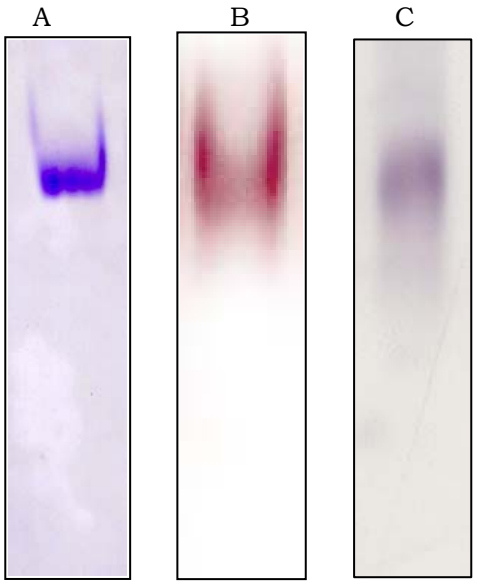

Figure 3.4. Native PAGE (7.5 % T, 2.7 % C) of field bean PPO. The gel was stained for protein Lane A, PPO activity. Lane B stained with catechol and MBTH and Lane C stained with TBC and *p*-phenylenediamine.

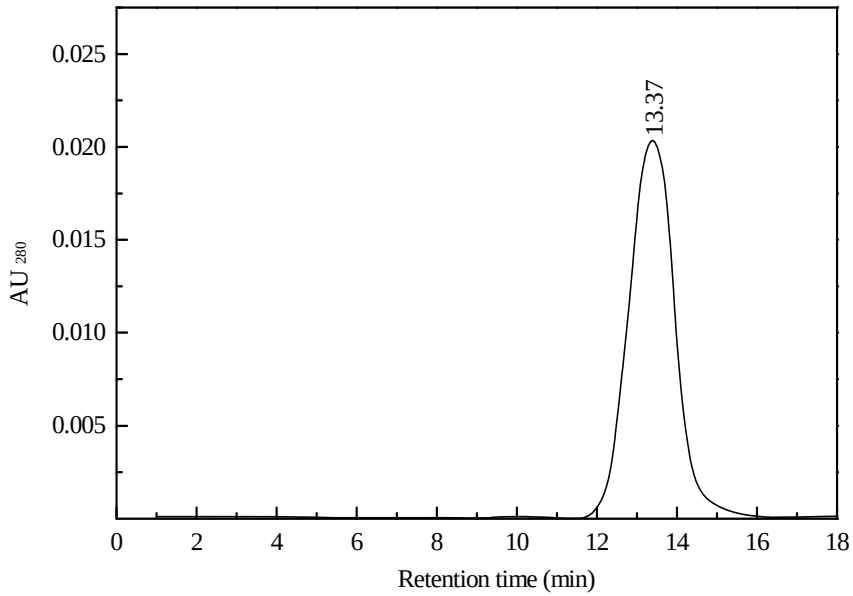

Figure 3.5. HPLC size exclusion chromatography profile of field bean seed PPO. Column used: Progel™-TSK G2000 SW_{XL} (7.8 mm id × 30 cm). PPO was eluted in 0.1 M phosphate buffer containing 0.1 M Na_2SO_4 at a flow rate of 0.5 mL/min. The detector was set at 280 nm.

SDS-PAGE (10 % T, 2.7 % C) of the purified PPO was carried out in a discontinuous buffer system. The Mr markers used were phosphorylase b (97400 Da), BSA (66300 Da), ovalbumin (43000 Da), carbonic anhydrase (29000 Da), soyabean trypsin inhibitor (20000 Da) and lysozyme (14300 Da). Protein staining using Coomassie blue R-250 showed a single subunit of 30000±1500 Da (Figure 3.6).

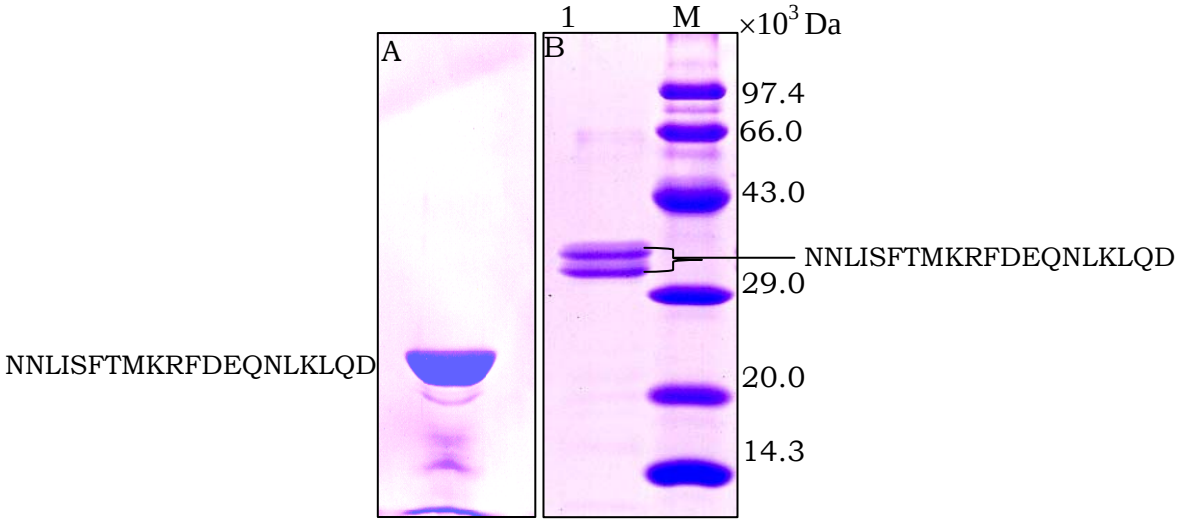

Figure 3.6. SDS-PAGE A (10 % T, 2.7 % C) and B, 12.5 % T 2.7% C) of field bean seed PPO. Lane 1 PPO, Lane M: phosphorylase b (97400 Da), BSA (66000 Da), ovalbumin (43000 Da), carbonic anhydrase (29000 Da), soybean trypsin inhibitor (20000 Da) and lysozyme (14300 Da).

The exact Mr. of the PPO was determined by MALDI-TOF on a Bruker Daltonics flex analysis (UK) Kompact Seq model which uses a 337 nm nitrogen laser desorption and 1.7 nm linear flight path. The instrument was calibrated over the mass range of 10000-150000 Da. The results showed two subunits of molecular weight 29808 and 31087 Da (Figure 3.7). These results were in contrast to the subunit structure noted earlier (Figure 3.6A). However on performing SDS-PAGE using a 12.5 % acrylamide indicates the presence of two closely moving protein bands (Figure 3.6B). In all further experiments the concentration of acrylamide used was 12.5 % T.

Amino-terminal sequence

SDS-denatured PPO was transferred to PVDF membrane and the two closely moving bands were excised and subjected to amino-terminal sequencing. The release of a single amino terminal amino acid aspergine

for both native and denatured PPO indicated the PPO to be homogenous. The sequence obtained after 20 cycles of automated Edman microsequencing was **NNLISFTMKRFDEQNLKLQD**. The first 20 residues of both the sub unit bands are identical in sequence, and also identical to the sequence reported by Paul and Gowda (2000). These results reflect the identical status of the subunits of field bean seed PPO.

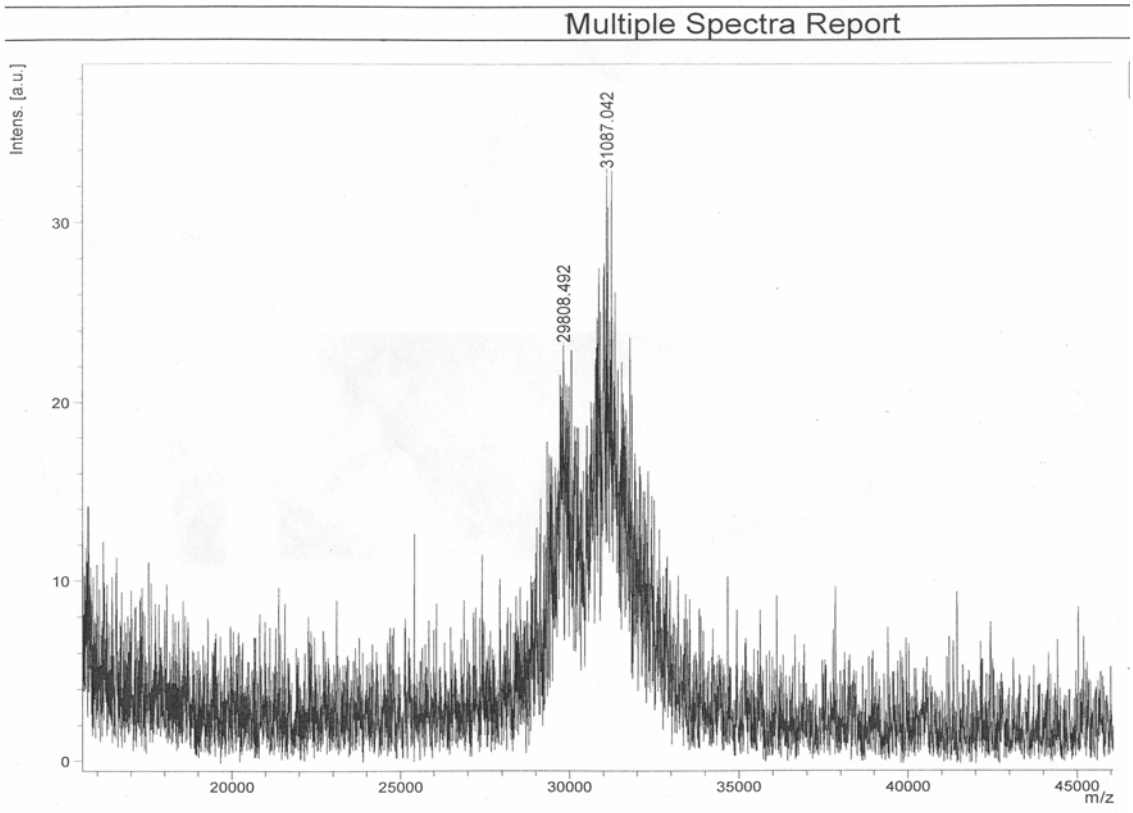

Figure 3.7. MALDI TOF profile of PPO subunits

Determination of isoelectric point (pI)

Isoelectric focusing of the PPO was carried out on a pre-cast Ampholine® PAG plate using pI markers (3.5 to 9.5). The pI of PPO was found to be 9.35. Figure 3.8 depicts the distance moved by proteins from cathode *vs* pI of marker proteins and PPO. This alkaline pI explains the absence of binding to DEAE-Sepharose at pH 8.2.

Glycoprotein staining and carbohydrate estimation

The purified PPO was subjected to glycoprotein staining after SDS-PAGE (12.5 % T, 2.7 % C). The two closely moving dark pink color bands

indicate the glycoprotein nature of PPO (Figure 3.9). The carbohydrate estimation was performed using glucose as the standard. The neutral carbohydrate content of PPO estimated by the phenol-sulfuric acid method was 8.0 %. This is in the range of 8-12 % estimated for plant PPOs.

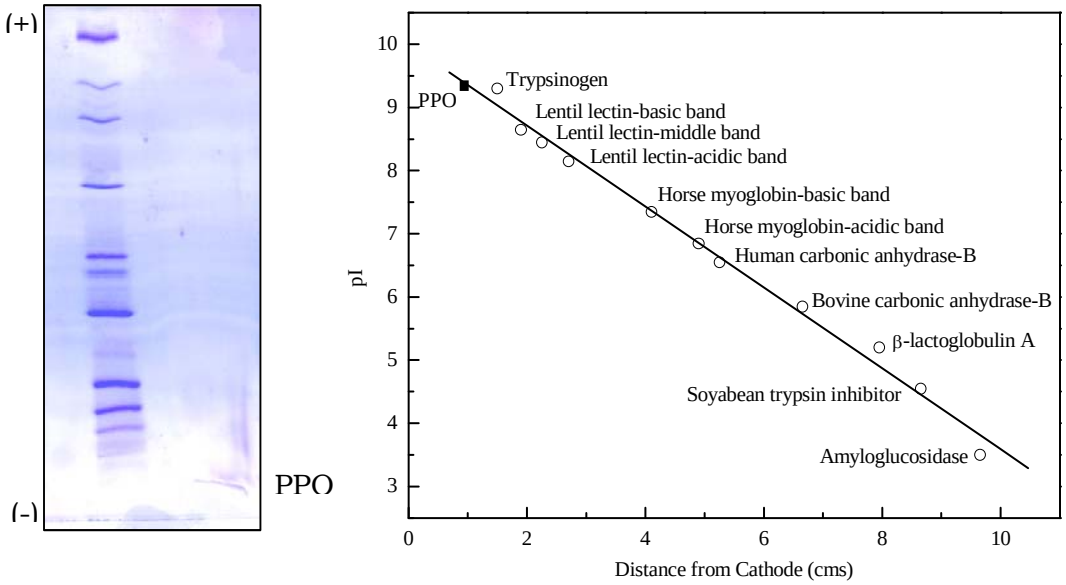

Figure 3.8. Determination of isoelectric point of PPO A) shows isoelectric focusing gel. B) The plot shows distance moved from cathode *vs* pI. Standard pI markers used are shown in the figure.

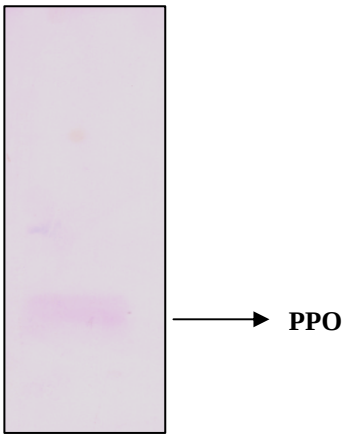

Figure 3.9. Glycoprotein staining of PPO. Staining was performed after SDS-PAGE (12.5 % T, 2.7 % C), using Schiff's reagent.

Effect of substrates/inhibitors on PPO activity

The effect of various substrates and inhibitors was tested on catalysis. Several *o*-diphenols with various substituents served as effective substrates of PPO (Table 3.2). Among them TBC was the best, followed by 4MC and CAT. The effect of varying TBC concentration on the velocity followed Michaelis-Menten kinetics (Figure 3.10). Similar results were obtained with the other substrates studied (results not shown). The K_m and V_{max} values were calculated from their respective Lineweaver-Burk double reciprocal plots. The kinetic constants are listed in Table 3.2.

Tropolone (2-hydroxy-2, 4, 6-cycloheptatrien-1-one) is one of the most potent inhibitors of PPO which is structurally analogous to the *o*-diphenol substrates of PPO and is an effective copper chelator. Tropolone inhibits PPO competitively. The K_i as determined by Dixon plot of 1/V against [I] is 5.7 ± 0.2 × 10^{-7} M (Figure 3.11). Several phenolic compounds were evaluated for minor differences in the structure of *o*-diphenols converted substrates to inhibitors and vice versa. To explain these differences, a detailed study of substrates and substrate analogs as inhibitors their inhibitor properties (Table 3.2) was undertaken and results presented in the is presented in next chapter (Chapter IV).

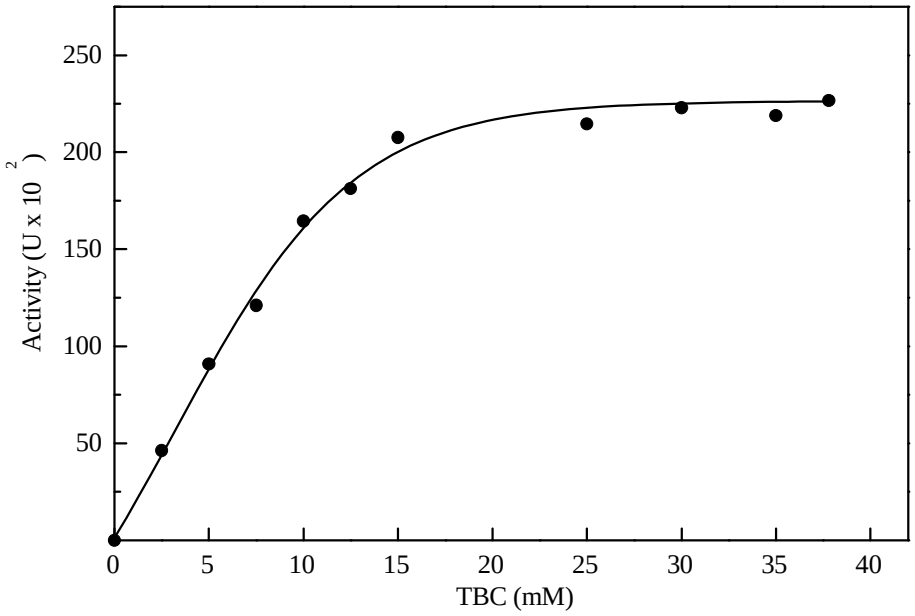

Figure 3.10. Effect of varying TBC concentration on the activity of field bean PPO.

Table 3.2. Kinetic measurements for PPO using various substrates

Compounds	K_m (mM)	V_{max} (U/mg)×10^3	pH optima	Wave length (nm)	K_i * (mM)	Type of inhibition
CAT	12.0±0.01	89.7±0.22	4.0	505	-	-
4MC	4.5±0.03	120.0±0.26	4.5	500	-	-
TBC	4.2±0.02	197.0±0.25	4.5	400	-	-
DOPAC	4.2±0.02	15.3±0.31	4.0	420	-	-
DOPA	2.9±0.04	33.5±0.32	5.0	505		
DPN	1.5±0.02	11.1±0.19	5.5	505	-	-
2, 3, 4-THBA	4.0±0.02	12.3±0.29	4.0	420	-	-
BA	-	-	-	-	3.5±0.02	competitive
*o*HBA	-	-	-	-	2.5±0.03	competitive
*m*HBA	-	-	-	-	2.0±0.04	competitive
*p*HBA	-	-	-	-	2.5±0.02	competitive
2, 3-DHBA	-	-	-	-	2.1±0.03	competitive
3, 4-DHBA	-	-	-	-	2.6±0.03	competitive
3, 4, 5-THBA	-	-	-	-	2.3±0.02	competitive

* The K_i was determined using TBC as the substrate.

Effect of pH on substrate and PPO activity staining

The pH optima of PPO was determined by measuring the activity at various pH using different buffers. The pH optima for various substrates was also determined (Table 3.2). The color developed for the detection of PPO in native-PAGE gels varies with varying substrate. Catechol, DOPA and 4-MC react with field bean PPO to form dark pink to maroon colored bands in the presence of MBTH. In the absence of MBTH, a dark brown band appears showing PPO activity. In contrast when TBC is used a bluish purple band is observed and MBTH is not required to capture the quinone. It is observed that with TBC alone the developed color varies with the pH (Table 3.3) changing from blue to brown with increasing pH. These results indicate that TBC as the substrate can be effectively used to detect PPO's with an acid pH optima (Figure 3.12).

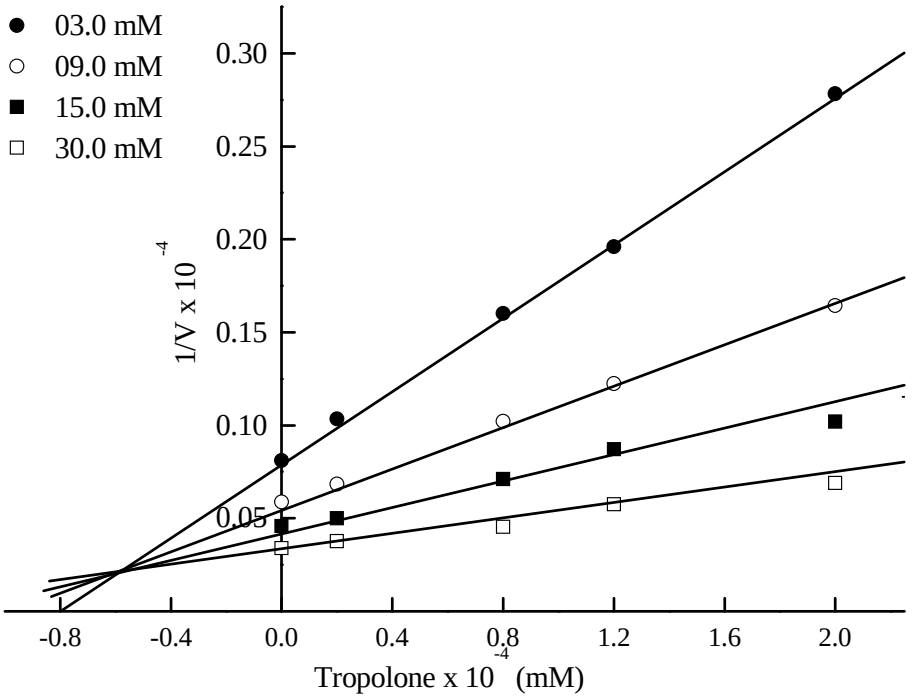

Figure 3.11. Dixon plot for tropolone inhibition of field bean PPO: TBC concentrations used are as shown.

Table 3.3. pH dependent activity of staining of purified field bean PPO.

SUBSTRATES	**Color of the PPO activity band**			
	pH 2.5	**pH 4.0**	**pH 5.0**	**pH 6.0**
CAT	Brown	Brown	Brown	Brown
DOPA	Brown	Brown	Brown	Brown
DPN	Brown	Brown	Brown	Brown
4-MC	Brown	Brown	Brown	Brown
TBC	Bluish purple	Bluish purple	Brown purple	Brown purple

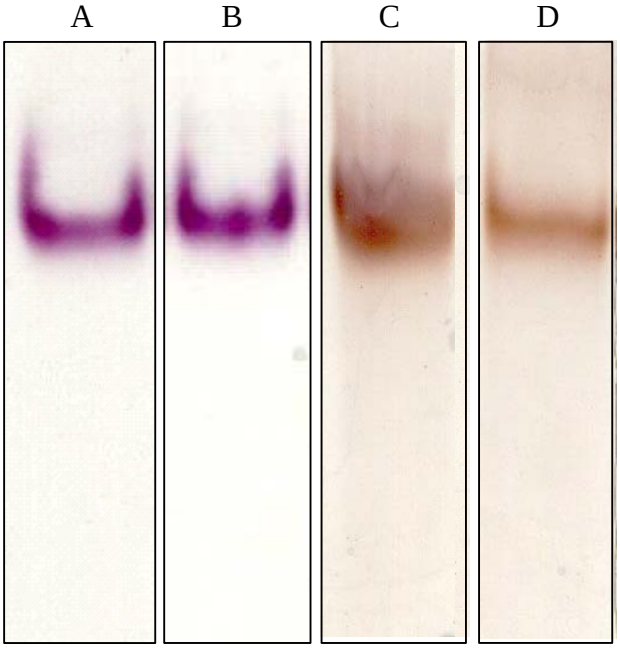

Figure 3.12. Effect of pH on the gel activity staining of field bean PPO using TBC as a substrate. A) pH 2.5; B) pH 4.5; C) pH 5.0 and D) pH 6.0.

Cross reactivity with PPO antibodies

The purified PPO was probed with the polyclonal antibodies developed against field bean PPO in a New Zealand white rabbit. Following SDS-PAGE (12.5 % T, 2.7 % C) the protein was transferred to nitrocellulose membrane and subjected to immunodetection as described under Section 2.2.22. Both the subunits of denatured PPO cross-reacted with the antibodies (Figure 3.12). These results indicate that the epitopes are present on both the subunits.

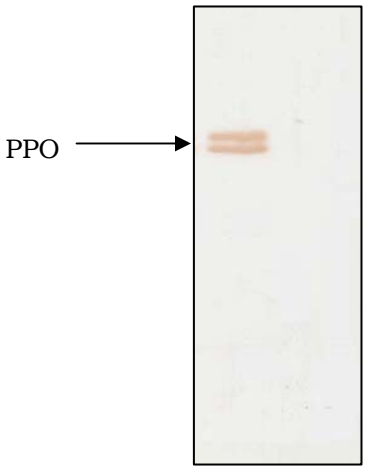

Figure 3.13. Immunodetection of field bean seed PPO. After SDS-PAGE (12.5 % T, 2.7 % C) PPO was transferred to nitrocellulose and probed with field bean PPO antibodies using a HRP conjugate.

Temporal expression of PPO

Temporal expression of PPO was examined across five seed germination stages and six seed developmental stages. Crude extracts of the cotyledons at varying stages of germination were prepared in 0.1 M Tris-HCl pH 7.0. The PPO activity after 24 h of germination (~80 %) had already begun to decrease, as compared to that of the imbibed seeds (Figure 3.14). The overnight imbibed seeds have been considered as 0 h of germination. PPO activity decreased dramatically after 48 h of germination. Thereafter, there was a decline with ~ 10 % of the activity retained in the cotyledons of the 4 day old seedlings. Interestingly it is observed that the decline in PPO activity of seeds germinated in light was lower than that in germinated in the dark. The residual PPO activity in the cotyledons of the 4 day old seedling germinated in light was higher (~20 %). The decline in the protein content was gradual over the entire period of germination (Figure 3.15B).

Native PAGE of the crude extracts followed by activity staining indicates a decrease in the intensity of staining for PPO (Figure 3.15). These results are in agreement with the activity measurements using TBC as the substrate. The gel stained for PPO shows that in the imbibed seeds and 24 h after germination PPO activity was indistinguishable. The very low intensity band of the 96 h cotyledon extract is commensurate with ~5 % activity measured.

PPO expression during seed development

During seed development a steep increase in the PPO activity with a concomitant increase in the protein content, is observed from 10 DAF. Although there is a significant change in protein content only a marginal change in the PPO content during the initial stages (0-10 DAF) is observed (Figure 3.16). The flower being considered 0 day. The extracts of the flower showed measurable PPO activity (~900 ± 2 U/g) when TBC was used as the substrate. Both, in the flower and at all stages of seed development, only a single isoform of PPO was detectable by native-PAGE, followed by activity staining with TBC and *o*-phenylenediamine (results not shown). The leaves of the *Dolichos lablab* plant also exhibit PPO activity. The PPO activity measured at the four leaf stage (~6000 U/g) was similar to that in the

mature leaf. All these results suggest that the expression of PPO is universal in all parts of the plant.

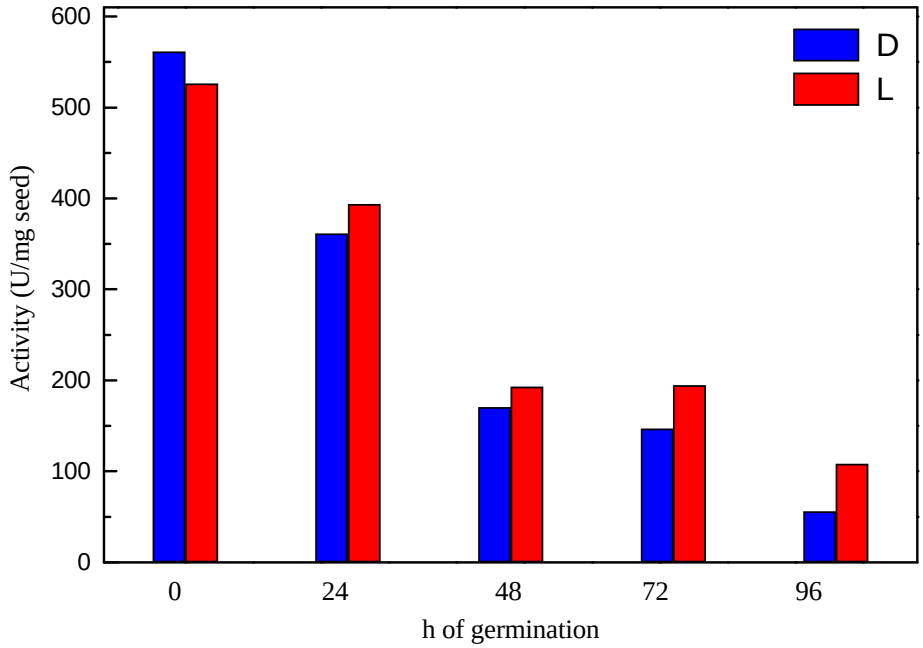

Figure 3.14. Effect of germination on the PPO activity of field bean seeds. D, germinated in dark and L, seeds germinated in light.

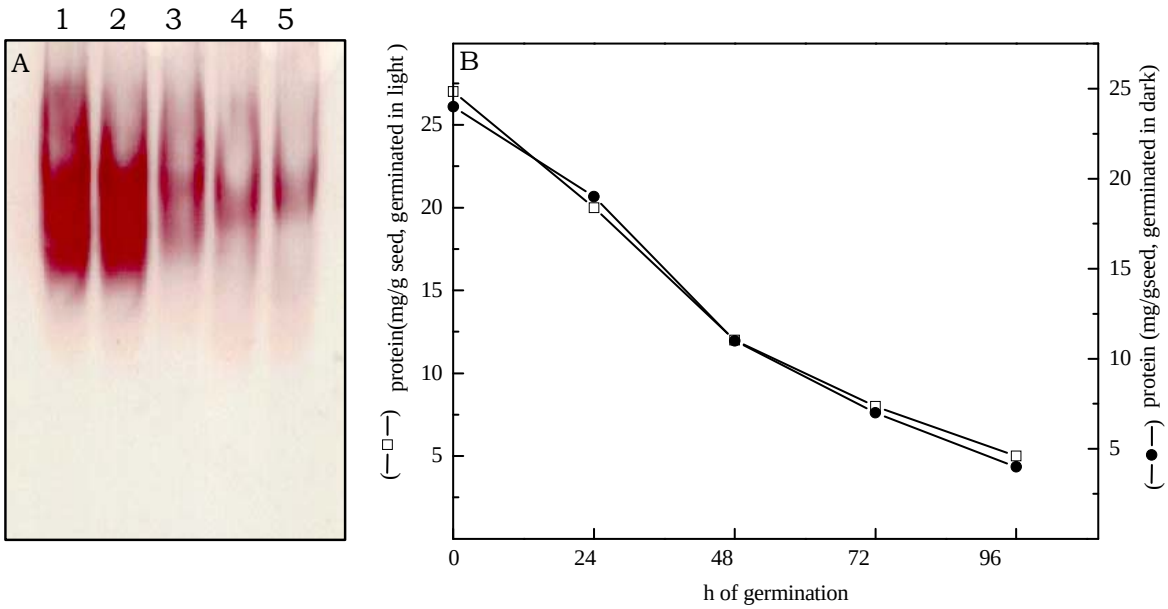

Figure 3.15. A) Native PAGE of the crude extracts from germinating field bean seeds stained for PPO activity with catechol and MBTH. Lanes 1-5 are extracts after 0, 24, 48, 72, 96 h of germination respectively (50 µg of protein was loaded in each lane). B) Effect of germination on the protein content of field bean seeds.

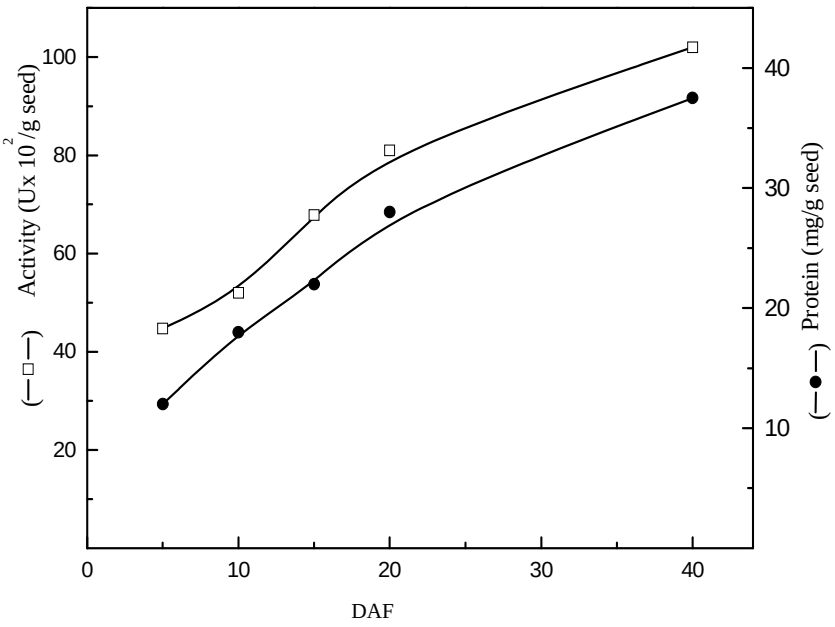

Figure 3.16. PPO expression profile during seed development. Extracts from seeds 5-40 DAF. A) PPO activity and protein; B) Native PAGE stained using catechol and MBTH.

Discussion

The initial biochemical investigations of PPO were carried out in 1895 on the mushroom *Russula nigricans,* the cut flesh of which turned red and then black on exposure to air (Bourquelot et al., 1895). It is only over the past three decades that the multifaceted protein, PPO has drawn the attention of a wide range of disciplines due to its importance in medicine. Bio-inorganic chemists used it as a model protein for the study of oxygen centers for metallo-oxygenases (Solomon et al., 1996). Limited structural studies on plant PPOs is due to the multiplicity of PPOs. More detailed knowledge of plant PPOs will provide valuable information that would aid in elucidating the physiological functions attributed to them.

PPO is ubiquitous in nature found among all the classes of living things including bacteria, fungi, algae, bryophytes, pteridophytes gymnosperms and angiosperms (Sherman et al., 1991). The characteristics of PPOs usually show no special features. Among angiosperms PPO was identified in all orders. PPO has been studied in many fruits and vegetables including peaches (Luh and Philthakphol, 1972), grape (Nunez-Delicado et al., 2005), apple (Murata et al., 1992), aerial roots of orchid Aranda (Ho et

al., 1999), parasitic plant cuscuta (Bar-Nun and Mayer, 1999), morula (*Sclerocarya birrea*, Mduli, 2005), loquat (Selles-Marchart et al., 2006) medicinal plants like ferula (Erat et al., 2006), oregganum (Dogan et al., 2005) oil bean (Chilaka et al., 1993), plantain (Ngalani et al., 1993), potato (Sanchez-Ferrer et al., 1993b), pineapple (Das et al., 1997), cabbage (Fujita et al., 1995), avocado (Espin et al., 1997b), ocimum (Donga et al., 2005) and tea stigma style (Neog et al., 2004). PPO has also been recently isolated from the microflora of bovine rumen (Beloqui et al., 2006).

However among the leguminous plants, PPO has been isolated from broad bean (*Vicia faba* L) leaves (Kenten, 1957), mung bean (*Vigna radiata*) leaf and seedlings (*Vigna mungo*) (Shin et al., 1997; Takeuchi et al., 1992). Early investigations in our laboratory, revealed the presence of a single PPO isoform by Native 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 structural analysis. The PPO from field bean seed was purified and partially characterized (Paul, 2000). In this study to make available a homogeneous preparation of PPO for structural studies the enzyme was purified and characterized in detail.

In this study, a combination of procedures, namely $(NH_4)_2SO_4$ fractionation, ion-exchange chromatography on DEAE-sepharose, hydrophobic interaction chromatography and size exclusion chromatography have been used to purify the enzyme to homogeneity. PPO does not bind to, DEAE-sepharose at pH 8.2 which can be explained by its highly basic pI. The pI of field bean PPO is 9.3. At pH 8.2 which is below the pI PPO is positively charged and therefore does not bind to the anion exchanger, and elutes in the buffer wash (Figure 3.1). Phenyl sepharose served as the hydrophobic interaction matrix with an inactive protein fraction eluting in the void volume (Figure 3.2). The size exclusion chromatography step on Sephadex G-150 (Figure 3.3) provided a homogenous PPO with a very high specific activity of 3.5×10^4 U/mg (Table 3.1). A single protein species is observed in native PAGE both by specific enzyme activity and protein staining (Figure 3.4). The homogeneity was also revealed by the release of a

single amino-terminal aspargine. All these results indicate that the preparation was homogenous and could be used for structural studies.

Molecular weight of plant PPOs are very diverse and variable. It is reckoned that a part of this variability is due to partial proteolysis of the enzyme during isolation. Multiplicity in the size of PPO has also been explained by genetic variability. The native Mr of field bean PPO is 120000 Da as revealed by analytical size exclusion chromatography (Figure 3.5). 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 larger than that reported for broad bean leaf PPO (Flurkey, 1989), 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. Previously Paul and Gowda, (2000) showed that field bean PPO was a homo-tetramer of 29000 ± 3000 Da. In contrast MALDI-TOF of the purified PPO showed two subunits (Figure 3.7). This difference be can explained by the observation that SDS-PAGE of PPO carried out using 10 % T (total acrylamide concentration) reveals a single subunit of Mr ~30000 ± 3000 Da. In contrast when 12.5 % or higher acrylamide concentration is used the protein resolves into two closely migrating subunits of ~29000 and ~31000 (Figure 3.6B). These results are in concurrence with MALDI-TOF results. The amino-terminal sequence for the first twenty residues of the two subunits are identical (Figure 3.6). These results suggest that the difference in mass is probably due to truncation at the carboxy-terminus. PPOs, display a complex, heterogeneous quaternary structure and the values reported cover a wide range. Flurkey, (1989) reported that the broad bean leaf PPO exists as doublets of Mr 61500 and 60000 Da and 44500 and 43000 Da, all of which have the same amino-terminal sequence. An association-dissociation phenomenon of pineapple PPO was reported in buffers of varying ionic strength (Das et al., 1997; Harel et al., 1973; 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 aggregate 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 (Paul, 2000).

Yoruk and Marshall (2003) reported that the optimum pH for maximum PPO activity for plant PPOs varies from about 4 to 8. The pH optima of field bean PPO for various phenolic substrates is in the range of 4.0-5.5 (Table 3.1). The pH optima is influenced by a number of experimental factors such as extraction methods, temperature, nature of the phenolic substrate and buffer system used during PPO assay (Whitaker, 1994). The optimum pH of 4.5 for field bean PPO with TBC is identical to that obtained for cherry and strawberry PPOs with 4-MC (Wesche-Ebeling and Montgomery, 1990; Fragnier et al., 1995). Egg plant PPO was shown to exhibit a similar optimum pH 5.0-6.5 for 4MC and TBC (Perez-Gilabert and Carmona, 2000). Several fruit PPOs including almond, apricot, peach and plum generally have maximal activities around pH 5.0 (Freigner et al., 1995). However, PPO from apricot and plum exhibit maximum activity in a wider acidic pH range. Apple and grape PPO have broad acidic pH optima with estimated values between 3.5 to 4.5 (Marques et al., 1995). The pH optimum of grape PPO varied among different varieties ranging from 3.5-7.5 (Yoruk and Marshall, 2003). The optimum pH is influenced significantly by a number of experimental factors. Therefore it is often difficult to extrapolate data when substrates vary. However the acidic pH optima for all the substrates indicate the involvement of a carboxylate residue at the active site. Klabunde et al., (1998) from their crystal structure implicated the role of Glu^{236} in catalysis. The chemical modification of carboxylate by EDAC and GME showed the complete loss of field bean PPO activity (Kanade, et al., 2006).

The pI of 9.3, for field bean PPO is similar to the 40000 Da. PPO of sweet potato (ibCO), which is 8.6. In contrast the pI of 39000 Da ibCO is 3.6 (Eicken et al., 1998). Potato tuber PPO has a pI of 6.5 (Marri et al., 2003). The pI of sorgum PPO is 9.0 and *Hevae brasiliensis* latex PPO is 9.2 (Wititsuwannakul et al., 2002). Most other PPOs show an acidic pI.

Fungal and higher plant enzymes act on a wide range of mono and diphenols. All catechol oxidases require the basic *o*-dihydroxyphenol

structure for oxidase activity with catechol as the simplest possible, but not necessarily the best substrate. Phenolic compounds are the primary substrates of PPO. Several diphenols were evaluated as substrates. Among the substrates TBC was oxidized to its quinone with a V_{max} of 1.97×10^5 U/mg (Table 3.2). The binding affinity was also the highest. Among the substrates the binding affinity was of the order TBC > 4MC > CAT (Table 3.2). In addition DOPA and DPN were also oxidized. 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 TBC, CAT and 4-MC and no affinity towards bulky *o*-diphenols such as caffeic acid, chlorogenic acid, catechin and also diphenolic oligomers (Paul and Gowda, 2000). 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 CAT, 4-MC, 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 well defined (Mayer and Harel, 1979). Our molecular docking studies indicate that diphenols bind to the active site by stacking of the phenyl ring against a His^{244} conserved in all PPOs (Kanade and Gowda, 2006). The differences in binding affinity between different phenols is due to their interactions with residue that line the substrate cavity (results presented in Chapter IV).

The impact of PPO-induced deleterious browning reactions in fruit and vegetables as well as sea foods is enormous, with yearly losses running into millons of dollars (Kim et al., 2000). Although a plethora of inhibitors have been reported for PPO, only a few inhibitors have potential use in food industry. Therefore, there still exists an interest to find additional effective and safe antibrowning agents. Among the reported inhibitors the substrate analogs (phenolic compounds) and Cu^{2+} chelators are potent competitive inhibitors. The effectiveness of substrate analogs (phenolic compounds) were studied. Tropolone, a substrate analog and an effective chelator is potent inhibitor with a K_i of $5.7 \pm 0.2 \times10^{-7}$ M with TBC as the substrate. Among the substrate analogs the aromatic carboxylic acids appeared to be more potent inhibitors with K_is ranging from 2.1 to 3.5 mM. The IC_{50} for PTU inhibition

of field bean PPO was 70 μM when CAT was used as a substrate (Table 3.2). Tropolone inhibits field bean PPO competitively in a classical manner (Figure 3.11), 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., 1993b) and mushroom tyrosinase (Kahn and Andrawis, 1985). Tropolone is reported to chelate the active site copper of PPO by slowly binding to the oxy-form of the enzyme (Valero et al., 1991). Tropolone does not inhibit laccase and therefore is useful in differentiating PPOs from laccases which also oxidize *o*-diphenols (Dawley and Flurkey, 2003). Diethyldithiocarbamate, a potent inhibitor of plant PPOs (Anosike and Ayaebene, 1982) also complexes the copper prosthetic group at the active center.

All the phenolic inhibitors studied (Table 3.2) were competitive inhibitors of TBC with close similarities in their K_i. A novel computational approach for analyzing inhibitory and non-inhibitory phenolics in order to predict which structure would be inhibitory to PPO has been adopted and presented in the next chapter (Chapter IV). Casanola-Martin et al., (2006) used atomic linear indices based classification as a model to distinguish between inhibitory and non inhibitory compounds of tyrosinase.

Patterns in the expression of PPO vary on spatial and temporal scales, exhibiting growth stage differences as well as variation in different tissues in a single plant. Germination has been suggested as an inexpensive and effective tool for improving the quality of legumes by enhancing their digestibility (Reddy et al., 1989) and reducing the antinutritional contents (Liener, 1994). On germination the PPO activity decreased (Figure 3.15B). However a residual activity of ~10 % at the end of 96 h of germination is suggestive of its protective role. PPOs have been implicated in plant defense and play a central role in insect herbivory. Therefore the residual PPO activity and the activity measured in the leaves (4 leaf stage) may be vital to defense.

PPO activity increases throughout the developmental stages from flower to fully mature seeds (40 DAF). Little information is available on the expression of PPO in plants. A steep increase in the PPO activity in the early

stages of seed development (10-20 DAF) is observed (Figure 3.16). Most insects attack pods at an early stage when they contain developing seeds. The increased levels of PPO in the early stages of seed maturation are therefore required. Unique patterns of PPO expression in tissues in a developmental specific manner have been reported for apple (Kim et al., 2001). A PPO transcript was detected in the apple flower, which dramatically increased in the early stages of fruit development, but decreased as the fruit ripened. It was however transcriptionally active in mature leaves. A high PPO activity was also reported in developing flowers, leaves and tubers and roots of potato (Thygeson et al., 1995). The PPO activity of potato tissue was monitored by PPO gene expression. Thygeson et al., (1995) concluded that control of PPO levels in potato plants is mediated at the level of transcription. As observed in Figure 3.16 the PPO activity of developing field bean seed increases with increasing protein concentration. This data is consistent with previous studies, in which PPO expression has been greatest in developing tissues (Doan et al., 2004). It is interesting to note that in the case of field bean PPO, the activity does not decline as the seed matures. The data suggests that PPO expression in field bean seed is mediated at the level of transcription i.e. protein expression. The control of PPO gene expression in potato, tomato and other fruits is complicated with different genes exhibiting distinct patterns of spatial and temporal expression. Six PPO genes are known in tomato (Newman et al., 1993). This should not be so with field bean PPO, considering into the fact that only a single isoform is present as compared to other plant PPOs.

PPO activity is very low in leaf of field bean. PPO activity increased throughout adult developmental stages from pre-flower through fruit ripening in four varieties of tomato (Felton et al., 1989). In potato, PPO activity was highest in the youngest leaves assayed and decreased with increase in leaf age (Thygesen et al., 1995). Among the various forms of tomato PPO, higher activity was generally observed in younger leaves of individual plants (Thipyapong et al., 1997). Various artificial induction methods on tomato expression of PPO isozymes, were studied in young *vs* old plant parts (Thiyapong and Steffens, 1997). A significant difference was observed in the PPO activity during sugar cane maturity. PPO activity was

high at the early development stage, decreased during maturation, and then remained relatively constant at different maturity stages (Yassin et al., 2002). In Fuji apple, PPO was expressed in early stages of fruit development and expression was dramatically reduced as the fruit ripened. In leaf, PPO was highly expressed in young and immature leaves and significantly induced in leaves and fruits when wounded (Kim et al., 2001).

A definite physiological function is yet to be attributed to PPO (Vaughn et al., 1988) but it seems likely that it has a role in plant defense against insect predation and fungal pathogenesis. The extremely high PPO activity in the mature field bean seed compared to that reported for potato, tomato, apple and other fruits is consistent with a role in the protection from predation during storage. High levels of PPO in the cotyledons even after 96 h germination and in the first four leaf stage also lend support to such a role, although the function of PPO is still not unequivocally known. A few reports have suggested a specific physiological function for PPO. Cho et al., (2003) showed that a PPO was responsible for the conversion of (+)-larreatricin into (+)-3'-hydroxylarreatricin in *Larrea tridentate* and that the conversion was specific for the (+)-enantiomer. Most of the reports on PPO indicate a function to defend plants against pathogens and insect attack. Recently, Li and Steffens (2002) have obtained direct evidence of such a role of PPO in plants. It is triggered by many factors such as biotic and abiotic, herbivory, fungal and bacterial infections, mechanical damage, insect regurgitation and by treatment with compounds (Melo et al., 2006).

The spatial and temporal expression of PPO is differentially regulated during vegetative and reproductive stages and in response to wounding. The molecular mechanism of differential regulation of PPO expression and its physiological significance needs to be investigated to understand molecular functions of PPO.

Chapter IV

Functional interaction of substrate/inhibitors of polyphenol oxidase: A molecular study

The control of PPO mediated browning is still a challenge to the food processing industry. Use of chemical agents as food additives is being revaluated by the Food and Drug Administration and use in some products has been banned resulting in continued efforts towards identifying naturally occurring inhibitors of PPO. Benzoic acid has long been known as a PPO inhibitor. Substitution around the aromatic nucleus has shown varied effects on the degree of PPO inhibition. 2, 3-dihydroxy benzoic acid displayed no inhibition whereas 2, 4-dihydroxy benzoic acid was a strong inhibitor. Hydroxylation and methylation of the aromatic rings decreased the inhibitory effect of fruit PPOs. Literature indicates that the type and degree of inhibition by aromatic carboxylic acids on PPO activity are dependent on the structure of both-the substrate and the inhibitor. In our continuing investigations on the effect of different dihydroxy phenols, trihydroxy phenols, benzoic acid and some of their derivatives on field bean PPO activity, we noted that subtle structural changes transformed biochemically evaluated substrates to inhibitors of PPO and vice versa. Similar observations reported for other PPOs have been attributed to differences in their molecular structure such as the position of the hydroxyl and carboxyl group, the bulkiness and the length of the side-chain, which lead to varied interaction between the active site and inhibitor binding site. The number of PPO inhibitors with very high inhibitory activity described in literature with diverse chemical structures, related and unrelated to *o*-diphenols are numerous. Yet the precise mechanism of PPO inhibition and binding still remains unclear. Because the structural differences between substrates and inhibitors of PPO are subtle, identifying them for their distinct specificity is trackable using only molecular modeling. An attempt has been made to correlate at the molecular level, by using molecular docking as to why some diphenols are substrates and others are inhibitors and vice versa. It is noted that only minor structural changes are required to transform substrates to inhibitors of PPO and vice versa.

RESULTS

Substrate specificity of field bean seed PPO

The diphenols used in this study were catechol (CAT, 2-100 mM), 4-

methyl catechol (4MC, 5-100 mM), t-butylcatechol (TBC, 2-40 mM), 3,4-dihydroxyphenylalanine (DOPA, 0.5-15.0 mM), dopamine (DPN, 0.25-25 mM), 3,4-dihydroxyphenylacetic acid (DOPAC, 5-100 mM). Among triphenols 2, 3, 4-trihydroxy benzoic acid (2, 3, 4-THBA, 2-25 mM), 2, 3, 4-trihydroxybenzaldehyde (2, 3, 4-THBZ, 2.5-25 mM), were used as a substrates (Table 4. 1). The activity of purified field bean PPO towards different phenolic compounds was determined (Table 4.1). The wavelength at which the corresponding oxidation product exhibited maximum absorption was determined and used to compute the PPO activity as described earlier (Table 3.2).

The values of K_m and V_{max} were obtained by evaluation of Lineweaver-Burk plots of these kinetic measurements. TBC and 4MC were the most readily oxidized substrates of field bean PPO as revealed by the high V_{max}. Field bean PPO obeys Michaelis-Menten kinetics and exhibits the phenomenon of inhibition by excess substrate for CAT and 4MC. CAT and 4MC differ from each other by the presence of a single $-CH_3$ group on the aromatic ring, yet the K_m differs by >2.5 fold. Among the substrates studied, the binding affinity (K_m) was strongest for DPN although the rate of oxidation was very low (Table 4.1).

Effect of benzoic acid and its derivatives on PPO activity

The effect of benzoic acid and closely related congeners such as *o*-hydroxybenzoic acid (*o*HBA); *m*-hydroxybenzoic acid (*m*HBA)*;* *p*-hydroxybenzoic acid (*p*HBA), dihydroxybenzoic acid like 2, 3-dihydroxybenzoic acid (2, 3-DHBA), 3, 4-dihydroxybenzoic acid (3, 4-DHBA), 2, 4-dihydroxybenzoic acid (2, 4-DHBA), 3, 5-dihydroxybenzoic acid (3, 5-DHBA), vanillic acid and trihydroxy benzoic acids like 3, 4, 5-trihydroxybenzoic acid (3, 4, 5-THBA, Figure 4.2) were tested as inhibitors of field bean PPO at different concentrations of CAT. The pattern of the Michaelis-Menten plots obtained were typical of a competitive type of inhibition. The results recorded in Table 4.2 were obtained by Lineweaver-Burk plots of $1/V$ against 1/[S], at four levels of inhibitor concentration. The inhibition constants K_i were confirmed by Dixon plots of $1/V$ against [I]

(Figure 4.3). The results showed that field bean PPO was strongly inhibited by monohydroxybenzoic acids.

Table 4.1. Kinetic measurements for field bean PPO using various substrates.

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Substrate	$V_{max} \times 10^3$ (U/mg)	K_m (mM)	$k_{cat} \times 10^3$
CAT	89.7±0.22	12.0±0.01	7.4
4-MC	120.0±0.26	4.5±0.03	26.6
TBC	197.0±0.25	4.2 ±0.02	46.9
DOPA	33.5±0.32	2.9±0.04	11.5
DPN	11.1±0.19	1.5±0.02	7.4
2, 3, 4-THBA	12.3±0.29	4.0±0.02	3.0
2, 3, 4-THB	11.3±0.40	4.5±0.04	2.5
DOPAC	15.3±0.31	4.2±0.02	3.6

Table 4.2. Inhibition of field bean PPO by benzoic acid and its derivatives.

Inhibitor	IC_{50} (mM)	K_i (M) 10^{-4}	Mode of inhibition
BA	1.2±0.03	0.30±0.02	Competitive
*o*HBA	3.6±0.01	0.44±0.02	Competitive
*m*HBA	1.1±0.02	0.40±0.03	Competitive
*p*HBA	0.21±0.03	0.22±0.01	Competitive
2, 3-DHBA	3.7±0.01	2.2±0.03	Competitive
3, 4-DHBA	2.8±0.01	1.0±0.02	Competitive
3, 4, 5-THBA	3.8±0.02	2.4±0.03	Competitive
PTU	0.068±0.002	0.014±0.003	Competitive

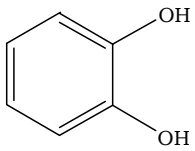
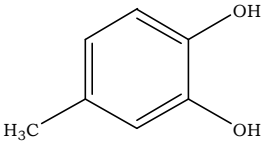
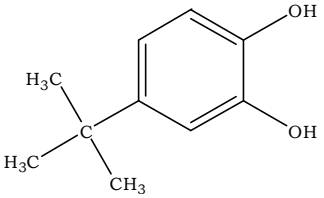
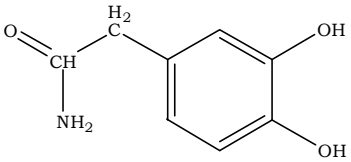
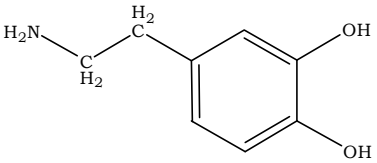
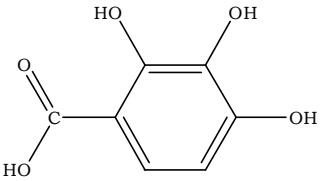
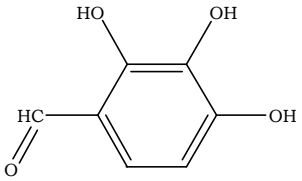
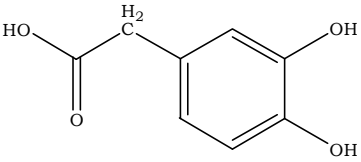
	
Catechol (CAT)	4-Methyl catechol (4-MC)
	
t-Butylcatechol (TBC)	3, 4-Dihydroxyphenylalanine (DOPA)
	
Dopamine (DPN)	2, 3, 4-Trihydroxynenzoic acid (2, 3, 4-THBA)
	
2, 3, 4-Trihydroxybenzaldehyde (2, 3, 4-THB)	3, 4-Dihydroxyphenylacetic acid (DOPAC)

Figure 4.1. Chemical structures of substrates.

Effect of benzaldehyde derivatives on field bean PPO activity

Inhibition of PPO by benzaldehyde derivatives was determined for 2, 5-dihydroxybenzaldehyde (2, 5-DHB), 3, 4-dihydroxybenzaldehyde (3, 4-DHB) 3, 4, 5-trihydroxybenzaldehyde (3, 4, 5-THB) and vanillin. These compounds like benzoic acid derivatives also showed strong inhibition (Table 4.3 and Figure 4.3). These results show that the aldehyde group is as

effective as the carboxyl group in inhibiting PPO. The IC_{50} values for PPO inhibition by the dihydroxy benzaldehydes were similar, suggesting that the mechanism involved in inhibition were similar. 3, 4, 5-THB was not as effective an inhibitor as dihydroxy benzaldehydes. 2, 3, 4-THB did not inhibit field bean PPO but it was oxidized, the V_{max} being 11.3×10^3 U/mg and K_m 4.5 mM.

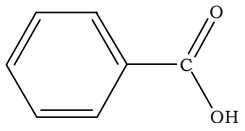
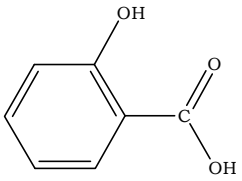
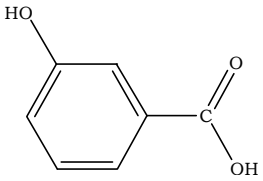
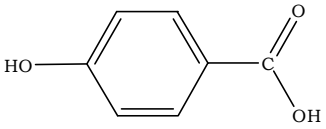
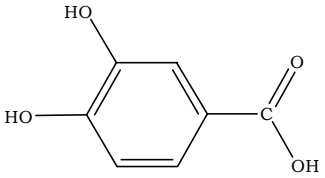
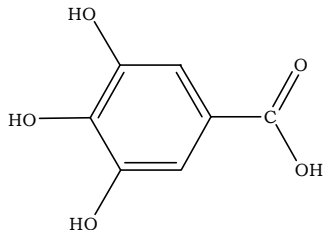
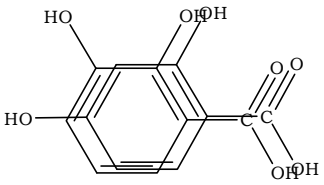
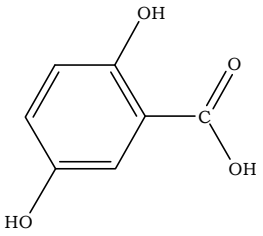
		
Benzoic acid (BA)	*o*-Hydroxybenzoic acid (*o*HBA)	*m*-Hydroxybenzoic acid (*m*HBA)
		
p-Hydroxybenzoic acid (*p*HBA)	2, 3-Dihydroxybenzoic acid (2, 3-DHBA)	3, 4-Dihydroxybenzoic acid (3, 4-DHBA)
		
3, 4, 5-Trihydroxybenzoic acid (3, 4, 5-THBA)	2, 4-Dihydroxybenzoic acid (2,4-DHBA)	3, 5-Dihydroxybenzoic acid (3, 5-DHBA)

Figure 4.2. Chemical structures of benzoic acid and derivatives of benzoic acid.

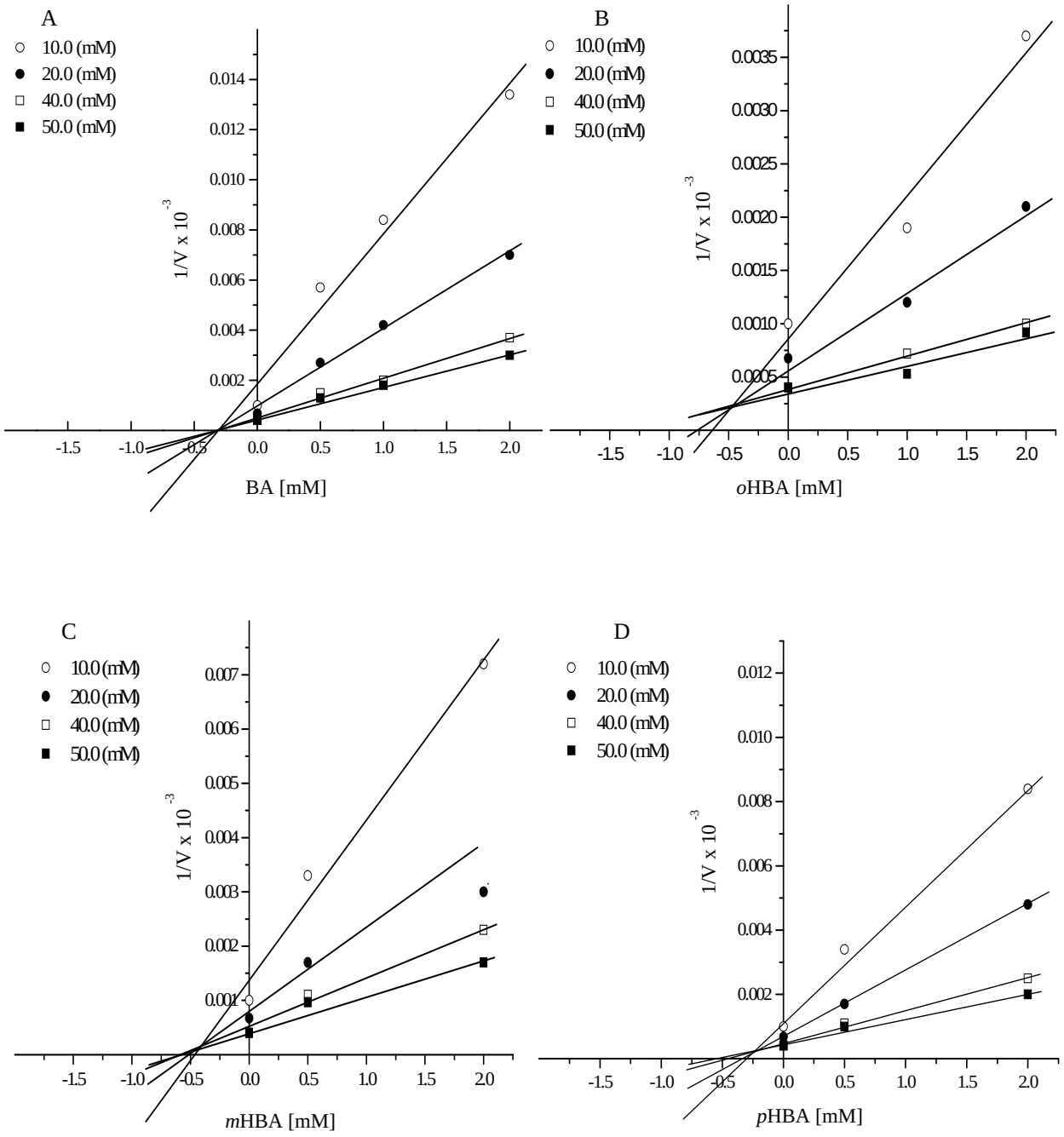


Figure 4.3. Dixon plots for the inhibition of field bean PPO. Enzyme was pre-incubated with varied inhibitor concentrations for 3 min and residual activity determined using CAT as the substrate. (A) BA; (B) *o*HBA; (C) *m*HBA; (D) *p*HBA. The substrate concentration used are indicated.

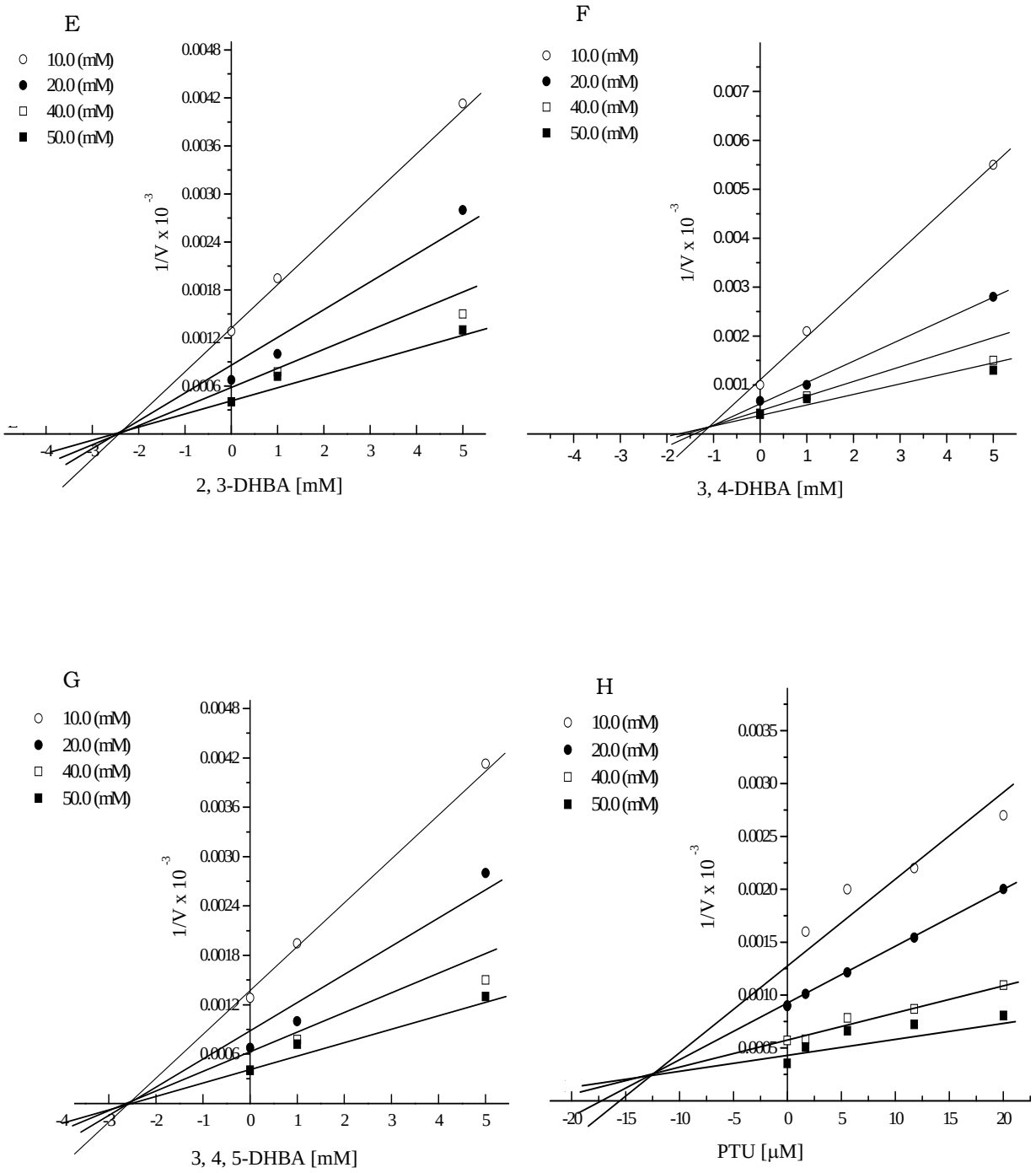


Figure 4.3. Dixon plots for field bean PPO inhibition by aromatic acids and PTU. PPO was pre-incubated with varied inhibitor concentrations for 3 min and residual activity determined using CAT as the substrate. (E) 2, 3-DHBA; (F) 3, 4-DHBA; (G) 3, 4, 5-THBA; (H) PTU. The substrate concentration used are indicated.

Effect of phenyl alkanoic acids on field bean PPO activity

The effect of some phenyl alkanoic acids such as *o*-toluic acid, phenyl acetic acid (PAA), 3 (*p*-hydroxyphenyl) propionic acid 3 (*p*-HPP), *3*-phenylpropionic acid (PPA), *4*-phenyl butyric acid (PBA), cinnamic acid and *p*-coumaric acid on PPO was determined. The results are summarized in Table 4.3. PAA exhibited maximum inhibition followed by (p-HPP) which was more potent than the other phenyl alkanoic acids studied.

Table 4.3. IC_{50} values for PPO inhibition by aromatic aldehydes and acids.

Compounds	**Inhibition IC_{50} (mM)**
2, 4-DHBA	1.2±0.02
3, 5-DHBA	1.6±0.02
2, 5-DHB	2.0±0.04
3, 4-DHB	1.7±0.04
3, 4, 5-THB	3.0±0.03
Vanillin	0.6±0.03
Vanilic acid	0.2±0.03
Cinnamic acid	1.8±0.03
p-Coumaric acid	2.2±0.04
o-Toluic acid	3.6±0.03
PAA	1.6±0.02
3 (p-HPP)	1.7±0.03
3-PPA	4.5±0.04
4-PBA	6.0±0.04

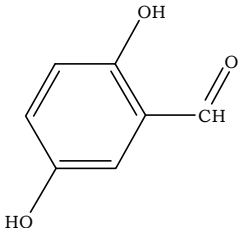
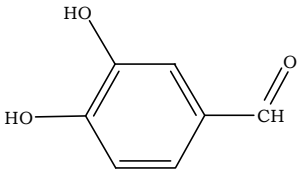
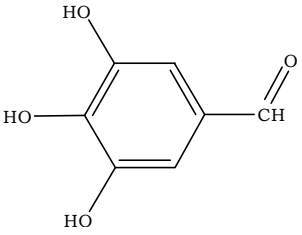
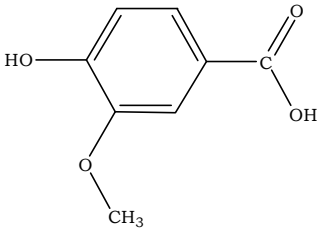
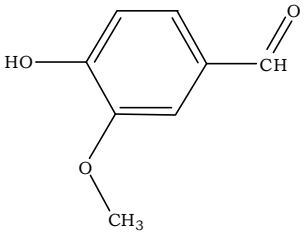
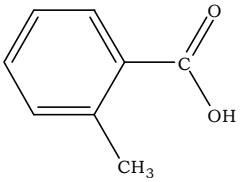
		
2, 5-Dihydroxybenzaldehyde (2, 5-DHB)	3, 4-Dihydroxybenzaldehyde (3, 4-DHB)	3, 4, 5-Trihydroxybenzaldehyde (3, 4, 5-THB)
		
Vanillic acid	Vanillin	*o*-Toluic acid

Figure 4.4. Chemical structures of aromatic aldehydes and acids.

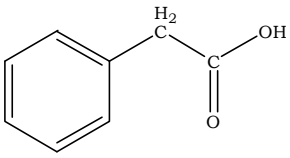
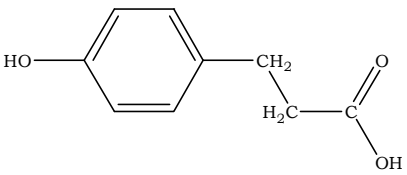
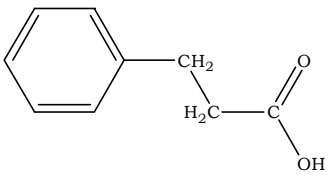
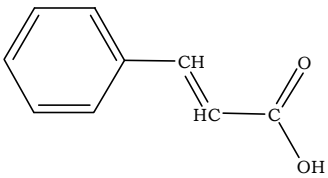
	
Phenylacetic acid (PAA)	*p*-Hydroxyphenylpropionic acid 3(*p*-HPP)
	
Phenylpropionic acid (PPA)	Phenylbutyric acid (PBA)

Figure 4.5. Chemical structure of phenyl alkanoic acids

Substrate /inhibitor specificity of sweet potato catechol oxidase (ibCO)

The oxidation of some phenolic substrates evaluated using purified ibCO is tabulated in Table 4.4. TBC was oxidized at the maximum rate and also showed the highest binding affinity. The oxidation of the substrates was of the order TBC > 4MC > CAT which is similar to that of field bean PPO (Table 4.2). Benzoic acid and closely related congeners such as *o*HBA, 2, 3-DHBA, 3, 4-DHBA and 3, 4, 5-THBA were tested as inhibitors of ibCO. The Michaelis-Menten plots obtained were typical of a competitive type of inhibition for *o*HBA and 2, 3-DHBA. The mode of inhibition was determined by Lineweaver-Burk plots of 1/V against 1/(S) (Figure 4.6). *o*HBA and 2, 3-DHBA showed competitive inhibition but 3, 4-DHBA and 3, 4, 5-THBA showed mixed inhibition. The K_i values are obtained from the secondary plots (Table 4.4). Among the inhibitors 3, 4, 5-THBA was the most potent.

Table 4.4. Experimental, predicted kinetic constants and predicted free energies of binding (ΔG_b) and interactions of phenolic compounds with sweet potato ibCO

Compound	EXPERIMENTAL		PREDICTED		Residues in Hydrogen bonding	Number of hydrophobic interactions	Aromatic-aromatic interactions
	Binding Constant ($\times10^{-3}$ M) (K_m/K_i)	V_{max} $\times10^3$ (U/mg)	Energy of Docking kcal/mol (ΔG_b)	Binding constant ($\times10^{-5}$ M)			
CAT	9.0±0.03	5.6±0.5	-5.97	4.07	-	4	$His^{88,244}$, Phe^{261}
4MC	5.2±0.02	2.08±0.3	-6.45	1.90	-	5	$His^{88,244}$, Phe^{261}
TBC	3.9±0.04	12.6±0.25	-7.3	0.43	-	5	$His^{88,244}$, Phe^{261}
DOPAC	6.0±0.03	0.86±0.3	-6.92	0.25	-	4	His^{244}, Phe^{261}
2, 3, 4-THBA	5.6±0.04	2.1±0.35	-6.95	0.81	-	2	His^{244}, Phe^{261}
BA	4.5±0.02		-6.5	2.2	-	3	$His^{240,244}$, Phe^{261}
*o*HBA	3.4±0.03		-6.54	1.59	His^{109} Phe^{261}	3	His^{244}, Phe^{261}
2, 3-DHBA	2.3±0.04		-6.71	1.11	His^{109}	2	$His^{88,244}$, Phe^{261}
3, 4-DHBA	4.3±0.03		-6.58	1.12	His^{109}	2	$His^{240,244}$, Phe^{261}
3,4,5-THBA	2.3±0.02		-7.09	0.51	His^{109} Asn^{260}	3	$His^{240,244}$, Phe^{261}

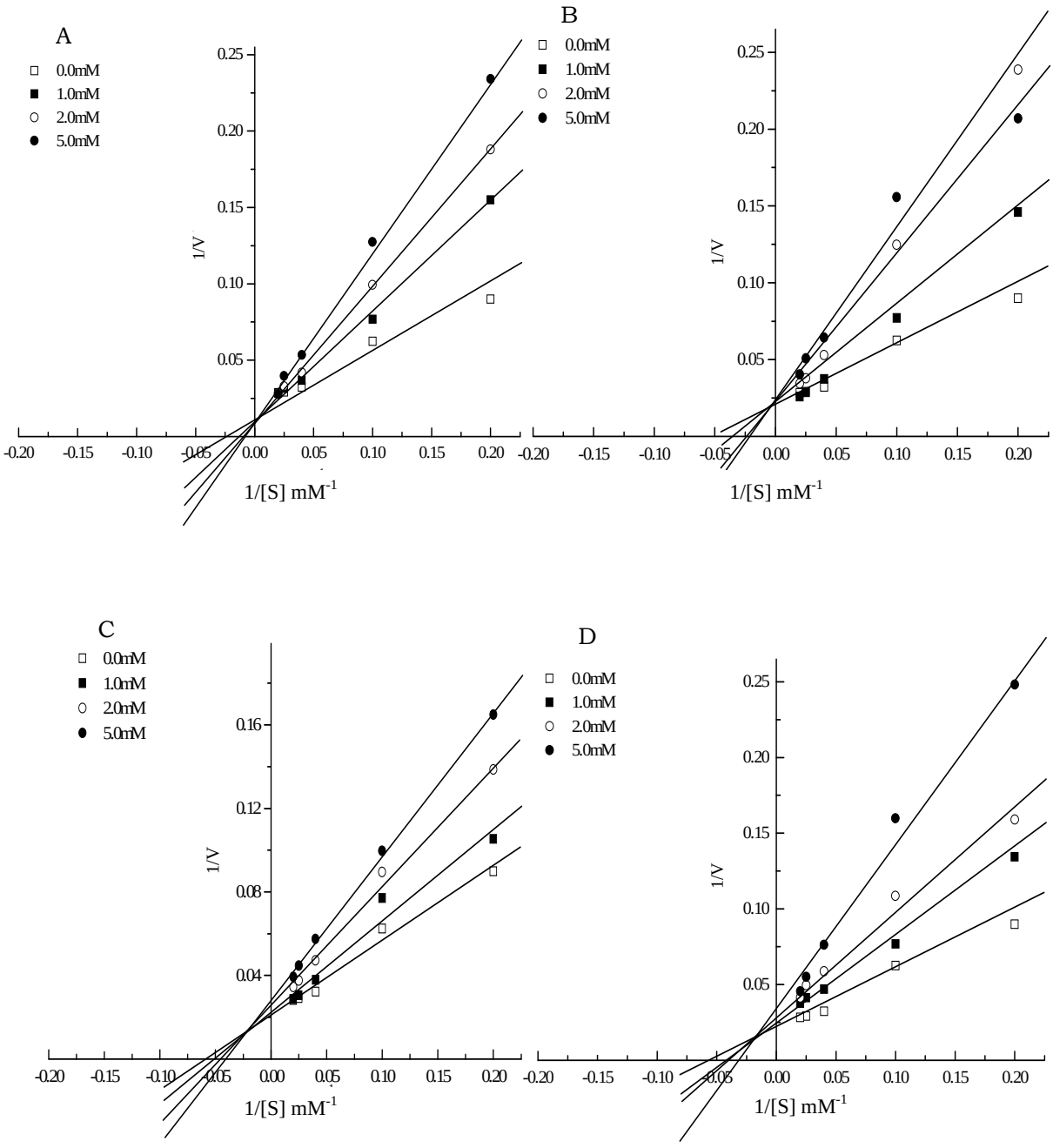


Figure 4.6. Lineweaver-Burk plots for the inhibition of ibCO. ibCO was pre-incubated with various inhibitor concentrations for 3 min and residual ibCO activity determined using CAT as the substrate. (A) *o*HBA; (B) 2, 3-DHBA; (C) 3, 4-DHBA and (D) 3, 4, 5-THBA. The substrate concentrations are indicated in the figure.

Rationale for molecular modeling

Distinct variations in the activity of field bean PPO and ibCO toward different types of substrates and inhibitors containing a common aromatic ring are observed (Table 4.1-to 4.4). The number of hydroxyl groups, their position in the aromatic ring of the substrate/inhibitor and the nature of the side chain exhibit a profound effect on the V_{max} and K_m of the two

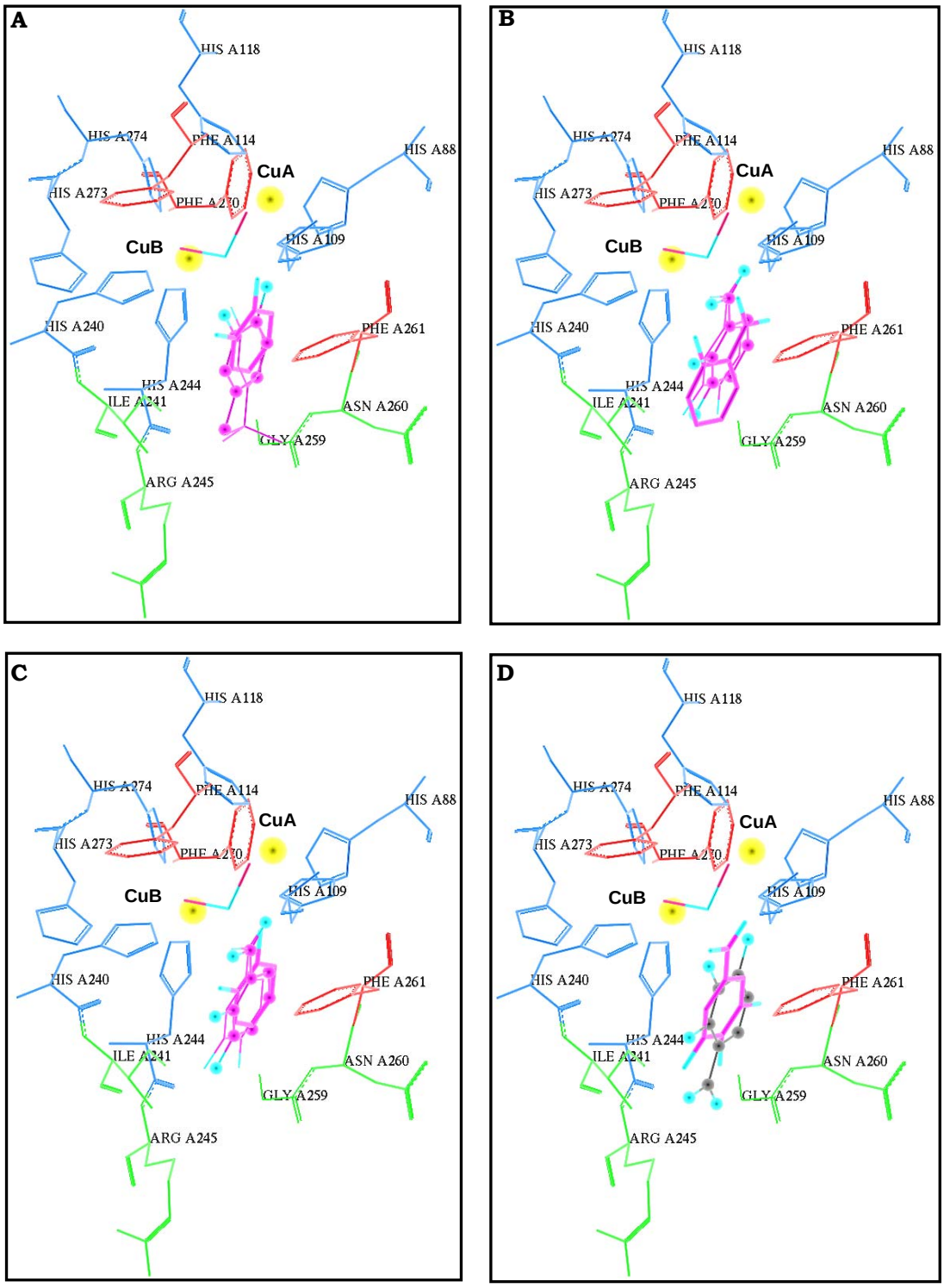
enzymes. Alkyl substitution by methyl and t-butyl group of *o*-diphenols leads to a considerable increase in the k_{cat} and tighter binding. As observed the rate of TBC oxidized is much higher than CAT (Table 4.1 and 4.4). The k_{cat} is several folds higher than that of CAT (Table 4.4). The most potent inhibitor of field bean PPO activity is exhibited by *p*HBA with a K_i of 2.2 mM. The two related phenolic analogues-2, 3-DHBA and 3, 4-DHBA did not alter the inhibitory potential. In contrast the substitution by a third hydroxy group to form 2, 3, 4-THBA renders it as a substrate for ibCO and field bean PPO. In direct contrast, a similar addition to form 3, 4, 5-THBA is still an inhibitor of both field bean PPO and ibCO. These results suggest that the apparent position of key groups (carboxylic and hydroxyl) are important in eliciting either substrate/inhibitor specificity. To elucidate the molecular determinants of substrate and inhibitors of PPO, molecular docking studies were carried out, using available X-ray structure of ibCO.

Molecular docking of substrates/inhibitors with ibCO

The compounds shown in Figures (4.1-4.3) are either substrates or inhibitors of ibCO and field bean PPO. To help rationalize and provide an explanation for the experimental data, computational docking studies were performed using Autodock 3.0. Autodock is a small-molecule docking program that uses grid-based methods for energy function to score docked ligands. The availability of the X-ray crystallographic structure of ibCO (PDB ID 1BUG) of sweet potato paved the way for these molecular modeling studies. PTU is a potent inhibitor (K_i =2.5 μM) that binds to the active site of ibCO (Klabunde et al., 1998). In the present study the water molecule and the inhibitor molecule were removed from the ibCO structure. Compounds, which have the same basic phenyl ring, were successfully docked to the active site of ibCO and comparisons made (Figure 4.7). Table 4.4 lists the results of the docking experiments, calculated free energy of binding (ΔG_b), inhibition binding constant and their corresponding experimentally determined constants. The binding mode of compounds CAT, 4MC and TBC indicated that TBC was the most favored substrate (Figure 4.7A). A careful inspection of the substrate-binding pocket also supported the experimental data. The phenyl ring of all the substrates was stacked perfectly against His ^{244} much like PTU and was co-ordinated to both the Cu^{2+} atoms with a metal-metal separation of 3.3, 4.2 and 4.2 Å for CAT, 4MC and TBC

respectively. The Cu-N distance was 2.8, 3.0 and 2.9 Å respectively. Additionally the methyl and t-butyl group of 4MC and TBC showed interactions with the alkyl side chain of Arg^{245}. Additional van der Waals interactions between the t-butyl group and the residues Ile^{241} and C_β of His^{244} (Figure 4.7A), in the hydrophobic cavity complemented strong binding. The complementarity of the alkyl side chain interaction and favorable van der Waals interactions improved the fitness of TBC and it is therefore strongly locked into the hydrophobic pocket. Both calculated and experimentally derived binding affinities (K_m) indicate that TBC has greater binding affinity. These results are also reflected by the estimated free energy of docking (Table 4.4).

Among the inhibitors evaluated the mono and dihydroxy benzoic acids are potent inhibitors. The binding mode of the reversible inhibitors (*o*HBA, 2, 3-DHBA, 3, 4-DHBA) of ibCO is shown in Figure 4.7B. Docking of these compounds indicate that the stacking of the aromatic ring is in concurrence with that of CAT (Figure 4.7C). However the carboxylic groups of all the three compounds are hydrogen bonded to His^{240} more or less like a salt bridge which, reckon tight binding. The distance between the metal-metal centers increased to 5.0 Å inhibiting the formation of the hydroxo-bridge essential to catalysis, which thereby renders them as inhibitors. The free energies of binding (ΔG_b) and theoretical K_i are similar to the experimental values, indicating that they inhibit ibCO to the same extent. These results correlate with the experimental results. The carboxyl groups of 3, 4, 5-THBA and aromatic ring superimpose exactly with 2, 3-DHBA and 3, 4-DHBA, and therefore are inhibitors. The hydroxy group at position 5 fits well into the open cavity of the substrate space in the active site (Figure 4.7E). 3, 4, 5-THBA is an inhibitor, and in direct contrast 2, 3, 4-THBA is a substrate. This minor structural difference that renders it as a substrate was probed by docking. When 2, 3, 4-THBA is docked, their exists a steric clash of the third hydroxy group with Ala^{259} and Phe^{260} residues that line the hydrophobic pocket unlike 3, 4, 5-THBA (Figure 4.7D). This steric hindrance does not permit the same orientation as 2, 3-DHBA, 3, 4-DHBA and 3, 4, 5-THBA. This unfavorable orientation and steric clash causes the flipping of the molecule, which mimics the orientation of TBC and 2, 3, 4-THBA therefore


A
HIS A118
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CuA
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CuB
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HIS A244
ILE A241
ASN A260
GLY A259
ARG A245
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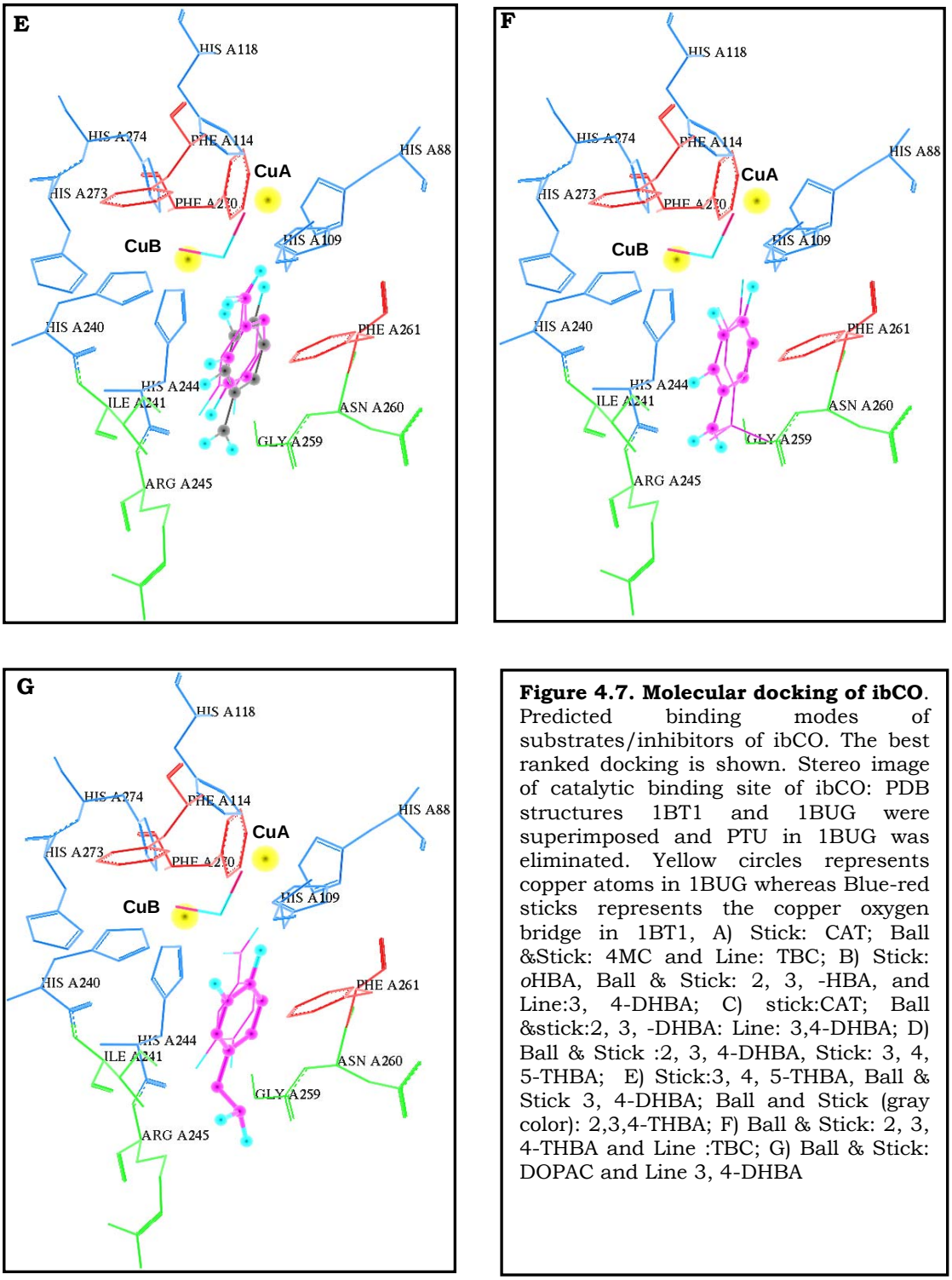

Figure 4.7. Molecular docking of ibCO. Predicted binding modes of substrates/inhibitors of ibCO. The best ranked docking is shown. Stereo image of catalytic binding site of ibCO: PDB structures 1BT1 and 1BUG were superimposed and PTU in 1BUG was eliminated. Yellow circles represents copper atoms in 1BUG whereas Blue-red sticks represents the copper oxygen bridge in 1BT1, A) Stick: CAT; Ball &Stick: 4MC and Line: TBC; B) Stick: *o*HBA, Ball & Stick: 2, 3, -HBA, and Line:3, 4-DHBA; C) stick:CAT; Ball &stick:2, 3, -DHBA: Line: 3,4-DHBA; D) Ball & Stick :2, 3, 4-DHBA, Stick: 3, 4, 5-THBA; E) Stick:3, 4, 5-THBA, Ball & Stick 3, 4-DHBA; Ball and Stick (gray color): 2,3,4-THBA; F) Ball & Stick: 2, 3, 4-THBA and Line :TBC; G) Ball & Stick: DOPAC and Line 3, 4-DHBA

behaves as a substrate (Figure 4.7F). These results are substantiated experimentally (Table 4.4). The calculated K_ms of TBC and 2, 3, 4-THBA were similar, indicating a very strong binding. A comparison of the docking of these two compounds indicated that the carboxyl group of the flipped 2, 3, 4-THBA occupies a position very similar to the t-butyl group of TBC and showed identical contacts with the alkyl side chain of Arg^{245} and van der Waals interactions. The metal-metal distance was 4.2 Å. All these interactions favor its orientation thus allowing Cu–Cu to bridge with oxygen for oxidation. DOPAC differs from 3, 4-DHBA by the presence of an additional $–CH_2$ group in the side chain. DOPAC was oxidized readily by ibCO and field bean PPO; in contrast to 3, 4-DHBA, which was inhibited by both PPO and ibCO (Table 4.1 and 4.4). This difference in binding was reckoned by the steric clashes observed at the active site. The increased length due to the additional $–CH_2$ group of DOPAC leads to several short contacts, resulting in steric clashes with the hydrogen bonded side chains, which ultimately render the flipping of the molecule. Therefore DOPAC becomes a substrate, with the -COOH group facing away for the CuA (Figure 4.7G). The theoretical K_m and ΔG_b indicated a high binding affinity (Table 4. 4).

Discussion

Catechol (*o*-dihydroxy phenol) the archetype of all plant PPOs is rapidly oxidized to its quinone at the expense of molecular oxygen. The nature of the side chain, number of hydroxyl groups and their positions in the benzene ring of the substrate has been shown to exhibit a major effect on the catalytic activity of PPOs (Harel et al., 1964; Park and Luh, 1985). The lack of structural information available for field bean PPO and the intriguing subtle structural differences in the substrates, which render them as inhibitors and vice versa motivated us to explore modeling studies with ibCO, to explain these experimental observations. For a better understanding of these differences using an automated molecular docking algorithm AutoDock and taking advantage of the only available crystal structure of a PPO (diphenol oxidase), ibCO, we for the first time have demonstrated that the observed difference in patterns of substrate/inhibitor relationship are due to marked changes in the interactions between the

compounds and orientation of the molecule at the active site of ibCO. Our results rationalize and support the experimental observations and afford a good explanation for the different behavior of substrates and inhibitors experimentally evaluated.

Plant PPOs are capable of oxidizing a wide range of *o*-dihydroxyphenols, the primary substrates with varied oxidation rates (V_{max}) and affinities of different orders of magnitude. The native concentrations of natural phenols vary from plant to plant (Mayer and Harel, 1979). Catechin, epicatechin and caffeic acid derivatives are believed to be common natural substrates of several fruit PPOs (Machiex et al., 1990). It appears that the substrate specificity of PPO is also dependent on species, cultivar and vegetative part of the plant (Yoruk and Marsha, 2003). Field bean seed PPO and ibCO oxidized TBC at a significantly faster rate than other structurally related compounds (Table 4.1 & 4.4), the order being TBC > 4MC > CAT. Furthermore trihydroxy phenols were also efficiently oxidized. Both field bean PPO and ibCO exhibit a greater affinity for *o*-dihydroxy phenols compared to the trihydroxy phenol (pyrogallol). The binding properties (K_m) and catalytic power (k_{cat}) of field bean PPO and ibCO increased with an increase in the size of the side chain in the aromatic ring of its substrates (Table 4.1 and 4.4). Tremolieres and Bieth, (1984) attributed similar observations to the electron donating capacity of a methyl group at the para position of 4MC. Decreased oxidation rates with different substrates were explained by the presence of electron attracting substituents such as > COOH (Duckworth and Coleman, 1970). The presence of the unsaturated-CH=CH-COOH led to an increased binding affinity in black poplar PPO. This phenomenon was rationalized by assuming that PPO possessed an extended substrate binding site (Tremolieres and Bieth, 1984). Previous reports on the substrate specificity of apple PPO (Walker, 1964; Harel et al., 1964) and other sources showed that 4MC, chlorogenic acid and catechin were readily oxidized. The substrate's side chain had a marked effect on the oxidation rate but more important was the spatial orientation of the essential vicinal dihydroxy groups with respect to the side chain (Walker and Wilson, 1975; Lerner et al., 1972). Lerner et al., (1972) based on the evidence that the binding sites for oxygen and phenolic substrate are independent, concluded that binding of oxygen induced a conformational change and could account

for the differences observed in the substrate interaction.

The pattern of inhibition expressed in this investigation indicate that the basic benzoic acid structure is a key element in eliciting potent inhibition of field bean PPO and ibCO. Either mono or di-hydroxylation of the benzoic acid nucleus increased inhibition and lowered K_i (Table 4.2 and 4.4). 3, 4-DHBA (protocatechuic acid) and the isomeric 2, 3-DHBA were equally potent inhibitors as evidenced by the similar K_i. These observations suggest that the benzoic acid nucleus is involved in inhibitor binding and hydroxylation increased the inhibition. These finding are in keeping with earlier reports on cherry PPO, which suggested that BA interfered with the binding of the substrate to the active site (Pifferi et al., 1974). Vamos-Vigyazo, (1981) suggested that for strong PPO inhibition, aromatic inhibitors require a free carboxylic group substituted directly on the benzene ring. BA and some substituted cinnamic acids were found to be competitive inhibitors of PPO from cherries, apples, pears and apricots. The K_i values were in the order BA > *o*-coumaric acid > *m*-coumaric acid > *p*-coumaric acid > cinnamic acid (Vamos-Vigyazo, 1981). The difference in the inhibitory strength of the benzoic acids and their derivatives and the effectiveness of all other inhibitor of PPO are speculative and interpreted on the basis of the occurrence of separate catalytic and inhibitor sites (Kahn, 1985) or the presence or absence of electron abstracting groups or the extended substrate binding sites (Tremoliers and Beth, 1985; Rompel et al., 1999; Passi and Nazzaro–Porro, 1981). Although 3, 4-DHBA and 2, 3-DHBA are potent inhibitors of both field bean PPO and ibCO (Table 4.2, 4.4) 2, 3, 4-THBA was rapidly oxidized to its quinones. In contrast 3, 4, 5-THBA (gallic acid) is a mixed type inhibitor. These results suggest that the spatial orientation of the hydroxy groups with respect to the carboxyl group dictates inhibitor/substrate specificity. Rompel et al., (1999) in their studies on substrate specificity of PPO from *Lycopus europaeus* suggest that a hydroxy group in the chromane ring of myricetin and quercitin stabilizes the produced quinone by mesomeric effects, which causes a decrease in the activation energy for the formation of an enzyme substrate complex. However none of the data and explanation of these observed differences has been proven at the molecular level.

Success of docking using Autodock, based on the Lamarckian genetic

algorithm has been recently demonstrated (Hetenyi and Spoel, 2002). This systematic docking study reproduced crystallographic information of eight different protein-ligand complexes. Therefore using Autodock we set out to explain the functional differences in the behavior of related phenolic compounds. As a starting configuration for the docking studies the bound PTU in the ibCO complex (PDB 1BUG) was replaced with the compounds shown in Figure 4.1-4.3. It is evident from the docking results (Table 4.4, Figure 4.7) that all compounds possessing a basic phenyl ring bind to ibCO. The phenyl moiety of all the phenolic compounds stacks perfectly against His^{244}. The aromatic interactions listed (Table 4.4) advocate that two interaction are involving Phe^{261} and His^{240} universal to all phenolic compounds irrespective of whether they are substrates or inhibitors. The other aromatic interactions occur between the phenyl ring and residues that line the hydrophobic cavity. These results reckon that any aromatic compound will bind to all plant PPOs. This is supported by the premise that the interaction involves His^{88}, His^{244} and Phe^{261} that are invariant residues in all plant PPOs (Hernandez-Romero et al., 2006). This is supported by the fact that PTU is not a phenol yet binds very strongly to ibCO (K_m=2.5 mM). Field bean PPO also shows a very similar binding affinity. A structural model of *p*-nitrophenol in coordination to the tyrosinase of *S. antibioticus,* derived based on ^1H and ^2D-NMR data binding to ibCO shows that one His residue is particularly sensitive to binding. This corresponded to His^{244} of ibCO (Tepper et al., 2005) and is consistent with our results showing the interaction with His^{244}. Among the residues involved in the aromatic interactions His^{244} is coordinated to CuB whereas His^{88} is coordinated to CuA. Phe^{261} located above the hydrophobic cavity has been termed as the 'gate residue' as it blocks the entrance to the hydrophobic cavity (Geredemann et al., 2002). To explain the absence of monophenolase of ibCO it is suggested that Phe^{261} blocks the direct approach and reorientation of monophenols to CuA, needed for its hydroxylation (Tepper et al., 2005). In fungal PPOs this residue is replaced by Leu or Pro. *Streptomyces* tyrosinase has a Gly at this position. These residues being small cannot block the entry and as expected monophenolase activity will be very high (Hernandez-Romero et al., 2006). The orientation of phenols with their hydroxyl group directed towards CuA appears to be necessary for the monophenolase activity (Decker and Tuczek, 2000). The gate residue shields CuA of ibCO, therefore the

monophenolase activity is restricted. It can therefore be assumed that the lack of monophenolase activity in field bean PPO is due this shielding. It may not be unreasonable to state that all PPOs that have a conserved Phe^{261} will not exhibit monophenolase activity.

The k_{cat} and binding affinity of TBC is > 4MC > CAT. The higher affinity of 4MC and TBC can be interpreted by the interaction with the alkyl side chains of Arg^{245} absent with catechol. The k_{cat} reported for 4MC is several folds higher than CAT for black poplar PPO (Tremolieres and Bieth, 1984), which shows an Arg residue corresponding to Arg^{245} (Hernandez-Romero et al., 2006). TBC, 2, 3, 4-THBA and DOPAC make additional van der Waals contact with the residues of the hydrophobic pocket increasing the binding affinity. Among them His^{244} is conserved whereas Ile^{241} is not. It is observed that in tomato and potato catechol oxidase, where this residue is Ile ^{241}, they exhibit a higher binding affinity.

A majority of the reversible inhibitors of plant PPO are substrate analogs. Among them, the aromatic acids are the most studied. The K_i for these compounds are in the millimolar range. As observed for the substrates, the perfect stacking of the phenyl moiety coupled with the aromatic interactions listed (Table 4.4), the bound analog is locked in the substrate cavity. The competitive nature of inhibition of all these compounds is not unexpected as the aromatic rings stack perfectly with the imidazole of His^{244} in the same fashion as CAT, the substrate used in the inhibition studies. All the inhibitors, *o*HBA, DHBAs and 3, 4, 5-THBA docked, superimpose on each other and show identical contacts (Figure 4.7B and D). The structural model of tyrosinase with its inhibitor *p*-nitrophenol showed that the phenyl moiety was tilted toward CuA. In this position the orientation favored efficient oxygen atom transfer (Tepper et al., 2005). In ibCO the two cupric ions CuA and CuB are bridged by a hydroxide ion at a distance of 1.8 Å. This forms the hydroxobridge essential for catalysis. Docking the substrate analogs (Figure 4.7B and D) increased the distance to 5.02 Å. The distance between the two Cu is 4.2 Å when ibCO binds to either the substrates TBC and 4MC or PTU. Although this distance is the same the sulfur atom of PTU replaces the hydroxobridge accounting for the potent inhibition (IC_{50} = 43 µM). PTU is also a very potent inhibitor of field bean PPO (IC_{50} = 70µM) suggesting a similar mechanism is operational. The universal hydrogen bond

between the inhibitor compounds and His^{109}, a coordinating ligand of CuA in plant PPOs further fixes the inhibitors. His^{109} liganded to CuA is covalently linked to a cysteine residue by an unusual thioether bridge. Molecular modeling showed that the flexibility of the His is sufficiently reduced because of this linkage (Gielens et al., 2007). The present results show that the flexibility is still sufficiently high in ibCO to allow the His^{109} to form a salt bridge like hydrogen bond with all the aromatic inhibitors. The crystallographic study of a metal free tyrosinase in complex with ORF378 (A caddie protein) has established that this linkage does not allow a bidentate intermediate, therefore monophenols cannot serve as either substrates or inhibitors (Matoba et al., 2006). Because of the universal interactions between the phenolic inhibitors and ibCO, it is expected that the binding affinities for the phenolic compound would be similar if not the same. This is justified by the experimental K_is listed (Table 4.4). The predicted inhibition constants are several orders lower than the experimental values although the relative trend is similar. The solvation and entropic effects have not been considered in this model. Moreover it is well established that the binding affinities depend on several in *vitro* conditions such as pH and ionic strength of the buffer, temperature, substrate used etc. These parameters are not accounted for in these models. The K_is calculated from Autodock or any other docking programmes are therefore best interpreted in a relative manner and not as absolute values.

Only a minor structural change is required to transform a substrate into an inhibitor. Experimentally 2, 3, 4-THBA is very effectively oxidized to its quinone. But 3, 4, 5-THBA is a competitive inhibitor. The docking study has provided a reasonably good explanation for this differential function which would otherwise have not been possible to explain. The steric hindrance caused between the 2(OH) and Ala^{264} and Phe^{261} of the cavity has evidently altered its orientation (Figure 4.7 C and D). But obviously the phenyl moiety still stacks and the aromatic interactions resemble the substrates. Both Ala^{264} and Phe^{261} are conserved in plant PPOs implicating that all PPOs would oxidise 2, 3, 4-THBA. As observed field bean PPO oxidizes 2, 3, 4, THBA though less efficiently than TBC. Similarly the addition of $–CH_2$ in DOPAC makes it bulky and the molecule is reoriented like the substrate making the same contacts as TBC. These results offer

explanations to the previous observations that phenolics acids with increased side chains length e.g. cinnamic acid, *p*-hydroxy phenyl propionic acid, 3, 4, dihydroxy phenyl propionic acid are oxidised more efficiently and with K_ms in the millimolar range (Tremolieres and Bieth, 1984). Klabunde et al., (1998) propose that Glu^{236} hydrogen bonded to a solvent molecule functions as general base/acid in the diphenol oxidation. We have also recently shown that a carboxylate group is vital to field bean PPO activity and a glutamate residue corresponding to that of Glu^{236} of ibCO is invariant in all catechol oxidases (Hernandez-Romero et al., 2006; Kanade et al., 2006). Mutation studies on this residue would confirm this observation. Two mutations other than the coordinating His at the CuA site of a mammalian tyrosinase did not alter the tyrosinase activity. The mutation of His^{390} at the CuB site abolished tyrosinase activity completely. Further His^{389} was responsible for the stereospecific recognition of *o*-diphenols not monophenols (Olivares et al., 2002). Such a His pair has been implicated to control the preference for carboxylated over decarboxylated substrates (Hernandez-Romero et al., 2006).

A plot of the predicted binding energies and our experimental data on the binding constants (K_m/K_i) shows a linear correlation (Figure 4.8). Our calculated difference in binding free energy between CAT (substrate) and BA (inhibitor) of 0.74 kcal/mol, which is near equivalent to the experimental difference of 0.81 kcal/mol. A similar trend was observed for CAT and the other inhibitors. The linear correlation of the experimental and predicted data indicates the suitability of the atomic approach to predict the relative binding energies of phenolic compounds that show subtle differences in structure. In conclusion, this study shows that the theoretical method based on flexible docking provides insights into the details of ibCO-substrate/inhibitor interactions and is a valuable complement to the limited PPO-substrate/inhibitor crystal structures available.

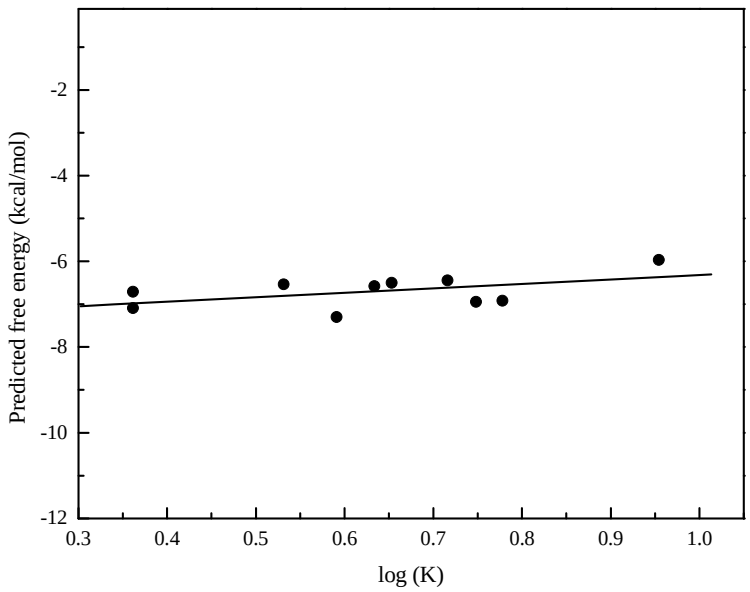

Figure 4.8. Correlation between Autodock predicted free energy and calculated binding constants (K_m or K_i) of ibCO.

These docking studies have afforded structural explanations for correlating subtle changes in the position and nature of the substitutions on diphenols to their functional properties as substrates and inhibitors. The results also demonstrate that minor changes in the diphenol substrates define their binding constants. It is clear that the computational analyses have provided important links between the structure and function that are otherwise difficult to obtain by experimental means. We believe that the data obtained by the Autodock studies are important for our continuing research efforts and provides useful hints to guide in the development and design of selective, potent inhibitors of PPO, to be used in the food and pharmaceutical industry.

Chapter V
Activation of field bean (*Dolichos lablab*) polyphenol oxidase by SDS and acid pH: Kinetics and mechanism

In plants, PPO is located in the chloroplast thylakoid membranes and often exists in multiple forms. An unusual and intriguing characteristic of the enzyme is its ability to exist in either a latent and/or an active form (Manson, 1965 and Whitaker, 1995). PPO is released from latency or activated by acid and base shock, anionic detergent such as SDS, polyamines, lipases, organic solvents and light etc. SDS as an activating agent is intriguing because very few enzymes are known to be activated by SDS in contrast to the many that are inactivated by it. This activation process alters its enzymatic and physical characteristics suggesting a limited conformational change, due to binding of small amounts of SDS, which could induce or initiate the activation of latent PPO (Moore and Flurkey, 1990). Studies to date on the activation phenomenon of PPO only advocate it being due to a limited conformational change. However, convincing structural and experimental details of the conformational changes at the molecular level that accompany the activation are yet to be elucidated. Field bean seed PPO is activated several fold by both SDS and acid-pH. In this chapter, the biochemical and biophysical changes that occur during activation, have been characterized and attempts are made to explain this phenomenon at the molecular level.

RESULTS

Activation of PPO by acid-pH

Field bean (*Dolichos lablab*) seed PPO was activated by exposure to acid-pH. The effect of pH on the activation of PPO was evaluated by incubating the purified PPO in buffers of pH 2.5, 4.0 and 7.0. At periodic intervals, aliquots were assayed for PPO using TBC as substrate. The results indicated that PPO activation occurred at pH 2.5 as well as at 4.0. However, the activation was more rapid at pH 2.5 reaching a maximum in 30 min (Figure 5.1). At pH 4.0 although a similar maximal steady state rate was achieved, the time taken to reach this maximum was between 2-4 h. The maximal activity of PPO, after exposure to pH 2.5 for 30 min, was $9.6 \pm 0.27 \times 10^4$ U/mg. PPO, activated at pH 2.5 although activated rapidly, was unstable loosing 97 % of its activity in 24 h. The activity of native PPO

assayed at pH 6.0, was 5.9 ±0.24 ×10^3 U/mg, which was less than 10 % of the activated forms. PPO activated by exposure to pH 2.5 for 10 min was used as 'acid-pH activated PPO' in all further studies. The activation of PPO and the subsequent decline (Figure 5.1) in the activity suggests that conformational changes take place in the enzyme upon exposure to a pH shock. Reversal of this activation was possible by exposure to pH 7.0.

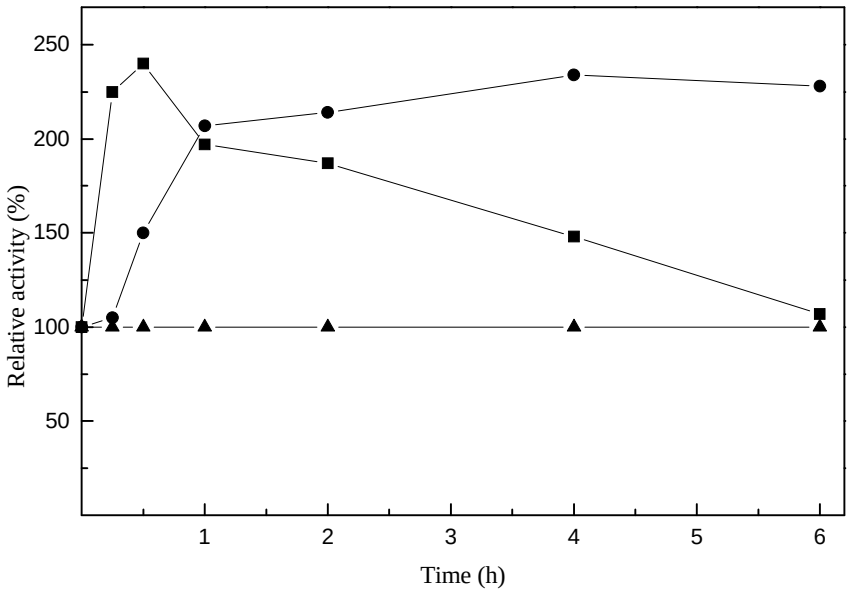

Figure 5.1. Activation of field bean PPO at varying pH. Native PPO was incubated in buffers pH 7.0, 4.0 and 2.5. Aliquots were assayed periodically. (—■—) pH 2.5, (—● —) pH 4.0 and (—▲—) pH 7.0.

To confirm that the activation and subsequent decrease in activity were indeed due to conformational changes, attempts were made to induce similar changes in the PPO by means other than the pH shock, such as by treatment with anionic detergent SDS and urea.

Effect of SDS on PPO activity

Field bean PPO, like other PPOs also exhibits latency and was activated by SDS, an anionic detergent, below its CMC, in contrast to many enzymes that are inactivated. The biochemical and biophysical properties of activated PPO were studied in the presence and absence of SDS. The effect of SDS on PPO activity was studied by incubating the PPO in 25 mM Tris-HCl, pH 7.0 at varying concentration of SDS for 30 min. The effect of increasing SDS concentration on the activity of purified field bean PPO is represented in Figure 5.2. The PPO activity was found to increase with

increasing SDS concentration. The most effective concentration of SDS was 1.25 mM where the measured activity was 1.32± 0.2 × 10^5 U/mg. At SDS concentrations below 0.3 mM the increase in activity was only 15-20 % to that of native PPO. However, a very sharp linear increase in PPO activity was observed from 0.3-1.25 mM with half the maximum activation at 0.64 mM SDS. The maximum steady state rate was achieved with 1.25 mM SDS. A further increase in the SDS concentration led to a near linear decrease in the PPO activity. Effect of time on SDS activation was studied by assaying the enzyme at different time intervals. The maximum activation was achieved in 30 min, further incubation with SDS led to a decrease in PPO activity (Figure 5.2B). On the basis of these observations, a concentration of 1.25 mM SDS at pH 7.0 for 30 min was used to activate PPO in all further studies.

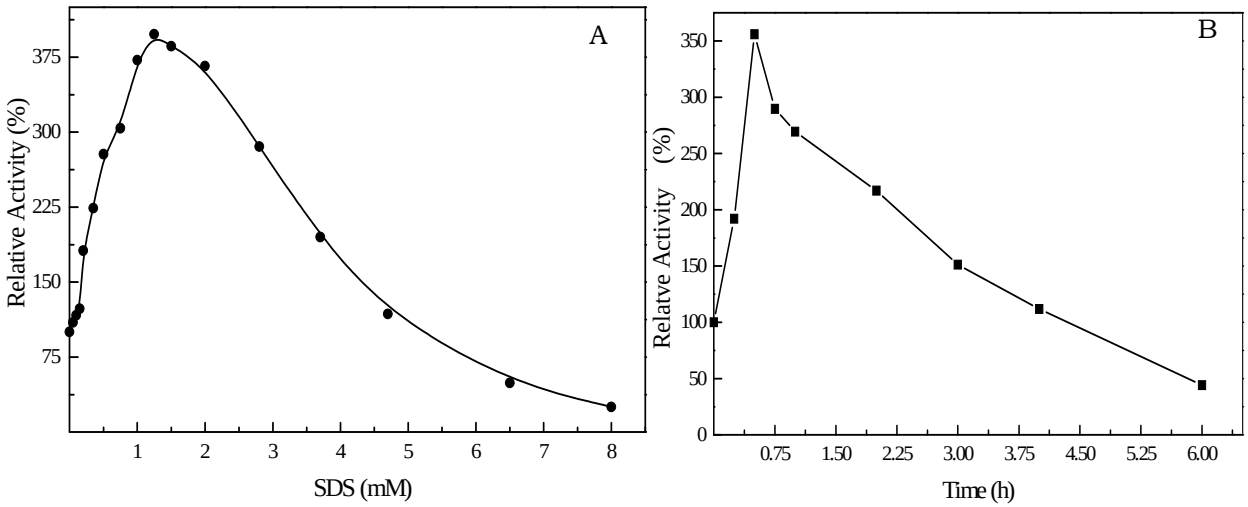

Figure 5.2 A. Effect of SDS concentration on field bean PPO activity. PPO was incubated in 25 mM Tris-HCl pH 7.0 containing 1.2 % NaCl (w/v) at indicated concentrations of SDS for 30 min and then assayed at pH 6.0 using TBC as substrate. Native PPO activity assayed at pH 4.5 using TBC in the absence of SDS was considered as 100 %. **B**; **Effect of incubation time on SDS activation of field bean PPO activity.**

Effect of pH on activated PPO activity

The effect of pH on the optimal activity of SDS and acid-pH activated PPO was evaluated using McIlvaines buffer (pH 2.5-7.5) and Tris-HCl (pH 8.0, 8.5). The pH optimum of the SDS-activated PPO was 6.0 with a

relatively high activity between pH 5.5-6.5 (Figure 5.3). The measurement of PPO activity of the acid-pH activated form also indicated a pH optimum of above 6.0, beyond which the activity decreased. At pH 4.5, the SDS and pH-activated forms of PPO exhibited only ~10 % of their maximum activity. In contrast, the native PPO exhibited maximum activity, at pH 4.5, for the oxidation of TBC. These results appear to suggest that field bean PPO exists in two forms, the native form with a pH optimum of 4.5 and an activated form with a pH optimum of 6.0. The similar shifts in the optimum pH for both SDS and acid-pH activated PPO suggested similarities in the activation mechanisms of SDS and acid-pH. This change in the pH optimum could be related to the displacement of a sensitive pKa value of the enzyme caused by interaction with SDS molecules. All further assays of the activated PPO were performed at pH 6.0.

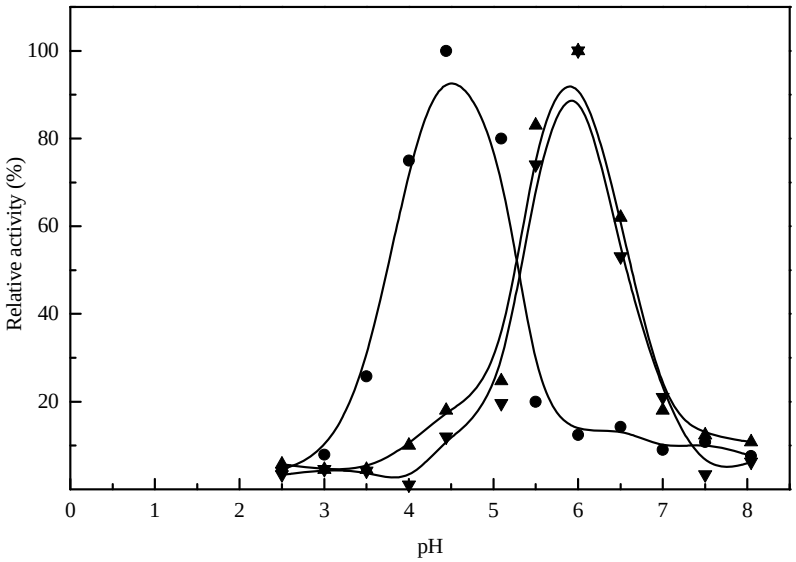

Figure 5. 3. Effect of pH on the activity of field bean PPO. Native PPO in 25 mM Tris-HCl pH 7.0 containing 1.2 % NaCl (w/v) (—● —), SDS-activated PPO (—▲—), Acid-pH activated PPO (—▼—). PPO assays using TBC as the substrate were performed in 0.1 M McIlvaine buffer, pH 2.5 –7.5 and 25 mM Tris-HCl pH 8.0 and 8.5 as described in methods.

Electrophoretic mobility

The electrophoretic mobility of PPO was examined after activation by SDS and acid-pH. Electrophoretic analysis was performed, as described by Angleton and Flurkey, (1984) using the method of Laemmli, (1970). There was no change in the electrophoretic mobility of the activated forms when compared to native PPO (Figure 5.4). A single enzymatically active band that

corresponds to a single protein band in the Coomassie brilliant blue stained gel was observed for both the SDS and acid-pH-activated PPO. Figure 5.4A shows protein staining of native PAGE. As observed there was no change in the mobility of the native, acid and SDS activated PPOs. Non-denaturing PAGE, in the presence of 0.1 % SDS, also shows no change in the electrophoretic mobility. These results imply that there is no change in the charge to mass ratio upon activation by either SDS or acid-pH treatment.

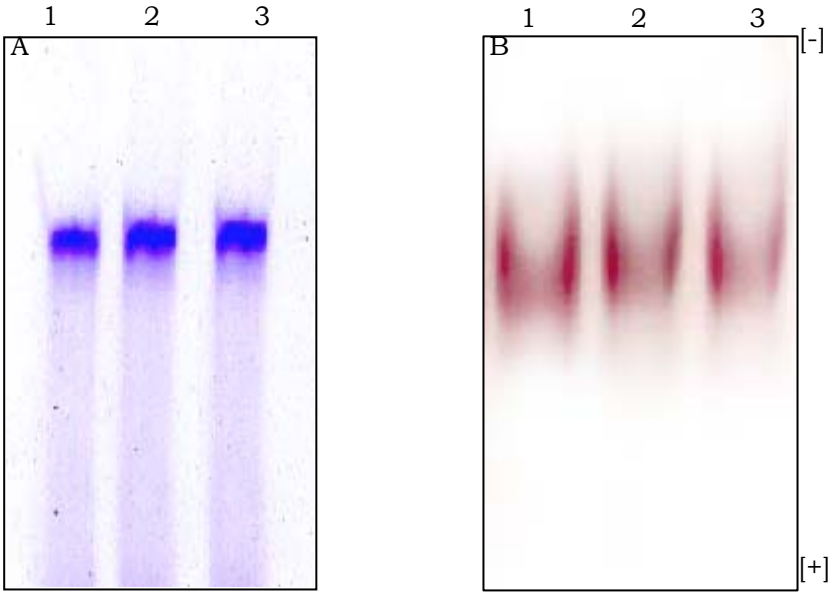

Figure 5. 4. Native-PAGE (7.5 % T, 2.7 % C) of native and activated PPO. A) Stained for protein using Coomassie Brilliant Blue and B) Stained for PPO activity using catechol and MBTH, Lane 1; Native PPO, Lane 2; SDS-activated PPO and, Lane 3; Acid-pH activated PPO.

K_m and V_{max} determinations

Activation of an enzyme could occur either due to an increase in the V_{max} or decrease of K_m or both. These parameters were evaluated for field bean PPO before and after activation by acid-pH shock and SDS treatment. K_m and V_{max} for PPO following activation were determined at pH 6.0 for TBC. The kinetic parameters were calculated from double reciprocal plots, which showed no indication of nonlinearity. Kinetic parameters K_m and V_{max} computed from nonlinear fitting to the Michaelis-Menten equation were similar to those obtained from double reciprocal plots. These parameters were compared to the kinetic parameters of native PPO determined at pH 4.5 for the K_m of PPO for its phenolic substrate TBC. The results revealed that the binding affinity towards TBC increased only marginally for both the activated forms. The SDS and acid-pH activated forms showed remarkably

higher V_{max} values towards the oxidation of TBC (Table 5.1). The overall catalytic efficiency (k_{cat}) increased by 6.5 and 5.8 fold for the SDS and acid activated forms respectively. The similarity in the changes by two different agents suggest that the mechanism of activation could be similar.

Inhibition by tropolone

Inhibition by tropolone, a potent competitive inhibitor of plant PPOs was carried out for native and the two activated forms of field bean PPO. The K_i value determined by Dixon plot, for competitive inhibition of activated PPOs by tropolone was far lower than that for native PPO (Table 5.1 and Figure 5.5). This lower K_i suggested the greater accessibility to the active site following acid-pH and SDS activation.

Table 5.1. Kinetic parameters of native and activated PPOs

Properties	**Native**	**SDS activated PPO**	**Acid-pH activated PPO**
pH optimum	4.5	6.0	6.0
K_m (TBC mM)	4.2 ±0.2	3.9±0.3	3.6±0.04
V_{max} ($\times 10^5$ U/mg)	1.97±0.25	12.03 ±0.29	9.77 ±0.24
$K_{i\ (tropolone)}$ ($\times 10^{-7}$ M)	5.7±0.2	1.8 ±0.04	3.6 ±0.22
V_{max} / K_m (k_{cat}) $\times 10^3$	0.47	3.1	2.7
E_a (kcal/mol)	32.2±0.29	28.3±0.20	21.8±0.25

Determination of activation energy

The activation energy (E_a) is defined as the minimum energy required for a fruitful reaction. By varying the concentration of TBC at various temperatures, V_{max} and K_m were calculated for native, SDS activated, acid– and pH activated PPOs. E_a was calculated using a plot of log (V_{max}/K_m) against 1/T, where T is the absolute temperature (Figure not shown). E_a values towards oxidation of TBC of native PPO was 32.3±0.29 kcal/mole

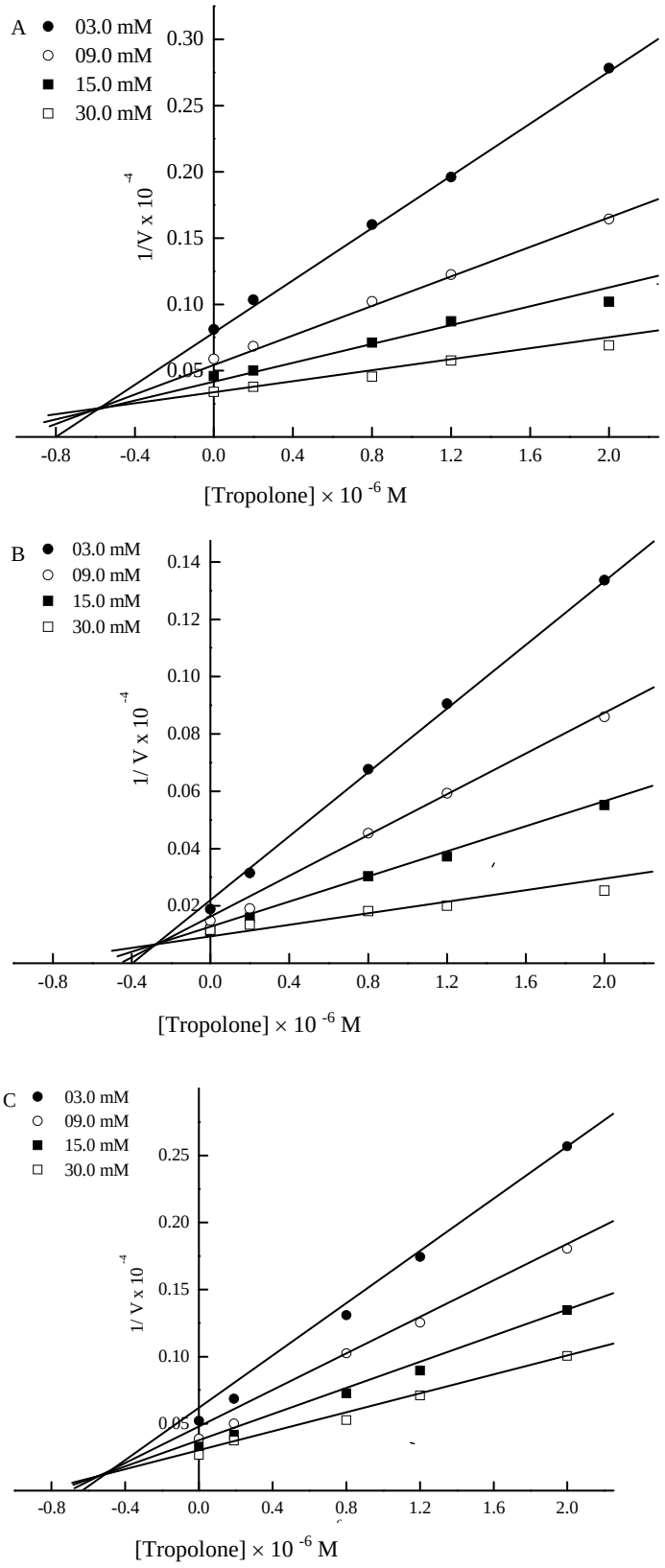

Figure 5.5. Dixon plot for determining the dissociation constants (K_i) of tropolone. A) Native; B) SDS-activated PPO and C) Acid-pH activated PPO. Substrate concentrations used are as indicated**.**

(135.2±0.29 kJ/mol) whereas for SDS-activated PPO it was 28.3±0.20 kcal/mole (118.9±0.20 kJ/mol) and for acid-pH activated PPO was 21.8±0.25 kcal/mole (91.6±0.25 kJ/mol). The plots were not biphasic and were without a sharp decline in the slope, indicating absence of any rate limiting step during oxidation at the temperatures studied. The lower values of E_a for the activated PPOs suggest that the native form was energetically more stable than the activated form.

Chemical modification of carboxylic group

A significant shift in the pH optima from 4.5 to 6.0 was observed for the SDS and acid-pH activated PPO (Figure 5.3). This shift is in the ionization range of carboxyl groups implicating them in catalysis. Klabunde et al., (1998) in their three-dimensional structure of a PPO from *Ipomea batatas* imply that Glu^{236} functions as a general base/acid in the catalysis of diphenol oxidation. An examination of plant PPO sequences (SWISSPROT release) and the multiple alignment of the catalytic region indicates that a Glu residue corresponding to Glu^{236} is invariant in all plant PPOs (Figure 5.6). The amino acid modifying agent EDAC specific to the carboxylic group of Glu and Asp was used to verify the presence of a glutamic acid residue at the active site of PPO.

A kinetic analysis of the inactivation of field bean and sweet potato PPO with various concentrations of EDAC was carried out (Figure 5.7). Incubation with GME alone had no effect on the enzyme activity of field bean and sweet potato ibCO. Both enzymes were fully stable in the presence of EDAC alone. The lack of inhibition by either of the reagents supports the direct involvement of the carboxylic groups, indicating that the inactivation measured was not due to cross-linking of other amino acids.

The semi-logarithmic plots of residual enzyme activity at various EDAC concentrations versus time were linear for both field bean PPO and sweet potato ibCO (Figure 5.7A), indicating that the inactivation followed pseudo first-order kinetics. A plot of the first-order inactivation rate constant (k_i) against EDAC concentration was also linear (Figure 5.7B). The second-order rate constants of field bean PPO and sweet potato ibCO were 0.099± 0.02 and 0.105±0.03 $M^{-1}\cdot min^{-1}$. A plot of log k_i versus log [EDAC] yielded a

slope of 1.25±0.21 (r> 0.99) and 1.10±0.12 (r> 0.99) for field bean PPO and sweet potato ibCO respectively (Figure 5.7C). The stoichiometry of the inactivation reaction was near 1.0 with respect to EDAC, indicating that a single carboxylate group was essential to both field bean PPO and sweet potato PPO activities. The concentrations of EDAC needed for inhibition (20–300 mM) were relatively high when compared with other studies. It could be partly due to the high level of glutamic acid and aspartic acid in field bean and sweet potato PPOs (Gowda & Paul, 2002, Klabunde et al., 1998). Similar conditions were used for the modification of glutathione transferase (100 mM) (Xia et al., 1993), oxalate oxidase (150 mM) (Kotsira and Clonis, 1998) and α-1, 4-glucan lyase (200 mM) (Nyvall et al., 2000).

```
   P43311   GRAPE       ---AGTLEHA PHNIVHKWTG L--A------
BAB64530 SANDPEAR       ---GGSIEGT PHGPVHLWTG D--N------
AAA69902 APPLE          ---GGSIEGT PHAPVHLWTG D--N------
S24758   FAVA BEAN      ---AGSIENV PHAPVHTWTG D--N------
Q06215   BROAD BEAN     ---AGSIENV PHAPVHTWTG D--N------
AAK13242 REDCLOVER      ---AGSLENI PHTPIHIWTG D--P------
AAC28935 APRICOT        -KYPGTIENM PHNNIHIWVG D--P------
AAK53414 QUAKING ASPEN  ---AGTIESS PHNNIHRWTG D--P------
BAB20048 SNAPDROGON     ---VGSIELV PHGMIHLWTG S--E------
BAA92317 SWEET POTATO   --GGGSIENI PHTPVHRWVG DVKP------
BAA08234 POKE WEED      ---AGSIENV PHGPVHVWTG D--P------
BAB89047 RICE           ---AGTVELQ PHNLMHVWVG DL-L------
AAA02877 POTATO         ---QGTIENI PHTPVHIWTG ----------
Q08307   TOMATO         ---QGTIENI PHTPVHIWTG ----------
CAA73103 TOBBACO        ---MGTIENI PHTPVHIWVG ----------
AAA85121 POTATO         ---PGTIENI PHGPVHIWSG TVRG------
AAC69365 D.KAKI         ---MGTIENI PHTPVHIWTG ----------
P43310   SPINACH        VSGAGILERM PHNSVHVWTR SNTI------
AAB94293 SUGARCANE      ---MGSLERM AHTAMHVWVG KAGAKPCDAA
AAM33417 BREAD WHEAT    ---PGSLENA AHTAVHIWVG ----------
AAK29783 PINEAPPLE      ---AGTLELV PHNTMHLWTG DP--------
BAA85119 EGG PLANT      ---PGTVEVI PHIPVH-WVG TARG------
BAA75621 SANDPEAR       ---GGSIEGT PHGSVHLWTG DN--------
BAA75624 LOQUT          ---GGSIEGT PHGPVHLWTG DN--------
AAA75625 CHINESEQUINCE…---GGSIEGT PHGPVHLWTG DN--------
BAA75623 PEACH          ---GGSIEGT PHGPVHLWTG DN--------
AG01409  BLACK TEA      ---AGSLENI PHGPVHIWCG DR--------
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Figure 5.6. Multiple amino acid sequence alignment of the catalytic region of plant PPOs. The invariant Glu and Trp are indicated by down arrows. The multiple alignment was generated using Multalin. The PPO sequences were retrieved from UniprotKB /Swiss-Prot release and from www.ncbi.nlm.nih.gov. Numbers on the left side are accession numbers of the proteins. The amino acid numbering is with respect to that of Sweet potato ibCO (Klabunde et al., 1998).

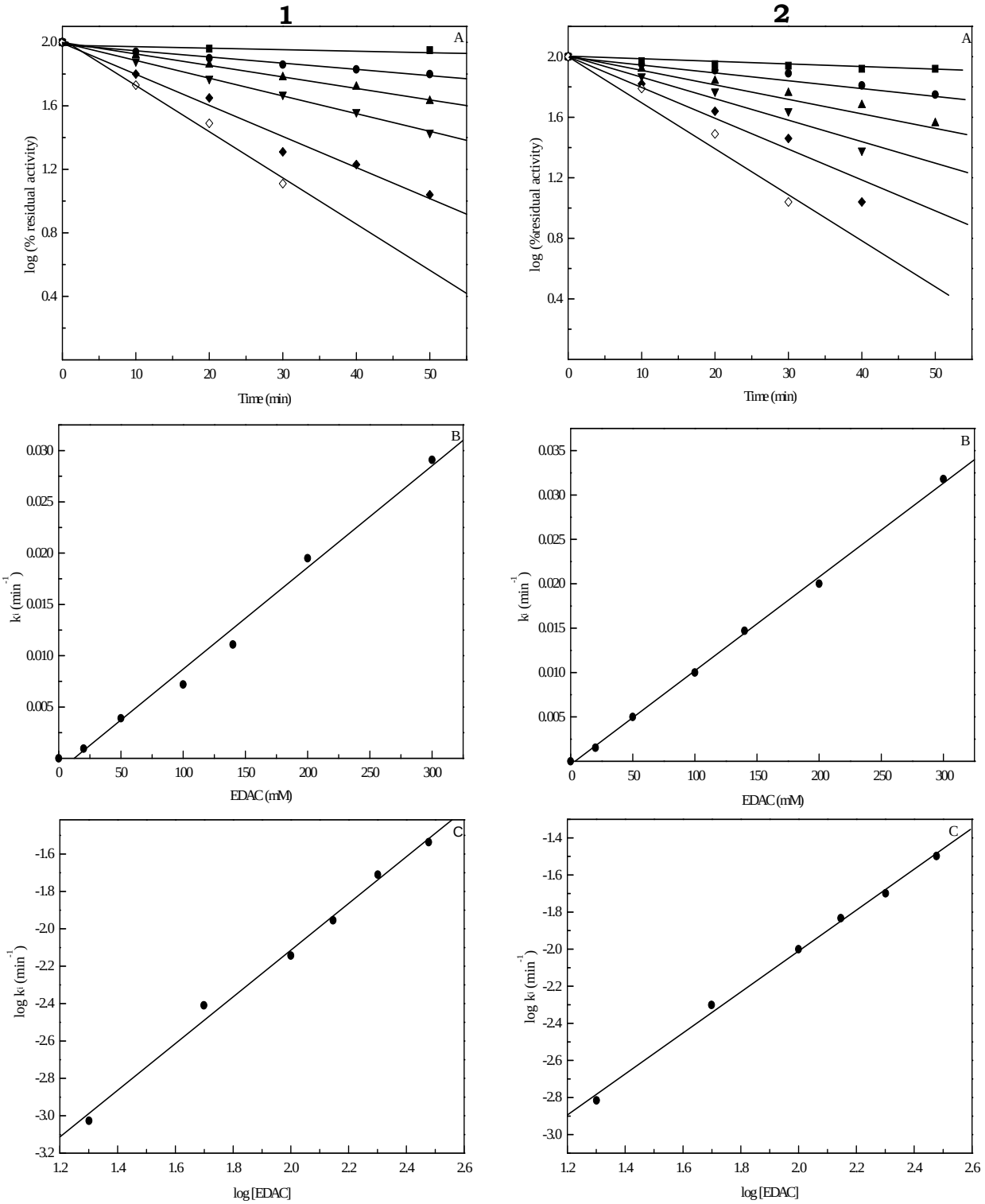


Figure 5.7. Inactivation of PPO by EDAC and GME. Panel (1) Field bean PPO, panel (2) Sweet potato ibCO. PPO was modified at different concentration of EDAC. (A) (—■—) 20mM, (—● —) 50 mM, (—▲—) 100mM, (—▼—) 140 mM, (—◆ —) 200 mM (—◇—) 300 mM and 275 mM GME in 50 mM sodium acetate buffer pH 4.8 at 25°C. (B) Plot of pseudo first order inactivation rate constant as a function of EDAC concentration. (C) Double logarithmic plot of pseudo first order inactivation rate constant as a function of EDAC concentration.

Subunit assembly and molecular dimensions of PPO
Size exclusion chromatography

The effect of SDS and acid-pH induced activation on the molecular dimensions of field bean PPO was evaluated by size exclusion studies on a TSK-G2000SW_{XL} column. The results are summarized in Figure 5.8. Native PPO of M_r ~ 120000 Da eluted as a single symmetrical peak with retention time of 13.39 min. In contrast, the SDS and acid-pH activated forms elute earlier with a retention time of 11.37 min (Figure 5.8). The retention time of 13.39 corresponds to the tetrameric form of native PPO (Paul & Gowda, 2000). This decrease in the retention time of the activated PPOs as compared to native PPO could be due to either aggregation or enhancement in the hydrodynamic radius.

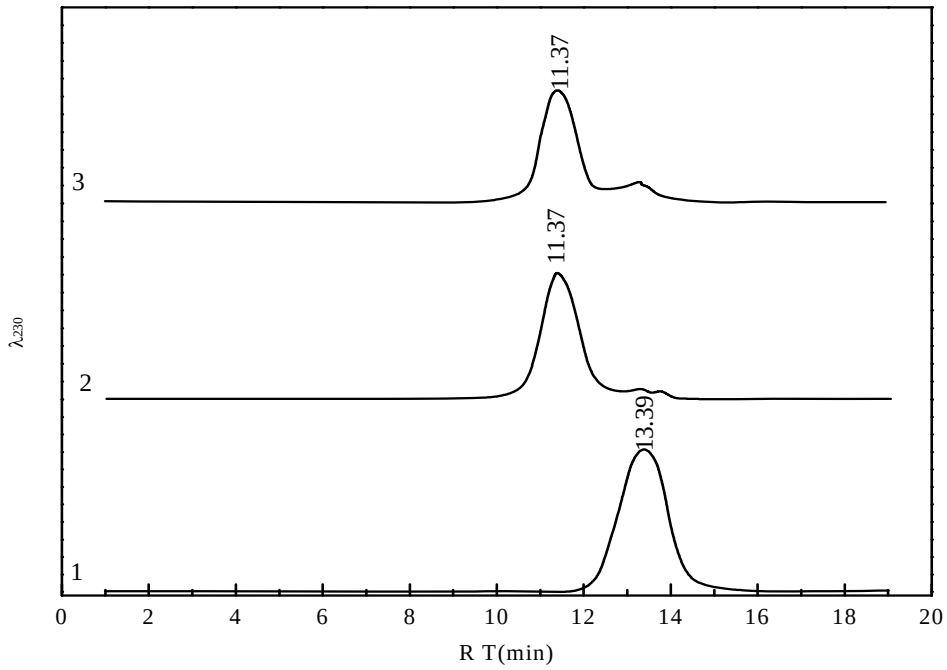


Figure 5.8. Evaluation of the molecular dimensions of field bean PPO. Size exclusion chromatography profile of field bean PPO on a TSK gel G2000 SW_{XL} (7.8 mm × 30 cm, 5μM) column, using a Waters HPLC system equipped with Waters 2996-photodiode array detector set at 230 nm. The column was pre-equilibrated with 0.1 M NaPi buffer pH 7.0 containing 0.1 M Na_2SO_4 at a flow rate of 0.5 mL/min. 1) Native PPO, 2) SDS activated PPO, eluted in buffer containing 1.25 mM SDS, and 3) Acid-pH activated PPO, eluted in 100 mM Glycine-HCl pH 2.5.

Determination of hydrodynamic radius

The conformational status of a protein can be ascertained by measuring the hydrodynamic radius (Stokes radius, R_s). Size exclusion chromatography was used to determine the Stokes radius. The hydrodynamic radii of native and activated PPOs were determined according

to Uversky (1993). A set of proteins of known molecular weight and Stokes radii were used to construct the calibration curve of log R_s vs migration rate ($1000/V_e$). The calibration curve was obtained using the TSK gel G2000 SW_{XL} (7.8 mm x 30 cm, 5μM) column. Native PPO eluted from the column with a retention time of 13.37 min and the calculated hydrodynamic radius was 49.1 Å (Figure 5.9). The hydrodynamic radius of SDS activated and acid-pH activated PPO was significantly increased to 75.9 Å. These results further evidenced that the significant decrease in retention volume of the activated PPOs was due to an enhanced hydrodynamic radii. The increase in the hydrodynamic radius as compared to native PPO could be due to either swelling of the molecules or due to a partial unfolding upon SDS or acid-pH treatment. The PPO eluting at 11.37 min cross-reacts with anti-PPO antibodies. Figure 5.8 shows that although a conformational change occurs by acid activation and SDS activation, the conformational change was incomplete with <10 % remaining in the native form. In the presence of SDS >95 % has an altered conformation with an increased Stokes, radius.

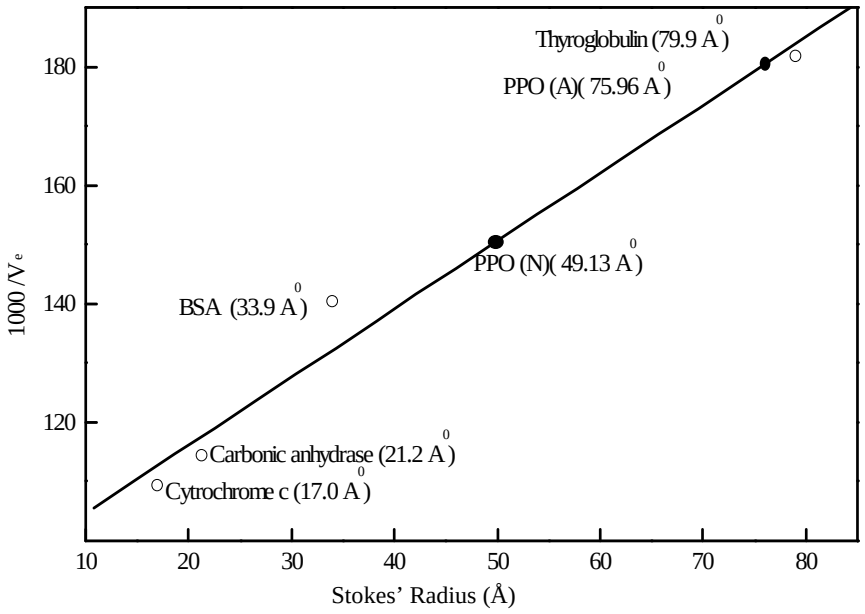


Figure 5.9. Determination of Stoke's radius. The proteins used to construct the calibration curve were Cytochrome C (17.0 Å), Carbonic anhydrase (21.2 Å), Bovine serum albumin (33.9 Å) and Thyroglobulin (79.9 Å).

Differential light scattering

Light scattering experiments were performed at 325 nm to ascertain whether the activation by SDS and acid-pH was due to aggregation or not. The light scattering measurement revealed a decrease in the absorbance as the SDS concentration increased suggesting that the activation was not due to aggregation (Figure 5.10). Furthermore, SDS-PAGE (10 % T, 2.7 % C, Figure 5.11) of the two activated forms showed similar subunit size ~ 30000 Da indicating they still exist as tetramers. When examined using higher acrylamide concentration (12.5 %& T) all the three forms showed two closely migrating subunits of size 29000 and 31000 Da by SDS-PAGE.

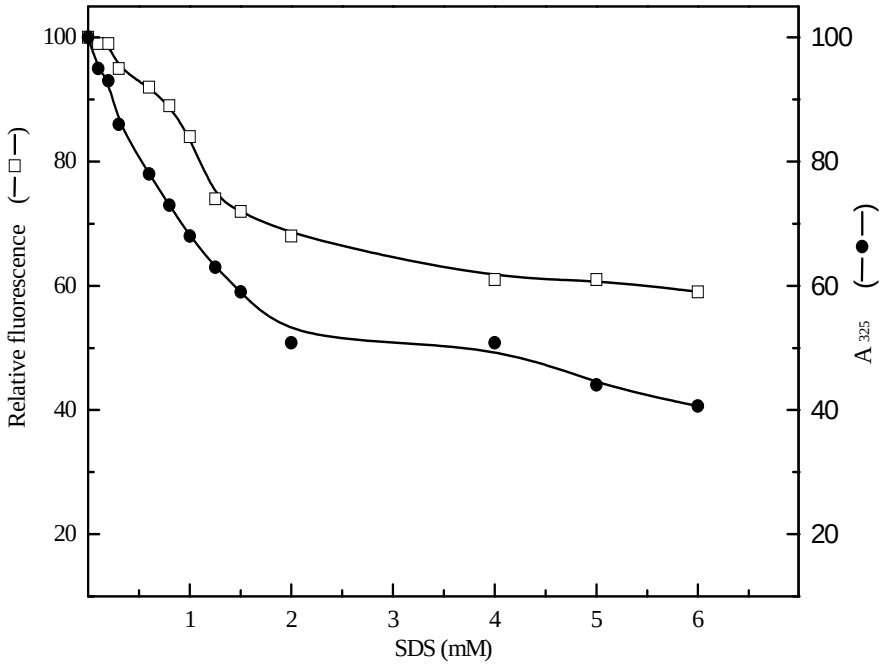


Figure 5.10. Light scattering measurements of PPO at 325 nm. (—●—) SDS activated field bean seed PPO and (—□—) Fluorescence emission of SDS activated PPO.

Glutaraldehyde cross-linking

Glutaraldehyde cross-linking studies were carried out to study the effect of SDS on the subunit configuration of PPO. Both native and SDS activated PPO at pH 6.0, were cross-linked using glutaraldehyde at pH 7.0. The cross-linked forms of native and activated PPO had only the tetrameric configuration suggesting that SDS brought about no change in the subunit configuration.

Dot blot analysis

The native and activated forms of PPO were immobilized on nitrocellulose membrane and probed with anti PPO antibodies. All the forms of PPO showed same intensity of cross reactivity suggesting that there was no significant conformational change (Figure 5.12).

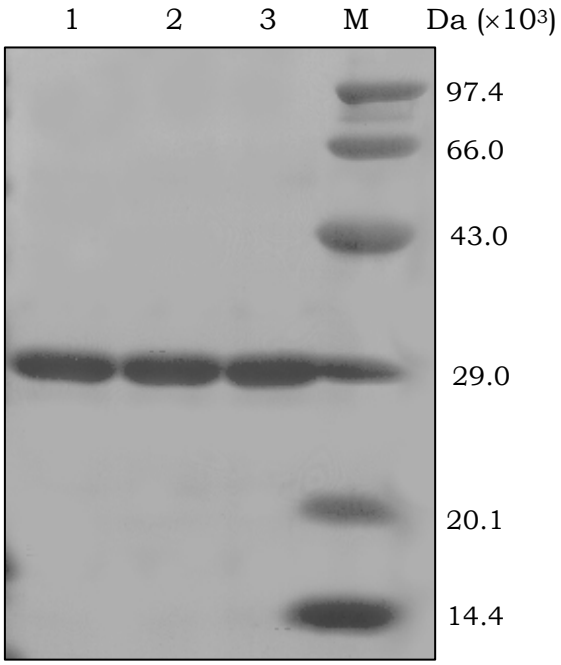


Figure 5.11. SDS-PAGE profile (10 % T, 2.7 % C) of PPO. Lane 1; Native PPO, Lane 2; SDS-activated PPO, Lane 3; Acid-pH activated PPO and Lane M; Molecular weight markers.

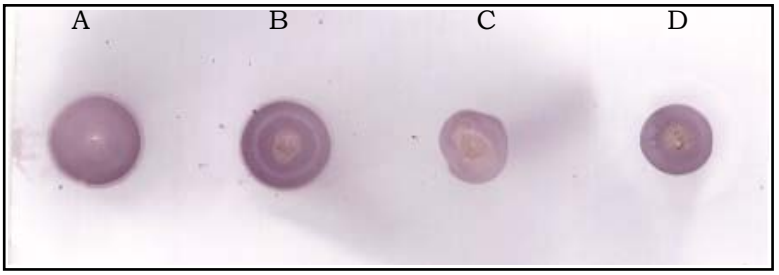


Figure 5.12. Dot bolt analysis of PPO. A; Native PPO; B; SDS activated PPO, C; PPO activated at pH 4.5, D; PPO activated at pH 2.5.

SDS and acid-pH activation induce partial unfolding

***Intrinsic fluorescence*:** The fluorescence spectra of native and activated field bean seed PPO were measured in a Shimadzu (Model RF-5000) spectrofluormeter. Intrinsic fluorescence was used to probe the perturbation in protein structure and conformation. Fluorescence emission was measured upon excitation at 295 nm to follow the changes in the microenvironment of the Trp residues and upon excitation at 280 nm, to follow the emission both from Tyr and Trp residues. PPO was activated for 30 min in presence of 1.25 mM SDS. The emission spectra of SDS activated, acid-activated at pH 2.5, 4.0 and native PPO was measured between 300-400 nm (Figure 5.13 A). The change in the fluorescence intensity was observed without any shift in the wavelength maxima. The emission spectrum of activated PPO was quite distinguishable from the native with an emission maximum at 330 nm, suggesting that the tryptophan residues were buried in a predominantly hydrophobic milieu shielded from the solvent. The intrinsic fluorescence spectra of the SDS activated PPO was quenched to a greater extent than the acid activated PPO. This quenching of the fluorescence significantly increased with increasing SDS concentration to 1 mM (Figure 5.13B). Activation of the enzyme by SDS also began to occur from 0.6-1.25 mM (Figure 5.2). The observed decrease in fluorescence beyond this concentration was marginal. These results provide strong evidence for the conformational change, which probably occurs during the initial binding and activation by SDS. The fluorescence intensity decreased by about 40 % after 6 h incubation as compared to 15 % after 30 min incubation (data not shown). Further binding of SDS beyond that required for maximum activity (1.25 mM) quenches the fluorescence and reflect gross conformational changes which could be due to inactivation (Figure 5.13B). The fluorescence data was consistent with a view that the two activated forms assume similar conformations but are different from that of the native form.

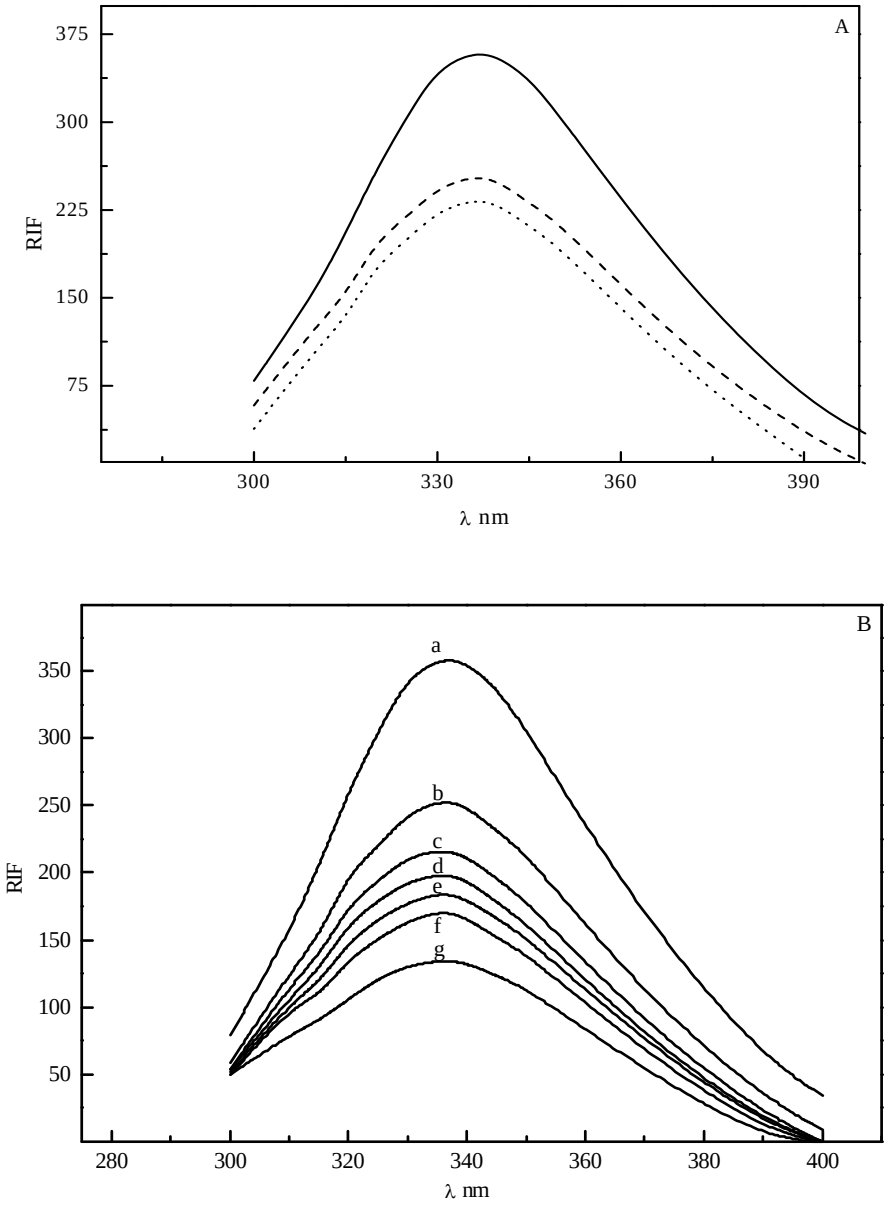


Figure 5.13. Fluorescence emission spectra of field bean seed PPO. (A). (——) Native PPO in 50 mM Tris-HCl buffer pH 7.0, (·····) PPO in 50 mM Tris-HCl buffer pH 7.0 containing 1.25 mM SDS, (----) PPO in 25 mM Glycine-HCl buffer pH 2.5. (B). Fluorescence spectra of SDS activated PPO; a to g increasing concentrations of SDS in mM 0.0, 1.0, 1.25, 2.0, 3.0, 4.0 and 5.0 respectively.

Acrylamide quenching of SDS activation: The topographical studies with proteins are carried out by solvent perturbation, chemical modification, quenching of fluorescence by the addition of very low molecular weight reagents like potassium iodide, oxygen and acrylamide. Tryptophan fluorescence is a good measure of the extent of exposure of these residues to the solvent. The quencher decreases the fluorescence intensity of the excited indole ring. Acrylamide is an excellent uncharged quenching probe that is

very sensitive to the exposure of tryptophan residues in proteins. Collisional quenching experiments with acrylamide were performed to assess the accessibility of tryptophan residues in the SDS activated form of PPO and corresponding Stern-Volmer constants (K_{sv}) were calculated. Stern-Volmer plots for SDS activated PPO (Figure 5.14) showed increasing slopes with increasing SDS concentrations, suggesting that the tryptophans in the activated forms were more accessible. Native PPO had a Stern–Volmer constant ~3.86 ± 0.4 M^{-1} (Table 5.2). The constant at 0.2 mM SDS are similar to native PPO suggesting that at low concentration no change occure. This data is consistent with the absence of activation observed at low SDS concentration (Figure 5.2). The higher Stern-Volmer constant, 4.22 ± 0.4 M^{-1} following SDS (1.25 mM) activation was indicative of a slightly disrupted tertiary structure with tryptophans that were more exposed to the solvent. The higher K_{sv} value was also consistent with a more open conformation of SDS-activated PPO. These results taken together with the fluorescence quenching implicate a subtle yet measurable change in the tertiary structure of PPO upon activation with SDS. The percentage exposure of tryptophans was also marginally increase in increasing with SDS concentration.

Table 5.2. Stern-Volmer constants of PPO

SDS (mM)	$K_s \times 10^3$ M^{-1}	f_a (Fractional accessibility, %)
0.00	3.86±0.4	47.55±0.4
0.20	3.67±0.2	51.33±0.6
0.75	4.19±0.3	50.95±0.5
1.25	4.22±0.4	51.56±0.5
1.50	4.26±0.5	52.85±0.6
2.00	5.15±0.6	56.94±0.3
3.00	5.65±0.1	61.38±0.4

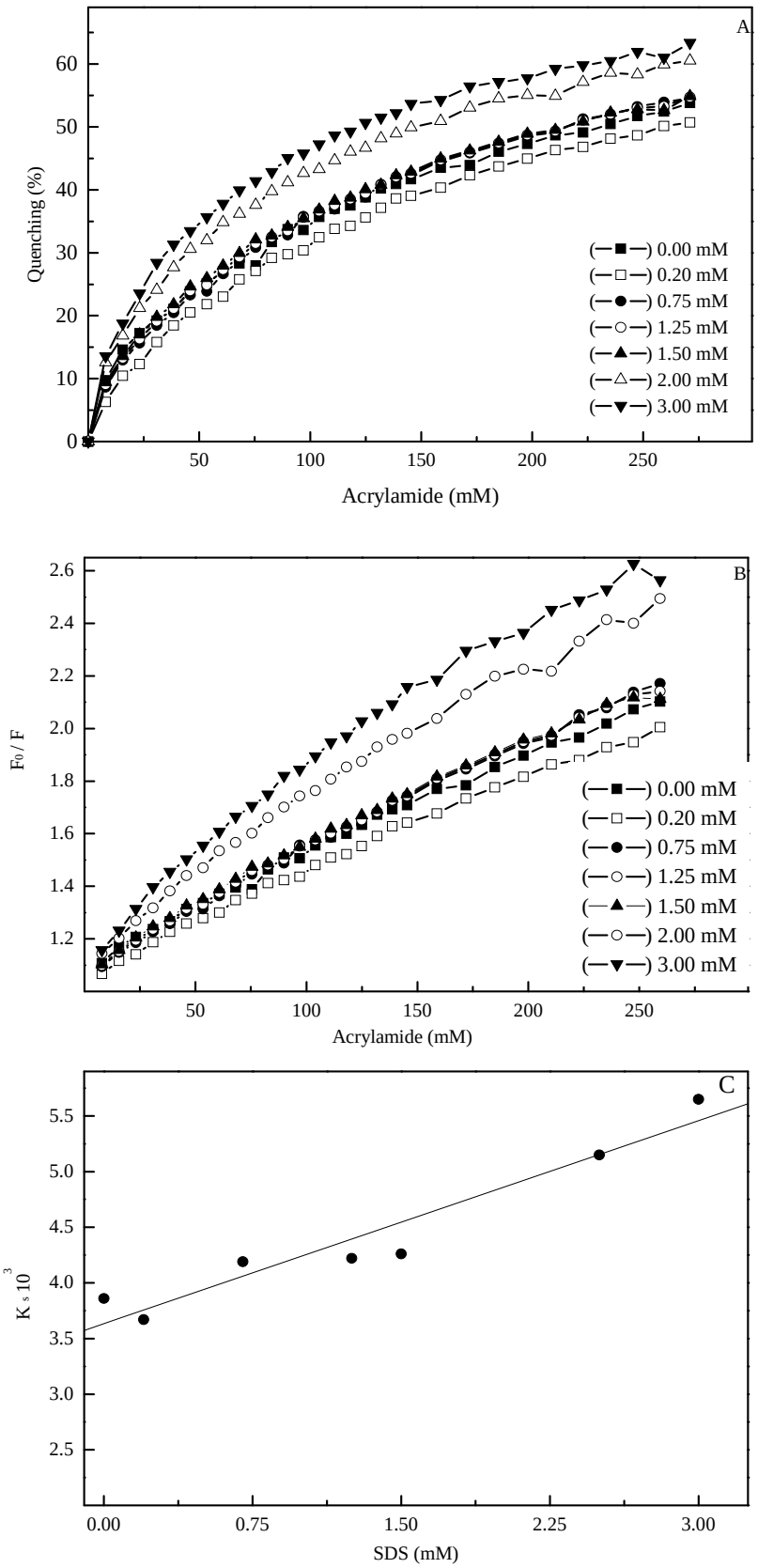


Figure 5.14. Acrylamide quenching of field bean PPO. A) Percentage quenching of PPO in 50 mM Tris-HCl buffer pH 7.0 containing varying concentration of SDS as mentioned in figure. B) Stern-Volmer plot C) Stern-Volmer constant with varying concentrations of SDS.

Circular dichroism studies

Secondary and tertiary structures of native and activated forms of PPO were analyzed from the far-UV and near-UV CD spectra as described under Section 2.2.25. Far UV-CD was used to examine the secondary structure of the native and activated forms of PPO (Figure 5.15A). The far UV-CD spectra of native PPO indicated the presence of 29 % helix consistent with the helix content reported for PPOs (Klabunde et al., 1998; Shi et al., 2003; Xiao et al., 2005). The far UV-CD spectra of the SDS and acid-pH activated forms showed a decrease in the intensity of the negative band at 208 and 222 nm suggesting changes in the secondary structure. Secondary structure analysis indicated a decreased α-helical content for both the SDS and acid-pH activated PPO.

The near UV-CD spectrum of the native PPO revealed that positive ellipticity peaks centered at 284 and 291 nm. This indicated a rigid asymmetric environment of the aromatic residues. The ellipticity values at these wavelengths decreased upon SDS activation and activation at pH 4.5 (Figure 5.15B). In contrast, the spectrum of PPO activated at pH 2.5 showed a shift with high positive ellipticity centered at 280 nm and an increased ellipticity at 291 nm. The positive ellipticity at 291 nm is attributable to tryptophan's environment. The differential changes observed at this wavelength were indicative of a change in the tryptophan environment as a result of the loss of tertiary structure. The loss in tertiary structure was apparently of a higher magnitude for the PPO activated at pH 2.5, compared to the SDS activated form.

Limited proteolysis

Limited proteolysis has been effectively used to monitor protein surface regions, ligand induced conformational changes and protein folding as well as unfolding. The vulnerability of a protein to degradation by a protease is governed by conformational parameters one of which is accessibility. Figure 5.16 shows the susceptibility of native and the activated PPOs to trypsin digestion as studied by RP-HPLC. An extensive proteolytic degradation of the activated PPOs occurs as evidenced by the increased

number of peptide fractions, in the digests of activated PPO (Figure 5.16, Panel B & C). The presence of multiple peptide peaks eluting early in the acetonitrile gradient are suggestive of increased protease accessibility, reckoned by a more open conformation and/or partial unfolding. The resistance to proteolytic digestion by native PPO points to a more compact conformation as compared to the SDS or acid-pH activated PPO.

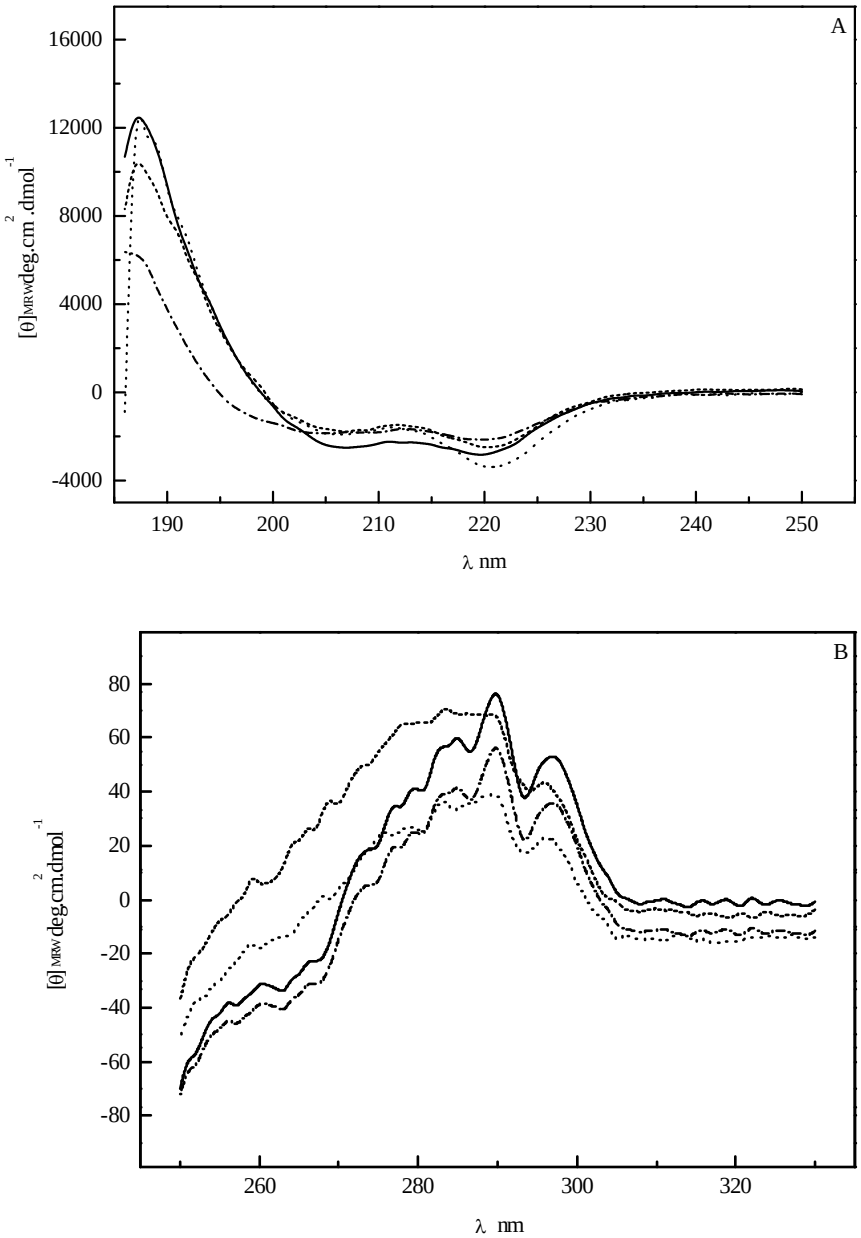


Figure 5.15. CD spectra of field bean PPO. A) Far-UV CD spectra (·····) Native PPO in 50 mM Tris-HCl buffer pH 7.0, (—·—) SDS-activated PPO, (——) pH 2.5 activated PPO, (---) pH 4.5 activated PPO. B) Near-UV CD spectra. (——) Native PPO in 50 mM Tris-HCl buffer pH 7.0, (—·—) SDS-activated PPO, (----) acid-pH activated PPO (pH 2.5) and (·····) acid-pH activated PPO (pH 4.5).

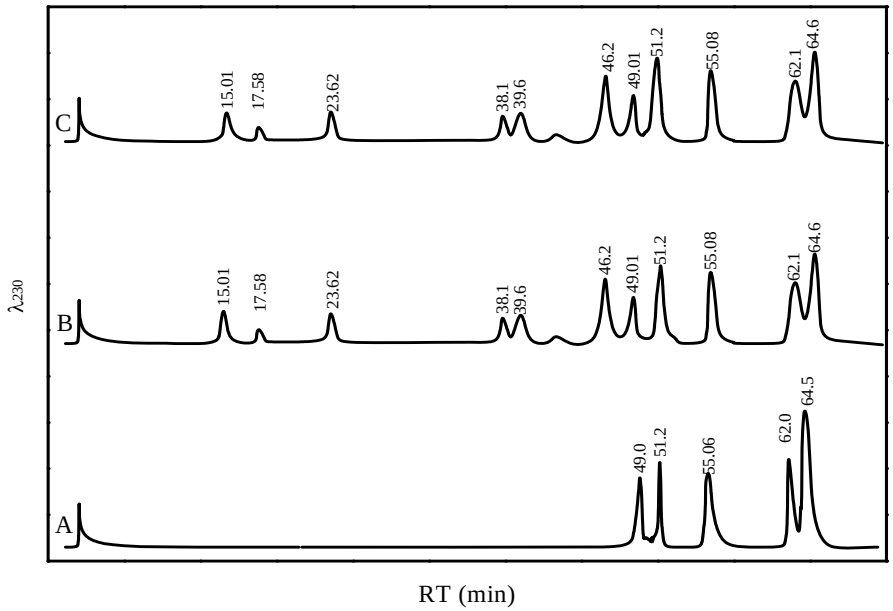


Figure 5.16. RP-HPLC profile of the tryptic digest of field bean PPO. Peptides were detected at 230 nm. A) Native field bean PPO, B) SDS-activated field bean PPO and C) Acid-pH activated field bean PPO.

Thermal stability of activated PPO

Structural changes, induced by either SDS or acid shock (pH 2.5) were further assessed by monitoring the effect of temperature (25-95 ^{0}C) on the catecholase activity of activated PPO and the results are summarized (Table 5.3). Native PPO was found to be more thermostable than the activated forms. The activity of the native enzyme remained stable between 20-60 ^{0}C beyond which the enzyme lost activity rapidly probably due to denaturation. In contrast the activated forms were more thermolabile with loss in activity occurring at ~ 40 ^{0}C. The loss in activity of the acid-pH activated form was gradual as compared to the SDS activated form. The greater thermostability of native PPO appeared to be probably due to the compact conformation as implicated by the smaller Stokes radius (Figure 5.17).

Native, SDS activated and pH 2.5 activated PPO exhibit a T_m of 70.4, 60.7 and 51.7 ^{0}C respectively (Table 5.3). These observations suggested that the activation resulted in increased thermolability probably due to the partial unfolding under the conditions of activation, as evidenced by the

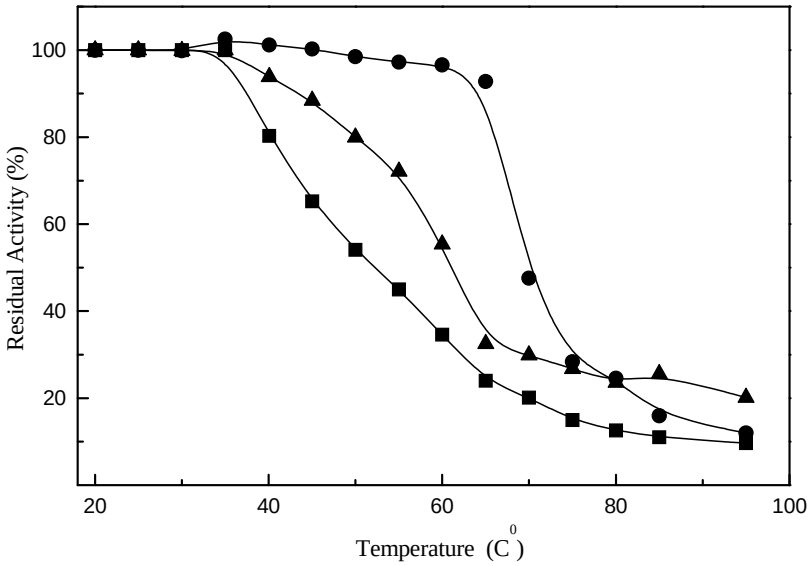


Figure 5.17. Thermal stability of field bean seed PPO. (—● —) Native PPO incubated in 25 mM Tris-HCl pH 7.0 containing 1.2 % NaCl (w/v), (—▲—) SDS activated PPO, incubated in 25 mM Tris-HCl pH 7.0 containing 1.25 mM SDS, (—■—) acid-pH activated PPO incubated in 25 mM Glycine-HCl pH 2.5 for 15 min at different temperatures ranging from 20 to 95°C.

changes in secondary and tertiary structure. The semi logarithmic plots of residual activity versus incubation time at different temperatures are characterized by single straight lines. Therefore the denaturation process can be attributed to a single exponential decay, for both the native and activated forms (Figure 5.18). The semi-logarithmic plots also indicated that the thermal inactivation of PPO, both in native and activated forms follows first order kinetics. The Arrhenius plots for irreversible denaturation, natural logarithm of k_r vs. reciprocal of the absolute temperature were linear for all native and activated PPOs in the temperature range evaluated. The Arrhenius activation energy (E_a) for thermal inactivation is calculated to be 39.8, 20.1, 13.4 kcal mol^{-1} for native, SDS-activated and acid-pH activated PPO, respectively. The half-life of native PPO at 60 ^{0}C was 85 min whereas that of the SDS activated PPO was 21 min, 4.0 fold lower. Concurrently the activation energy decreased by 19.8 kcal mol^{-1} with an increase in entropy (ΔS^*) from - 47.5 to +7.6 cal K^{-1} mol^{-1}. The decrease in E_a and increase in ΔS^* were found to be much greater for the acid-pH activated PPO indicating that it was more thermolabile than the SDS activated form (Figure 5.18

Table 5.3). The dramatic change in ΔS* indicated that the transition to the activated state presents a more disordered structure than the native PPO. The significant changes in the entropy and the difference in the slopes of the Arrhenius plots indicated that the activation of PPO was due to a conformational change as evidenced earlier by the significant changes in the hydrodynamic radii, intrinsic fluorescence, CD spectra and limited proteolysis data.

Table 5.3. Thermal inactivation parameters of native, SDS and acid-pH activated PPO.

	Incubation temp. (°C)	**Half life (Min.)**	**T_m (°C)**	**$k_r^{\#}$**	**ΔG* kcal mol^{-1}**	**ΔH*# kcal mol^{-1}**	**ΔS* cal K^{-} mol^{-1}**
Ea=167.2 kJ/mol (39.8 kcal mol^{-1})							
Native	60	85.00	70.4	3.3	23.3	39.1	47.5
	65	54.00		5.7	23.2	39.1	47.0
	70	24.00		11.8	23.2	39.1	46.4
	75	10.00		30.7	22.9	39.1	46.6
Ea=84.4 kJ/mol (20.1 kcal mol^{-1})							
SDS-Activated	50	47.00	60.7	7.27	22.0	19.5	8.2
	55	28.00		13.9	22.0	19.5	7.8
	60	21.00		22.7	22.0	19.5	7.6
	65	10.00		28.7	22.0	19.4	7.9
Ea=56.3 kJ/mol (13.4 kcal mol^{-1})							
Acid-pH Activated	40	31.00	51.7	5.9	21.5	15.8	18.4
	45	20.00		10.3	21.5	15.8	18.2
	50	14.00		13.7	21.6	15.7	18.4
	55	08.00		30.0	21.5	15.7	17.7

reaction rate constant.

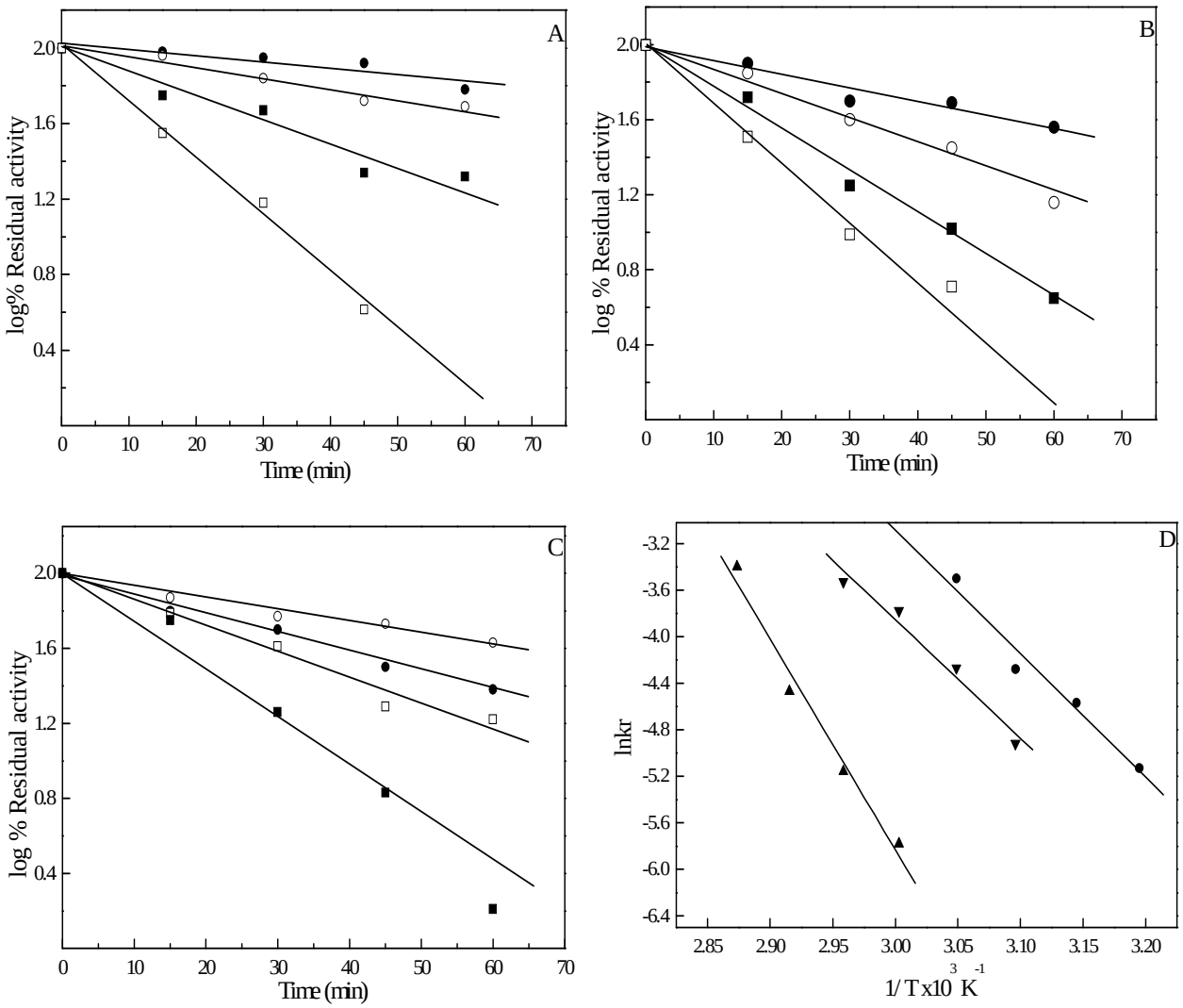


Figure 5.18. Kinetics of thermal inactivation of field bean seed PPO. (A) Native PPO incubated in 25 mM Tris-HCl pH 7.0 containing 1.2 % NaCl (w/v), (—● —) 60 °C, (—○—) 65 °C, (—■—) 70 °C, (—□—) 75 °C. (B) SDS-activated PPO incubated in 25 mM Tris-HCl pH 7.0 containing 1.25 mM SDS (—● —) 50 °C, (—○—) 55 °C, (—■—) 60 °C and (—□—) 65 °C. (C) Acid-pH activated PPO incubated in 25mM Glycine-HCl pH 2.5 (—○—) 40 °C, (—● —) 45 °C, (—□—) 50 °C and (—■—) 55 °C. Samples were incubated at the desired temperatures under respective conditions. Aliquots of the enzyme were removed at the indicated time intervals and assayed using TBC as the substrate. (D) Arrhenius plots of PPO inactivation. (—▲—) Native PPO, (—▼—) SDS activated PPO and (—● —) acid-pH activated PPO.

Effect of urea

If the activation and subsequent decrease in activity were indeed due to conformational changes due to partial unfolding as evidenced by the biophysical and biochemical measurements described above, it should be possible to induce similar changes in the PPO by treatment with urea. The effect of varying urea concentrations on PPO activity was evaluated. The

results indicated a sharp increase in the oxidation of TBC with increasing urea concentration (Figure 5.19). The maximum urea concentration required to bring about a two-fold increase in activity is 0.07 M.

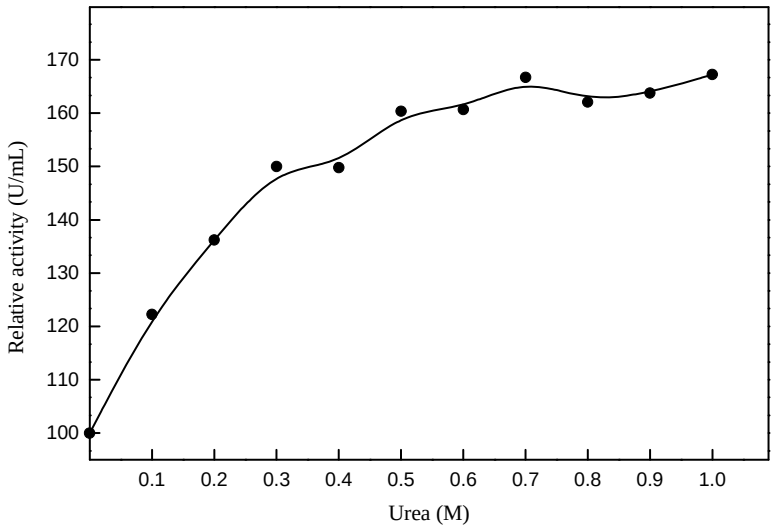


Figure 5.19. Effect of Urea on field bean PPO activity. PPO was incubated in 25 mM Tris-HCl pH 7.0 containing 1.2 % NaCl (w/v) at indicated concentrations of SDS for 30 min and then assayed at pH 6.0 using TBC as substrate.

Discussion

Most enzymes, with a few exceptions are inactivated when exposed to extremes of pH and anionic detergents like SDS. One of the exceptions is PPO (Flurkey, 1986; Lerner et al., 1972; Wang and Constable, 2003). It is well documented that plant-PPOs are activated by a plethora of physical and chemical treatments (Moore and Flurkey, 1990). The molecular mechanism by which SDS binding or acid shock mediates activation of plant PPOs is yet unknown. Most models invoke some form of allosterism based on observations that detergent binding often results in a conformational change in proteins. Kenten, (1958) observed SDS induced activation of a crude preparation of broad bean PPO and attributed this to a limited conformational change. Extending these studies to homogenous preparations of broad bean leaf PPO, Moore and Flurkey, (1990) suggested that at the levels of SDS needed for activation, a minor conformational change may occur, which leads to opening or unlocking of the active site, resulting in an enhanced enzyme activity. Using a homogeneous preparation

of field bean PPO that exists as a single isoform, the biochemical and structural changes associated with PPO activation by SDS and acid shock have been investigated. In plants, PPO functions as an inducible anti-nutritive defense against herbivore insects and pathogen attack. During herbivore insect-feeding PPO reacts with endogenous polyphenolic substrates and the resulting quinones alkylate essential amino acids of the insect dietary protein, making them nutritionally unavailable to the insect (Kessler and Baldwin, 2002). Therefore any increase in PPO levels or activity would be very useful to the plant in its defense against herbivory.

Homogenous field bean PPO, as evidenced by the release of a single amino-terminal asparagine, exist as a single isoenzyme of molecular mass ~120000 Da (Paul and Gowda, 2000) and is a heterodimer. A marked increase in field bean PPO activity occurred after exposure to either pH 2.5 or 4.5 when compared to pH 7.0. This activation process at pH 2.5 was transient, which decreased significantly after 30 min (Figure 5.1). Activation of field bean PPO also occurred in the presence of SDS (Figure 5.2). The concentration of SDS required for half the maximal activation is 0.6 mM, similar to that reported for plant PPOs (Moore and Flurkey, 1990; Kenten, 1957; Chazarra et al., 1996). The SDS concentration of 1.25 mM required for maximum PPO activity was below the determined CMC of SDS (Paul, 2000). The activation of field bean PPO increased linearly with the SDS concentration up to 1.25 mM and decreased thereafter (Figure 5.2). Moore and Flurkey (1990) observed that broad bean leaf PPO was activated by SDS in a sigmoidal manner below the CMC and attributed it to a co-operative interaction between SDS binding and activation. The activity of *Vicia faba* leaf tyrosinase was also enhanced six fold by exposure to pH 3.0 for 2 min followed by neutralization (King and Flurkey, 1987). In contrast, the amount of SDS needed to activate a *Xenopus* tyrosinase was related to the maximum number of detergent monomers in solution (Wittenberg and Tripplett, 1985).

In the present study, the activation by either SDS or acid-pH introduced similar changes in the V_{max} values with little change in the K_m for TBC (Table 5.1). The changes in the kinetic parameters were more obvious when the catalytic efficiency, k_{cat} of the activated PPOs was compared to

native field bean PPO. The activation by SDS decreased the E_a of TBC oxidation thereby enhancing the catalytic efficiency. SDS was the better activating agent than acid-pH treatment as evidenced by the higher k_{cat} (Table 5.1). This increase in catalytic efficiency in both the SDS and acid-pH activation could be attributed to the increase in the catalytic power similar to SDS-activated potato leaf PPO (Sanchez-Ferrer et al., 1993a) and table beet PPO (Escribino et al., 1997). This increased PPO turnover (k_{cat}) by acid activation could be seen as a mechanism that significantly enhanced the production of quinones, which are implicated in cross-linking of insect dietary proteins. Mild denaturing conditions had led to activation in *Vicia faba* tyrosinase while stronger denaturing conditions caused irreversible loss in enzyme activity (King and Flurkey, 1987).

The increase in the catalytic efficiency of field bean PPO is associated with a shift in the pH optimum from 4.5 to 6.0 (Figure 5.3). The activity of native field bean PPO at pH 6.0 is 10 % of the activated form (Figure 5.3). The low pH optimum of 3.5 reported for a broad bean leaf PPO was abolished in the presence of SDS (Moore and Flurkey, 1990). A latent mushroom tyrosinase was reported to show no activity at the optimum pH of the SDS-activated form (Espin et al., 1999a). The observed shift in the pH optima of field bean PPO (Figure 5.3) could be related to the displacement of sensitive pKa values at the catalytic site. Displacement of the pH optimum of *Vicia faba* leaf PPO towards higher pH values as SDS concentration increased has been reported. Jiminez and Garcia-Carmona, (1996) have opined that among the binding centers for SDS there was one responsible for pH dependence of PPO activity, the dissociation constant being 0.52 mM. The pH optimum of 4.5 for native PPO reflects the role of a carboxyl group at the active site. In the crystal structure of sweet potato PPO Klabunde et al., (1998) observed that Glu^{236} was hydrogen bonded to a solvent molecule close to the di-metal active site, which donates a proton and thereby functions as a general base/acid in the oxidation reaction of diphenols. Robert et al., (1995) hypothesized that the acidic pK_a of palmito PPO referred to an acidic amino acid at the active center. Interestingly in this study the multiple alignment of several plants PPO protein sequences also showed that a Glu residue,

corresponding to that of Glu^{236} of sweet potato PPO, was invariant in all plant PPOs, implicating a vital role for this residue in catalysis (Figure 5.6). Chemical modification of Glu residues of field bean and sweet potato PPO resulted in the abolition of catecholase activity, following pseudo-first order kinetics (Figure 5.7). The observed stoichiometry of near 1 for the inactivation of sweet potato and field bean PPO by EDAC was implicative of a single core carboxylate involved in catalysis. The shift in the pH optimum from 4.5 to 6.0 upon activation could therefore be explained by perturbations in the ionization constant of the crucial γ-carboxyl group of Glu, as a consequence of changes in the microenvironment caused by the observed conformational changes. The pKa values of sensitive groups can often be substantially perturbed from the normal values and are even more perturbed in the enzyme-substrate complex. The pKa of Glu^{270} of carboxypeptidase (Petra et al., 1971) and Glu^{35} of lysozyme (Parsons et al., 1972) titrate with anomalously high pK_a s of 7.0 and 6.5 respectively.

No change in the molecular mass, subunit dissociation or protein aggregation was noticed in the activated PPOs when examined by SDS-PAGE (Figure 5.11). Glutaraldehyde cross-linking and light scattering at 325 nm also indicated that upon activation, the field bean PPO maintained the same quaternary structure. The activation of field bean PPO however was accompanied by a substantial increase in the hydrodynamic size. The Stokes radius increased from 49.1±2 Å to 75.9±0.6 Å. Similar electrophoretic mobilities in native PAGE of the activated PPOs suggest that the charge to /mass ratio remained unaltered (Figure 5.4). The increase in the hydrodynamic radii of the activated field bean PPO can thus be attributed to a more “open conformation” in and around the active site. This structural change thereby unlocks the catalytic site for easy access by the substrates resulting in the increased turnover of PPO (Table 5.1). A similar increase in the Stokes radii of the SDS and acid-pH activated PPO was suggestive of similar conformational changes between the two forms (Figure 5.8). The increase in the Stokes radius of a broad bean PPO was previously speculated to arise from a conformational change (Lerner et al., 1972; Lerner and Mayer, 1975). Moore and Flurkey, (1990) interpreted that the small increase

in the size of broad bean leaf PPO could be due to the bound SDS molecules. The activation and kinetic behavior of a latent thylakoid–bound grape (*Vitis vinifera*) PPO was interpreted in terms of a pH induced slow transition, which did not affect the active site of the enzyme (Valero and Garcia-Carmona, 1992a; Valero and Garcia-Carmona, 1992b). Moore and Flurkey, (1990) by sedimentation coefficient measurements found little or no differences in the molecular mass, of broad bean leaf PPO either in the presence or absence of SDS.

The far-UV CD spectrum of field bean PPO indicated 29 % helical content for native PPO (Figure 5.15). The decrease in α-helical content of SDS and acid-pH activated PPO suggest subtle changes in secondary structure upon activation. Near-UV CD studies also showed a loss in tertiary structure upon SDS/acid-pH activation. However a significantly higher loss of secondary structure occurred at pH 2.5. The subtle differences observed at 291 nm relate to a change in the tryptophan environment (Figure 5.14). The striking similarity between the changes in the far-UV CD spectrum of SDS and acid-pH activated field bean PPO points to similar perturbations in the secondary structure. These results taken together with the decreased intrinsic fluorescence and increased Trp accessibility to collisional quenching are in accordance with a partial unfolding of field bean PPO leading to a more open conformation that enhances catalysis. The extensive proteolysis of SDS/acid-pH activated PPO by trypsin also supports a more unfolded or open conformation for the activated forms under these conditions thereby increasing their susceptibility to proteolytic digestion. Akhtar and Bhakuni, (2003) had also noticed that the more open conformation of glucose oxidase (GOD) rendered it more susceptible to proteolytic digestion than the compact deglycosylated GOD.

Data on changes in the tertiary structure of plant PPOs following activation by SDS/acid-pH are yet to be reported therefore this is the first study. The near-UV and far-UV CD data (Figure 5.15) pointed to a localized change stemming from the fact that the CD-spectra retained their original shape with minor alterations in intensity. Given that the observed conformational changes were localized and not global as evidenced by CD

data, it is possible to speculate that these changes were centered in and about the catalytic site. The increased catalytic efficiency (k_{cat}), shift in the optimum pH caused by a change in the microenvironment of Glu and increased inhibition by tropolone (Table 5.2) further strengthen the fact that the conformational change involves the catalytic region of the enzyme. In addition to the conserved Glu the presence of a conserved and invariant Trp (Figure 5.6) in the dimetal catalytic CuB site of PPOs (Klabunde et al., 1998), together with the observed increase in solvent accessibility of the SDS and acid-pH activated field bean PPO, support the localized conformational change anchored around this site. Acyl carrier protein, a small acid protein adopts a partially unfolded structure at neutral pH, but has a tight fold at acidic pH (Park et al., 2004). The presence of both secondary and tertiary structure indicated that the activated field bean PPOs were not in the molten globule conformation.

In order to probe the effects of these observed conformational changes, thermal stability of the native PPO *vs* activated PPO was evaluated. The decrease in the T_m for the activated field bean PPO suggests decreased thermal stability of the enzyme caused by the partial unfolding under these conditions as compared to the more compact native PPO. The thermal stability curves showed a relatively higher stability of native field bean PPO in comparison to the activated field bean PPO (Figure 5.17). The semi-logarithmic plots of residual activity *vs* incubation time characterized by a single exponential decay (Figure 5.18) suggest that the same thermal inactivation mechanism operates for the native and activated forms. The observed single exponential decay for field bean PPO further evidenced its homogeneity as reported (Paul and Gowda, 2000). The biphasic nature of heat inactivation isotherms of PPO isolated from different sources have been explained by the presence of more than one isoenzyme in the preparation (Robert et al., 1995). Effectively a biphasic rate of inactivation most likely points to the enzyme being heterogeneous. The calculated thermodynamic parameters over the temperature range studied for native field bean PPO indicated a negative ΔS whereas the activated PPOs exhibited a positive value. These results further indicated that native PPO presents a more

ordered structure than the acid-pH or SDS-activated PPO and SDS-activated PPO were more ordered than acid-pH activated PPO. These data further support the fact that the localized secondary and tertiary structural perturbations that accompany SDS/pH activation culminated in a partially unfolded thermolabile PPO.

The partial unfolding of field bean PPO induced by SDS or acid-pH leads to the opening or unblocking of the active site thereby accelerating the oxidation of the *o*-diphenols and enhancing the catalytic efficiency several fold. This conformational change alters the microenvironment of a core Glu residue at the active site resulting in a shift in the pKa value of the carboxyl group as reflected by the shift in pH optimum and chemical modification of an essential carboxylate. Whether this structural change is a mimic of the *in vivo* regulatory mechanism of activation upon insect or pathogen attack is yet unknown. PPO an inducible plant defence protein against insect herbivory is considered as an defense related antinutritive oxidative enzyme The effect of high PPO levels against insect herbivory is proposed to reside in the propensity of PPO generated *o*-quinones to covalently modify and cross link dietary proteins during feeding resulting in reduced insect nutrition and performance (Kessler and Baldwin, 2002). Over-expression and high levels of PPO in transgenic *Poplar* leaves have been demonstrated to enhance resistance to herbivory by caterpillars (*Malacosoma disstrica)* (Wang and Constable, 2004). Wounding of plant tissue by either insect herbivory or mechanical injury evokes a jasmonic acid burst consequently lowering the pH, which is amplified by insect feeding (Kessler and Baldwin, 2002). Elicitors such as salicyclic acid and oxalic acid induce defense related enzyme activities such as peroxidase and PPO against *Alternaria alternata*, a fungal pathogen (Tian et al., 2005). Such an acidic milieu would activate PPO resulting in high activity and enhanced turnover. Cipollini et al., (2004) have demonstrated increased PPO, peroxidase, N-acetylglucoaminidase and trypsin inhibitor activity of *Arabidopsis thaliana* and reduced insect growth following jasmonic acid treatment. Therefore the activation and labile nature of field bean PPO could be an adaptive defense strategy to maximize the supply of PPO mediated *o*-quinones to covalently modify proteins and ensure

a decreased nutrition to the feeding predator. This activation is therefore critical to the in vivo physiological defense mechanism postulated for plant PPOs.

Chapter VI
Field bean PPO is a
D-galactose specific enzymic lectin

To attribute the physiological properties of PPO to its function requires the information on the three dimensional structure The first three-dimensional structure of a PPO is that from *Ipomea batatas* (Klabunde et al., 1998). The single form of field bean seed PPO, a catecholase renders it suitable for both primary structure determination and three-dimensional analysis. To better understand and correlate at the molecular level the structure activity relationship, studies on the isolation of a full-length cDNA encoding field bean PPO were initiated. However all our cloning strategies devised by employing degenerate primers designed to the identified amino-terminal sequence (Chapter III) and the well conserved Cu^{2+} binding regions of plant PPOs did not yield any suitable cDNA fragments. Internal peptide sequences were generated by sequencing peptides obtained by TPCK-trypsin cleavage and the partial primary structure was elucidated. cDNA was obtained using degenerate primers designed to the internal sequence. In this chapter the results of the cloning and sequencing are presented and discussed. The results show that field bean PPO is a galactose specific lectin. The multifunctional role of a single protein referred to as PPO-haemagglutinin is also presented and discussed.

RESULTS

RNA isolation

Total RNA was prepared from mid mature field bean (*Dolichos lablab*) seeds stored at -70 ^{0}C, following the conventional lithium chloride method (Figure 6.1A). The presence of two bands of 18S and 5.8S RNA indicated that the preparation was suitable for further studies.

cDNA synthesis and cloning

Degenerate oligonuleotide primers were designed based on the determined amino-terminal sequence (Chapter III) and labeled as indicated below

PPDF-1 5' AAYAAYYTNTHWSNTTYACNATG 3'

PPDF-2 5' ATHWSNTTYACNATGAARMGNTTYG 3'

PPDF-3 5' TTYACNATGAARMGNTTYGAYGARC 3'

PPDF-4 5’ ATGAARMGNTTYGAYGARCARAA 3’

Degenerate primers listed below were also designed to the well-conserved regions of Cu^{2+} binding region of plant PPOs.

PCOP-F (sense) 5’ TGGYTHTTCTTBCCBTTCCAYMG 3’

PCOP-R (antisense) 5’ CKRGGAAVGGVAAGAADARCCA 3’

PNVDR-R (antisense) 5’ CCACATBCKRTCDACATT 3’

cDNA was generated from 8 µg of field bean seed total RNA using M-MLV reverse transcriptase by following the manufacturer’s protocol. The synthesis of cDNA was confirmed by amplification of the 26SRNA and β-actin (Singh et. al., 2004). The positive amplification indicated the synthesis of the first strand (Figure 6.3A).

The schematic representation of 5’ and 3’ rapid amplification of cDNA ends (RACE) is shown in Figure 6.2. The putative 5’ cDNA end was amplified by 35 cycles of PCR, using an initial denaturation at 94 °C for 5 min, followed by three-step cycles (40 cycles, 94 °C for 30 sec, 54 °C for 30 sec and 72 °C for 1 min) and further extended at 72 °C for 10 min. The 3’ cDNA end was amplified by 35 cycles of PCR, comprising of an initial denaturation at 94 °C for 5 min, followed by three-step cycles (35 cycles, 94 °C for 30 sec, 64 °C for 20 sec and 72 °C for 45 sec) and further extended at 72 °C for 10 min. Reaction were performed in a total volume of 50 µL containing 30 pmol of each primer, 0.2 µM dNTPs and 1.5 U of *Taq* polymerase in the corresponding buffer (Figure 6.3).

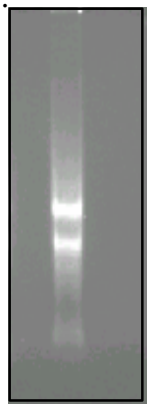


Figure 6.1. Total RNA isolated from the field bean (*Dolichos lablab*) seeds.

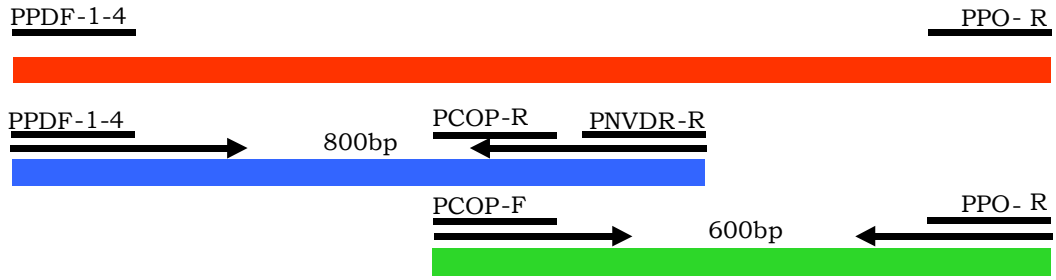


Figure 6.2. Pictorial representation of strategies used in the amplification of PPO gene

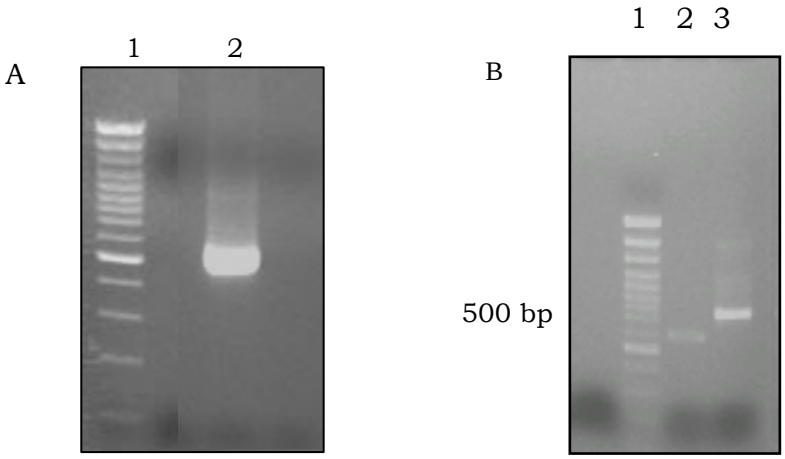


Figure 6.3. Amplification of 5'and 3' cDNA ends. A) Amplification of 26 S RNA, Lane 1; 100 bp ladder, Lane 2; 534 bp. B) Amplification using *Taq* DNA polymerase, Lane 1; 100 bp ladder, Lane 2; 600 bp 3' cDNA end, Lane 3; 800 bp 5' cDNA end.

The cDNA fragments obtained by PCR amplification were purified by gel elution. The purified PCR fragments were reamplified using same PCR protocol replacing *Taq* polymerase with Phusion polymerase with its appropriate buffer. The fragments were cloned into *PvuII* site of pRSET C vector (Figure 6.4). Positive clones of transformed DH5α *E. coli* chemically competent cells were isolated from ampicillin LB plates. The recombinants were assessed by the DNA shift method. The DNA sequence was determined by cycle sequencing using Big Dye Chemistry (Applied Biosystems, Foster City, CA).

Among the positive clones, none of the sequences obtained showed homology to any of the reported plant PPO sequences. Several other cloning strategies did not yield any positive clones. To overcome this problem, it was

decided to use internal peptide sequences by generated enzymatic cleavage followed by Edman sequencing.

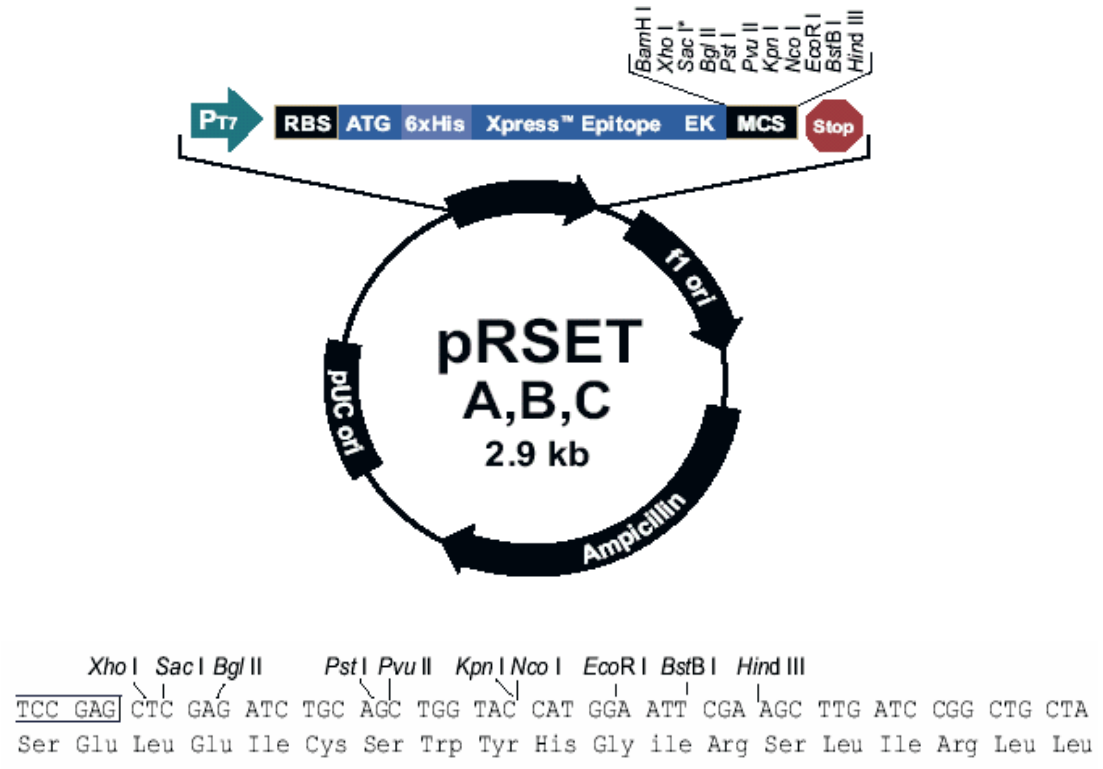


Figure 6. 4. Vector map of pRSETC vector. Multiple cloning sites of the vector are shown in the figure.

Internal peptide sequences of PPO

Cleavage with TPCK-trypsin, separation of peptides and sequence determination: The cleavage of the field bean PPO protein was carried out using catalytic amounts of TPCK-trypsin. The enzymatic digest was separated on a Phenomenex ODS RP-HPLC column (250 × 4.6 mm, 5μ) and peptides fractionated using a linear gradient of 0.1 % TFA in water and 70 % acetonitrile containing 0.05 % of TFA. Peptide fractions were collected over several runs. The peptide fractions were subjected to Edman analyses on an automated sequenator. The sequences of the peptides obtained by Edman degradation are listed (Table 6.1). The major peptide sequences terminate at either K or R indicating the specificity of trypsin. A BLAST search of these

Table 6.1. Sequence of peptides obtained from TPCK-trypsin cleavage and their homology

Sequence of peptides	**Homology (%)**	Name and accession number
NAWDPETYHIGIDVNSK	85-90	*Sophora japonica* bark and seed lectin (AAB51458, AAB51441) *Phaseolus leptostachyus* (CAH60215) *Phaseolus maculates* (CAH60256) *Phaseolus filiformis* (CAH60216) *Vigna unguiculata* (CAF18557) *Phaseolus oligospermus* (CAH60173) *Phaseolus microcarpus* (CAH60170) *Vigna linearis var.latifolia* (CAD 43280)
FSIGRAFYTTPIRVWDK	75-80	*Phaseolus oligospermus* (CAH 60172) *Phaseolus vulgaris* (CAD 29133) *Phaseolus coccineus* (CAD27654 *Phaseolus acutifolius* (AAA82181)
ATVADGLAFALVPVGAQPR	90-95	*Phaseolus maculates* (CAH60256) *Phaseolus filiformis* (CAH60216) *Vigna unguiculata* (CAF 18557) *Phaseolus oligospermus* (CAH60173) *Phaseolus microcarpus* (CAH60170 *Phaseolus costaricensis* (CAH 60989) *Phaseolus parvulus* (CAH 60255) *Phaseolus augusti* (CAH 59200) *Phaseolus vulgaris* (CAD 28674) *Phaseolus linearis* (CAD43280)
	80	*Dolichos biflorus* (DBL, DB 58)

peptides indicated a high degree of homology to a group of D-galactose-specific legume lectins including the well studied DBL and DB58 of *Dolichos biflorus* (Schnell and Etzler, 1988). The maximum identity (93 %) was observed with a group of uncharacterized lectins such as *Phaseolus*

filiformus and Phaseolus parvulus. Only the proteins showing >70 % identity are listed (Table 6.1). Degenerate oligonuleotide primers were designed targetting the obtained internal sequence.

cDNA synthesis using primers targeting internal peptide sequences

The designed primers for the sequence **NAWDP and HIGIDV** of the peptide NAWDPETWYHIGIDVNSK,are listed below

HIGIDV-5' ANACRTCDATNCCDATRTG 3' (Antisense)

HIGIDVF -5' ADGYRAAAGACCAWGADWSVA 3' (Sense)

HIGIDVF1 5' AATGCTGCATGGGATCCAG 3' (Sense)

SWSFR 5' GADGYRAAAGACCAWGAD 3' (Antisense)

SWSFF 5'T CGCTCCAGTGGTAGCTGAG 3' (Sense)

CTER-R- 5' CTAGAGGATYTTGTTGAGG 3' (Antisense)

These primers were used in combination with the primers designed to the amino-terminal sequence

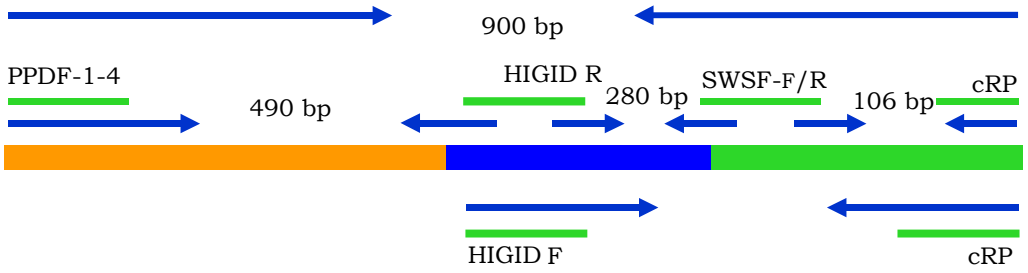


Figure 6.5. Pictorial representation of the amplification strategies using the internal peptide sequence.

The schematic representation of amplification of the complete gene is shown in the Figure 6.5. A gene fragment of 490 bp was amplified using the primer pair PPDF-1-4 and HIGID-R corresponding to the amino-terminal sequence and internal peptide respectively by 35 cycles of PCR, comprising of an initial denaturation at 94 °C for 5 min, followed by three-step cycles (40 cycles, 94 °C for 30 sec, 54 °C for 30 sec and 72 °C for 1 min) and further extended at 72 °C for 10 min (Figure 6.6A). The PCR reaction were performed in a reaction volume of 50 µL containing 30 pmol of each primer, 0.2 µM dNTPs and 1.5 U of *Taq* polymerase in the corresponding buffer. The

490 bp product was purified and sequenced by dideoxy chain termination on a 310 Genetic Analyzer (Applied Biosystems, USA, Figure 6.7A). Based on the sequence of the 490bp amplified product, a specific sense primer for the sequence HIGIDVF used in combination with a degenerate primer coding for the highly conserved SWSF sequence towards the carboxy-terminus of legume lectins. A 280 bp amplicon was amplified by 35 PCR cycles using the conditions reported above (Figure 6.7B). The amplified fragment was sequenced. Based on the obtained sequence, a specific sense primer, for the sequence SWSF along with a degenerate primer with very low degeneracy coding for the carboxy-terminal six amino acids of galactose specific legume lectins aligned by CLUSTALW. (CTER) was used. The 110 bp product obtained by 30 cycles of PCR was sequenced (Figure 6.6C and 6.7C).

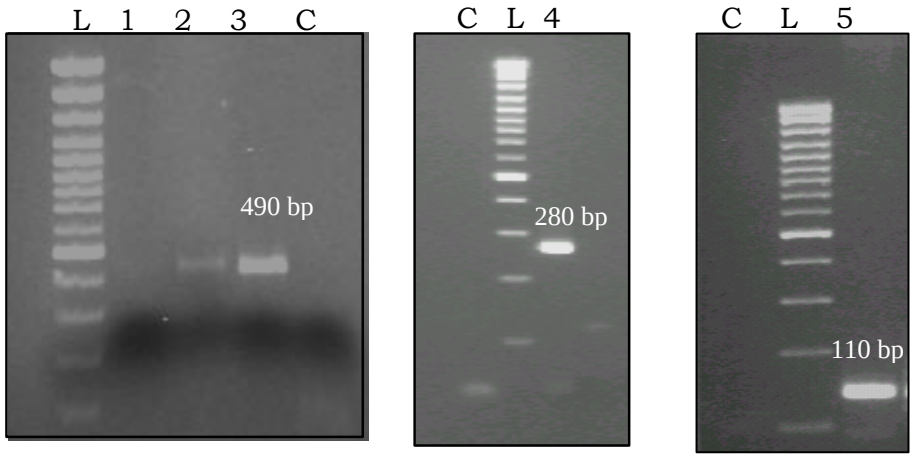


Figure 6.6. Agarose gel electrophoresis of the amplification products obtained using varying degenerate primers. Lane L; 100 bp ladder, Lane 1; Primer pair PPDF2 and HIGIDV, Lane 2; Primer pair PPDF3 and HIGID, Lane 3; Primer pair PPDF 4 and HIGIDV, Lane 4; primer pair HIGIDVF and SWSF, Lane 5; Primer pair SWSF and CRP, Lane C; PCR control.

By overlapping and combining all the sequence a total of 786 bp sequence was obtained and submitted to Gene Bank (Accession Number EF204527). The deduced amino acid sequence for the 786 bp is shown in Figure 6.8. The amino acid sequence of the TPCK-derived peptides are underlined. A BLAST search showed the maximum homology to the D-galactose specific lectins.

A

5′ATGAAAAGATTCGATGAACAGAATTTGAAGTTACAAGACATTGTTAATATAAACTCAACC
AACCACTTCTCCTTCAATTTCACCGGCGTTTTGATGAGCAGAACCTCCATCCTCCAACGTGT
CGCCAAAGTCTCCAGCGGCACGTTACGACTCACCAACGTTAGTGCCAAGGGCGTTCCCTTGG
CGTTCTCTATTGGATGCGCTTTCTATACCACCCCCATCCGTGTCTGGGACAAATCCACTGGC
AGCGTCGCCAGCTGGGCCACCTCCTTCACATTCAACATTAACGCTCCCAACAAGGCAACTAC
TGCCGATGGCCTCGCCTTTGCTTTGGTACCCGTCGGGGCTCAGCCCAGAACTTCGTTTGGAT
ATCTAGGTCTCTTCGACACCNCTGACAACAACAGCTCCGTCCAGACTCTTGCTGTCGAGTTT
GACAACCATCGC**AATGCCTGGGACCCCGAAACCTACCACATCGGCATAGACGTTAACAGTAA
AA** 3′

B

5′AATGCCTGGGACCCCGAAACCTACCACATCGGCATAGACGTTAACAGTAAAATCAGATCC
AAAAGTACTAAGTCGTGGATTTTGCAGAATCGTGAACTGGGCAAAGTTGTGATACAATTTCG
TGCTGCTACTAATGTGTTAAATGTTTATTTGACTTATCCTAATTCAATTAATTATACTCTTA
GTGAAATTGTGCCTTTGAAGGATGTTGTTCCTGAGTGGGTGAGGGTTGGTTT**CTCAGCTACC
ACTGGAGCG** 3′

C

5′**CTCAGCTACCACTGGAGCGA**TTCATTCATGGTCTTTCGCTTCAGACTTGGAAACAACCAC
ATCTGAAGGTTTGAGTCTCGCCAACATCGTCCTCAACAAGATCCTC 3′

Figure 6.7. DNA sequence of PCR fragments A. 490 bp, B; 280 bp and C; 110 bp products. Primer sequences are underlined and overlapping sequences obtained from the three are shown in bold.

A recent report of a D-galactose specific lectin from the Indian lablab bean, a different cultivar of *Dolichos lablab* shows an identical amino-terminal sequence of ten residues. These observations with the high sequence homology of the deduced sequence of D-galactose lectins together raise the question as to whether the PPO is per se a galactose specific lectin. To provide a rationale answer and explain these unusual results the purified (Chapter III) was evaluated for its haemagglutinating activity. The purified field bean PPO exhibited haemagglutinating activity against human RBCs. The specific activity for agglutination was 1224 U/mg of protein. Therefore, to unequivocally prove that the PPO is indeed a haemagglutinin the protein was purified 1) as described in chapter III and 2) by D-galactose sepharose affinity chromatography commonly used to purify D-galactose specific lectins. PPO and haemagglutinating activity were followed simultaneously at every step of purification. Earlier reports speculate that all D-galactose specific lectins are enzymic lectins that exhibit ∝-galactosidase activity.

Therefore ∝-galactosidase activity was also followed during the purification protocol.

```
atgaaaagattcgatgaacagaatttgaagttacaagacattgttaatataaactcaacc
 M  K  R  F  D  E  Q  N  L  K  L  Q  D  I  V  N  I  N  S  T
aaccacttctccttcaatttcaccggcgttttgatgagcagaacctccatcctccaacgt
 N  H  F  S  F  N  F  T  G  V  L  M  S  R  T  S  I  L  Q  R
gtcgccaaagtctccagcggcacgttacgactcaccaacgttagtgccaagggcgttccc
 V  A  K  V  S  S  G  T  L  R  L  T  N  V  S  A  K  G  V  P
ttggcgttctctattggacgcgctttctataccacccccatccgtgtctgggacaaatcc
 L  A  F  S  I  G  R  A  F  Y  T  T  P  I  R  V  W  D  K  S
actggcagcgtcgccagctgggccacctccttcacattcaacattaacgctcccaacaag
 T  G  S  V  A  S  W  A  T  S  F  T  F  N  I  N  A  P  N  K
gcaactactgccgatggcctcgcctttgctttggtacccgtcggggctcagcccagaact
 A  T  T  A  D  G  L  A  F  A  L  V  P  V  G  A  Q  P  R  T
tcgtttggatatctaggtctcttcgacaccnctgacaacaacagctccgtccagactctt
 S  F  G  Y  L  G  L  F  D  T  X  D  N  N  S  S  V  Q  T  L
gctgtcgagtttgacaaccatcgcaatgcctgggaccccgaaacctaccacatcggcata
 A  V  E  F  D  N  H  R  N  A  W  D  P  E  T  Y  H  I  G  I
gacgttaacagtaaaatcagatccaaaagtactaagtcgtggattttgcagaatcgtgaa
 D  V  N  S  K  I  R  S  K  S  T  K  S  W  I  L  Q  N  R  E
ctgggcaaagttgtgatacaatttcgtgctgctactaatgtgttaaatgtttatttgact
 L  G  K  V  V  I  Q  F  R  A  A  T  N  V  L  N  V  Y  L  T
tatcctaattcaattaattatactcttagtgaaattgtgcctttgaaggatgttgttcct
 Y  P  N  S  I  N  Y  T  L  S  E  I  V  P  L  K  D  V  V  P
gagtgggtgagggttggtttctcagctaccactggagcgattcattcatggtctttcgct
 E  W  V  R  V  G  F  S  A  T  T  G  A  I  H  S  W  S  F  A
tcagacttggaaacaaccacatctgaaggtttgagtctcgccaacatcgtcctcaacaag
 S  D  L  E  T  T  T  S  E  G  L  S  L  A  N  I  V  L  N  K
atcctc
 I  L
```

Figure 6.8. The 786 bp nucleotide and deduced amino acid sequence of PPO. The underlined sequences correspond to the amino acid sequence obtained from Edman sequencing of the tryptic peptides of field bean PPO (Table 6.1).

Extraction and Purification

The crude extract of field bean seed powder was prepared as described earlier (Chapter II Section 2.2.3). The crude extract exhibited a specific activity of 1275 U/mg for PPO and 944 HAU against human RBCs. A glucose/mannose specific lectin has been previously reported in field bean (Gowda et al., 1994). The measured haemagglutinating activity therefore includes that of the glucose/mannose specific lectin also. Therefore to quantify the haemagglutinating activity of the D-galactose specific lectin alone, the haemagglutination assay was carried out in the presence of 0.5 M

glucose. The activity thus measured was 47.4 HAU. These results show that the D-galactose lectin was only 5 % of the total lectin of field bean seeds. The ratio of PPO/HAU was 26.9 (Table 6.2).

Ammonium sulfate fractionation

The crude extract was saturated to 40 % $(NH_4)_2SO_4$ (22.6 g/100 mL) by addition of finely powdered solid $(NH_4)_2SO_4$ at 4 °C. The solution was allowed to stand for 2 h at 4 °C for complete precipitation. The precipitate obtained after centrifugation at 15000 × g for 45 min at 4 °C was discarded and supernatant was subjected to 80 % $(NH_4)_2SO_4$ (25.8 g/100 mL) precipitation. The solution was allowed to stand overnight at 4°C for complete precipitation of proteins. The precipitate was collected by centrifugation at 15000 × g for 45 min at 4 °C. The 40-80 % precipitate was re-dissolved in 10 mM Tris-HCl buffer, pH 8.2 containing 1.2 % NaCl (w/v) and dialyzed against the same buffer (5 × 500 mL). The specific activity for haemagglutinating activity was 124.3 HAU and PPO/HAU was 23.0. A purification of 2.24 fold was obtained in this step with a 48.6 % yield from the previous step for both PPO and lectin (Table 6.2).

DEAE-sepharose chromatography

Ion exchange chromatography on DEAE-sepharose was used to remove some inactive proteins. PPO eluted as a single unbound protein peak in the column wash. The hemagglutinating activity was coincident with the same fraction (Figure 6.9A). Although the yield of the haemagglutination titer is considerably lower than that of PPO, the two activities coelute. Figure 6.9B depicts the native-PAGE profile of 50 µL fractions across the DEAE-sepharose peak. A single staining region specific for PPO is observed. The intensity of the PPO activity bands coinside with both the A_{280} and hemagglutinating activity. The active fractions were pooled as shown. The pooled fraction showed a specific activity of 7416 U/mg for PPO and 784 HAU (Table 6.2). A purification of 6.0 fold was obtained in this step with a 60 % yield from the previous step.

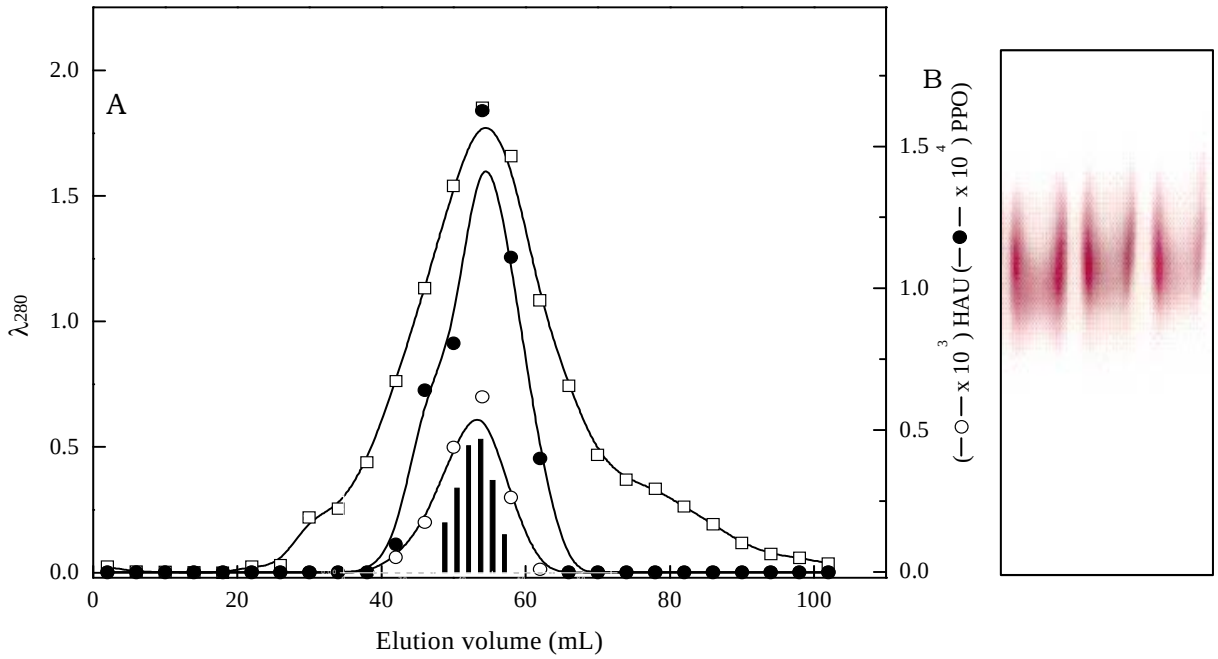


Figure 6.9. A) DEAE-sepharose chromatography elution profile of PPO and haemagglutinin activities. Protein (—□—), PPO (—●—), HAU (—○—), bar graph-α-galactosidase, activities respectively. B) Native-PAGE of fractions collected across the peak and stained for PPO activity with catechol and MBTH. From left to right lanes correspond to V_e of 50, 54 and 58 mL respectively. The fraction pooled were from V_e 46-62 mL.

Size exclusion chromatography

The pooled fraction of the previous step was concentrated and subjected to size exclusion chromatography on a Superdex-200 column (Amersham Biosciences) by FPLC. The PPO, hemagglutinating and α-galactosidase activities are coincident on the descending shoulder of the major protein peak (Figure 6.10A). SDS-PAGE of 100 μL portions of the three peak fractions across the PPO-hemagglutinin activity is shown in Figure 6.10B. All the fractions show the presence of two very similar sized polypeptides of Mr~29000 and 31000Da. The intensity of the protein staining is coincident with both the peak hemagglutination and PPO activity. The specific activity of PPO and hemagglutination increased by 25.3 fold. Table 6.2 summaries the overall purification procedure.

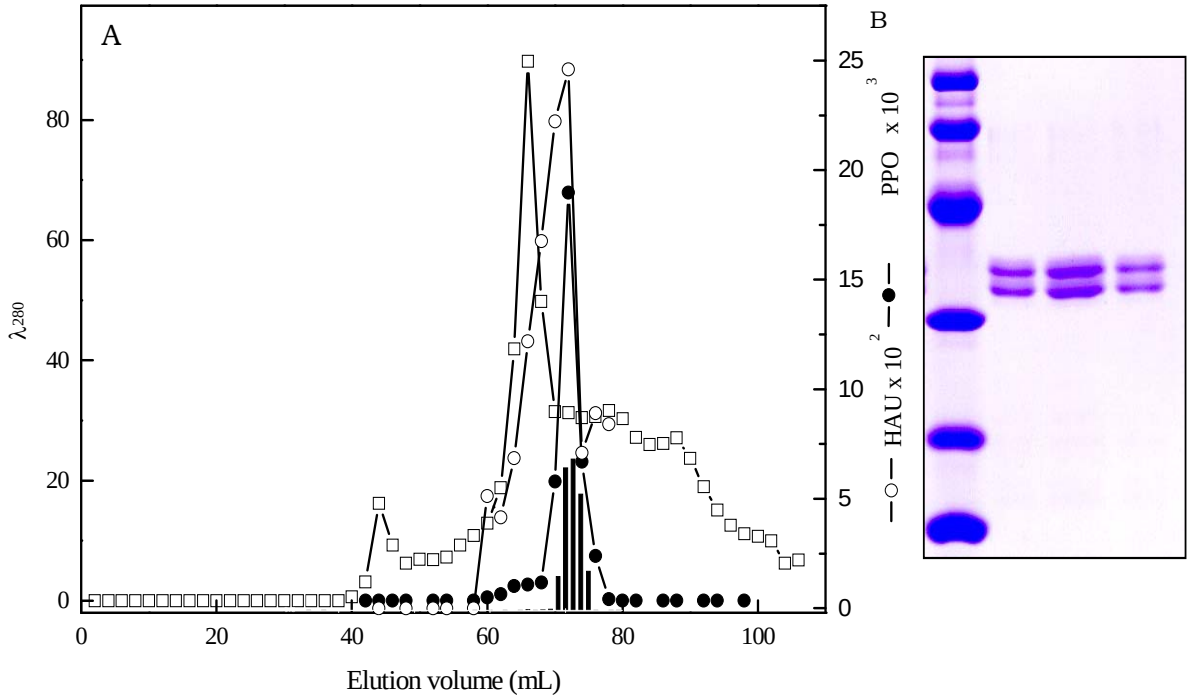


Figure 6.10. A) Size exclusion chromatography (Superdex 200) elution profile. Protein (—□—), PPO (—●—), HAU (—○—) and bar graph α-galactosidase, activities respectively. B) SDS-PAGE of fractions collected across the peak with molecular weight markers 97400 Da; phosphorylase b, 66000 Da; ovalbumin, 43000 Da; albumin, 29000 Da; carbonic anhydrase, 20100 Da; soybean trypsin inhibitor and 14300 Da; lysozyme. From left to right the lanes correspond to V_e of 70, 72 and 74 mL respectively. The fractions pooled were from V_e 68-78 mL.

Galactose sepharose affinity chromatography

The protein was also purified by D-galactose affinity chromatography. The 40-80 % ammonium sulfate precipitate as described earlier was dissolved in TBS containing 1.5 M $(NH_4)_2SO_4$ and applied to the column, which was pre-equilibrated with TBS containing 1.5 M $(NH_4)_2SO_4$. The column was washed with the same buffer and bound protein eluted with TBS minus $(NH_4)_2SO_4$. The PPO, haemagglutinating activity and α-galactosidase activity co-elute as a single symmetrical peak (Figure 6.11A). Figure 6.11B shows the SDS-PAGE profile of 100 µL portions of the peak fractions. All the fractions show the presence of two very similar sized polypeptides of Mr~29000 and 31000Da. The intensity of the protein staining is coincident with both the peak haemagglutination and PPO activity. The specific activity of PPO and haemagglutinating increased 22.2 fold. The purification of protein by galactose-sepharose affinity chromatography is summarized in the Table 6.4.

Table 6.3 Purification of PPO-haemagglutinin by the conventional method *

Step	Total protein (mg)	Total PPO activity ($U \times 10^5$)	Specific activity (U/mg)	HAU#	Fold Purification	Yield (%)	PPO/ HAU
Crude	474.6	6.0	1275.8	47.4	-	100	26.0
40-80 % $(NH_4)_2SO_4$ fractionation	102.8	2.9	2864.9	124.5	PPO 2.24 HAU 2.62	48.6	23.0
DEAE-Sepharose Chromatography	7.1	1.4	20058.8	784.0	PPO 15.7 HAU 16.5	23.6	25.2
Superdex-200 Chromatography	3.0	0.98	32265.7	1261	PPO 25.3 HAU 26.6	16.2	25.6

*These are the results of a typical purification starting from 10 g of defatted field bean powder. These values are reproduced in three separate purifications. #HAU was determined in the presence 0.5 M D-glucose

Table 6. 4. Purification of PPO-haemagglutinin by galactose sepharose affinity chromatography *

Step	Total protein (mg)	Total PPO activity ($U \times 10^5$)	Specific activity (U/mg)	HAU#	Fold Purification	Yield (%)	PPO/ HAU
Crude	474.6	6.0	1275.8	47.4	-	100	26.9
$(NH_4)_2SO_4$ precipitation (40-80 %)	102.8	2.9	2864.9	124.5	PPO 2.24 HAU 2.62	48.6	23.0
D-Galactose sepharose affinity Chromatography	4.64	1.31	28294.5	1240	PPO 22.2 HAU 26.16	21.7	26.2

*These are the results of a typical purification starting from 10 g of defatted field bean powder. These values are reproduced in three separate purifications. #HAU was determined in the presence of 0.5 M D-glucose.

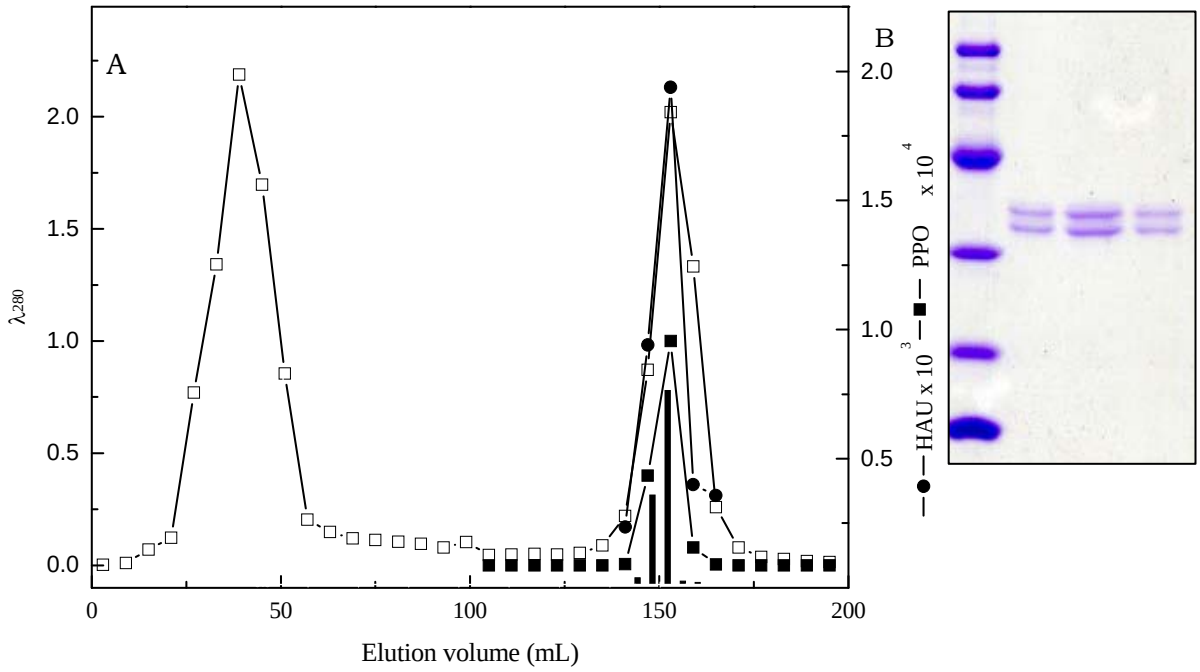


Figure 6.11. A) Galactose-sepharose elution profile of PPO activity and haemagglutinin activities. Protein (—□—), PPO (—■—), HAU (—○—) and bar graph-α-galactosidase, activities respectively. Arrow shows the start of elution with TBS. **B)** shows SDS-PAGE loaded with 100 µL samples collected across the peak molecular weight markers 97400 Da; Phosphorylase b, 66000 Da; Ovalbumin, 43000 Da; albumin, 29000 Da; carbonic anhydrase, 20100 Da; soybean trypsin inhibitor, 14300 Da; lysozyme. From left to right the lanes correspond to V_e of 148, 151 and 154 mL respectively. The fractions pooled were from Ve 148-154 mL.

The two activities co-purify through all the steps of purification. The specific activity of PPO 28294.5 U/mg obtained by galactose affinity chromatography is comparable to that of 32265.7 U/mg obtained by the earlier procedure. As observed the ratio of PPO/HAU is near constant in all the steps of purification (Table 6.3 and 6.4).

Erythrocyte haemagglutination overlay assay

An erythrocyte overlay assay was performed to demonstrate the agglutinating activity of the affinity chromatography purified protein. The purified protein was separated by native PAGE and electroblotted onto a nitrocellulose membrane. The membrane was incubated with human A^{+ve} blood group erythrocytes. Erythrocyte binding as a single band was observed (Figure 6.12A). Further in order to show that the same protein is associated with PPO activity the membrane was washed to remove formalin and then stained for PPO activity with catechol and MBTH. The appearance of a pink

colored band is reminiscent of PPO activity and is coincident with the erythrocyte binding region (Figure 6.12B). This result confirms that the two activities are associated with a single protein. Here after this protein is referred to as the PPO-haemagglutinin.

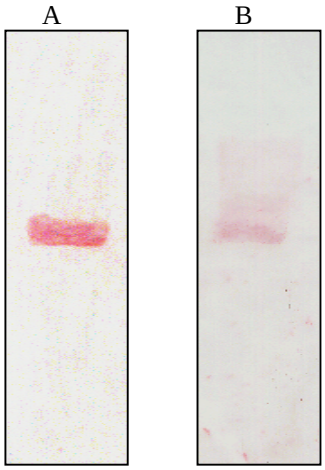


Figure 6.12. Erythrocyte haemagglutination overlay assay. Lane A; PPO-haemagglutinin blotted onto nitrocellulose membrane after native-PAGE and then incubated with human A^+ erythrocytes. Lane B shows the same membrane washed to remove formalin and then stained for PPO activity with catechol and MBTH

Molecular weight

The apparent M_r of PPO-haemagglutinin purified by galactose affinity chromatography was determined by analytical gel filtration on a Progel™-TSK G2000 SW_{XL} HPLC column, SDS-PAGE (Laemmli, 1970) and also by MALDI-TOF. The M_r of the purified PPO-haemagglutinin estimated by HPLC gel filtration was 120000±2000Da from a plot of log M_r versus V_e/V_o retention time. SDS-PAGE (12.5 % T, 2.7 % C) of the purified PPO-haemagglutinin was carried out in a discontinuous buffer system. Protein staining using Coomassie blue R-250 showed a two subunits of 29000±1300 Da and 31000±1500 Da (Figure 6.13). This suggests that the PPO-hemagglutinin purified by D-galactose sepharose affinity is a heterodimer. The exact molecular weight of the two subunits as determined by MALDI-TOF for the D-galactose affinity chromatography purified PPO-hemagglutinin was 29808.492 and 31087.042 Da. The mass spectrum is consistent with that obtained by SDS-PAGE indicating a pair subunits differing by a mass of 1279 Da, which could arise from a deletion of 10 or 11 residues. All these results are identical to the PPO purified to homogeneity.

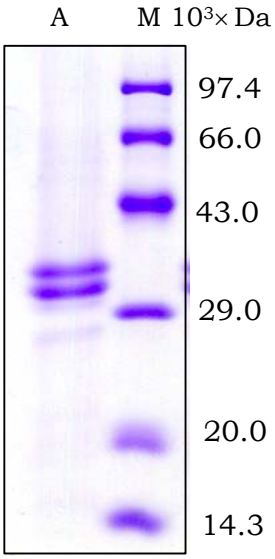


Figure 6.13. SDS-PAGE (12.5 % T, 2.7 % C) profile of the PPO-haemagglutinin. Lane A; PPO-haemagglutinin and Lane M; Molecular weight markers.

Blood group specificity

Examination of the haemagglutination ability of the PPO-haemagglutinin with different erythrocyte types (human, rabbit and sheep) revealed some interesting observations. The protein showed strong haemagglutinating activity when tested against native human blood groups (A, AB, B and O, Table 6.4). The PPO-haemagglutinin agglutinated all of these erythrocytes showing no apparent blood group specificity. However it was characterized by its slight preference towards the A group (Table 6.4). Like all other lectins, the sensitivity was enhanced following erythrocyte treatment with trypsin. The specific activity of 628 HAU/mg toward rabbit erythrocytes shows it to be less specific. The PPO-haemagglutinin did not show any agglutination against sheep erythrocytes.

Sugar inhibition studies

The carbohydrate binding specificity of the PPO-haemagglutinin was studied by carrying out sugar inhibition studies as described under Section 2.2.5d. Several sugars (all sugars of D configuration) were examined for their ability to inhibit the agglutination reaction. The results are summarized in Table 6.5. Among the sugars tested for inhibition, galactose and its

derivatives galactosamine, N-acetylgalactosamine and lactose were found to inhibit the agglutination of human erythrocytes. The minimum inhibitory concentrations of these sugars indicate that it is a D-galactose specific lectin (Table 6.5). An eight fold higher concentration of galactosamine was required to inhibit agglutination as compared to D-galactose and N-acetylgalactosamine. The higher binding capacity of N-acetylgalactosamine as compared to galactosamine indicates a preference for the acetylated form.

Table 6.4. Agglutination of erythrocytes by purified PPO-haemagglutinin.

Erythrocytes	HAU (U/mg)
Human A	1261
Human B	628
Human AB	1261
Human O	312
Rabbit	628
Sheep	0

Table 6.5. Minimum inhibitory concentration of sugars require to inhibit haemagglutination.

Sugars	**Minimum concentration required for inhibition (mM)**
D-Galactose	3.9
D-Galactosamine	31
N-acetyl galactosamine	3.9
D-Glucose	NA
D-Rhamnose	NA
D-Arabinose	NA
D-Fructose	NA
L-Fucose	NA
3-O-Methyl D-glucopyranose	NA

NA: no agglutination even at 0.5 M concentration.

PPO and haemagglutinin activity domains are different

The catalytic activity (PPO) and the sugar binding activities (haemagglutination) were observed to be independent of each other. A set of PPO inhibitors and D-sugars were tested against both PPO activity and haemagglutinating activity (Table 6.6). Tropolone is a potent copper chelator. A very low concentration of tropolone is sufficient to bring about 50 % inhibition of PPO (IC_{50} 8.0×10^{-4} M). At the same concentration the haemagglutinating activity was unaltered. Other copper chelators like diethyldithiocarbamate (IC_{50} 9.3×10^{-6} M), 2-mercaptobenzimidazole (IC_{50} 2.9×10^{-5} M) and 2-mercaptonenzothiazole (IC_{50} 8.0×10^{-7} M) inhibit PPO activity at very low concentration. However these Cu^{2+} chelators did not have any effect on the haemagglutination activity. In the presence of D-galactose and N-acetylgalactosamine, sugars that inhibit the agglutination at concentrations as high as 10 mM had no effect on the PPO mediated oxidation of TBC (Table 6.6). These results demonstrate that the PPO and haemagglutinating activity is independent of each other.

Metal analysis

Carbohydrate binding activity of legume lectins depends on the presence of metal ions. Whereas PPO activity depends on the presence of Cu^{2+} at the active site. Metal analysis showed the presence of 2 mol copper/mol of protein. The Mn^{2+} content was 0.40 mols/mol protein. The presence of Cu^{2+} in the PPO-haemagglutinin is responsible for the PPO activity. The complete inhibition of haemagglutination in the presence of EDTA indicates the presence of calcium.

Cross reactivity

Antibodies raised against field bean PPO were used to examine a number of pure, well-characterized plant lectins for immunological cross-reactivity. Several galactose specific lectins (SBA, DBL PHA-M) showed very strong cross reactivities. Greater cross reactivity was observed with DBL, which is a well characterized galactose specific lectin. The cross reactivity studies with DBL, SBA and PHA, revealed that these lectins have similar antigenic determinants and structural features. Cross reactivity was also

observed with the glucose/mannose specific lectin of field bean. Both the sub units reacted with the antisera suggesting the antibody is not subunit specific. No cross reactivity was observed with ConA and the lectin of *Limulus polyphemus* (Figure 6.14).

Table 6.6. Effect of D-sugars and PPO inhibitors on haemagglutination and PPO activity.

Inhibitor	Concentration (mM)	Relative activity (%)	
		PPO	HAU
Tropolone	0.8*	50	100
Diethyldithiocarbamate	0.0093*	50	100
	0.100	0	100
2-Mercaptobenzimidazole	0.0293*	50	100
	0.100	0	100
2-Mercaptobenzthiazole	0.0008*	50	100
	0.10	0	100
EDTA	0.20**	98	0
D-Galactose	3.9**	99	0
	100	98	0
N-Acetyl-D-galactosamine	3.9**	97	0
	100	97	0
D-Galactosamine	31**	99	0
	100	96	0
D-Lactose	500**	98	0

* IC_{50} concentration required to bring about 50 % inhibition of PPO. **MIC Minimum Inhibitory Concentration of agglutination.

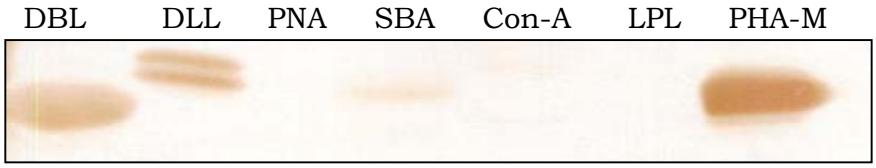


Figure 6.14. Cross reactivity of legume lectins with antibodies raised against field bean PPO purified by the conventional procedure. *Dolichos biflorus* (DBL), *Dolichos lablab* (DLL), *Arachis hypogea* (PNA), G*lycine max* (SBA), *Canavulia ensiformis* (ConA), *Limulus polyphemus* (LPL) and *Phaseolus vulgaris* (PHA-M)

Determination of ∝-galactosidase activity

The galactose specific lectins have been reported to exhibit ∝-galactosidase activity. Hence the ∝-galactosidase activity was also followed during purification. The ∝-galactosidase activity of PPO-haemagglutinin was determined using PNPA as the substrate as explained in methods. The ∝-galactosidase activity of the purified PPO-haemagglutinin was 0.93 U/mg. The pH optima was found to be 5.5.

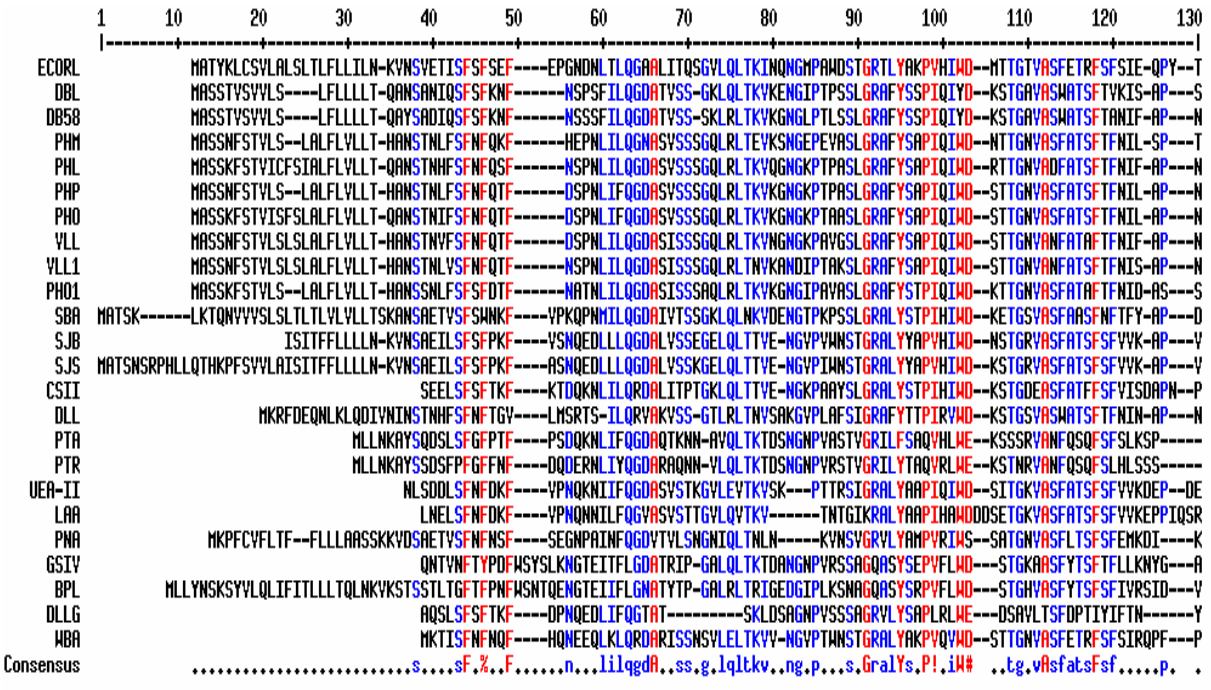
Multiple sequence alignment

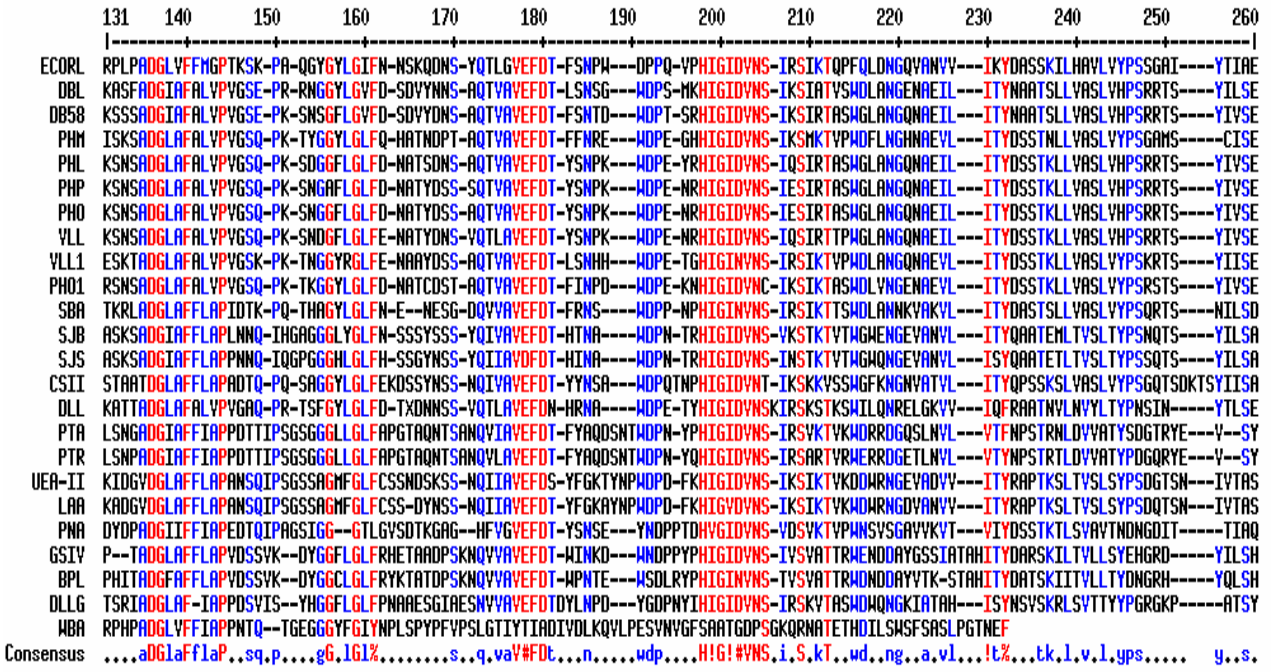
A comparison of the deduced PPO-haemagglutinin protein sequence with the sequences of other legume lectins retrieved from the Swiss-Prot protein sequence data bank and protein sequences derived from legume cDNA sequences in the Genebank was made using MULTALIN. The results show that PPO-haemagglutinin shows maximum homology to the structural group of tetrameric galactose specific lectins. The alignment of PPO-haemagglutinin with legume lectins shows a high degree of homology to DBL (Table 6.7, Figure 6.15). The deduced sequence shows 55 % indentity with the uncharacterized lectins from Phaseolus species *leptostachyus*, *P.oligosperm, P. microcarpus, P. maculates*, *P. filiformis* and *P. parvulus,* and 50, 51, 54, 49, 51, 40, 54 and 58 % identity with *Sophora japonica* seed and bark lectin (Van Demme, et al., 1997), DBL and DB58 (Schnell and Etzler, 1987, 1988), *Vicia faba* lectin (Hemperley, et al., 1979), soybean agglutinin (Vodkin, et al., 1983), Con A (Carrington, et al., 1985), *Erythrina corollodendron* (EcorL lectin; Arango, et al., 1990), phytohaemagglutinin (Voelker, et al., 1986) and *Lens culinaris* lectin (Foriers, et al., 1981)

respectively. The residues that form the double metal binding site lectins corresponding to Glu^{8}, Asp^{10}, Tyr^{12}, Asn^{14}, Asp^{19}, His^{24}, in ConA is conserved in this PPO-haemagglutinin corresponding to Glu^{143}, Asp^{145}, Asn^{150}, Asp^{153}, and His^{158}. The residue of EcorL implicated in Ca^{2+} binding that are essential for sugar binding viz Ala^{88}, Asp^{89}, Gly^{107}, Phe^{131} and Asn^{133} from its metal binding loop and Val^{106}, Asn^{107} and Ala^{218}, from the sugar interaction site are also conserved in the PPO-haemagglutinin. Ala^{104}, Asp^{105}, Gly^{124} and Asn^{149} are conserved and conserved in place of Phe^{131} is a His^{147}. The consensus sequence LQRD for targeting proteins to the vacuole is also conserved (Leu^{18}-Arg^{20}). The theoretical calculations indicate a pI of 9.45. This is good correlation with the experimentally determined pI of 9.35 for PPO (Chapter III). The PPO-haemagglutinin has two glycosylation sites Asn^{18} and Asn^{53}, which are close to the amino-terminus. The theoretical calculations predict a predominantly β-structure characteristic of lectins (Figure 6.16).

Table 6.7 Comparison of deduced amino-acid sequence with galactose specific lectin.

Species	**Identity (%)**	**Reference**
Phaseolus leptostachyus,	55	Accession No CAH60215
Phaseolus oligosperm	55	Accession No CAH60173
Phaseolus maculates	55	Accession No CAH60256
Phaseolus parvulus	55	Accession No CAH602151
Sophora japonica	50	Van Demme, et al., 1997
Dolichos biflorus (DBL and DB58)	51	Schnell and Etzler, 1987, 1988
Vicia faba	54	Hemperley, et al.,1979
SBA (soybean agglutinin)	49	Vodkin, et al., 1983
ConA	51	Carrington, et al.,1985
Erythrina corollodendron (EcorL)	40	Arango, et al.,1990
Phytohaemagglutinin	54	Voelker, et al., 1986


1 10 20 30 40 50 60 70 80 90 100 110 120 130
ECORL MATYKLCSVLALSLTLFLLILN-KVNSVETISFSFSEF----EPGNDNLTLQGAALITQSGVLQLTKINQNGMPAWDSTGRTLYAKPVHIWD--MTTGTVASFETRFSFSIE-QPY--T
DBL MASSTVSVVLS----LFLLLLT-QANSANIQSFSFKNF------NSPSFILQGDATVSS-GKLQLTKVKENGIPTPSSLGRAFYSSPIQIYD--KSTGAVASWATSFTVKIS-AP---S
DB58 MASSTVSVVLS----LFLLLLT-QAYSADIQSFSFKNF------NSSSFILQGDATVSS-SKLRLTKVKGNGLPTLSSLGRAFYSSPIQIYD--KSTGAVASWATSFTANIF-AP---N
PHM MASSNFSTVLS--LALFLVLLT-HANSTNLFSFNFQKF------HEPNLILQGNASVSSSGQLRLTEVKSNGEPEVASLGRAFYSAPIQIWD--NTTGNVASFATSFTFNIL-SP---T
PHL MASSKFSTVICFSIALFLVLLT-QANSTNHFSFNFQSF------NSPNLILQGDASVSSSGQLRLTKVQGNGKPTPASLGRAFYSAPIQIWD--RTTGNVADFATSFTFNIF-AP---N
PHP MASSNFSTVLS--LALFLVLLT-HANSTNLFSFNFQTF------DSPNLIFQGDASVSSSGQLRLTKVKGNGKPTPASLGRAFYSAPIQIWD--STTGNVASFATSFTFNIL-AP---N
PHO MASSKFSTVISFSLALFLVLLT-QANSTNIFSFNFQTF------DSPNLIFQGDASVSSSGQLRLTKVKGNGKPTAASLGRAFYSAPIQIWD--STTGNVASFATSFTFNIL-AP---N
VLL MASSNFSTVLSLSLALFLVLLT-HANSTNVFSFNFQTF------DSPNLILQGDASISSSGQLRLTKVNGNGKPAVGSLGRAFYSAPIQIWD--STTGNVANFATAFTFNIF-AP---N
VLL1 MASSNFSTVLSLSLALFLVLLT-HANSTNLVSFNFQTF------NSPNLILQGDASISSSGQLRLTNVKANDIPTAKSLGRAFYSAPIQIWD--STTGNVANFATSFTFNIS-AP---N
PHO1 MASSKFSTVLS--LALFLVLLT-HANSSNLFSFSFDTF------NATNLILQGDASISSSAQLRLTKVKGNGIPAYASLGRAFYSTPIQIWD--KTTGNVASFATAFTFNID-AS---S
SBA MATSK------LKTQNVYVSLSLTLTLVLVLLTSKANSAETVSFSWNKF----VPKQPNMILQGDAIVTSSGKLQLNKVDENGTPKPSSLGRALYSTPIHIWD--KETGSVASFAASFNFTFY-AP---D
SJB ISITFFLLLLN-KVNSAEILSFSFPKF----VSNQEDLLLQGDALVSSEGELQLTTVE-NGVPVWNSTGRALYYAPVHIWD--NSTGRVASFATSFSFVVK-AP---V
SJS MATSNSRPHLLQTHKPFSVVLAISITFFLLLLN-KVNSAEILSFSFPKF----ASNQEDLLLQGDALVSSKGELQLTTVE-NGVPIWNSTGRALYYAPVHIWD--KSTGRVASFATSFSFVVK-AP---V
CSII SEELSFSFTKF----KTDQKNLILQRDALITPTGKLQLTTVE-NGKPAAYSLGRALYSTPIHIWD--KSTGDEASFATFFSFVISDAPN--P
DLL MKRFDEQNLKLQDIVNINSTNHFSFNFTGV----LMSRTS-ILQRVAKVSS-GTLRLTNVSAKGVPLAFSIGRAFYTTPIRVWD--KSTGSVASWATSFTFNIN-AP---N
PTA MLLNKAYSQDSLSFGFPTF----PSDQKNLIFQGDAQTKNN-AVQLTKTDSNGNPVASTVGRILFSAQVHLWE--KSSSRVANFQSQFSFSLKSP-----
PTR MLLNKAYSSDSFPFGFFNF----DQDERNLIYQGDARAQNN-VLQLTKTDSNGNPVRSTVGRILYTAQVRLWE--KSTNRVANFQSQFSLHLSSS-----
UEA-II NLSDDLSFNFDKF----VPNQKNIIFQGDASVSTKGVLEVTKVSK---PTTRSIGRALYAAPIQIWD--SITGKVASFATSFSFVVKDEP--DE
LAA LNELSFNFDKF----VPNQNNILFQGVASVSTTGVLQVTKV------TNTGIKRALYAAPIHAWDDDSETGKVASFATSFSFVVKEPPIQSR
PNA MKPFCVFLTF--FLLLAASSKKVDSAETVSFNFNSF----SEGNPAINFQGDVTVLSNGNIQLTNLN-----KVNSVGRVLYAMPVRIWS--SATGNVASFLTSFSFEMKDI----K
GSIV QNTVNFTYPDFWSYSLKNGTEITFLGDATRIP-GALQLTKTDANGNPVRSSAGQASYSEPVFLWD---STGKAASFYTSFTFLLKNYG---A
BPL MLLYNSKSYVLQLIFITLLLTQLNKVKSTSSTLTGFTFPNFWSNTQENGTEIIFLGNATYTP-GALRLTRIGEDGIPLKSNAGQASYSRPVFLWD---STGHVASFYTSFSFIVRSID---V
DLLG AQSLSFSFTKF----DPNQEDLIFQGTAT---------SKLDSAGNPVSSSAGRVLYSAPLRLWE---DSAVLTSFDPTIYIFTN------Y
WBA MKTISFNFNQF----HQNEEQLKLQRDARISSNSVLELTKVV-NGVPTWNSTGRALYAKPVQVWD--STTGNVASFETRFSFSIRQPF---P
Consensus .....s....sF.%..F......n...lilqgdA..ss.g.lqltkv..ng.p...s.GralYs.P!.iW# .,tg.vAsfatsFsf.....p. .


131 140 150 160 170 180 190 200 210 220 230 240 250 260
ECORL RPLPADGLVFFMGPTKSK-PA-QGYGYLGIFN-NSKQDNS-YQTLGVEFDT-FSNPW---DPPQ-VPHIGIDVNS-IRSIKTQPFQLDNGQVANVV---IKYDASSKILHAVLVYPSSGAI----YTIAE
DBL KASFADGIAFALVPVGSE-PR-RNGGYLGVFD-SDVYNNS-AQTVAVEFDT-LSNSG---WDPS-MKHIGIDVNS-IKSIATVSWDLANGENAEIL---ITYNAATSLLVASLVHPSRRTS----YILSE
DB58 KSSSADGIAFALVPVGSE-PK-SNSGFLGVFD-SDVYDNS-AQTVAVEFDT-FSNTD---WDPT-SRHIGIDVNS-IKSIRTASWGLANGQNAEIL---ITYNAATSLLVASLVHPSRRTS----YIVSE
PHM ISKSADGLAFALVPVGSQ-PK-TYGGYLGLFQ-HATNDPT-AQTVAVEFDT-FFNRE---WDPE-GHHIGIDVNS-IKSMKTVPWDFLNGHNAEVL---ITYDSSTNLLVASLVYPSGAMS-----CISE
PHL KSNSADGLAFALVPVGSQ-PK-SDGGFLGLFD-NATSDNS-AQTVAVEFDT-YSNPK---WDPE-YRHIGIDVNS-IQSIRTASWGLANGQNAEIL---ITYDSSTKLLVASLVHPSRRTS----YIVSE
PHP KSNSADGLAFALVPVGSQ-PK-SNGAFLGLFD-NATYDSS-SQTVAVEFDT-YSNPK---WDPE-NRHIGIDVNS-IESIRTASWGLANGQNAEIL---ITYDSSTKLLVASLVHPSRRTS----YIVSE
PHO KSNSADGLAFALVPVGSQ-PK-SNGGFLGLFD-NATYDSS-AQTVAVEFDT-YSNPK---WDPE-NRHIGIDVNS-IESIRTASWGLANGQNAEIL---ITYDSSTKLLVASLVHPSRRTS----YIVSE
VLL KSNSADGLAFALVPVGSQ-PK-SNDGFLGLFE-NATYDNS-VQTLAVEFDT-YSNPK---WDPE-NRHIGIDVNS-IQSIRTTPWGLANGQNAEIL---ITYDSSTKLLVASLVHPSRRTS----YIVSE
VLL1 ESKTADGLAFALVPVGSK-PK-TNGGYRGLFE-NAAYDSS-AQTVAVEFDT-LSNHH---WDPE-TGHIGINVNS-IRSIKTVPWDLANGQNAEVL---ITYDSSTKLLVASLVYPSKRTS----YIISE
PHO1 RSNSADGLAFALVPVGSQ-PK-TKGGYLGLFD-NATCDST-AQTVAVEFDT-FINPD---WDPE-KNHIGIDVNC-IKSIKTASWDLVNGENAEVL---ITYDSSTKLLVASLVYPSRSTS----YIVSE
SBA TKRLADGLAFFLAPIDTK-PQ-THAGYLGLFN-E--NESG-DQVVAVEFDT-FRNS----WDPP-NPHIGINVNS-IRSIKTTSWDLANNKVAKVL---ITYDASTSLLVASLVYPSQRTS----NILSD
SJB ASKSADGIAFFLAPLNNQ-IHGAGGGLYGLFN-SSSYSSS-YQIVAVEFDT-HTNA----WDPN-TRHIGIDVNS-VKSTKTVTWGWENGEVANVL---ITYQAATEMLTVSLTYPSNQTS----YILSA
SJS ASKSADGIAFFLAPPNNQ-IQGPGGGHLGLFH-SSGYNSS-YQIIAVDFDT-HINA----WDPN-TRHIGIDVNS-INSTKTVTWGWQNGEVANVL---ISYQAATETLTVSLTYPSSQTS----YILSA
CSII STAATDGLAFFLAPADTQ-PQ-SAGGYLGLFEKDSSYNSS-NQIVAVEFDT-YYNSA---WDPQTNPHIGIDVNT-IKSKKVSSWGFKNGNVATVL---ITYQPSSKSLVASLVYPSGQTSDKTSYIISA
DLL KATTADGLAFALVPVGAQ-PR-TSFGYLGLFD-TXDNNSS-VQTLAVEFDN-HRNA----WDPE-TYHIGIDVNSKIRSKSTKSWILQNRELGKVV---IQFRAATNVLNVYLTYPNSIN-----YTLSE
PTA LSNGADGIAFFIAPPDTTIPSGSGGGLLGLFAPGTAQNTSANQVIAVEFDT-FYAQDSNTWDPN-YPHIGIDVNS-IRSVKTVKWDRRDGQSLNYL---VTFNPSTRNLDVYATYSDGTRYE---V--SY
PTR LSNPADGIAFFIAPPDTTIPSGSGGGLLGLFAPGTAQNTSANQVLAVEFDT-FYAQDSNTWDPN-YQHIGIDVNS-IRSARTVRWERRDGETLNYL---VTYNPSTRTLDVYATYPDGQRYE---V--SY
UEA-II KIDGYDGLAFFLAPANSQIPSGSSAGMFGLFCSSNDSKSS-NQIIAVEFDS-YFGKTYNPWDPD-FKHIGIDVNS-IKSIKTVKDDWRNGEVADVY---ITYRAPTKSLTVSLSYPSDGTSN---IVTAS
LAA KADGYDGLAFFLAPANSQIPSGSSAGMFGLFCSS-DYNSS-NQIIAVEFDT-YFGKAYNPWDPD-FKHIGYDVNS-IKSIKTVKWDWRNGDVANVV---ITYRAPTKSLTVSLSYPSDQTSN---IVTAS
PNA DYDPADGIIFFIAPEDTQIPAGSIGG--GTLGVSDTKGAG--HFVGVEFDT-YSNSE---YWDPPTDHVGIDVNS-VDSVKTVPWNSVSGAVVKVT---VIYDSSTKTLSVAVTNDNGDIT-----TIAQ
GSIV P--TADGLAFFLAPVDSSVK--DYGGFLGLFRHETAADPSKNQVVAVEFDT-WINKD---WNDPPYPHIGIDVNS-IVSVATTRWENDDAYGSSIATAHITYDARSKILTVLLSYEHGRD-----YILSH
BPL PHITADGFAFFLAPVDSSVK--DYGGCLGLFRYKTATDPSKNQVVAVEFDT-WPNTE---WSDLRYPHIGINVNS-IVSVATTRWDNDDAYVTK-STAHITYDATSKIITVLLTYDNGRH-----YQLSH
DLLG TSRIADGLAF-IAPPDSVIS--YHGGFLGLFPNAAESGIAESNVVAVEFDTDYLNPD---YGDPNYIHIGIDVNS-IRSKVTASWDWQNGKIATAH---ISYNSVSKRLSVTTYYPGRGKP-----ATSY
WBA RPHPADGLVFFIAPPNTQ--TGEGGGYFGIYNPLSPYPFVPSLGTIYTIADIVDLKQVLPESVNVGFSAATGDPSGKQRNATETHDILSWSFSASLPGTNEF
Consensus ....aDGlaFflaP..sq.p....gG.lGl%.....s..q.vaY#FDt...n.....wdp....H!G!#VNS.i.S.kT..wd..ng..a.vl...!t%...tk.l.v.l.yps..... y..s.

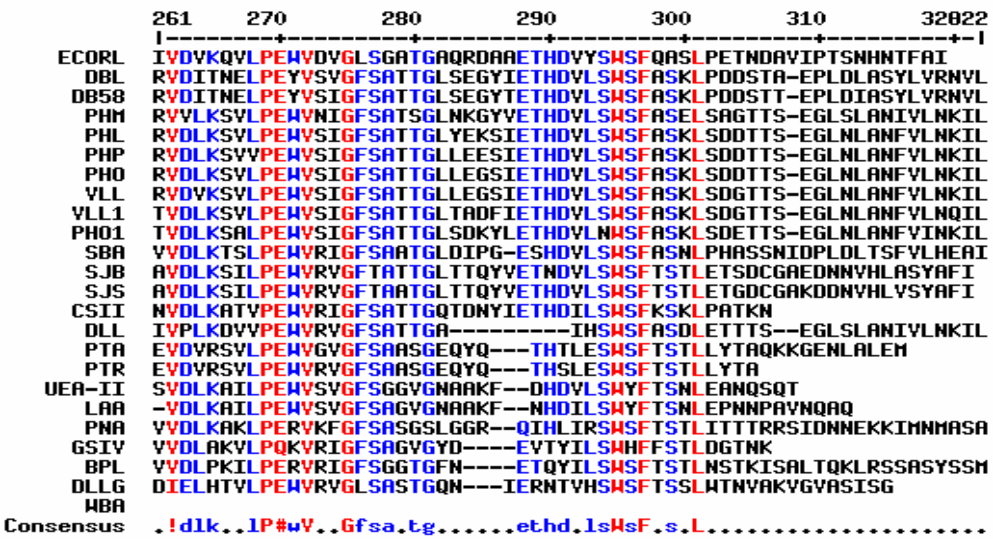


Figure 6.15. Multiple alignment of deduced amino acid sequence with ECORL: *Erythrina corollodendron* (coral tree, P16404); DBL: *Dolichos biflorus* (seed lectin, P05045); DB58: *Dolichos biflorus* (stem lectin, 19588); PHM: *Phaseolus maculates* (CAH 60256); PHL: *Phaseolus leptostachyus* (CAH 602151); PHP; *Phaseolus parvulus* (CAH 602151); PHO: *Phaseolus oligospermus* (CAH 60173.1); VLL: *Vigna linerais* (CAD 43280.1); VLL1: *Vigna linerais var lanearis* (CAD 43279.1); PHO1; *Phaseolus oligospermus* (CAH 60172.1); SBA: Soybean agglutinin (*Glycine max*, P05046); SJB: *Sophoro japonica* (bark lectin, P93538); SJS: *Sophoro japonica* (seed lectin, P93535); CSII: *Cytisus scoparius* (Scotch broom, P29257); DLL: *Dolichos lablab* (galactose specific lectin); PTA: *Pterocarpus rotundifolius* (AAT 57665.1); UEA-II: *Ulex europeus* (furze, P22973); LAA: *Laburnum alpinum* (Scotch laburnum, P23558); PNA: Peanut agglutinin (*Arachis hypogea,* P02872); GSIV: *Griffonia simplicifolia* (P24146); BPL: *Bauhinia purpurea* (Camel's foot tree, P16030); DLLG: *Dolichos lablab* glc/man specific lectin, A45891); WBA: *Psophocarpus tetragonolobus* (Winged bean, U60765).

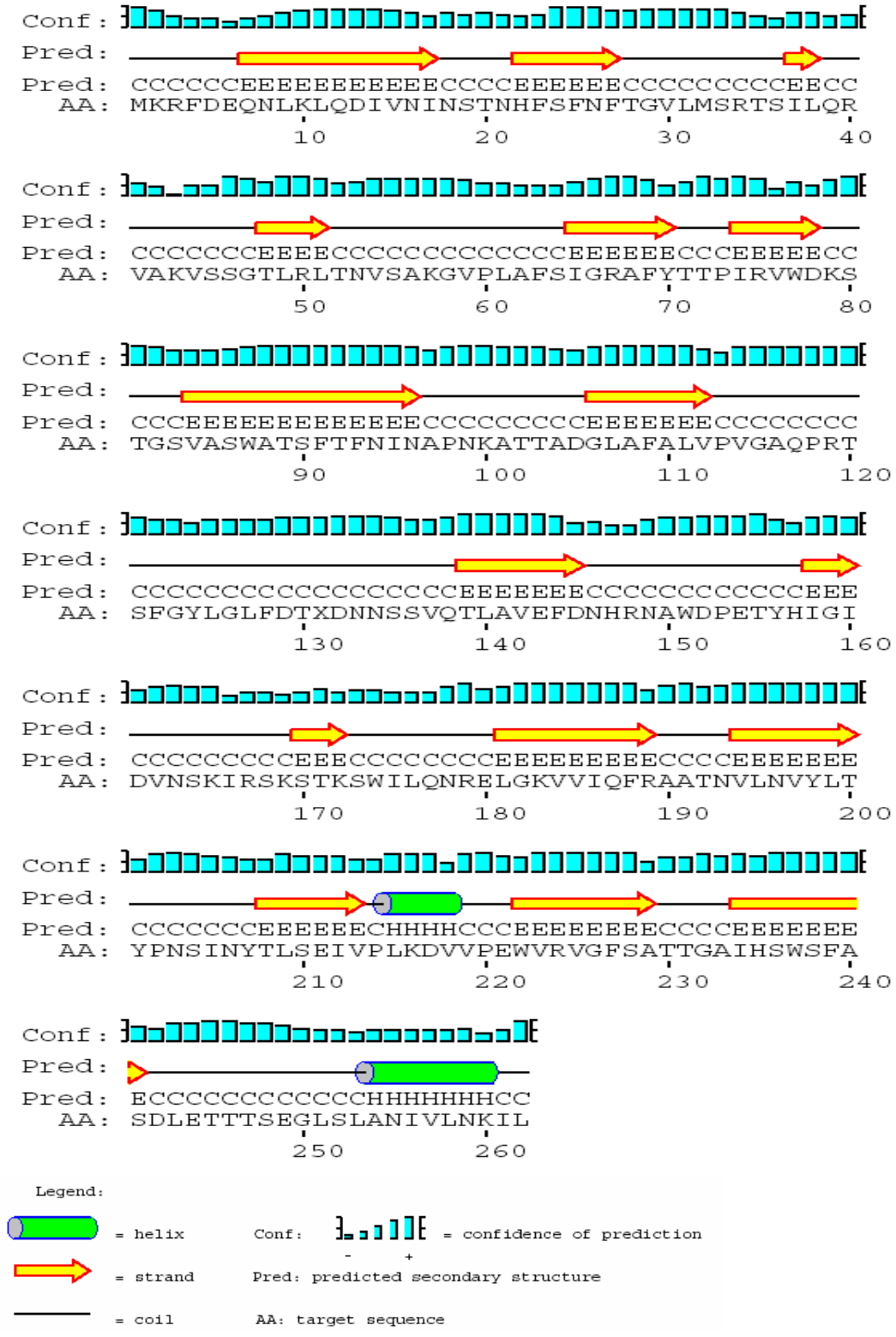


Figure 6.16. Secondary structure prediction of PPO-haemagglutinin. The deduced amino-acid sequence was used to predict the secondary structure. (www.expasy.org, server http://bioinfz.cs.ucl.ac.uk)

Homology modeling of PPO-haemagglutinin

The entire computational analysis was performed on a Pentium 4, 3.20 GHz processor operating on a Windows XP professional. The amino acid sequence used was the deduced sequence of 262 residues (Figure 6.17). The finest accessible template structure to carry out homology modeling of lectin was sought by a phylogenetic analysis of galactose specific lectins of Leguminosae. The PPO-haemagglutinin sequence was optimally aligned with galactose specific lectins using a preliminary conventional pair wise sequence alignment tool employing EBLOSUM 62 (Smith and Waterman, 1981). The resulting file was uploaded to the SWISS-MODEL automated homology model-building server for model computation. All tertiary structural analysis, including viewing of 3-D structures, superimpositions and residue distance determinations were performed using the Swiss-PDB viewer program (SPdbV version 3.7) of EXPASY (http:/www.expasy.ch). The ensuing structure was energy minimized using Deep View and the process was repeated. The homology model shows mainly beta strands (Figure 6.17) and is concurrent with that theoretically predicted. The model superimposed perfectly on the monomer of DBL from *Dolichos biflorus*.

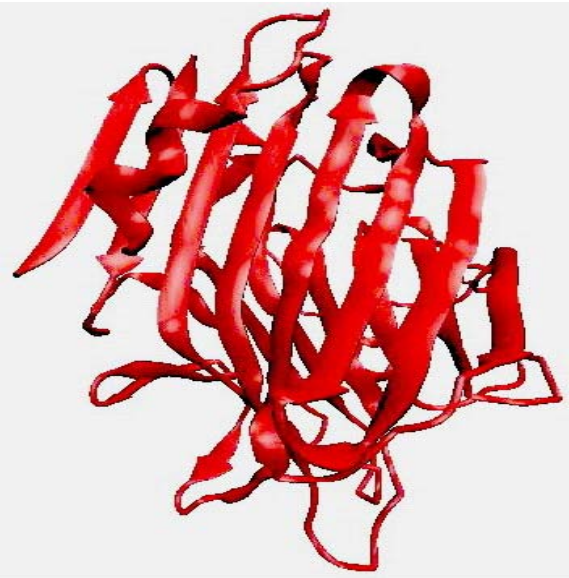


Figure 6.17. Homology model of the PPO-haemagglutinin. The DBL crystal structure was used as template , PDB ID 1LUL.

Discussion

Although the first PPO, a mushroom tyrosinase was discovered a century and half ago, only one three-dimensional structure of a plant PPO is known. PPO has been isolated and purified from a variety of plant sources, but pigment contamination and the occurrence of multiple forms have frequently hampered its characterization. During the structural characterization of a glucose mannose specific lectin from field bean (*Dolichos lablab*), seeds, Gowda et al., (1994) observed severe browning of the crude extracts. The presence of a single PPO form by native PAGE followed by activity staining was observed in the extracts. The absence of multiplicity of PPO in field bean seeds rendered it ideal for structure-activity studies. Consequently the PPO was purified and characterized (Paul and Gowda, 2000). To explain the structure-activity relationships and identify the active site residues, cloning and over expressing the gene for this PPO was undertaken. Using combinations of degenerate primers designed based on the amino-terminal sequence of the first twenty residues of PPO (Chapter III) and the highly conserved Cu^{2+} binding regions of PPO, which have been used by others to obtain the cDNA sequence, isolation of the PPO gene was attempted.

A RT-PCR approach was adopted to obtain the PPO gene sequence. The putative amplified RACE products (Figure 6.3) were cloned into the *PvuII* site of pRSET C (Figure 6.4) and the clones sequenced. None of sequence showed homology with any of the PPO sequence known. With all the optimization using different combinations of primers it was not possible to isolate the PPO gene using the determined amino-terminal sequence.

Simultaneously an attempt was made to obtain the partial protein sequence. The internal peptide sequences for peptides obtained were included. A BLAST search of these sequences indicated a high degree of homology to a group of D-galactose-specific legume lectins including the well studied DBL and DB58 of *Dolichos biflorus* (Schnell and Etzler, 1988). The maximum identity (93 %) was observed with a group of uncharacterized lectins such as *Phaseolus filiformus and Phaseolus parvulus* (Table 6.1).

Therefore primers were designed for the obtained internal sequence. A 490 bp 5' gene fragment was amplified using PPDF3, PPDF4 and RLECT primers (Figure 6.6A). The sequence homology showed that it resembled the not so well characterized lectins from *Phaseolus sps*. Based on the sequence of the amplified product, specific primers were designed and used in combination with the degenerate primers to obtain a full length fragment of 786 bp. The complete sequence of 786 bp has been deposited in the Gene Bank (Accession number EF 204527). This cDNA included an open reading frame of 262 amino acids with a predicted molecular weight of 28915 Da. This is in close agreement to that reported for a single subunit of PPO. The deduced sequence show significant identity with the lectins from *Phaseolus sps* and DBL and DB58 of *Dolichos biflorus* and EcorL of *Erythrina corollodendron.* The biochemical properties based on the derived sequence such as pI and amino acid composition were similar to experimental values. The peptide sequences obtained by Edman degradation are identical to the derived sequence (Figure 6.8). These results advocate that the field bean PPO could be a D-galactose binding lectin. This conclusion is further supported by a recent report on a D-galactose binding lectin from an Indian lablab bean, a different cultivar of *Dolichos lablab* (Vadaka, et al., 2006). The amino-terminal sequence of the first 10 residues and the subunit architecture are identical to that of the field bean PPO (Chapter III).

To positively conclude that the PPO is indeed a galactose specific lectin and vice versa the protein was purified by the conventional protein purification procedure described in chapter III and by D-galactose sepharose affinity chromatography and the two activities followed simultaneously.

At all the steps of purification both the PPO and haemagglutinating activities coeluted (Figure 6.9-6.11). Further, the gel activity staining band and SDS-PAGE show that the affinity purified protein is identical to the purified PPO (Figure 6.12). The biochemical characteristics of the protein purified by these independent methods are identical, which also show that these two proteins are the same. The observed ratio of PPO to HAU is almost constant between all the steps of purification (Table 6.2 and 6.3). A similar

increase in the specific activities of PPO and haemagglutination further proves that the activities are associated with a single species.

The two activities occur at two independent loci of the protein. This conclusion is supported by the fact that PPO activity is not affected in the presence of high concentrations of D-galactose and N-acetylgalactosamine (Table 6.6). Tropolone a reported potent inhibitor of PPO (Paul and Gowda, 2000) has no effect on agglutination. All plant PPOs contain copper at the active site required for the oxidation of diphenols (Mayer, 2006) and therefore are inhibited by copper chelators. No noticeable change in the haemagglutination activity is observed in the presence of the copper chelators tropolone, diethyldithiocarbamate, 2-mercaptobenzthiozole and 2-mercaptobenzimidazole. 2-Mercaptobenzthiozole at a concentration of 125 fold greater than the IC_{50} for PPO inhibition has no effect on haemagglutination (Table 6.6). EDTA a calcium chelator shows complete inhibition of haemagglutination but has no effect on PPO activity. These results indicate that the purified protein contains Ca^{2+} required only for erythrocyte binding and not for PPO activity. In addition metal analysis indicates the presence of Cu^{2+} and Mn^{2+}. All legume lectins posses two bound metal ions (one calcium ion and one transition metal ion mainly Mn^{2+}) in the vicinity of the sugar binding site. The presence of these two metal ions is vital for the sugar binding capabilities of lectins (Hameryck, et al., 1996). In addition to these two metal ions, PPO-haemagglutinin, also contains Cu^{2+} which although not essential for sugar binding, is essential to PPO activity like all other PPOs. Gilbride and Pistole, (1981) reported that a D-galactose binding lectin from *Limulus polyphemus* showing hemocyanin activity contains Cu^{2+}.

The PPO activity in the presence of bound erythrocytes (Figure 6.12) further supports the existence of two independent sites, one for PPO activity and the other for the sugar binding activity. Souza et al., (2005) in an erythrocyte overlay assay showed the absence of erythrocyte binding in the presence of D-galactose for *Synadenium carinatum* latex lectin. All these observation put together lead to the conclusion that the field bean (*Dolichos lablab*) seeds D-galactose specific lectin has a multifunctional role.

The PPO-haemagglutinin binds to D-galactose sepharose only in the presence of high concentration of salts (1.5 M $(NH_4)_2SO_4$). Unlike other sugar affinity purification methods, the bound PPO-haemagglutinin desorbed in the absence of galactose with buffer minus $(NH_4)_2SO_4$. The *Dolichos biflorus* seed lectin (DBL) is a strict N-acetylgalactosamine specific lectin (Etzler et al., 1981) in that agglutination is inhibited by N-acetylgalactosamine but not by galactose (Etzler and Kabat, 1970). Although grouped under galactose/ N-acetylgalactosamine lectins DBL does not bind to D-galactose sepharose. In contrast, the two other galactose specific lectins from stem (DB58) and leaf of *Dolichos biflorus* and soybean agglutinin bind to D-galactose sepharose. The low binding affinity of DBL is attributed to the substitution of a conserved aromatic residue (Phe^{131} in DB58 and Phe^{131} in SBA) by an aliphatic Leu^{127} (Hamelryck, et al., 1996). A mutant Leu^{127} Phe showed that 50 % inhibition of binding occurred at 28.4 mM galactose as against 120.2 mM for the native lectin. The conserved aromatic residues (Phe^{128} in SBA, Phe^{131} in EcorL, Tyr^{125} in PNA and Phe^{126} in WBAI) in the four Gal-binding lectins stack perfectly against the C-3, C-4, C-5 and C-6 patch of Gal (Weis and Drickamer, 1996). From the derived amino acid sequence of PPO-haemagglutinin this residue is His^{147}. His is neither an aromatic nor an aliphatic amino acid. However, it is believed that in the presence of high $(NH_4)_2SO_4$. concentration His behaves like an aromatic residue. This may explain why PPO-haemagglutinin binds to D-galactose sepharose only in the presence of $(NH_4)_2SO_4$ and can be desorbed easily in buffer minus salt. The presence of His in this position occurs in the bark and seed lectin of *Sophora japonica*. However, the purification has been carried out using N-acetylgalactosamine sepharose (Hankins et al., 1987) and therefore no comparisons can be made.

The PPO-haemagglutinin is a heterotetramer and consists of two subunits of 29000 and 31000 Da (Figure 6.13). The amino-terminal sequence of the first 20 residues are identical. The difference in Mr of the two subunits as determined by MALDI-TOF is equivalent to 11 residues. This is similar to that of the seed lectin DBL in which the two subunits differ from each other by 11 amino acid residues. The smaller subunit II (241 amino

acid residue) is posttranslationaly formed from subunit I 253 amino acid residues) by removal of 12 residues from its carboxyl terminus (Roberts and Goldstein, 1982; Young, et al., 1995). Therefore, it is not unreasonable to assume that in the case of PPO-haemagglutinin, such a carboxyl terminal truncation is responsible for the subunit architecture observed.

The D-galactose specific PPO-haemagglutinin is distinct from the glc/man specific lectin of the same seed and unrelated in structure, supporting the previous conclusions of Hankins et al., (1979) that at least two distinct nonhomologous classes of proteins with haemagglutinating activity exist in legume seeds. The subunit architecture, amino acid sequence and sugar inhibition show that the PPO-haemagglutinin differs from that of glucose/mannose specific lectin present in these seeds (Gowda et al., 1994).

As seen from the purification, an ∝-galactosidase activity coelutes with the PPO-haemagglutinin. Several legume lectins have been shown to exhibit ∝-galactosidase activity, which is responsible for the clot dissolution. The haemagglutinating and enzyme activities have been attributed to the same catalytic site. The purified PPO-haemagglutinin is a multifunctional protein having PPO activity, galactose binding specificity and ∝-galactosidase activity.

Both in mung beans and soybean seeds it has been conclusively demonstrated that the α-galactosidase and haemagglutinin activities are associated with a single species (Campillo et al., 1981; Campillo and Shannon, 1982). The soybean α-galactosidase-haemagglutinin can be reversibly converted by pH changes from its tetrameric form, which shows enzymic and lectin activity to the monomeric form, which displays only enzymic activity. In its monomeric form, it is enzymatically active and displays a different pH optima and carbohydrate specificity. The PPO-haemagglutinin also undergoes conformation changes when exposed to acid pH with no change in the quaternary structure. However, a large change in the hydrodynamic radius with a change in PPO pH optima was observed (Kanade et al., 2006). PPO-haemagglutinin associated with exceptionally

high levels of PPO activity. Changes in the agglutinating power could not be studied with activated forms of PPO-haemagglutinin as the RBCs agglutinate in the presence of SDS alone. Comparison of the activities of PPO from other species is not possible due to the diverse nature of substrate used to assay the enzyme. The inhibition data (Table 6.6) indicates that the sugar binding and the PPO activities exist on different loci. The well-characterized galactose specific lectin DBL of horsegram seeds has recently been shown to exhibit intrinsic lipoxygenase activity far higher than any of the known sources (Roopashree et al., 2006).

A similar quaternary structure described for other galactose/ N-acetylgalactosamine lectins; PHAl (Hamelryck, et al., 1996), SBA (Dessen, et al., 1995) *Vicia villosa* isolectin (Osinaga, et al., 1997). The SBA tetramer consists of carboxy-terminally truncated subunit and intact subunits (Mandal, et al., 1994). The antibodies raised against field bean PPO (Paul, 2000) show a very strong cross reactivity toward the PPO-haemagglutinin purified by affinity chromatography and the galactose specific lectins (SBA, DBL, PHA-M; Figure 6.14). The homology model (Figure 6.17) is very similar to the monomeric structures of DBL, SBA, PHA-L and EcorL. The multiple sequence alignment shows maximum homology to the tetrameric galactose/ N-acetylgalactosamine lectins (Figure 6.15). In the absence of a crystal it is tempting to speculate that the three-dimensional structure of the PPO-haemagglutinin would be similar to that of other galactose/ N-acetylgalactosamine lectins. The architecture of the PPO-haemagglutinin monomer consisting of is similar to that pointed out by Banerjee and coworkers (Banerjee, et al., 1996). The main hydrophobic core is located between the back and front. These studies are in progress in this laboratory.

Observing that the galactose specific lectins of *Vigna radiata*, soybean, *Phaseuolus vulgaris* and mung bean exhibit an associated enzymic function, Hankins et. al., (1979) opined that it was not unreasonable to assume that most if not all galactose specific legume lectins have enzymatic function. Arcelin (Romero Andreas, et al., 1986; Hartweck, et al., 1991; Goossens, et al., 1994) and an α-amylase inhibitor (Moreno and Chrispeels, 1989) are considered to be truncated forms of the lectin PHA in which loops that play a

sugar binding role are missing, abolishing the sugar binding properties. Both arcelin (Osborn, et al., 1988) and α-amylase inhibitor protect the bean seed against predation by pests. Both the field bean PPO-haemagglutinin and horsegram DBL-lipoxygenase are galactose specific lectins, which aptly justify the previous assumption. All these observations coupled lead to an important conclusion that a distinct class of bifunctional proteins termed "enzymic lectins" exist in legume seeds. All the reported enzyme activities associated with the lectin also play an important role in plant defense against predators as inferred from toxicity to insects, and inhibition of fungal pathogenesis. The physiological implications of the presence of two orders of plant defense exhibited by a single molecule need further investigation.

Chapter VII
Summary and conclusions

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. 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. The main enzyme involve in the enzymatic browning is polyphenol oxidase which catalyzes both the hydroxylation of monophenols to diphenols (monophenolase/cresolase) and also oxidation of *o*-diphenols to *o*-quinones (diphenolase/catecholase. 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.

In the present investigation entitled "**Molecular characterization and expression of an oxidase from field bean (*Dolichos lablab*)**" has been undertaken to understand the structure function relationship and kinetics of inactivation and activation and determination of functional determinants and sequence. The following are the salient features of the present investigation.

- The single isoform of field bean seed PPO was purified by buffer extraction, $(NH_4)_2SO_4$ fractionation, DEAE-sepharose anion exchange chromatography, phenyl sepharose chromatography and size exclusion chromatography.
- The PPO was purified with a yield of 20 %. The specific activity 35553 U/mg of the purified protein was 29 fold higher than that of the crude extract (Table 3.1).
- Homogeneity of PPO was analyzed on native PAGE, for both protein and activity staining (Figure 3.4).
- The Mr of PPO was determined by analytical gel filtration chromatography, SDS-PAGE and MALDI-TOF (Figure 3.5, 6 and 7).
- The pI found to be 9.35 (Figure 3.8) and sugar composition was 8 %.
- The PPO activity decreased dramatically to after 48 h of germination (Figure 3.14) and in flower and at all stages of seed

development only a single isoform of PPO was expressed (Figure 3.15).

- A series of phenolic compounds experimentally evaluated for their binding affinity and inhibition constants were computationally docked to the active site of catechol oxidase.
- Docking studies suggested two distinct modes of binding, dividing the docked ligands into two groups. Remarkably, the first group corresponds to ligands determined to be substrates and the second group corresponds to the reversible inhibitors.
- The phenyl ring of the substrate/inhibitor was stacked perfectly against His^{244} and was co-ordinated to both the Cu atoms. Higher reaction rates and binding are reckoned by additional interactions of the substrates with key residues that line the hydrophobic cavity. (Figure 4.7A).
- The docking results suggest that inhibition of oxidation stems from an interaction between the aromatic carboxylic acid group and the apical His^{109} one of the four co-ordinates of the trigonal pyramidal co-ordination polyhedron of CuA. (Figure 4.7B)
- The spatial orientation of the hydroxyl in relation to the carboxylic group either allows a perfect fit in the substrate cavity leading to inhibition or due to a steric clash flips the molecule vertically facilitating oxidation. (Figure 4.7D and G).
- This is the first study, which explains at the molecular level the determinants of substrate and inhibitor specificity of a catechol oxidase.
- The enzyme is activated many fold either in presence of the anionic detergent sodium dodecyl sulfate below its CMC or on exposure to acid-pH (Figure 5.1 and 5.2).
- The enhancement of k_{cat} upon activation is accompanied by a marked shift in the pH optimum for the oxidation of *t*-butyl catechol from 4.5 to 6.0, an increased sensitivity to tropolone. (Table 5.1, Figure 5.3).
- Chemical modification of field bean and sweet potato PPO with N- (3-dimethylaminopropyl-) N'-ethylcarbidiimide followed by kinetic analysis, leads to the conclusion that both the enzymes possess one carboxylate essential to activity (Figure 5.7).
- The Stokes radius of the native enzyme is found to increase from

49.1±2 Å to 75.9±0.6 Å. (Figure 5.8).

- The activation by SDS and acid-pH results in a localized conformational change that is anchored around the catalytic site of PPO (Figure 5.13, 5.14 and 5.15).
- The activation led altered susceptibility to proteolytic degradation and decreased thermostability (Figure 5.16 and 5.17).
- The structural change that accompanies the activation of PPO can play a vital role in the regulation of PPO mediated physiological defense against attack by insect or pests.
- cDNA was generated from 8 µg of field bean seed total RNA and cloned. Among the positive clones, none of the sequences obtained showed homology to any of the reported plant PPO sequences. Several other cloning strategies did not yield any positive clones.
- BLAST search of internal peptide sequence (Table 6.1) shows high degree of homology to a group of D-galactose-specific legume lectins.
- The gene sequence of 786 bp obtained was (Figure 6.7) submitted to Gene Bank (Accession Number EF204527).
- The purified field bean PPO showed haemagglutinating activity against human RBCs. The specific activity for agglutination was 1275 U/mg of protein.
- The crude extract exhibited a specific activity of 1275 U/mg for PPO and 944 HAU against human RBCs.
- The protein was purified by DEAE-sepharose anion exchange chromatography, size exclusion chromatography on a superdex-200 column (Amersham Biosciences) by FPLC and by galactose affinity chromatography (Table 6.2 and 3).
- The two activities co-purify through all the steps of purification. The specific activity of PPO 28294.5 U/mg obtained by galactose affinity chromatography is comparable to that of 32265.7 U/mg obtained by the earlier procedure. The ratio of PPO/HAU is near constant in all the steps of purification.
- Erythrocyte haemagglutination overlay assay revealed that the two activities are associated with a single protein and this protein is referred to as the PPO-haemagglutinin (Figure 6.4).
- The exact molecular weight of the two subunits as determined by

MALDI-TOF PPO-hemagglutinin was 29808.492 and 31087.042 Da and the subunits differing by a mass of 1279 Da, which arise from a deletion of 10 or 11 residues.

- The PPO-hemagglutinin showed strong haemagglutinating activity against native human blood groups (A, AB, B and O, Table 6.4).
- The D-galactose and its derivatives galactosamine, N-acetyl galactosamine and lactose were found to inhibit the agglutination of human erythrocytes. (Table 6.5).
- The catalytic activity (PPO) and the sugar binding activities (haemagglutination) were observed to be independent of each other (Table 6.6).
- Antibodies raised against field bean PPO were cross reacted with well-characterized galactose specific lectins (SBA, DBL PHA-M), revealed that these lectins have similar antigenic determinants and structural features (Figure 6.14).
- The deduced sequence shows 55 % identity with the uncharacterized lectins from *Phaseolus* species *leptostachyus*, *P.oligosperm, P. microcarpus, P. maculates*, *P. filiformis* and *P. parvulus*
- The homology model shows mainly beta strands (Figure 6.17). The model superimposed perfectly on the monomer of DBL from *Dolichos biflorus*.

The results in the thesis describe the purification of PPO from field bean (*Dolichos lablab*) and temporal expression of PPO during germination and seed development. The study also shows that the experimental and theoretical explanation of functional determinants of substrate/inhibitor of PPO. The local conformational change during activation which implicates critical to the in vivo physiological defense mechanism postulated for plant PPOs is presented. The multifunctional properties of PPO were a discussed.

REFERENCES

Akthar, S. M. and Bhakuni, V. (**2003**)**,** *Arch. Biochem. Biophys.*, 413, 221.

Albisu, I., King, R. D. and Kovlov, I. A., (**1989**), *J. Sci. Food Agric.*, 37, 775.

Angleton, E. L. and Flurkey, W. H. (**1984**) *Phytochem.*, 23, 2723.

Anosike, E. O. and Ayaebene, A. O. (**1982**), *Phytochem.*, 21,1889.
Anosike, E. O. and Ojimelukwe, R. C. (**1982**), *J. Exper. Bot.*, 33, 487.
Arango, R. Rozenblatt, S. and Sharon, N. (**1990**), *FEBS Letters*, 264, 109.

Asada, N., Fukumitsu, T., Fujimoto, K. and Masuda, K. I. (**1993**), *Insect. Biochem. Mol. Biol.*, 23, 515.

Ashie, I. N. A., Simpson, B. K. and Smith, J. P. (**1996**), *Crit. Rev. Food Sci. Nutri.*, 36, 1.

*Babbel, G. R. (**1974**), *Bot. Gaz.,*135, 297.

Bachem, C., Speckmann, G., Vanderline, P., Verheggen, F., Hunt, M., Steffens, J. and Zabeau, M. (**1994)**, *Biotechnology*, 12, 1101.

Banerjee, R., Das, K., Ravishankar, R., Suguna, K., Surolia, A. and Vijayan, M., (**1996**), *J. Mol. Biol.*, 259, 281.

Balzarini, J., Neyts, J., Schols, D., et al., (**1992**), *Antiviral Res.,* 18, 191.

Bar Nun, N. and Mayer, A. M. (**1999**), *Phytochem.*, 50, 710.

Barrientos, L. G. and Gronenborn, A. M. (**2005**), *Mini Rev. Med. Chem.* 5, 21.

Baruah, P. and Swain, T. (**1959**), *J. Sci. Food Agric.* 10, 125.

Battaini, G., Monzani, E., Casella, L., Lonardi, E., Tepper, A. W.J.W., Canters, Gerard W. and Bubacco, L. **(2002)** *J. Biol. Chem.,* 277, 44606.
Beloqui, A., Pita, M., Polaina, J., Martinez-Arias, A., Golyshina, O. V., Zumarraga, M., Yakimov, M. M., Garcia-Arellano, H., Alcade, M., Fernandez, V. M., et. al., (**2006**), *J. Biol. Chem.*, 281, 22933.

Benjamin, N. D. and Montgomery, M. W. (**1973**), *J. Food Sci.*, 38,799.

Ben-Shalom, N. Kahn, V. Harel, E. and Mayer, A. M. (**1977**), *Phytochem.* 28, 245.

Bertrand, G. (**1896**), *CRC. Acad. Sci. Paris.*, 122, 1215.

*Bora, P. S., Holschuh, H. J., da Silva vasconelos, M. A. (**2004**), *Ciene. Technol. Aliment.*, 4, 267.

Boss, P. K., Gardener, R. C., Janseen, B. J. and Ross, G. S. (**1994**), *Plant Mol. Biol.*, 27, 429.

Boss, P. K., Gardner, R. C., Jansse, B. J. and Ross, G. S. (**1995)**, *Plant Mol. Biol.*, 27, 429.

*Bourquelot, E., Bertrand, A. A. (**1895**), *R. Soc. Biol.*, 47, 582.

Bradford, M.M. (**1976**), *Anal. Biochem.*, 72, 248.

Briganti, S., Camera, E. and Picardo, M. (**2003**), *Pigment Cell Res.*, 2, 101.

Bull, A. T. and Carter, B. C. (**1973**), *A. J. Gen. Microbiol.*, 75, 61.

*Burdoch, G. A. (**1995**), Fenaroli's Handbook of Flavor Ingradients, CRC Press: Boca Raton.

Burton, K. S., Love, M. E. and Smith, J. F. (**1993**), *Enzyme and Microbial Technol.*, 15, 736.

Cambie, R. C. and Bocks, S. M. (**1966**), *Phytochem.*, 5, 391.

Campillo, E. D. and Shannon, L. M. (**1982)**, *Plant Physiol.*, 69, 628.
Campillo, E. D., Shanon, L. M. and Hankins, C. N. (**1981**), *J. Biol. Chem.*, 256, 7177.

Carrington, D. M., Auffret, A., et al., (**1972**), *Proc. Natl. Acad. Sci.* USA, 69, 2580.

Cary, J. W., Lax, A. R. and Flurkey, W. H., (**1992**), *Plant. Mol. Biol.*, 20, 245.

Casanola-Martin, G. M., et al., (**2006**), *Bioorg. Med. Chem. Letters*, 16, 324.

Cash, J. N., Sistrunk, W. A. and Strette, C. A. (**1976**), *J. Food Sci.*, 41, 1398.

Challice, J. S. and Williams, A. H. (**1970**), *Phytochem.*, 9, 1261.

Chazarra, S., Cabanes, J., Escribano, J. and Garcia-Canovas, F. (**1996**), *J.Agric.Food Chem.,* 44, 984.

Chazzara, S., Cabanes, J., Escribano, J. and Garcia-Carmona, F. (**1997**), *Biochim. Biophys. Acta*, 1339, 297.

Cheng, T. M., Huang, P. C., Pan, J.P., Lin, K. Y. and Mao, S. J. T. (**2007**), *J. Chromatgraphy B*, 849, 331.

Chevalier, T., de Rigal, D., Mbeguie-A-Mbeguie-D, Gauillard, F., Richard Forget, F., and Fils-Lycaon, B. R. (**1999**), *Plant Physiol.*, 119, 1261.

Chilaka, F. C., Anosike, E. O. and Egubuna, P. C. (**1993**), *J. Sci. Food Agric.*, 61, 125.

Cho, M. H., Syed, G. A., Helms, G. L., Hishiyama, S., Eichinger, D., Davin, L. B. and Lewis, N. G. (**2003**), *Proc. Nat. Acad. Sci.,* USA. 100, 10641.

Chrispeels, M. J. and Raikhel, N. V. (**1991**), *The Plant Cell,* 3, 1.

Ciopraga, J., Gozia, O., Tudor, R., Brezuica, L., Doyle, R. J. **(1999),** *Biochim. Biophys. Acta.*, 1428, 424.

Cipollini D., Enright S., Traw M.B. and Bergelson, (**2004**), J. *Mol. Ecol.,* 13, 1643.

Coetzer, C., Corsini, D., Love, S., Pavek, J. and Tumer, N. (**2001**), *J.Agric. Food Chem.*, 49, 652.

Cosetang, M. Y. and Lee C. Y. (**1987**), *J. Food Sci.*, 52, 985.

*Craft, C. C. (**1966**), *Am. Pot. J.*, 43, 112.

Cuff, M. E., Miller, K. I., van Holde, K. E. and Hendrickson, W. A. (**1998)**, *J. Mol. Biol.*, 278, 855.

Czaininski, Y. and Catesson, (**1974)**, A. M. in *Electron microscopy of enzymes*, Hayat, M. A., Ed., Van-Nostrand Reinhold, New York, Vol.2, p66.

Das, J. R., Bhat, S. G. and Gowda, L. R. (**1997**), *J. Agric. Food Chem.*, 45, 2031.

Dawley, R. M. and Flurkey, W. H. (**1993**), *J. Food Sci.*, 58, 609.

Decker, H. and Tuczek, F. (**2000**), *Trends Biochem. Sci.*, 25, 392.

Dessen, A., Gupta, D., Sabesan, S., Brewer, C. F. and Sacchettini, J. C. (**1995**), *Biochemistry*, 34, 4933.

Dicko, M. H., Hilhorst, R., Gruppen, H., Laane, C., van Berkel, H. J. H. and Voragen, A. G. J. **(2002)** *Anal. Biochem.,* 306**,** 336.

Ding, C. K., Chachin, K., Ueda, Y. and Imahori, Y. (**1998**), *J. Agric. Food Chem.*, 46, 4144.

Dixon, M. (**1942**), *Biochem. J.*, 146, 85.

Doan, A., Ervin, G. and Felton, G. (**2004**), *Biochem. System. Ecol.*, 32, 117

Dogan, S., Arslan, O. and Ozen, F. (**2005a**), *Food Chem.*, 91, 341.

Dogan, S., Turan, Y., Erturk, H. and Arslan, O. (**2005b**), *J. Agric. Food Chem.*, 53, 776.

Dry, I. B. and Robinson, S. P. (**1994**), *Plant Mol. Biol.*, 26, 495.

DuBios, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (**1956**), *Anal. Chem.*, 28, 350.

Duckworth, H. W. and Coleman, J. E. (**1970**), *J. Biol. Chem.*, 245, 1613.

Eicken, C., Zippel, F., Buldt-Karentzopoulos, K. and Krebs, B. (**1998**), *FEBS Lett.*, 436, 293.

Erat, M., Sakiroglu, H. and Kufrevioglu, O. I. (**2006**), *Food Chem.*, 95, 503.

Escribano, J., Cabanes, J. and Garcia-Carmona, F. (**1997**), *J. Sci. Food Agric.*, 73, 34.

Escribano, J., Gandid-Herrero, F., Caballero, N. and Pedreno, M. A. **(2002)** *J.Agric.Food Chem.,* 50, 6123.

Espin, J. C. and Wichers, H. J. (**1999a**), *J. Agric. Food Chem.*, 47, 3518.

Espin, J. C. and Wichers, H. J. (**1999b**), *J. Agric. Food Chem.*, 47, 3503.

Espin, J. C., Garcia-Ruiz, P. A., Tudela, J. and Garcia-Canovas, F. (**1998b**), *Biochem. J.*, 331, 547.

Espin, J. C., Garcia-Ruiz, P. A., Tudela, J., and Garcia-Canovas, F. (**1998a**), *J. Agric. Food Chem.*, 46, 2469.

Espin, J. C., Leeuwen, J. and Wichers, H. J. (**1999**), 47, 3509.

Espin, J. C., Morales, M., Garcia-Ruiz, P. A., Tudela, J. and Garcia-Canovas, F. (**1997a**), *J. Agric. Food Chem.*, 45, 1084.

Espin, J. C., Morales, M., Varon, R., Tudela, J. and Garcia-Canovas, F. (**1995a**), *Anal. Biochem.*, 231, 237.

Espin, J. C., Morales, M., Varon, R., Tudela, J. and Garcia-Canovas, F. (**1995b**), *J. Agric. Food Chem.*, 43, 2807.

Espin, J. C., Trujano, M. F., Tudela, J. and Garcia-Canovas, F. (**1997b**), *Phytochem.*, 45, 667.

Espin, J. C., Varon, R., Fenoll, L. G., Gilabert, M. A., Garcia-Ruiz, P. A., Tudela, J. and Garcia-Canovas, F. (**2000**), *Eur. J. Biochem.*, 267, 1270.

Etzler, M. E. and Kabat, E. A. (**1970**), *Biochemistry*, 9, 869.
Etzler, M. E., et al., (**1981**), *J. Biol. Chem.*, 256, 2367.

Fenoll, et al., (2001), *Biochim. Biophys. Acta.*, 1548,1.

Ferrer, P. H. and Walker, J. H. L. (**1996**), *J. Food Biochem.*, 20, *15.*

Flurkey, W. H. (**1986**), *Plant Physiol.*, 81, 614.

Flurkey, W. H. (**1989**), *Plant Physiol.*, 91, 481.

Foriers, A., Lebrum, E., Van Rapenbusch, R., De Neve, R. and Strosberg, A. D. (**1981**), *J. Biol. Chem.*, 256, 5550.

Foriers, A., Wuilmart, C., Sharon, N. and Strosberg, A. D. (**1977**), *Biochem. Biophys. Res. Commun*, 75, 980.

Fraignier, M. P., Marques, L., Fleuriet, A. and Macheix, J. J. (**1995a**), *J. Agric. Food Chem.*, 39, 33.

Fraignier, M. P., Marques, L., Fleuriet, A. and Macheix, J. J.(**1995b**), *J. Agric. Food Chem.*, 43, 2325.

Frankos, V. H., et al., (**1991**), *Regul. Toxicol. Pharmacol.*, 14, 202.

Frieden, E., Osaki, S. and Kobayashi, H. (**1965**), *J. Gen. Physiol.*, 49, 213.

Friedman, M. and Bautisata, F. F. (**1995**), *J. Agric. Food Chem.*, 43, 69.

Froderman, S. R. and Flurkey, W. H. (**1997**), *Phytochem.*, 45, 15.

Fujita, S. and Tono, T. (**1988**), *J. Sci. Food Agric.*, 46, 115.

*Fujita, S. and Tono, T. (**1981**), *Nippon Shokuhin Kogyo Gakkaishi*, 28, 600.

Fujita, S., bin Saari, N., Maegawa, M., Tetsuka, T., Hayashi, N., and Tono, T. (**1995**), *J. Agric. Food Chem.*, 43, 1138.

Fujita, S., Tono, T. and Kawahara, H. (**1991**), *J. Sci. Food Agric.*, 55, 643.

Galeazzi, M. A. M. and Sgarbieri, V. C. (**1981**), *J. Food Sci.*, 1404.

Galeazzi, M. A. M. and Sgarbieri, V. C., (**1993**), *J. Food Sci.*, 609.

Gandia-Herrero, F., Escribano, J. and Garcia-Carmona, F. (2005), *Planta*, 222, 307

Gandia-Herrero, F., Jimenez, M. and Cabanes, J. (**2003**), *J. Agric. Food Chem.* 51, 7764.

Gandia-Herrero, F., Jimenez-Atienzar, M., Cabenae, J., Garcia-Carmona, F. and Escribano, J. (**2005b**), *J. Agric. Food Chem.*, 53, 6825.

Gandia-Herrero, F., Jimenez-Atienzar, M., Garcia-Carmona, F. and Escribano, J. (2005a), *Biol. Chem.*, 386, 601.

Garcia-Borron, J. C. and Solano, F. (**2002**), *Pigment Cell Res.*, 15, 162.

Gatehouse, A.M., Powell, K.S., Peumans, W.J., Van Damme, E.J. and Gatehouse, J.A. (**1995**). Insecticidal properties of plant lectins:their potential in plant protection. In: Pusztai, A., Bardocz, S. (Eds.), Lectins: Biomedical Perspectives, Taylor & Francis, London, pp. 35–58.

Gauillard, F. and Richard-Forget, F. (**1997**), *J. Sci. Food Agric.*, 74, 49.

Gaykema, W. P. J.; Hol, W. G. J.; Vereijke, J. M.; Soster, N. M.; Bak, H. J.; Baintema, J. J. (**1984)**, *Nature*, 309, 23.

Gerdemann, C., Eicken, C. and Krebs, B. (**2002**), *Acc. Chem. Res.*, 35, 183.

Gielens, C., Idakieva, K., Maeyer, M., Van den Bergh, Siddiqui, N. I. and Compernolle, F. (**2007**), *Peptides,* 28**,** 790.

Gilbride, K. J. and Pistole,T. G. (**1981**), *Develop. Compr. Immunol.*, 5, 347.

Golan-Gordhirsh, A., Osuga, D. T., Chen, A. O., (**1992)** in *The Bioorganic chemistry of enzymatic catalyses, a homage to Myron L. Bender,* Whitaker, J. R., D'souza, F. J., Eds., CRC Press, Boca Raton, p 61.

Golbeck, J. A. and Cammarata, K. V. (**1981**), *Plant Physiol.*, 67, 977.

Gonzalez, E. M., DeAncos, B. and Cano, M. P., (**1999**), *J. Agric. Food Chem.*, 47, 4068.

Goossens, A., Geremia, R., Bauw, G., Van Montagu, M and Angenon, G. (**1994**), *Eur. J. Biochem.*, 225, 787.

Gowda, L. R. and Paul, B. (**2002**), *J. Agric. Food Chem.*, 50, 1608.

Gowda, L. R., Savithri, H. S. and Rao, D. R. (**1994**), *J. Biol. Chem.*, 269, 18789.

Gunata, Y. Z., Sapis, J-C.; Moutounet, M. (**1987**), *Phytochem.*, 26, 1573.

Halaouli, S., Asther, M., Sigoillot, J C., Hamdi, M. and Lomascolo, A. (**2006**), *J. Appl. Microbl.*, 100, 219.

Halim, D. H. and Montgomery, M. W. J. (**1978**), *Food Sci.*, 43, 603.

Hamelryck, T. W., Loris, R., Bouckaert, J., Dao-Thi, A., Strecker, G.,

Imberty, A., Fernandez, E. and Etzler, M. E. (**1996**), *J. Mol. Biol.*, 286, 1161.

Hankins, C. N. and Shannon, L. M. (**1978**), *J.Biol. Chem.*, 253, 7791.

Hankins, C. N., Kindinger, J. and Shannon, L. M. (**1987**), *Plant Physiol*, 83, 825.

Hankins, C. N., Kindinger, J. and Shannon, L. M. [**1979**], *Plant Physiol*, 64, 104.

Harel, E., Mayer, A. M. and Lehman, E. (**1973**), *Phytochem.*, 12, 2649.

Harel, E., Mayer, A. M. and Shain, Y. (**1964**), *Physiol. Plant*, 17, 921.

Harel, E., Mayer, A. M. and Shain, Y. (**1965**), *Phytochem.*, 4, 783.

Hartweck, L. M., Vogelzang, R.D. and Osborn, T. C. (**1991**), *Plant Physiol.*, 97, 204.

Hatakeyama, T., Suenaga, T., Eto, S., Niidome, T. and Aoyagi, H., (**2004**), *J. Biochem.*, 135, 65.

Hazes, B., Magnus, K. A., Bonavontura, J., Dantu, X., Kalk, K. H., Hol, W. G., (**1993)**, *J. Protein Sci.*, 2, 597.

Hearing, V. J. (**1973**), *Nature (London)*, 245, 81.

Heimdal, H., Larsen, L. M., and Poll, L. (**1994**), *J. Agric. Food Chem.*, 42, 1428.

Hemperley, J. J., Hopp, T. P., Becker, J. W. and Cunningham, B. A. (**1979**), *J. Biol. Chem.*, 254, 6803.

Hermodson, M. A. and Mahoney, W. C. (**1983**), *Methods Enzymol.* 91,352.

Hernandez-Romero, D., Sanchez-Amat, A. and Solono, F. (**2006**), *FEBS J.* 273, 257.

*Herrmann, K. (**1974**), *Erwerbsobstbau*, 16, 1.

Hetenyi, C. and Spoel, D. V. D. (**2002**), *Protein Sci.* 11, 1729.

Himmelwright, R. S., Eickman, N. C., LuBien, C. D., Lerch, K. and Solomon, E. I. (**1980)**, *J. Am. Chem. Soc.*, 102, 7339.

Ho, K. K., (**1999**), *Plant Physiol. Biochem.*, 37, 841.

Hoare, D. G. and Koshland, D. E. (**1967**), *J. Biol. Chem.*, 242, 2447.

*Hochstein, P. and Cohen, G., (**1962**), *Ann. NY. Acad. Sci. Paris.*, 254, 914.

*Holst, R. W. and Yopp, J. H. (**1976**), *Phycologia*, 15, 119.*

Hunt, M. D., Newman, S. M., Eannetta, N. T.; Yu, H. and Steffens, J. C. (**1993**), *Plant Mol. Biol.*, 21, 59.

Iyengar, I. and McEvily, A. (**1992**), *Trends Food Sci. Technol.*, 3, 60.

Janovitz-Klapp, A. H., Richard, F. C. and Nicolas, J. J. (**1989**), *Phytochem.*, 28, 2903.

Janovitz-Klapp, A. H., Richard, F. C., Goupy, P. M. and Nicolas, J. J. (**1990a**), *Phytochem.*, 28, 2903.

Janovitz-Klapp, A. H., Richard, F.C., Goupy, P.M. and Nicolas, J. J. (**1990b**), *J.Agric. Food Chem.*, 38, 1437.

Jayaraman, K. S., Ramanuja, M. N., Vijayaraghavan, P. K. and Vaidhyanathan, C. S. (**1987**), *Food Chem.*, 24, 203.

Jeffery, S. C. (**1999**), *Trends Bichem. Sci.* 24, 8.

Jen, J. J. and Kahler, K. R. (**1974**), *Hortscience*, 9, 590*.

Jiang, H., Shi, C., Xie, Y., Xu, X. and Liu Q. (**2003**), *Ind. J. Biochem. Biophys.,* 40, 350.

Jiang, Y. (**1999**), *Food Chem.*, 66, 75.

Jimenez, M. and Garcia-Carmona, F. (**1996**), *Phytochem.*, 42, 1503.

Jimenez, M. and Garcia-Carmona, F. (**1999**), *J. Agric. Food Chem.*, 47, 56.

Jimenez-Atiener, M., Pedreno, M. A. and Garcia-Carmona, F. (**1991**), *Biochem Int.,* 25, 861.

Jimenez-Atienzar, M., Escribano, J., Cabanes, J., Gandia-Herrero, F. and Garcia-Carmona, F. (**2005**).*Plant. Physiol. Biochem.*, 43, 866.

Jolley, R. L. and Nelson, R. M. and Robb, D. A. (**1969**), *J. Biol. Chem.*, 244, 3251.

Kader, F., Rovel, B., Girardin, M. and Metche, M. (**1997**), *J. Sci. Food Agric.*, 73, 513.

Kahn, V. (**1985**), *J Food Sci.*, 50, 111.

Kahn, V. and Andrawis, A. (**1985**), *Phytochem.*, 24, 905.

Kahn, V. and Andrawis, A. (**1986**), *Phytochem.*, 25, 333.

Kahn, V. and Pomeratz, S. H. (**1980**), *Phytochem.*, 19, 379.
Kanade, S. R. and Gowda, L. R. (**2006**) *FASEB J.* 20 (4) A 473

Kanade, S. R., Paul, B., Rao, A. G. A. and Gowda, L. R. (**2006**), *Biochem. J.,* 395, 551.

Kapitany, R. A. and Zebrowski, E. J. (**1973**), *Anal Biochem.,* 56, 361.

Kato, C., Uritani, I., Saijo, R. and Kakeo, T., (**1976**), *Plant Cell Physiol.*, 17, 1045.

Katz, Y. and Mayer, A. M. (**1969**), *Israel J. Botany*, 18, 11.

Kenten, R. H. (**1958**), *Biochem. J.*, 68, 244.

Kenten,R. H. (**1957**), *Biochem. J.*, 67, 300.

Kermasha, S., Goetghebeur, M., Monfette, A., Metche, M. and Rovel, B. (**1993)**, *Phytochem.*, 34, 349.

Kessler, A. and Baldwin I. T. (**2002**), *Annu. Rev. Plant Biol.,* 53, 299.

Khan, M.T.H., Choudhary, M. I., Khan, K. M., Rani, M.and Atta-ur-Rahman, (**2005**), *Bioorg. Med. Chem.*, 13, 3385.

Khatib, S., Nerya, O., Musa, R., Shmuei, M., Tamir, S., and Vaya, J. (**2005**), *Bioorg. Med. Chem.*, 13, 433.

*Kim, J. Y., Marsahl, M. R. and Wei, C. (**2000**), Polyphenoloxidase. In Seafood Enzymes utilization and influence on post harvest seafood quality,

PP 271, marcel Dekker, New York*.

Kim, J. Y., Sea, Y. S., Kim, J. E., Sung, S. K., Song, K. J., et al., (**2001**), *Plant Sci.*, 161, 1145.

King, R. S. and Flurkey, W. H. (**1987**), *J. Sci. Food Agric.,* 41, 231.

Kirschner, K. and Bisswanger, H. (**1976**), *Annu. Rev. Biochem.*, 45, 143.

Klabunde, T., Eicken, C.; Sacchettini, J. C. and Krebs, B., (**1998**), *Nature Struct. Biol.*, 5, 1084.

Kotsira, V. P. and Clonis, Y. D., (**1998**), *Arch. Biochem. Biophys.*, 356, 117.

Koussevitzky, S., Neeman, E. and Harel, E. (**1998**), *J. Biol. Chem.*, 273, 27064.

Koval, I. A., Gamez, P., Belle, C., Selmeczi, K. and Reedijk, J. (**2006**), *Chem. Soc. Rev.*, 35, 814.

Kruger, R. C. (**1955**), *Arch. Biochem. Biophys.*, 57, 52.

Kubo, I and Kinst-Hori, I. (**1999**), *Planta.,* 65, 19.

Kubo, I. and Kinst-Hori, I. (**1988**), *J. Agric Food Chem.*46, 5338.

Kubo, I. and Kinst-Hori, I. (**1998**), *J. Agric Food Chem.,* 46, 5338.

Kubo, I., Kinst-Hori, I., Chaudhuri, S. K., Sanchez, Y. and Ogura, T., (**2000**), *Biorg. Med. Chem.*, 8, 1749.

Kutner, R. and Wagreich, H. (**1953)**, *Arch. Biochem. Biophys.*, 11, 341.

Kuwabara, T. (**1995),** *FEBS Lettres*, 371, 195.

Kuwabara, T. and Katoh, Y. (**1990**), *Plant Cell Physiol.*, 40, 1029.

Kuwabara, T., Masuda, T. and Aizawa, S. (**1997**), *Plant Cell Physiol.*, 38, 179.

Laemmli, U. K. (**1970**), *Nature,* 227, 680.

Lanker, T., Flurkey, W. H. and Hughes, J. P. (**1988**), *Phytochem.*, 27, 3731.

Laveda, F., Nunez_Delicado, E., Garcia Carmona, F. and Sanchez-Ferrer, A. (**2001**), *J. Agric Food Chem.*, 49, 1003.

Laveda, F., Nunez-D.E., Garcia-Carmona F. and Sanchez-Ferrer A. (**2000**), *Arch. Biochem. Biophys.,* 379, 1.

Lax, A. R., Vaughn, K. C. and Templeton, G. E. (**1984**), *J. Heredity*, 75, 285*.

Le Bourvellec, C., Le Quere, j. M., Sanoner, P., Drilleau, J. F. and Guyot, S. (**2004**), *J. Agric. Food Chem.*, 52, 122.

Lee, S. E. Kim, M. K., Lee, S. G., Ahn, Y. J. and Lee, H. S. (**2000**), *Food Sci. Biotechnol.*, 9, 330.

Lee, C. Y. and Whitaker, J. R. (**1995**), Eds American Chemical Society, Washington DC, pp2.

Lee, P. M., Lee, K. H. and Karim, M. I. A. (**1991**), *J. Sci. Food Agric.*, 55, 643.

Lehrer, and Leavis, (**1978**), *Methods Enzymol.*, 49, 222.

Lerch, K. (**1982**), *J. Biol. Chem.*, 257, 6414

Lerch, K. and Ettlinger, L. (**1972**), *Eur. J. Biochem.*, 31, 472.

Lerner, A. B. (**1953**) *Advn. Enzymol.*, 14, 73.

Lerner, A. R. and Mayer, A. M. (**1975**), *Phytochem.*, 14, 1955.

Lerner, H. R. and Mayer, A. M. (**1976**), *Phytochem.*, 15, 57.

Lerner, H. R., Mayer, A. M. and Harel, E. (**1972**), *Phytochem.,* 11, 2415.

Li, L. and Stefens, J. C. (**2002**), *Planta*, 215, 239.

Liener, I. E. (**1994**), *Crit. Rev. Food Sci. Nutr.,* 34, 31.

Lim, J. Y., Ishiguro, K. and Kubo, I. (**1999**), *Phytother. Res.*, 13, 371.

Lima, A. W. O. and Bora, S. P. (**2003**), *J. Food Biochem.,* 27**,** 237.

Lineweaver, H. and Burk, D. (**1934**), *J. Am. Chem. Soc.*, 56, 658.

Lopez-Serrano, D., Sanchez-Amat, A. and Solano, F. (**2002**), *Pigment Cell Res.,* 15, 104.

Lourenco, E. J., Neves, V. A. and Silva, M. A. (**1992**), *J. Agric. Food Chem.*, 40, 2369.

Luh, B. S. and Philthakphol, B. J. (**1972**), *J. Food Sci.*, 27, 264.

Macheix, J. J., Fleuriet, A. and Billot, J. (**1990**) in *Fruit Phenolics*, CRC Press:Boca Raton, FL, p295.

Macheix, J. J., Sapis, J. C. and Fleuriet, A. (**1991**), *Crit. Rev. Food Sci. Nutr.*, 30, 441.

Mahoney, W. C. and Hermodson, M. A. (**1980**), *J. Biol. Chem.*, 255, 1119.

Malesset-Bras, M. (**1962**), *C. R. Acad.Sci.* Paris. 254, 914

Mandal, D. K., Nieves, E., Bhattacharyya, L., Or, G. A., et al., (**1994**), *Eur.J. Biochem.*, 221, 547.

Manson, H.S. (**1965**), *Annu. Rev. Biochem.,* 34,595.

Marques, L., Fleuriet, A. and Macheix, J. J. (**1995**), *Plant Physiol. Biochem.*, 33, 193.

Marques, L., Fleuriet, A., Macheix, J. J., (**1995**), *Plant Physiol. Biochem.*, 33, 193.

Marri, C., Frazzoli, A., Hochkoeppler, A. and Poggi, V. (**2003**), *Phytochem.*, 63, 745.

Martinez, M. V. and Whitaker, J. R. (**1995**), *Trends Food Technol.*, 6, 195.

Martinez-Cayuela, M. Rodriguez-Vico, F., Faus, M. J. and Gil, A. (**1989**), *J. Plant Physiol.*, 133, 660.

Marusek, C. M., Trobough, N. M., Flurkey, W. H.,and Inlow, J. K. (**2006**), *J.Inorg. Chem.*, 100, 108.

Mason, H. S. (**1955**), *Adv. Enzymol.*, 16, 105

Mathew, A. G. and Parpia, H. A. B., (**1971**), *Adv. Food Res.*, 19, 75.

Matoba, Y., Kumagi, T., Yamamoto, A., Yoshitsu, H. and Sugiyama, M. (**2006**), *J. Biol. Chem.* 281, 8981.

Matsudaira, P. (**1987**) *J. Biol. Chem.*, 262, 10035.

*Mayer, A. M. (**1965**), *Israel J. Botany*, 13, 74.

*Mayer, A. M. (**2004**), *Isr. J. Plant Sci.*, 52, 279.

Mayer, A. M. (**2006**), *Phytochem.*, 67, 2318

Mayer, A. M. and Friend, J. (**1960**), *Nature*, 185, 464.

Mayer, A. M. and Harel, E. (**1979**), *Phytochem.*, 18, 193.

Mayer, A. M. and Harel, E. (**1981**), in *Recent Advances in Biochemistry of Fruits and Vegetables*, Friend, J, Rhodes, M. J. C., Eds., Academic press, Londres, pp161.

Mazzafera, P. and Robinson, S. P. (**2000**), *Phytochem.*, 55, 285.

McEvily, A. J., Iyengar, R., Otwell, W. S., (**1992)**, *Crit. Rev. Food Sci. Nutr.*, 32, 253.

Mdluli, K. and Owusu-Apenten, R. (**2003**), *J. Food Biochem.*, 27, 67.

Mduli, K. M. (**2005**), *Food Chem.*, 92, 311.

Melo, G. A., Shimizu, M. M. and Mazzafera, P. (**2006**), *Phytochem.*, 67, 277.

Menon, I. A. and Haberman, H. F. (**1970**), *Arch.Biochem.Biophys.* 137, 231.

Menon, S., Fleck, R. W., Yong, G. and Strothkamp, K. G. (**1990**), *Arch Biochem. Biophys.*, 280, 27.;

Mikkelsen, R. B., Tang, D. H. and Triplett, E. L. (**1975**), *Biochem. Biophys. Res. Commun.*, 63, 980.

Moore, B. M. and Flurkey, W. H. (**1990**), *J. Biol. Chem.,* 265, 4482.

Moreno, J. and Chrispeels, M. J. (**1989**), *Proc. Natl. Acad. Sci.* U. S. A., 86, 7885.

Morris, G. M., Goodsell, D. S., Halliday, R. S., Huey, R., Hart, W. E., Belew, R. K. and Olson, A. J. (**1998**), *J. Comput.Chem.*, 19, 1639.

Muchuweti, M., Mupure, C., Ndlala, A. and Kasiyamhuru, A. (**2006**), *J. Sci. Food Agric.*, 86, 328.

Murata, M., Haruta, M., Murai, N., Tanikawa, N., Nishimura, M., Homma, S. and Itoh, Y. (**2000**), *J. Agric. Food Chem.*, 48, 5243.

Murata, M., Kurokami, C. and Homma, S., (**1992**), *Biosci. Biotechnol. Biochem.*, 56, 1705.

Murata, M., Tsurutani, M., Hagiara, S. and Homma, S. (**1997**), *Biosci. Biotech. Biochem.*, 61, 1495.

Nagai, T. and Suzuki, N. (**2001**), *J. Agric Food Chem.*, 49, 3922.

Nagi, T. and Suzuki, N. (**2003**), *J. Food Chem and Tchnol.*, 68, 16

*Nakamura, K., Amano, Y. and Kagami, M. (**1983**), *Am. J. Enol. Vitic.*, 34, 122.

Nakayama, T. (**2002**), *J.Biosci. Bioeng.*, 94, 487.

Neog, B., yadav, R. N. S. and Singh, I. D. (**2004**), *J. Indian Inst. Sci.*, 84, 47.

Newman, S. M., Eannetta, N. T., Yu, H., Prince, J. P., Carmen de Vicente, M., Tanksley, S. D. and Steffens, J. C. (**1993**), *Plant Mol. Biol.*, 21, 1035.

Ngalani, J. A., Signoret, A. and Crouzet, (**1993**), *J. Food Chem.*, 58, 341.

Nkya, E., Koung, C., Li, Y., Yang, C., Hayashi, N. and Fujita, S. (**2003**), *J. Agric. Food Chem.*, 51, 5467.

Nozue, M., Souri, M., Arakawa, D. and Kojima, M. (**1998**), *J. Plant Physiol.*, 153, 552.

Nunez-Delicado, E., Serrano-Megias, M., Perez-Lopez, A. J. and Lopez-Nicolas, J. M. (**2005**), *J. Agric. Food Chem.*, 53, 6087.

Nyvall, P., Pedersen, M., Kenne, L. and Gacesa, P. (**2000)**, *Phytochem.*, 54, 139.

Oba, K., Iwatsuki, N., Uritani, I., Alvarez, A. M., Garcia, V. V., (**1992**), *Biosci.*

Biotech. Biochem., 57, 1027.
Okot-Kotber, M., Liavoga, A., Yong, K.. and Bagorogoza, K. (**2002**), *J. Agric. Food Chem.,* 50, 2410.
Olivares, C., Garcia-Borron, C. and Solano. F. (**2002**), *Biochemistry,* 41¸ 676.

Osaki, S., (**1963**), *Archiv. Biochem. Biophys.*, 100, 378.

Osborn, T. C., Alexander, D. C., Sun, S. S. M., Cardona, C., and Bliss, F. A. (**1988**), *Science*, 240, 207.
Osinaga, E., Tello, D., Batthyany, C., Bianchet, M., Tavares, G., Duran,, R., et al., (**1997**), *FEBS Letters*, 412, 190.
Osuga, D., van der Schaff, A., Whitaker, J. R., in *Protein Structure Function Relationships in Foods*, Yada, R. V. and Jackman, R. L. (**1994**), Smith, J. L., Eds., Blackie Academic and Professional, Glasgow, Scotland., 62.

Ozen, A., Colak, A., Dincer, B. and Guner, S. (**2004**), *Food Chem.*, 85, 431.

Palmer, J. K. (**1963**), *Plant Physiol.*, 38, 508.

Parish, R. W. (**1972**), *Eur. J. Biochem.*, 31, 446.

Park, E. Y. and Luh, B. S., (**1985)**, *J. Food Sci.*, 50, 679.
Park, S. J., Kim, J., Son, W. and Lee, B. J. (**2004)**, *J. Biochem.,* 35, 337.
Parsons, S. M. and Raftery, M. A. (**1972**), *Biochemistry* 11, 1623.

Partington, J. C., Smith, C. and Bolwell, G. P. (**1999**), *Planta*, 207, 449.

Partington, J. C.; Bolwell, G. P., *Phytochem.*, **1996**, 42, 1499.

Passi, S. and Nazzaro-Porro, M. (**1981)**, *Br. J. Dermatol.*, 104, 659.
Paul, B. (**2000**), Ph.D. thesis Purification and structural characterization of polyphenol oxidase from field bean (*Dolichos lablab).* Mysore University. Mysore India.

Paul, B. and Gowda, L. R. (**2000**), *J.Agric Fooc Chem.*, 48, 3839.

Perez-Gilabert, M. and Garcia-Carmona, F. (**2000**)**,** *J. Agric. Food Chem.*, 48, 695.

Perez-Gilabert, M. and Garcia-Carmona, F. (**2001**), *Biochem. Biophys. Res. Commim.*, 285, 257.

Petra, P. H. and Neurath, H. (**1971**), *Biochemistry*, 10, 3171.

Peumans, W.J.and Van Damme, E.J.M. (**1995**), *Plant Physiol.*, 109, 347.

Pifferi, P. G., Baldassari, L. and Cultrera, R. (**1974**), *J. Sci. Food Agric.*, 25, 263.

Pomerantz, S. H. (**1963)**, *J. Biol. Chem.*, 238, 2351.

Pomerantz, S. H. and Murthy, V. V. (**1974**), *Arch. Biochem. Biophys.*, 160, 73.

Prabhakaran, K. (**1968)** *Nature*, 218, 473.

Pruidze, G. N., Mchedlishvili, N. T., Omiadze, N. T., Gulua, L. K. and Pruidze, N. G. (**2003**), *Food Res. Inter.*, 36, 587.

Ramasarma, T. (**1994**), *Curr. Sci.*, 67, 24.

Rapeanu, G., Loey, A. V., Smout, C. and Hendrickx, M. (**2006**), *Food Chem.*, 94, 253.

Rathjen, A. H. and Robinson, S. P. (**1992**), *Plant Physiol.*, 99, 1619.

Reddy, N. R., Sathe, S. K., Salunkhe, D. K. (**1989**), Carbohydrates. In CRC handbook Food Legumes,Nutritional Chemistry of World, Proceessing Technology and Utilization, Salunkhe, D. K. Kadam, S. S. Eds, CRC Press, Boca Raton, Fl, Vol.1. PP65.

Rescigno, A., Sollai, F., Rinaldi, A. C. Soddu, G. and Sanjust, E. (**1997**), *J. Bichem. Biophys. Methods.*, 34, 155.

Ricard, J., Noat, G. and Nari, J. (**1984**), *Eur. J. Biochem.*, 145, 311.

Ridgway, T. J. and Tucker, G. A. (**1999**), *Enzyme Microbial Technol.*, 24, 225.

Ritcher, H., Lieberei, R. and von Schwartzenberg, K. (**2005**), *Plant Biol.*, 7, 283.

Rivas, N. J. and Whitaker, J. R. (**1973**), *Plant Physiol.*, 52, 501.

Robb D. A., Mapson, L. W. and Swain, T. (**1965**), *Phytochem.*, 4, 731.

Robert, C. M., Cadet, F. R., Rouch, C. C., Pabion, M. and Richard-Forget, F. (**1995**), *J. Agric. Food Chem.*, 43, 1143.

Robert, M. Z., Tatiana, E., Zelljohn, H. M. and John, J. W. (**1969**), *Anal Biochem.*, 30,148.

Roberts, D. D. and Glodstein, I. J. (**1982**), *J. Biol. Chem.*¸257, 11274.

Robinson, S. P. and Dry, I. B. (**1992**), *Plant Physiology*, 92, 312.

Rocha, A. M. C. N and Morais, A. M. M. B. (**2001**), *Food Control*, 12, 85.

Rodriguiz-Lopez , J. N., Tudela, J., Varon, R., Garcia-Carmona, F. (1992), *J. Biol. Chem.*, 267, 3801.

*Romero Andreas, J., Yandel, b. S., and Bliss, F. A. (**1986**), *Theor. Ppl. Genet.*, 72, 123.

Rompel, A., Fischer, H., Meiwee, D., Buldt-Karentzopoulos, K., Dillinger, R. Tuezek, F., Witzel, H. and Krebs, B. (**1999**), *J. Biol. Inorg. Chem.*, 4, 58.

Roopashree, S., singh, S. A., Gowda, L. R. and Rao, A. G. A. (**2006**), *Biochem. J.*, 395, 629.

Rouge´, P., Richardson, M., Ranfaing, P., Yarwood, A. and Sousa-Cavada, B., (**1987**) *Bio. Syst. Econ.* 15, 341.

Ruis, H. (**1972)**, *Phytochem.*, 11, 53.

Sachde, A. G., Al-Bakir, A. Y. and Abdul-Raheem, J. A. K., (**1989)**, *J. Food Biochem.*, 12, 241.

Shivakumar, N. and Rao, D. R. (**1986**), *J. Biosci.*, 10, 95.

Sambrook, J. and Russel, D. R. (**2001**), Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press Cold Spring Harbor NewYork.

Sanchez-Ferrer, A., Bru, R. and Garcia-Carmona, F. (**1990**), *Anal. Biochem.*, 184, 279.

Sanchez-Ferrer, A., Garcia-Carmona, F. (**1992**), *Biochem. Edu.*, 20, 235.

Sanchez-Ferrer, A., Levada, F. and Garcia-Carmona, F. (**1993a**), *J. Agric.*

Food Chem., 41, 1583.

Sanchez-Ferrer, A., Levada, F. and Garcia-Carmona, F. (**1993b**), *J. Agric. Food Chem.*,41, 1225.

Sanchez-Ferrer, Rodrigeuz-Lopez, J. N., Garcia-Canovas, F Garcia-Carmona, F. (**1995**), *Biochim. Biophys. Acta,*. 1247, 1.

Sanjust, E., Cecchini, G., Sollani, F., Curreli, N. and Rescigno, A. (**2003**), *Arch Biochem. Biophys.*, 412, 272.

Sapers, G. M. (**1993)**, *Food Technol.*, 47, 75.

Sato, M. (**1962)**, *Phytochem.*, 8, 353.

Sayavedra-Soto, L. A. and Montgomery, M. W. (**1986)**, J. *Food Sci.*, 51, 1531.

Schnell, D. J. and Etzler, M. E. (**1987**), *J. Biol. Chem.*, 262, 7220.
Schnell, D. J. and Etzler, M. E. (**1988**), *J. Biol. Chem.*, 263, 14648.

Segel, I. H. (**1976**), Biochemical calculations, Second Ed., pp 273-281, John Wiley and Sons, New York.

Selles-Marchart, S., Casado-Vela, J. and Bru-Martinez, R. (**2006**), *Arch Biochem. Biophys.*, 446, 175.

Seo, S., Sharma, V. and Sharma, N. (**2003**), *J. Agric. Food Chem.*, 51, 2837.

Sharon, O. and Kahn, V. (**1979),** *J. Sci. Food Agric.*, 30, 634.

Sheprovitszy, Y. G. and Brudvig, G. W. (**1996**), *Biochemistry*, 35 16255.

Sherman, Y. O., Vaughn, K. C. and Duke, S. O. (**1991**) *Phytochem.*, 30, 2499.

Shi, C., Dai, Y., Xie, Q., Liu, Y. and, Xu, X. (**2003**)**,** *J. Mol. Str.,* 644**,** 139.

Shin, R., Froderman, T., Flurkey, W. H. (**1997**), *Phytochem.*, 45, 15.

Shoenbein, (**1856**), *Phil. Mag.* 11, 137

Shomer, I. N., B., Harel, E. and Mayer, M. (**1979**), *Ann. Bot.*, 44, 261.

Siddiq, M., Sinha, M. K., Cash, J. N. and Hanum, T. (**1996**), *J. Food Biochem.*, 20, 111.

Siddiq, M., Sinha, N. K. and Cash, J. N. (**1992)**, *J. Food Sci.*, 57, 1177.

Singh, K., Raizada, J., Bhardwaj, P., ghawana, S., Rani, A., Singh, H., Kaul, K. and Kumar, S., (**2006**), *Anal. Biochem.*, 335, 330

Smith, T. F. and Waterman, M. S. (**1981**), *J. Mol.Biol.*,147, 195.

Soderhall, I. (**1995**), *Phytochem.*, 39, 33.

Sojo, M.; Nunez-Delicado, E.; Garcia Carmona, F.; Sanchez-Ferrer, A., (**1998a**), *J. Agric. Food Chem.*, 46, 4924.

Sojo, M., Nunez-Delicado, E., Garcia Carmona, F., Sanchez-Ferrer, A., (**1998b**), J. *Agric. Food Chem.*, 46, 4931.

Sokolenko, A., Fulgosi, H., Gal, A., Altschmied, L., Herrmann, R. G. (**1995**), *FEBS Letters,* 371, 176.

Soler-Rivas, C., Arpin, N., Oliver, J. M. and Wicher, H. J. (**1997**), *Mycol. Res.,* 101, 375.

Solomon, E. I., Sundaran, U. M. and Machonkin, T. E., (**1996**), *Chem. Rev.*, 96, 2563.

Sommer, A., No'eman, E., Steffens, J. C., Mayer, A. M. and Harel, E. (**1994**), *Plant Physiol.*, 105, 1301.

Souza, M. A., Amancio-Pereira, F., Cardoso, C. R. B., Silva, A. G., Silva, E. G., Andrade, L. R., Pena, J. D. O., Lanza, H. and Afonso-Cardoso, S. (**2005**), *Braz. Arch. Biol. Technol.*, 48, 705.

Speicher, D. W. (**1989**). In: Techniques in protein chemistry. Hugli T. E.(Eds) Academic Press, NewYork, 24

Stefens, J. C., Harel, E. and Hunt m. D. (**1994**), Polyphenoloxidase. In Ellis, B e. et al. (Eds), genetic enginerring of plant secondary metabolism. Plenum Press, New York, pp275-312.

Strack, D., Vogt, T. and Schliemann, W. (**2003**), *Phytochem.*, 62, 247.

Sugumaran, M. (**1990**), in *Defense Molecules*, Marchalonis, J. J.; Reinisch, E. L., Eds., Wiley-Liss, New York, p47.

Sugumaran, M. (**1988**), *Adv. Insect.Physiol.*, 21, 179.

Sugumaran, M. and Nellaiappan, K. (**1991**), *Biochem. Biophys. Res. Commun.*, 176, 1371.

Sullivan, M. L., Thoma S. L. and Samac, D. A. (**2004**), *Plant Physiol.*, 136, 3234.

Suseelan, K. N., Bhatia, C. R. and Mitra, R. (**1997**), 50, 211.

Suseelan, K. N., Mitra, R., Bhatia, C. R. and Gopalkrishana, T. (**2004**), *Plant Foods Hum. Nutri.*, 59, 123.

Swain, T., Mapson, L. W. and Robb, D. A. (**1966**), *Phytochem.*, 5, 469.

Takeuchi, W., Takahashi, H., Kojima, M. (**1992**), *Biosci. Biotech. Biochem.*, 56, 1134.

Tanfel, K. and Voigt, J. (**1963**), *Ernaehrungsforschung*, 8, 406.
Tepper, A. W. J. W., Bubacco, L. and Canters, G. W. (**2005**), *J. Am. Chem. Soc.* 127, 567.

Thipyapong, P., Hunt, M. D., Steffens, J. C. (**2004**), *Planta*, 220, 105.

Thipyapong, P., Joel, D. M., Steffens, J. C. (**1997**), *Plant Physiol.*, 113, 707.

Thygsen, P. W., Dry, I. B. and Robinson, S. P. (**1995**), *Plant Physiol.*, 109, 525.

Tian, S., Wan, Y., Qin, G. and Xu, Y. (**2005**),.*Appl. Microbiol Biotechnol*, 13,1.

Tocher, R. D. and Meeuse, B. J. D. (**1966**), *Can. J. Botany*, 44, 551.

Tolbert, N. E. (**1973**), *Plant Physiol.*, 51, 234.

*Tono, T., Fujita, S., Kawasaki, H. and Li, Z. F. (**1986**), *Nippon Nogeikagaku*

Kaishi, 60, 705.

Towbin, H., Stachelin, T. and Gordon, J., (**1979**), *Proc. Natl. Acad. Sci., USA*, 76, 4350.
Tremolieres, M. and Beith, J. G. (**1984**), *Phytochem.*, 23, 501.

Uversky, V. N., *Biochemistry*, **1993**, 32, 13288.

Vadaka, L. L., Rao, R. N. and Nadimpalli, S. K. (**2006**), *Protein Expr. Purif.*, 45, 296.

Valero, E. and Garcia–Carmona, F. (**1998**), *J. Agric. Food Chem.*, 46, 2447.

Valero, E., Garcia-Moreno, M., Varon, R. and Garcia-Carmona, F. (**1991**), *J. Agric. Food Chem.*, 39, 1043.

Valero, E.and Garcia-Carmona, F. (**1992**), *Biochem. J.*, 286, 623.
Vamos-Vigyazo, (**1981**), *CRC Crit. Rev. Food. Sci. Nutr.*, 15, 49.

Van Demme, E. J., Barre, A., Rouge, P. and Peumans, W. J. (**1997**), *Plant Mol. Biol.*, 33, 523.

van Gelder, C. W. G. Flurkey, W. H. Wichers, H. J., (**1997**), *Phytochem.*, 45, 1309.

van Leeuwen, J. and Wichers, H. (**1999**), *Mycol. Res.*, 103, 413.

van Poucke, M. (**1967**), *Physiol. Plant*, 20, 932.

Vaughn, K. C. and Duke, S. O. (**1984**), *Physiol. Plant*, 60, 102.

Vaughn, K. C., Lax, A. R. and Duke, S. O., (**1988**), *Physiol. Plant*, 72, 659.

Vijayan, M.and Chandra, N., (**1999**). *Curr. Opin. Struct. Biol.* 9, 707–714.
Vodkin, L. O., Rhodes, P. R. and Goldberg, R. B. (**1983**), *Cell*, 34, 1023.
Voelker,T. A., Stawick, P. and Chrispeels, M. J. (**1986**), *EMBO J.*,5, 3075.

Volbeda, A. and Hol, W. G. J. (**1989**), *J. Mol. Biol.*, 209, 249.

Volke, R., Harel, E., Mayer, A. M. and Gan-Zvi, E. (**1977**), *J. Exp. Botany*, 28, 820.

Walker, J. R. L. (**1964**), *Aust. J. Biol. Sci.*, 17, 360.

Walker, J. R. L. (**1975**), *Enzyme Technol. Dig.*, 4, 89.

Walker, J. R. L. and Wilson, E. L. (**1975**), *J. Sci. Food Agric.*, 26, 1825.

Walker, J. R. L. and Ferrar, P. H. (**1995**), *Chemistry and Industry*, 20, 836.

Walker, J. R. L. and Wilson, E. L. (**1975)**, *J. Sci. Food Agric.*, 26, 1825.

Wang J. and Constabel P. C. (**2004**), *Planta* 220, 87.

Wang, J. and Constabel, P. C. (**2003**), *Phytochem.,* 64, 115.

Wang, N. and Hebert, D. N. (**2006**), *Pigment Cell Res.*, 19, 3.

Weis, W. I. and Drickamer, K. (**1996**), *Annu. Rev. Biochem.*, 65, 441.

Wesche-Ebeling, P., Montgomery, M. W., (**1990**), *J. Food Sci.*, 55, 1315.

Whitaker, J. R. (**1994**) Principles of Enzymology for thre food sciences, second Ed. PP 271-556, Marcel Dekker, New York.

Whitaker, J. R. (**1996**) Polyohenoloxidase. In Food Industry, (O. R.Fennema,) PP 492-494, Marcel Dekker, New York.

Whitaker, J. R. and Lee, C. Y., (**1995**), in *Enzymatic Browning and its Prevention*, Whitaker, J. R., Eds., American Chemical Society, Washington DC, p8.

Wichers, H. J., Gerritsen, Y. A. M. and Chapdon, C. G. (**1996**), J., *Phytochem.*, 43, 333.

Wichers,H. J., Recourt, K., Hendrinks, M., Ebbelaar, C. F. M., Biancone, G., Hoeberichts, F. A. et al., (**2003**), *Appl. Micribiol. Technol.,* 61, 336.

Wilcox, D. E., Porras, A. G., Hwang, Y. T., Lerch, K., Winkler, M. E. and Solomon, E. I., (**1985**), *J. Am. Chem. Soc.*, 107, 4015.

Witcop, C. J., (**1984)**, in *Genodermatoses: Clinics in Dermatology*, Goodman, R. M., Ed., J. B. Lippincot. Philadelphia, Vol 2, p70.

Wititsuwannakul, D., Chareonthiphakorn, N., Pace, M. and

Wititsuwannakul, R. **(2002)**, *Phytochem.* 61, 115.
Wittenberg, C. and Triplett, E. L. (**1985**), *J. Biol. Chem.* 260, 12542.

Wong, J. H. and Ng, T. B. (**2005**), *Arch. Biochem. Biophys.*, 439, 91.

Wong, J. H., Clarence, C. T. and Ng. T. B. (**2006**), *Biochim. Biocphys. Acta.*, 1760, 808.

Xia, C., Meyer, D. J., Chen, H., Reinemer, P., Huber, R., and Ketterer, B. (**1993**), 293, 357.
Xiao H., Xie Y., Liu, Q., Xu X. and Shi, C. (**2005**), *Spetrochimica Acta Part A* 61,2840.

Yalovskg, S., Paulsen, H., Michaeli, D., Chitnis, P. R. and Nechushtai, R. (**1992**), *Proc. Natl. Acad. Sci.* USA.89, 5616.

Yang, C., Fujita, S., Kohno, K., Kusubayashi, A., Ashrafuzzaman, M. D. and Hayashi, N. **(2001),** *J.Agric.Food Chem.*, 49, 1446.

Ye, X. Y., Ng. T. B., Tsang, P. W. and Wang, J. (**2001**), *J. Protein Chem.*, 20, 367.

Yopp, J. H. (**1976**), *Phycologia*, 15, 119.
Yoruk, R. and Marsha, M. R. (**2003**), *J. Food Biochem.* 27, 361.

Yoshida, H., Tanaka, Y. and Nakayama, K. (**1974**), *Agric. Biol. Chem.*, 38, 627.

Young, N. M., Watson, D. C., Yaguchi, M., Adar, R., Arango, R., Rodriguez-arango, E., Sharon, N., Blay, P. K. S. and Thibault, P. (**1995**), *J. Biol. Chem.*, 270, 2563.

Zhang, J., Chen, Q., Song, K. and Xie, J. (**2006**), *Food Chem.*, 95, 579.

Zhang, X. and Flurkey, W. H. (**1997**), *J. Food Sci.* 62 97.

Zhang, X. and Flurkey, W. H. (**1999**), *J. Food Biochem.*, 23, 95.

Zhang, X., van Leeuven, J., Wichers, H. J. and Flurkey, W. H. (**1999**), *J. Agric. Food Chem.,* 47, 374.

Zhou, P., Smith, N. L. and Lee, C. Y. (**1993)**, *J. Agric. Food Chem.*, 41, 532.

Zor, T. and Selinger, Z., (**1996)**, *Anal. Biochem.*, 236, 302.

*Taken from cross reference, original paper not seen.