KINETIC CHARACTERIZATION OF ASCORBATE PEROXIDASE FROM *Vigna radiata*

A PROJECT REPORT

Submitted by

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in partial fulfillment for the award of the degree

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The report of the project work submitted by the above students in partial fulfillment for the award of Bachelor of Technology degree in Biotechnology of Anna University was confirmed to be the report of the work done by the above students and then evaluated.

(INTERNAL EXAMINER) **(EXTERNAL EXAMINER)**

Dedicated to our Beloved Parents

&

Respected Guide

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Abstract

ABSTRACT

One of the paradoxes of life on this planet is oxygen, the molecule that sustains aerobic life, fundamentally essential for energy metabolism and respiration, when activated to reactive oxygen species (ROS) leads to cellular damage during highly stressed conditions. In chloroplasts of higher plants, oxygen consumption in the absence of electron acceptors is accompanied by the production of H_2O_2 and other activated forms of oxygen. Chloroplasts contain several protective systems which are effective against various forms of activated oxygen but they lack catalase, and the disposal of H_2O_2 is accomplished by means of Ascorbate Peroxidase (APX). APX (EC - 1.11.1.11) is an oxidoreductase that uses ascorbic acid as the electron donor to scavenge the hydrogen peroxide. A detailed knowledge of the enzyme is not available especially for leguminous plants and hence the present study focuses on APX from *Vigna radiata*. Screening of Ascorbate Peroxidase (APX) activity was done in five legumes including *V. radiata* and one spice namely *Brassica nagra*. Maximal APX activity was observed in *Vigna radiata* and *Trigonella foenum-graecum*. Further purification and experiments were carried out in *V. radiata* since it is lesser studied among the two. Ammonium sulphate precipitation of the homogenate showed maximum APX activity in 40-60% fraction. Ion exchange chromatography using DEAE-Cellulose column has been carried out with the dialysate and the fractions with maximum APX activity were pooled for gel filtration chromatography using Sepharose 6B. The elution resulted in purified form of the enzyme which was used for its characterization. The enzyme exhibited sigmoidal kinetics indicative of allosteric interactions with its substrate molecules, ascorbate and hydrogen peroxide.

Table of Contents

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS & SYMBOLS

APX	–	Ascorbate peroxidase
Asa	–	Ascorbate
BSA	–	Bovine Serum Albumin
cAPX	–	Cytosolic Ascorbate peroxidase
CcP	–	Cytochrome c peroxidase
chlAPX	–	Chloroplastic Ascorbate peroxidase
DEAE	–	Diethyl aminoethyl
DHA	–	Dehydroascorbate
EC	–	Enzyme Commission
EDTA	–	Ethylenediamine tetraacetic acid
FMS	–	Formulated mineral salts
GSH	–	Glutathione (reduced)
HRP	–	Horse radish peroxidase
IEC	–	Ion-exchange Chromatography
mAPX	–	Microsomal Ascorbate peroxidase
MDHA	–	Monodehydroascorbate
mitAPX	–	Mitochondrial Ascorbate peroxidase
$O_2^{\cdot -}$	–	Superoxide
$OH^{\cdot}$	–	Hydroxyl radical
ROS	–	Reactive Oxygen Species
PS I	–	Photo System I
sAPX	–	Stromal Ascorbate peroxidase
SEC	–	Size-exclusion Chromatography
SOD	–	Superoxide Dismutase
tAPX	–	thylakoid membrane-bound Ascorbate peroxidase

Introduction

1. INTRODUCTION

One of the paradoxes of life on this planet is oxygen, the molecule that sustains aerobic life, fundamentally essential for energy metabolism and respiration, when activated to reactive oxygen species (ROS) leads to cellular damage (Marx, 1985). ROS are usually small molecules formed by partial reduction or activation of stable oxygen molecule as a natural byproduct during normal metabolism. The ROS are highly reactive and toxic as they oxidize biomolecules leading to their destruction and disruption of membrane organization. Consequently, the evolution of all aerobic organisms has been dependent upon the development of efficient ROS-scavenging mechanisms involving enzymes like peroxidases, catalase, superoxide dismutase etc. But during conditions of environmental stress, the level of ROS increases dramatically while plants try to exert the following protective responses by adjusting their metabolism at the molecular level so that minimum damage is done:

- Increases in levels of antioxidant enzymes and metabolites and;
- Induction of protection-related secondary metabolite genes.

If the plants are able to express these responses adequately, stress-induced damages are prevented. However, if these protective responses are inadequate and are unable to cope with the incidence and the level of stress, injury occurs. This happens when there is an imbalance between the production of ROS and the biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage resulting in a condition called oxidative stress. Recent research has revealed that ROS has also been involved in the control and regulation of biological processes such

as programmed cell death, hormonal signaling, stress responses, and development. These studies extend our understanding of ROS and suggest a dual role of ROS in plant biology as toxic byproducts of aerobic metabolism and key regulators of metabolic and defense pathways.

1.1. REACTIVE OXYGEN SPECIES

ROS are highly reactive due to the presence of unpaired valence shell electrons which are produced as a result of normal aerobic metabolism by the reduction or activation of molecular oxygen. The ROS includes oxygen ions, free radicals, and peroxides, both organic and inorganic. Some of these ROS, especially hydrogen peroxide, are key signalling molecules, while others appear to be extremely detrimental to biological systems, effects that are dependent on the concentrations that are perceived by the cells.

A radical (also called a "free radical") is a cluster of atoms one of which contains an unpaired electron in its outermost shell of electrons. This is an extremely unstable configuration, and radicals quickly react with other molecules or radicals to achieve the stable configuration of 4 pairs of electrons in their outermost shell. For example, a primary ROS is superoxide, which is formed by the one-electron reduction of molecular oxygen. Superoxide anion is a charged molecule and cannot cross biological membranes and hence it is to be removed at the site of its generation. The generation of superoxide anion in chloroplasts is believed to take place in normal unstressed photosynthetic conditions also, by photoreduction of O_2 at PS I and PS II, when the energy from the triplet excited state of chlorophyll

is transferred to O_2 (Asada & Takahashi, 1987). This is the reaction catalysed by NADPH oxidase, with electrons supplied by NADPH:

$$O_2 + e^- \longrightarrow O_2^{-\bullet} \text{(superoxide)}$$

$$2O_2 + NADPH \longrightarrow 2O_2 + NADP^+ + H^+$$

Following the formation of superoxide anions is a probable cascade of ROS production which is as shown in the *Figure 1.1.1.* Further reduction of oxygen results in hydrogen peroxide formed by the dismutation of superoxide, which can be either spontaneous or enzymatic through the action of a family of enzymes called superoxide dismutase (SOD). Hydrogen peroxide is formed by the dismutation of the superoxide radicals produced through the auto oxidation of the photo reduced, primary electron acceptor in photosystem I and the photo-reduced ferridoxin (Asada and Takahashi 1987). Therefore, under physiological conditions, once superoxide is formed the presence of hydrogen peroxide becomes almost inevitable.

$$2O_2^{-\bullet} + 2H^+ \longrightarrow H_2O_2 + O_2$$

Hydrogen peroxide is converted to hydroxyl radicals when exposed to ultraviolet light or when it comes in contact with a range of transition metal ions, of which the most important is iron. Hydroxyl radicals are charged and extremely reactive, with a short half-life, and will probably react with the first molecule they encounter.

In neutrophils, myeloperoxidase catalyses the formation of hypochlorous acid (HOCl), while the superoxide anion radicals react with nitric oxide to form a relatively reactive and more toxic molecule,

peroxynitrite. The extracellular SODs might be directly involved in nitric oxide (NO) metabolism besides dismutation of superoxide anion radicals. The extracellular SOD in the apoplast might inhibit the formation of toxic peroxynitrite by dismutating the $O_2^{-\bullet}$ into H_2O_2.

$$NO^{\cdot} + O_2^{-\bullet} \longrightarrow OONO^{-} \text{ (peroxynitrite)}$$

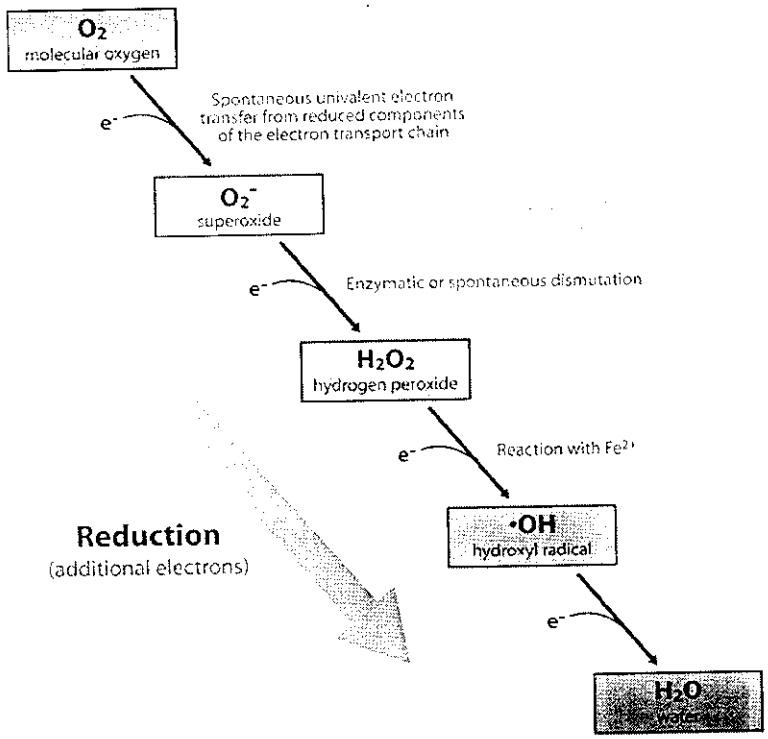


Figure 1.1.1. Cascade of ROS production - Sequential reduction of molecular oxygen

1.1.1. Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is produced predominantly in plant cells during photosynthesis and photorespiration, and to a lesser extent, in the respiration processes. It does not exhibit radical properties and is the most

stable of the reactive oxygen species (ROS), and therefore plays a crucial role as a signaling molecule in various physiological processes. Intra and intercellular levels of H_2O_2 increase during environmental stresses.

Hydrogen peroxide is a strong nucleophilic oxidizing agent and its role in oxidation of –SH group in enzymes and other proteins is considered to be a major mode of its phytotoxic action. Hydrogen peroxide interacts with thiol-containing proteins and activates different signaling pathways as well as transcription factors, which in turn regulate gene expression and cell-cycle processes. Genetic systems controlling cellular redox homeostasis and H_2O_2 signaling are starting to be unraveled. In addition to photosynthetic and respiratory metabolism, the extracellular matrix (ECM) plays an important role in the generation of H_2O_2, which regulates plant growth, development, acclimatory and defence responses. During various environmental stresses the highest levels of H_2O_2 are observed in the leaf veins. Early in the evolution of oxygenic photosynthesis on earth, H_2O_2 could have been involved in the evolution of modern photosystem II.

Hydrogen peroxide is not very abundant in nature. It is formed mainly by the action of sunlight on water and is thus found in traces in rain and snow. Though H_2O_2 is weakly reactive, the single bond between the two oxygen atoms is easily broken resulting in its ready fragmentation into hydrogen radical and a hydroperoxyl radical or into two hydroxyl radicals. Because these latter molecules are highly reactive (with hydroxyl radicals being the most reactive of all the ROS), H_2O_2 is used as a powerful oxidizing agent.

Just as H_2O_2 has the ability to harm microorganisms, it also has the ability to kill our body's cells. The damaging power comes from the transition to highly reactive hydroxyl radicals that indiscriminately react with a wide variety of organic substrates causing peroxidation of lipids, cross-linking and inactivation of proteins, and mutations in DNA. It is widely accepted that H_2O_2 is a toxin *in vivo* at concentrations above 50 μM and above. To counteract stress caused by H_2O_2, organisms have evolved a wide variety of defence mechanisms. For instance, the reactions leading to H_2O_2 are minimized by sequestering the metal ions that would otherwise act as catalysts denaturing and damaging proteins.

Ferritin, transferrin, hemosiderin and heme are examples of proteins that enclose iron and thus play a role in protecting the cell against oxidative damage. Other tools in the combat against oxidative stress are enzymes that are employed to rapidly dismutate H_2O_2 to water. Superoxide dismutases (SOD), catalases, peroxidases (especially glutathione peroxidases, GPX), and thioredoxin-linked systems are examples of such enzymes. Many other molecules serve as antioxidants including vitamins (e.g. vitamin E, vitamin C, provitamin A, also called beta-carotine), hormones (e.g. melatonin) and cofactors such as coenzyme Q.

1.2. PRODUCTION AND SCAVENGING OF ROS IN CELLS - THE ROS CYCLE

The steady state level of ROS in the different cellular compartments is determined by the interplay between multiple ROS-producing pathways, and ROS-scavenging mechanisms. These are in turn controlled by the ROS-

signal transduction pathway and constitute the "basic ROS cycle". During normal growth and development this pathway monitors the level of ROS, produced by aerobic metabolism, and controls the expression and activity of ROS-scavenging pathways (*Figure 1.2.1.*).

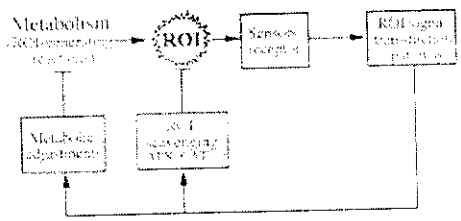


Figure 1.2.1. The Basic ROS Cycle which modulates the cellular levels of ROS during normal metabolism

The basic ROS cycle may also perform fine metabolic tuning, e.g., suppression of photosynthesis, to reduce the production rate of ROS. The ROS are produced by either reactions of normal aerobic metabolism, such as photosynthesis and respiration, or pathways enhanced during abiotic stresses, such as photorespiration. In recent years new sources of ROS were identified in plants, including NADPH oxidases, amine oxidases, and cell wall-bound peroxidases. These are tightly regulated and participate in the control of processes such as programmed cell death, stress response, and pathogen defense.

Under optimal growth conditions the production of ROS in cells is estimated at a constant rate of $240\mu Ms^{-1}$ O_2^-, and a steady state level of

0.5μM H_2O_2. However, stresses that disrupt the cellular homeostasis of cells result in the enhanced production of ROS of up to 720 μMs^{-1} O_2^-, and a steady state level of 5-15μM H_2O_2. These stressed conditions include drought and desiccation, salt, chilling, heat shock, heavy metals, UV radiation, air pollutants such as ozone and SO_2, mechanical stress, nutrient deprivation, pathogen attack, and high light. The enhanced production of ROS during stress can pose a threat to cells, and many stress conditions enhance the expression of ROS-scavenging enzymes. However, it is also thought that during stress ROS are actively produced by cells (e.g., by NADPH oxidase), and act as signals for the induction of defense pathways. Thus, ROS may be viewed as cellular byproducts of stress metabolism, as well as secondary messengers involved in the stress-response signal transduction pathway. This view, of the "extended ROS cycle", is presented in *Figure. 1.2.2.*

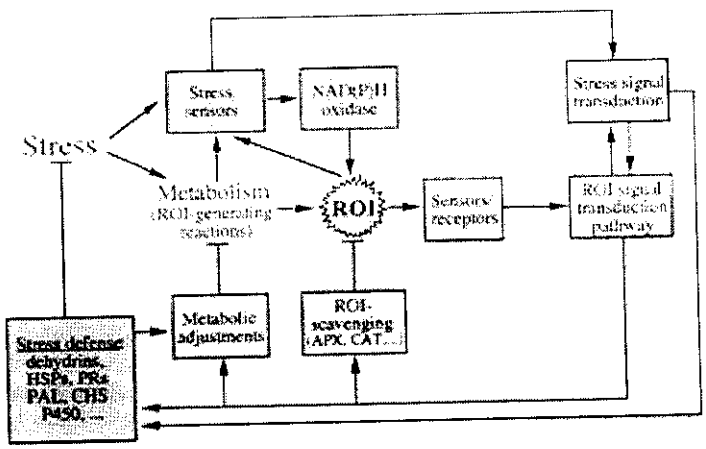


Fig. 1.2.2. The extended ROS cycle - operates in plants during biotic or abiotic stresses. . HSPs, heat shock proteins; PR, pathogenesis related proteins; PAL, phenylalanine ammonia-lyaze; CHS, chalcone synthase; P450, cytochrome P450. Note: ROS is also commonly termed ROI.

Because ROS are toxic but also participate in key signaling events, plant cells require different mechanisms to regulate their intracellular ROS concentrations by scavenging of ROS. Major ROS scavenging mechanisms of plants include superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT). The balance between SOD and APX (and/or CAT) activity in cells is considered to be crucial for determining the steady-state level of O_2^- and H_2O_2. This balance, together with sequestering of metal ions such as Fe and Cu by ferritin and copper-binding proteins, is thought to be important to prevent the formation of the highly toxic $HO^\cdot$ via the metal-dependent Haber–Weiss or the Fenton reactions. Antioxidants such as ascorbic acid and glutathione, found at very high concentrations in chloroplasts and other cellular compartments (5-20mM ascorbic acid and 1-5mM glutathione), are also important for the defense of plants against oxidative stress. Consequently, mutants with suppressed ascorbic acid levels, and transgenic plants with suppressed ROS-scavenging enzymes, are hypersensitive to pathogen attack and abiotic stress conditions. In addition, over-expression of ROS-scavenging enzymes increases the tolerance of plants to abiotic stresses. ROS production can also be decreased in cells by the alternative channeling of electrons in the electron transport chains of the chloroplasts and mitochondria by a group of enzymes called alternative oxidases.

1.2.1. BIOTIC AND ABIOTIC STRESSES

ROS play a central role in the defense of plants against pathogens. During this response, ROS are produced by plant cells via the enhanced enzymatic activity of plasma membrane-bound NADPH oxidases, cell wall-bound peroxidases and amine oxidases in the apoplast. H_2O_2 produced

during this response is thought to diffuse into cells through aquaporins and together with salicylic acid (SA) and nitric oxide (NO) activate many of the plant defenses, including the induction of programmed cell death. The activity of APX and CAT is suppressed during this response by salicylic acid and nitric oxide, the expression of APX is post-transcriptionally suppressed, and the expression of CAT is down regulated at the steady-state mRNA level. Thus, the plant simultaneously produces more H_2O_2 and diminishes its own capability to scavenge H_2O_2, resulting in the over-accumulation of H_2O_2 and the activation of PCD. This response serves as an excellent example to show how the steady state level of ROS can dramatically increase in cells when the basic ROS cycle is severed.

1.3. ROS – MACROMOLECULAR AND CELLULAR DAMAGE

Reactive oxygen species can cause macromolecular damage leading to destruction of cells and thereby injury of plant tissue. Macromolecular damage by ROS includes peroxidation of lipids, oxidation of proteins and mutatios in the mitochondrial DNA.

Peroxidation of polyunsaturated fatty acids by ROS attack can lead to chain breakage and shortening which will increase membrane fluidity and permeability. When isolated, mammalian mitochondria are exposed to oxidative stress, the membrane phospholipids, diphosphatidylglycerol is damaged presumably via peroxidation of the polyunsaturated fatty acids 18:2 and 18:3 – the main fatty acids of the lipid. This results in inhibition of cytochrome c oxidase, which appears to require this phospholipid for activity (Paradies *et al.*, 2000).

The peroxidation of membrane lipids can have numerous effects, including:

- Increased membrane rigidity
- Decreased activity of membrane-bound enzymes (e.g. sodium pumps)
- Altered activity of membrane receptors
- Altered permeability

In addition to the effects on phospholipids, radicals can also directly attack membrane proteins and induce lipid-lipid, lipid-protein and protein-protein cross linking, all of which obviously have effects on membrane function.

Proteins can be damaged by ROS either through direct chemical interaction or indirectly involving end products of lipid peroxidation resulting in loss of function. A number of amino acids can be modified for example, cysteine can be oxidized to cystine and both proline and arginine are converted to glutamyl semialdehyde. In some cases, the damaged amino acids are repaired *in situ*, whereas in other cases the entire protein is removed and degraded (Dean *et al.*, 1997; Møller and Kristensen, 2004). A number of oxidized proteins have been identified in isolated plant mitochondria, presumably because they are particularly susceptible to oxidative damage *in vivo*. A further number of oxidized proteins were identified after oxidative treatment of a matrix fraction (Kristensen *et al.*, 2004).

Breakdown products of lipid peroxidation, notably 4-hydroxy-2-nonenal (HNE), affect several mitochondrial processes. Decarboxylating dehydrogenases, such as glycine decarboxylase, are inhibited by HNE because it specifically binds to, and inactivates, lipoic acid, an essential cofactor for these enzymes (Millar and Leaver. 2000). HNE inhibits the alternative oxidase, so an increasing proportion of the enzyme may become inactivated during oxidative stress. The induction of alternative oxidase gene expression during stress might therefore be necessary to maintain the activity of the enzyme in the face of increasing inactivation rather than to upregulate its activity (Winger *et al.*, 2005). Interestingly, HNE stimulates the uncoupling protein, which will prevent over reduction of the electron transport chain and thus lower ROS production.

Oxidative damage of proteins is initiated by reactive oxygen species and accumulation of such proteins over time is a major contributor to the functional decline that is characteristic of aging according to the Free-radical theory. This does not appear to be the case in *Arabidopsis*, where the amount of proteins with free carbonyl groups increases during the vegetative phase, but decreases dramatically during the flowering and senescence phases (Johansson *et al.*, 2004).

Finally, ROS can cause mutations in mitochondrial DNA (mitDNA). During aging in mammals, mutations in mitDNA accumulate faster than in nuclear DNA which might be because mitDNA is closer to the site of ROS synthesis and/or because mitDNA is not protected by histones. Excision and replacement of the modified nucleotides can repair the DNA damage. However, plant mitDNA does not have a particularly high rate of mutation;

in fact, rearrangements are more common. Little is known about the ROS-induced DNA modifications in plant mitochondria.

1.4. SCAVENGING OF ROS

The conversion of the superoxide into hydrogen peroxide by the enzyme superoxide dismutase does not solve the problem because hydrogen peroxide being an ROS must also be detoxified. There are five potential enzymes (or enzyme systems) for removing hydrogen peroxide in plants *(Figure. 1.4.1.)*.

- Catalase is normally considered to be a peroxisomal enzyme, but it has been found in heart mitochondria as well. Catalase activity is also found in plant mitochondria, but proof that it is not a contaminant is lacking. Catalase has one of the highest turnover rates of all enzymes; one molecule of catalase can convert millions of molecules of hydrogen peroxide to water and oxygen per second.

- The ascorbate/glutathione cycle comprises of four enzymes namely APX, MDAsAR, DAsAR and GR and two low-molecular-weight compounds including ascorbate (vitamin C) and glutathione, a sulfhydryl-containing tripeptide. It is the main ROS-detoxifying system in the chloroplast, but it has also been reported present in plant mitochondria (Jimenez *et al.*, 1997; Chew *et al.*, 2003).

- Thioredoxin, a small sulfhydryl-containing protein, and thioredoxin reductase regulate the activity of a number of Calvin cycle enzymes in the chloroplast stroma. Recently, a number of mitochondrial proteins

able to interact with, and possibly be regulated by, thioredoxin were identified (Balmer *et al.*, 2004). Specifically, thioredoxin has been shown to be involved in the regulation of the alternative oxidase (Gelhaye *et al.*, 2004). Both thioredoxin and thioredoxin reductase have the ability to reduce hydrogen peroxide.

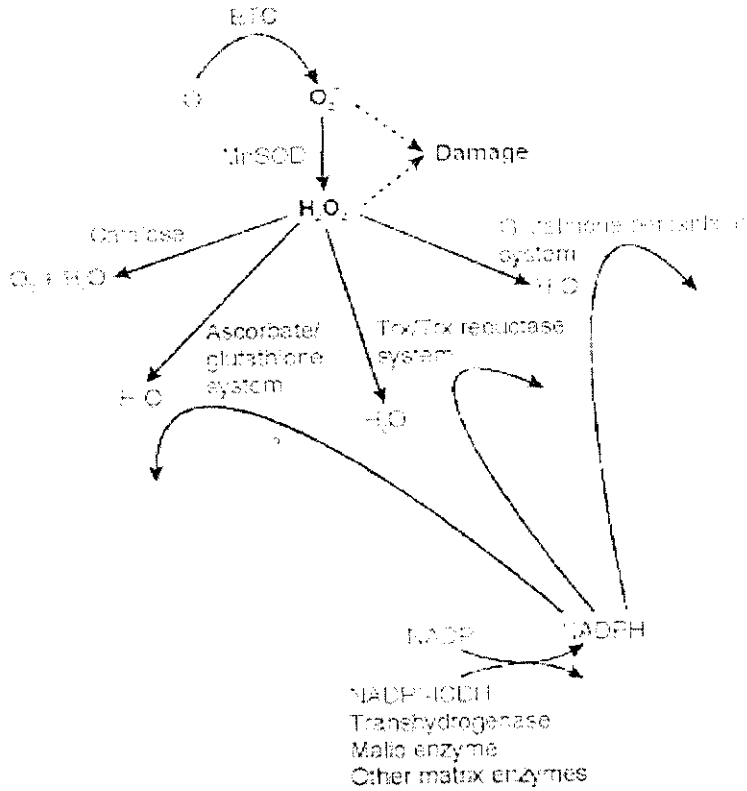


Figure 1.4.1. Summary of the enzyme systems involved in ROS detoxification in plant mitochondria. ETC, electron transport chain; ICDH, isocitrate dehydrogenase; MnSOD, Mn-superoxide dismutase; Trx, thioredoxin.

- Peroxiredoxin, another small sulfhydryl-containing protein, is involved in detoxification of ROS such as hydrogen peroxide and various other peroxides. *Arabidopsis* plants, where the mitochondrial peroxiredoxin has been knocked out, are more sensitive to strong abiotic stresses than are wild-type plants (Finkemeier *et al.*, 2005).

- The main hydrogen peroxide-removing enzyme in mammalian mitochondria is the glutathione peroxidase. It uses reduced glutathione as a direct source of reducing equivalents. Although reported to be present in plants, this enzyme has not yet been found in plant mitochondria.

The last four hydrogen peroxide-removing enzymes use sulfhydryl groups as donors of reducting equivalents and, in all four cases, these sulfhydryl groups are regenerated by reduction with NADPH. This is a good example of the many uses of NADPH in mitochondria (Møller and Rasmusson, 1998).

Objectives

2. OBJECTIVES

The present study focuses on the antioxidant enzyme, Ascorbate peroxidase that uses ascorbic acid as the electron donor to breakdown hydrogen peroxide.

The model system - the germinating seedlings of *Vigna radiata*, was chosen for the present study on account of its high metabolic rate, short generation time and high levels of ascorbate peroxidase activity. The following are the aim of this study:

- To purify Ascorbate Peroxidase (APX) from *Vigna radiata* to homogeneity.
- To study the molecular characteristics of purified APX from *Vigna radiata.*
- Studying the effect of inhibitors and stability of APX.
- To study the kinetic parameters of APX.

Literature Review

3. LITERATURE REVIEW

3.1. PEROXIDASES

Peroxidases are haem-containing enzymes found in bacteria, fungi, plants and animals that use hydrogen peroxide as the electron acceptor to catalyze a number of oxidative reactions. They catalyze the oxidation of cellular components by either hydrogen peroxide or organic hydroperoxides:

$$AH_2 + H_2O_2\,(ROOH) \longrightarrow A + 2\,H_2O\,(ROH + H_2O)$$

Most haem peroxidases catalyze the univalent oxidation of the electron donor (AH_2) by hydrogen peroxide, forming the primary radical product (AH) twice:

$$2\,AH_2 + H_2O_2 \longrightarrow 2\,AH + 2H_2O$$

$$2\,AH \longrightarrow AH_2 + A$$

The stable oxidation product (A) is produced by the interaction of the primary product. With respect to physiological function, the peroxidases are divided into two groups:

i. peroxidases, where the oxidation products of the electron donors have physiological roles, and
ii. peroxidases whose function lies in the scavenging of hydrogen peroxide or organic hydroperoxides

On the basis of sequence similarity, fungal, plant and bacterial haem-peroxidases can be viewed as members of a superfamily consisting of the following three major classes (Welinder, 1992)

i) Class I consists of the intracellular peroxidases which include yeast cytochrome c peroxidase (CcP), ascorbate peroxidase (APX) and bacterial catalase-peroxidases (Welinder, 1991)

ii) Class II consists of secretory fungal enzymes (e.g. manganese peroxidase and lignin peroxidase) and

iii) Class III contains the secretory plant peroxidases such as horseradish peroxidase (HRP).

The above classifcation is based on sequence comparisons and enzyme localization rather than on function; thus APX, which preferentially reacts with a small substrate molecule, ascorbate, is in the same class as CcP, which reacts with another protein, cytochrome c.

In plants, the peroxidases participating in the lignin biosynthesis, degradation of indloe-3-acetic acid, or biosynthesis of ethylene, belong to the former group. The plant peroxidases of this group use a wide range of electron donors and can be referred to as guaiacol peroxidases, because guaiacol has been widely used as the electron donor for the assay. Examples of this group of peroxidases in mammals are thyroxide peroxidases, active in the biosynthesis of thyroxin from thyroglobulin, and myeloperoxidase in neutrophills, which has bactericidal activity by producing hypochlorite from chloride. Plant guaiacol peroxidases also play similar fungicidal and bactericidal roles and have a role in wound healing. Hydrogen peroxide or organic hydroperoxides for these peroxidases will be produced by the reactions of the one-(superoxide forming) and two-electron (hydrogen peroxide-forming) oxidases under strict regulation in response to

environmental and cellular signals, so that the peroxides are not over produced.

3.2. ASCORBATE PEROXIDASE - SCAVENGER OF HYDROGEN PEROXIDE

Ascorbate peroxidase (1.11.1.11) is a haem-peroxidase and is the main enzyme responsible for hydrogen peroxide removal in the chloroplasts and cytosol of higher plants. In chloroplasts of higher plants, oxygen consumption in the absence of electron acceptors is accompanied by production of hydrogen peroxide and activated forms of oxygen. Chloroplasts contain several protective systems (such as superoxide dismutase (SOD), alpha-tocopherol and carotenoids), which are effective against various forms of activated oxygen. However, they lack catalase, and the disposal of H_2O_2 is accomplished by other means, one such is through the activity of APX.

Ascorbate peroxidase has a marked preference for ascorbic acid as a reducing substrate, whereas classical plant peroxidases oxidize phenolic compounds at a much higher rate. Ascorbate peroxidase with ascorbate as its reducing substrate is believed to scavenge excess H_2O_2 formed in plant cells under normal and stress conditions, as do glutathione peroxidases in mammals and NAD(P)H peroxidase in bacteria.

3.2.1. CLASSIFICATION OF ASCORBATE PEROXIDASE

The ascorbate peroxidases belong to the oxidoreductases class of enzyme and have the following enzyme classification number EC - 1.11.1.11

1Oxidoreductases

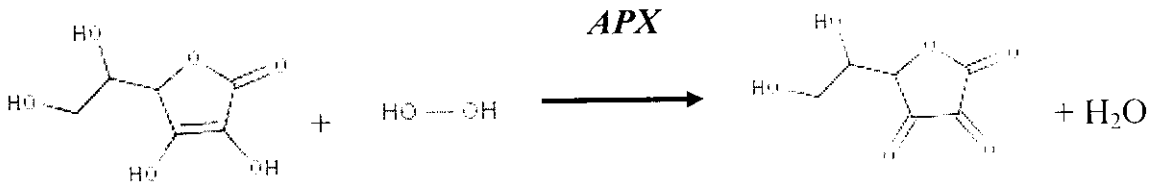
1.11 Acting on a peroxide as acceptor

1.11.1 Peroxidases

1.11.1.11 L-ascorbate peroxidase

3.2.2. ASCORBATE PEROXIDASE MOLECULAR REACTION

The enzyme utilises the antioxidant, ascorbic acid (Vitamin C) to scavenge hydrogen peroxide produced by superoxide dismutase. Ascorbic acid is a strong antioxidant that is effective in scavenging superoxide (O2-'), hydroxyl (OH') radicals and singlet oxygen. It can also remove H_2O_2 in the following reaction which is catalysed by ascorbate peroxidase:



3.2.3. ASCORBATE – GLUTATHIONE CYCLE

- Ascorbate Peroxidase utilizes ascorbate (AsA) as its specific electron donor to reduce H_2O_2 to water with the concomitant generation of monodehydroascorbate (MDHA), a univalent oxidant of AsA.

- MDHA is spontaneously disproportionated to AsA and dehydroascorbate (DHA). MDHA is also directly reduced to AsA by the action of NAD(P)H-dependent MDHA reductase.

- DHA reductase utilizes glutathione (GSH) to reduce DHA and thereby regenerate AsA. The oxidized GSH is then regenerated by GSH reductase, utilizing reducing equivalents from NAD (P) H. (*Figure 3.2.3.1.*)

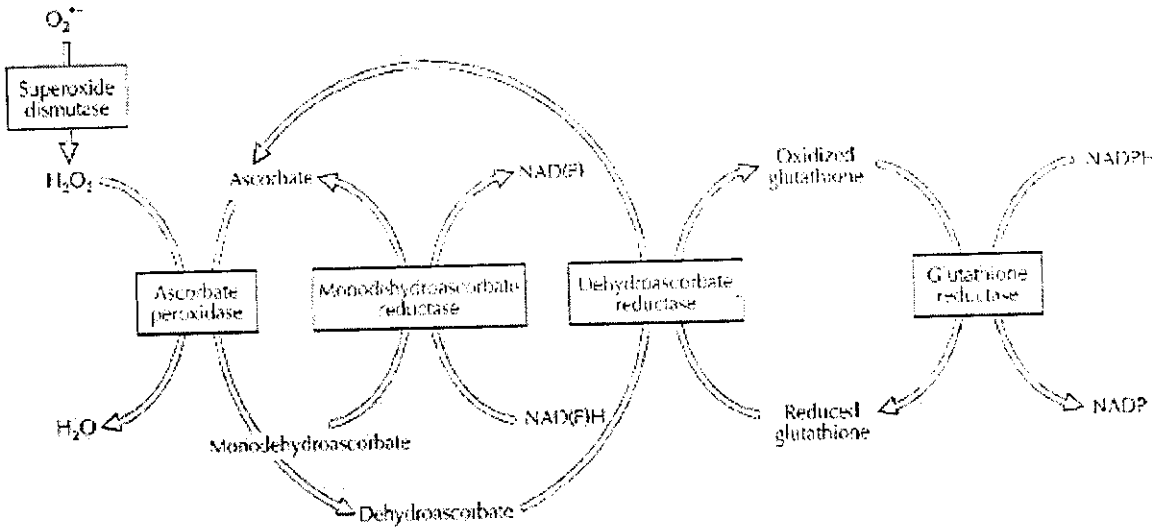


Figure 3.2.3.1. Ascorbate – Glutathione Cycle

Thus, ascorbate peroxidase in combination with the effective AsA–GSH cycle functions to prevent the accumulation of toxic levels of H_2O_2 in photosynthetic organisms (Asada, 1992, 1997). In the chloroplasts of higher plants, in addition to the AsA–GSH cycle located in the stroma the water–water cycle, which is the photoreduction of oxygen to water in PSI by the electrons derived from water in PSII, participates in the detoxification of AOS and the dissipation of the energy of excess photons (Asada, 1999).

Recent studies on the response of Ascorbate Peroxidase expression to some stress conditions and pathogen attack indicate the importance of

Ascorbate Peroxidase activity in controlling the H_2O_2 concentration in intracellular signaling.

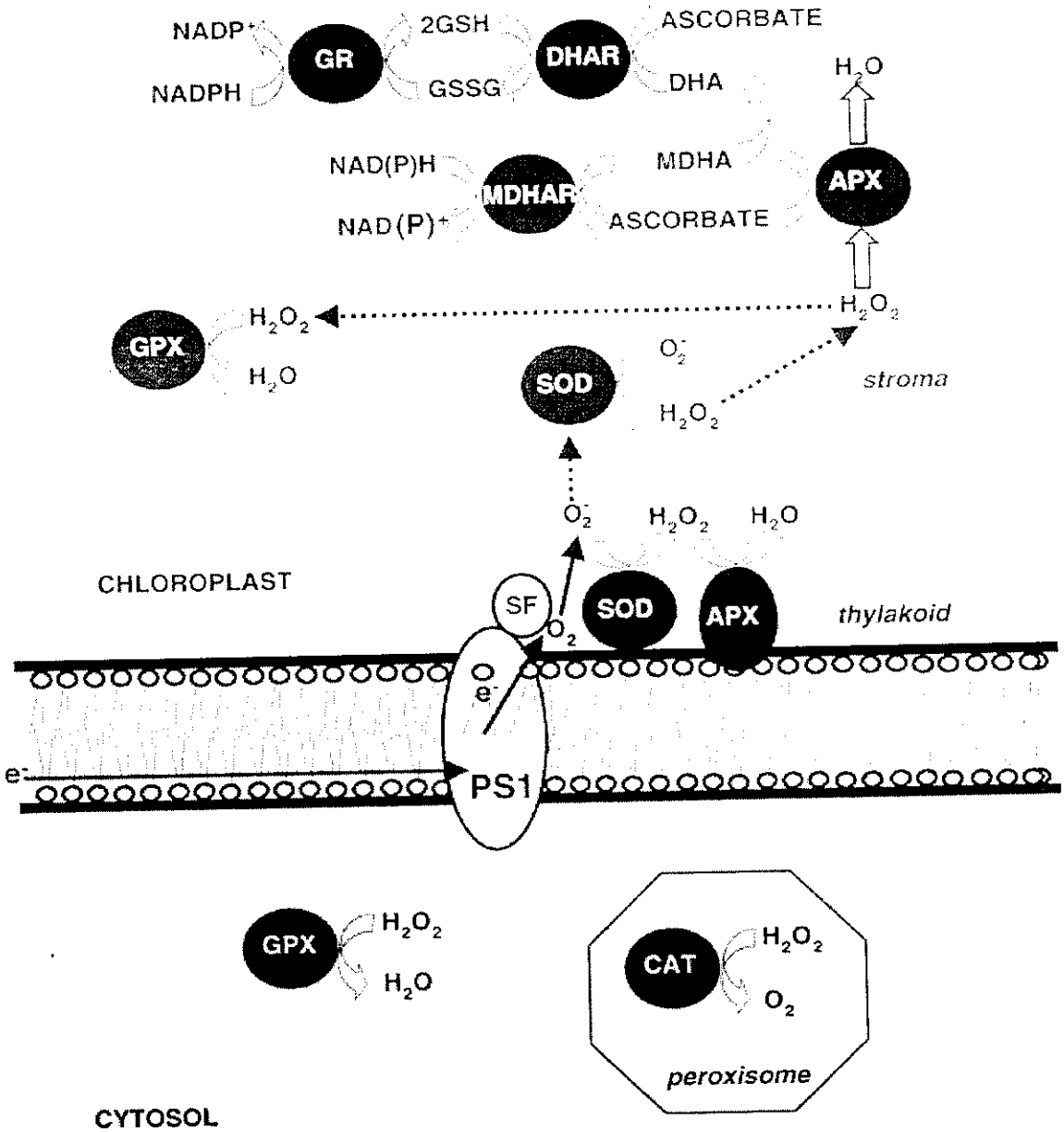


Figure 3.2.3.2. ROS scavenging system in higher plants.

3.2.4. PHYLOGENIC DISTRIBUTION OF ASCORBATE PEROXIDASE

The enzyme was first found in a soluble form by Kelly and Latzko (1979) and later as a thylakoid-bound form by Groden and Beck (1979). Ascorbate peroxidase has been found in the angiosperms so far surveyed: leaves of pea (Gerbling *et al.*, 1984; Mittler & Zilinskas, 1991a), spinach (Nakano and Asada, 1981, Tanaka *et al.*, 1991), duckweed and sycamore (Drotar *et al.*, 1985), maize (Nakano and Asada, 1987), as well as root nodules of legumes (Dalton *et al.*, 1987), endosperms of castor bean (Klapheck *et al.*, 1990), tea leaves (Chen and Asada, 1989), komatsuna (*Brassica rapa)* (Ishikawa *et al.*, 1996), potato tuber (Leonardis *et al.*, 2000) and rice seedlings (Sharma and Dubey, 2004).

Ascorbate peroxidase has also been detected in eukaryotic algae including *Euglena* (Shigeoka *et al.*, 1980), *Chlamydomonas* (Yokota *et al.*, 1988; Miyake *et al.*, 1991), *Zooxanthella* (Lesser and Shiek, 1989), *Chlorella vulgaris* (Takeda *et al.*, 1998) and *Galdieria partita* (Sano *et al.*, 2001). Certain cyanobacteria also show ascorbate peroxidase activity (Tel-Or *et al.*, 1986; Mittler and Tel-Or, 1991c; Miyake *et al.*, 1991), but several species lack the enzyme which has been attributed to the absence or low level of AsA in these organisms The ascorbate peroxidase-lacking cyanobacteria decompose hydrogen peroxide only by catalase, and on peroxidase dependent decomposition using a photoreductant as the electron donor is observed when the fate of H_2O_2 is followed (Miyake *et al.*, 1991). Thus, the system scavenging hydrogen peroxide by using peroxidase was first acquired during the evolution of cyanobacteria, which allowed a

lowered steady state concentration of hydrogen peroxidase in cells as compared with scavenging by disproportionation with catalase.

In addition to photosynthetic organisms, ascorbate peroxidase has been found in the protozoan *Trypanosoma cruzi* (Boveris *et al.*, 1980), in the salivary gland of insects such as *Helicoverpa zea* (Mathews *et al.*, 1997), but not in the fungi and mammals. The only animal source of ascorbate peroxidase is the bovine eye tissue which was detected (Kaul *et al.*, 1988) and later purified and characterized, (Wada *et al.*, 1998), wherein its enzymatic properties were found to be different from those of the plant enzymes.

3.2.5. ASCORBATE PEROXIDASE ISOENZYMES

Ascorbate Peroxidase isoenzymes are distributed in at least five distinct cellular compartments (Chen and Asada, 1989 ; Miyake *et al.*, 1993 ; Yamaguchi *et al.*, 1995a,b; Bunkelmann and Trelease, 1996; Ishikawa *et al.*, 1996a,b, 1998; Jiménez *et al.*, 1997; Leonardis *et al.*, 2000).

- Stromal Ascorbate Peroxidase (sAPX)
- Thylakoid membrane-bound Ascorbate Peroxidase (tAPX) in chloroplasts,
- Microbody (including glyoxysome and peroxisome) membrane-bound APX (mAPX)
- Cytosolic Ascorbate Peroxidase (cAPX)
- Mitochondrial membrane-bound Ascorbate Peroxidase (mitAPX)

Two isozymes of ascorbate peroxidase have been found in tea leaves, and their properties were compared (Chen and Asada, 1989). One of them

(chloroplastic isozyme) is localized and scavenges hydrogen peroxide in chloroplast, and the other (cytosolic isozyme) is the major isozyme in non-photosynthetic tissues and seems to be localized in other cellular components that the chloroplast.

The chloroplastic and cytosolic isozymes of ascorbate peroxidase are distinguished from each other in the following properties (Chen and Asada, 1989, 1990):

- As compared with the cytosolic isozyme, the chloroplastic isozyme has a very short life time in the ascorbate-depleted medium.
- The sensitivities of the chloroplastic isozyme to thiol reagents and to suicide inhibitors are higher than those of the cytosolic isozyme.
- The chloroplastic isozyme is more specific to ascorbate as the electron donor than the cytosolic isozyme - for example, the cytosolic isozyme can oxidize pyrogallol at an appreciable rate.

According to these criteria, the ascorbate peroxidases purified so far can be referred to either the chloroplastic or cytosolic isozymes. The algal ascorbate peroxidase (Shigeoka *et al.*, 1979) as well as the enzymes from pea leaves (Gerbling *et al.*, 1984, Mittler and Zilinskas, 1991a) and root nodules of legumes (Dalton *et al.*, 1987) are supposed to be of the cytosolic type, because they oxidize pyrogallol at a higher rate than ascorbate. Ascorbate peroxidase in spinach chloroplasts loses its activity in less than a minute in an ascorbate-depleted medium, and this is the case also for the purified enzymes from spinach and tea leaves (Nakano and Edwards, 1987, Chen and Asada, 1989).

3.2.6. EVOLUTION OF ASCORBATE PEROXIDASE ISOENZYMES

A phylogenetic tree constructed using the deduced amino acid sequences of the catalytic active domains of APX isoenzymes, not including the transit peptide and the membrane-spanning sequences, shows that APX isoenzymes in higher plants and algae can be divided into four groups (cAPX I, cAPX II, chlAPX, and mAPX) (*Figure 3.2.6.1.*). The earliest event in APX evolution resulted in the appearance of separate groups: a cAPX I group, a chlAPX group, and mAPX and cAPX II groups. It is likely that cAPX I, chlAPX, and mAPX share common features conserved among plant species, whereas the cAPX II group may have evolved from cAPX in a species-specific manner. chlAPX isoenzymes are divided into two types that arose by alternative splicing from a single gene and by different genes at a very recent stage.

APX isoenzymes in higher plants show high homology (70–90%) within each group. Furthermore, the four groups of APX isoenzymes show 50–70% homology with each other. The Arg-38, Asn-71, Glu-65, and Asp-208 residues around the distal His-42 and proximal His-163 residues of pea cAPX are conserved in all APX groups as well as the entire plant peroxidase family. These residues are essential for binding of the ligand haem (Welinder, 1992).

Trp-179 is conserved in most APX groups and is the third participant in a hydrogen-bonding network together with the proximal His-163 and Asp-208 residues; however, the residue in the cAPX II group is changed to Phe, which is the predominant residue at this position in the Class II and Class III peroxidases of higher plants (Jespersen *et al.*, 1997).

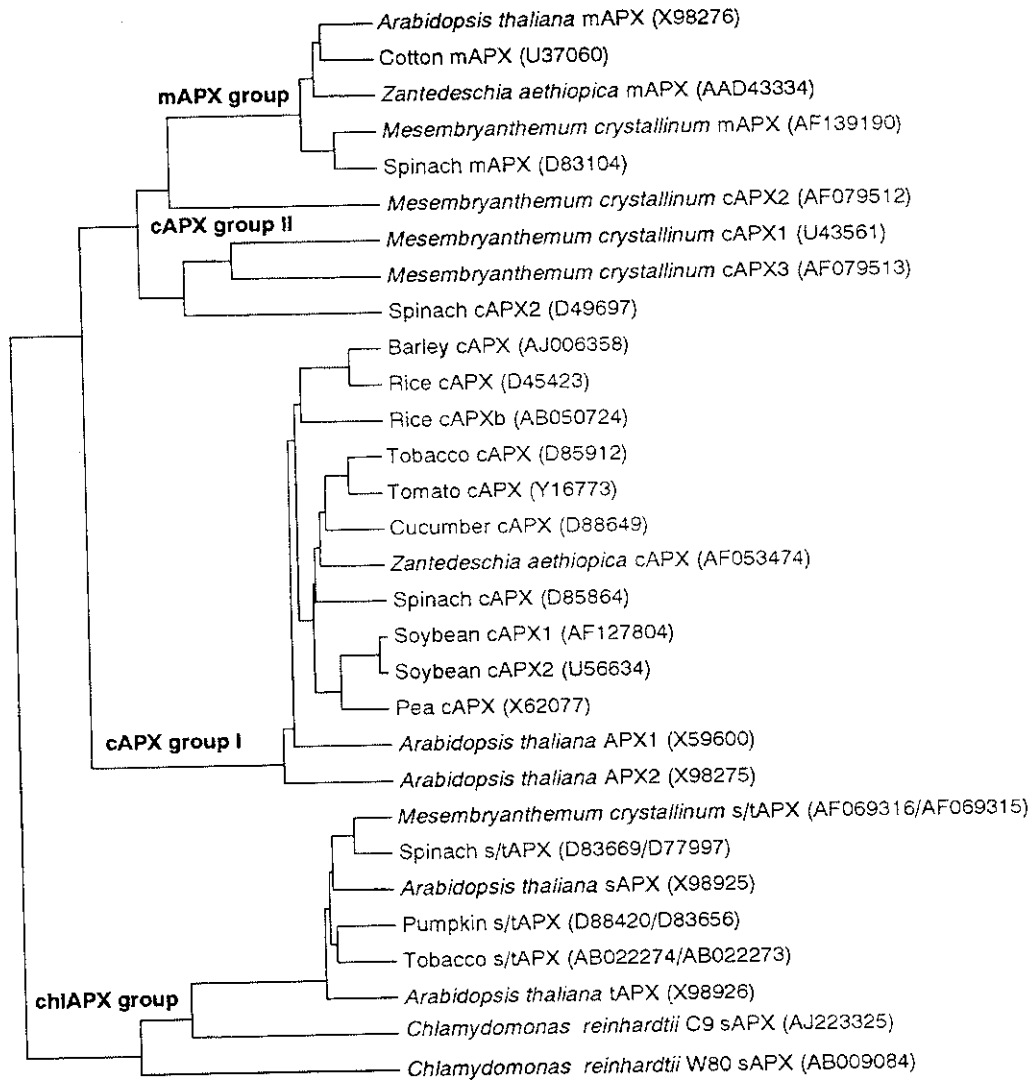


Figure 3.2.6.1. Phylogenetic tree for APX isoenzymes

The change from Trp-41 to Phe is also found in the cAPX II group as well as in Class II and III peroxidases. Phe-175 is conserved in all APX groups except for a Trp in the chlAPX group, suggesting that the Trp residue may be the major determinant of the greater specificity toward AsA of chlAPX isoenzymes. Four additional regions in the internal sequences and seven residues in the C-terminal extension of the catalytic domain are

observed in chlAPX isoenzymes, but not in the other APX isoenzymes. Therefore, chlAPX isoenzymes generally have larger molecular weights than cAPX and mAPX, suggesting that these additional sequences may be associated with the greater instability of chlAPX isoenzymes.

3.2.7. ENZYMATIC PROPERTIES OF ASCORBATE PEROXIDASE ISOENZYMES

The molecular and enzymatic properties of APX isoenzymes are different from those of other haem-peroxidases such as GP. APX isoenzymes have high specificity for AsA as the electron donor, which is especially the case for the chloroplastic APX (chlAPX) and mitAPX isoenzymes (Yoshimura *et al.*, 1998; Asada, 1999; Leonardis *et al.*, 2000). One of the most characteristic properties of APX is its instability in the absence of AsA. Under conditions where the concentration of AsA is lower than 20 μM, APX activity is rapidly lost. The half-inactivation times of chlAPX and mitAPX are less than 30 seconds, while those of cAPX and mAPX are about 1 hr or more (Chen and Asada, 1989; Miyake *et al.*, 1993; Ishikawa *et al.*, 1998; Yoshimura *et al.*, 1998; Leonardis *et al.*, 2000). The instability of APX seems to be one reason that APX was not found for a long time in photosynthetic organisms.

ChlAPX isoenzymes exist in a monomeric form, but cAPX is a dimer consisting of identical subunits with a molecular mass of 28 kDa (Mittler and Zilinskas, 1991a; Miyake *et al.*, 1993). As for chlAPX isoenzymes, the molecular mass of tAPX is about 4.5 kDa larger than that of sAPX (33.2 kDa); the difference in molecular mass between tAPX and sAPX is related to the requirement for membrane binding (Chen and Asada, 1989; Miyake *et*

al., 1993; Ishikawa *et al.*, 1996a). The molecular masses (31 kDa) of mAPX and mitAPX are similar (Yamaguchi *et al.*, 1995a; Ishikawa *et al.*, 1998; Leonardis *et al.*, 2000).

3.2.8. CRYSTAL STRUCTURES OF ASCORBATE PEROXIDASE

The crystal structure of recombinant pea cytosolic ascorbate peroxidase has been determined for data between 8 to 2.2 Å resolution (Patterson and Poulos, 1995) which is as shown in the *Figure 3.2.8.1.* The refined model consists of four ascorbate peroxidase monomers consisting of 249 residues per monomer assembled into two homodimers, with one heme group per monomer. The ascorbate peroxidase model confirms that the pea cytosolic enzyme is a noncovalent homodimer held together by a series of ionic interactions consists of four ascorbate peroxidase monomers consisting of 249 residues per monomer assembled into two homodimers, with one heme group per monomer. The ascorbate peroxidase model confirms that the pea cytosolic enzyme is a noncovalent homodimer held together by a series of ionic interactions The active site structures are also the same, including the hydrogen-bonding interactions between the proximal His ligand, a buried Asp residue, and a Trp residue, whose indole ring is parallel to and in contact with the proximal His ligand just under the heme ring. This proximal Trp residue is thought to be the site of free radical formation in cytochrome c peroxidase compound I and is also essential for enzyme activity. The corresponding Trp in ascorbate peroxidase, Trp179, occupies exactly the same position. The most interesting, and possibly functionally important, difference between the two peroxidases is the presence of a cation binding site in ascorbate peroxidase located approximately 8 A from the alpha-carbon atom of Trp179.

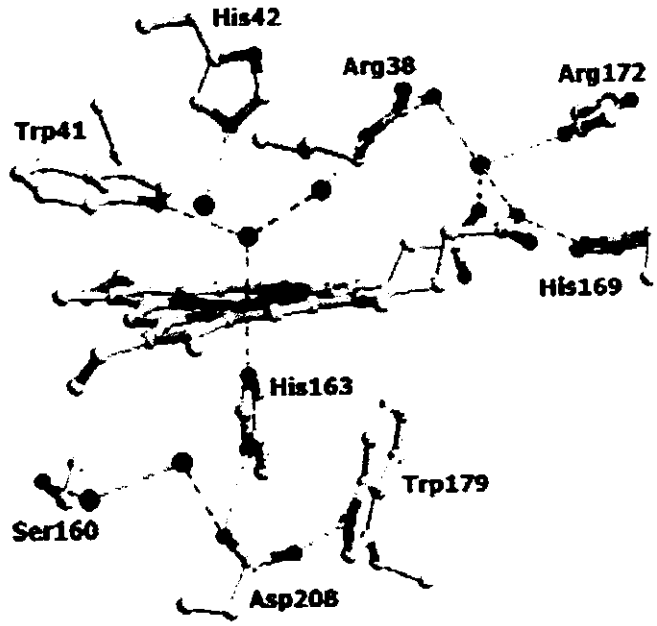


Figure 3.2.8.1. X-ray crystal structure of the active site residues of recombinant pea ascorbate peroxidase

Wada *et al.,* (2003) determined the crystal structure of stromal ascorbate peroxidase (sAPX) from tobacco leaves at 1.6 Å. The overall structure of sAPX was found to be similar to that of cytosolic APX (cAPX) from pea and cytochrome c peroxidase (CCP) from yeast with a substantial difference in a loop structure located in the vicinity of the heme. The side chain of Arg169 in sAPX corresponding to His169 in cAPX and His181 in CCp extended in the opposite direction from the heme, forming two hydrogen bonds with carbonyl groups in the loop structure and this characteristic conformation of Arg169 owing to the loop structure has been attributed to the rapid inactivation sAPX.

Sharp *et al.,* (2003) determined the crystal structure of ascorbate peroxidase – ascorbate complex, defining the ascorbate-binding interaction

and proposed a new mechanism for electron transfer challenging the existing views of substrate oxidation in peroxidases.

Yadav *et al.*, (2008) investigated the Trp-208 residue of ascorbate peroxidase enzyme from *Leishmania major* through point mutagenesis of the Trp-208 with Phe. This enzyme which is considered to be a hybrid between CcP and APX was found to require the Trp-208 residue for electron transfer from cytochrome c to heme ferryl but was not indispensable for ascorbate or guaiacol oxidation.

3.2.9. ARTIFICIAL SUBUSTRATES OF ASCORBATE PEROXIDASE

In addition to oxidizing AsA, cAPX and mAPX of higher plants and algal APXs can also oxidize artificial electron donors such as pyrogallol or guaiacol at appreciable rates (Chen and Asada, 1989; Ishikawa *et al.*, 1995, 1996b; Yoshimura *et al.*, 1998; Asada, 1999). Ascorbate peroxidase can also oxidize hydroxamic acid and phenol derivatives, the primary radical products of which inactivate the enzyme (Chen and Asada, 1990).

3.2.10. INHIBITORS OF ASCORBATE PEROXIDASE

APX, a haem-containing enzyme whose prosthetic group is protoporphyrin, is inhibited by cyanide and azide (Shigeoka *et al.*, 1980; Chen and Asada, 1989). APX is also inhibited by thiol-modifying reagents such as p-chloromercuribenzoate and suicide inhibitors such as hydroxylamine, p-aminophenol, and hydroxyurea (Chen and Asada, 1990).The p-aminophenol radicals oxidize ascorbate (AsA) to produce monodehydroascorbate radicals. Kinetic analysis indicates that p-

aminophenol radicals also oxidize monodehydroascorbate to dehydroascorbate. Incubation of AsA peroxidase from tea leaves and hydrogen peroxide with p-aminophenol, p-cresol, hydroxyurea, or hydroxylamine results in the inactivation of the enzyme. No inactivation of the enzyme was found upon incubation of the enzyme with these compounds either in the absence of hydrogen peroxide or with the stable oxidized products of these compounds. The enzyme was protected from inactivation by the inclusion of AsA in the incubation mixture. The radicals of p-aminophenol and hydroxyurea were produced by AsA peroxidase as detected by their ESR signals. These signals disappeared upon the addition of AsA, and the signal characteristic of monodehydroascorbate was found.

Thus, AsA peroxidase is inactivated by the radicals of p-aminophenol, p-cresol, hydroxyurea, and hydroxylamine which are produced by the peroxidase reaction, and it is protected from inactivation by AsA via the scavenging of the radicals. Thus, these compounds are the suicide inhibitors for AsA peroxidase. Isozyme II of AsA peroxidase, which is localized in chloroplasts, is more sensitive to these compounds than isozyme I. In contrast to AsA peroxidase, guaiacol peroxidase was not affected by these various compounds, even though each was oxidized by it and the corresponding radicals were produced.

Sharma and Dubey, (2004) purified two isoenzymes, APX1 and APX2 from *Oryza sativa* and reported that Ni2+ activated the enzymes at a concentration below 0.01mM while inhibited at a higher concentration.

3.2.11. COFACTORS OF ASCORBATE PEROXIDASE

The cofactor for APX has been found to be haem in almost all the species including *Euglena gracilis* Z (Shigeoka *et al.*, 1980), *Arachis hypogea*, pea, soybean, *Vicia faba, Vicia sativa, Vigna unguiculata*, etc., (Dalton *et al.*, 1987), Spinach (Nakano & Asada, 1987), maize (Koshiba, 1993), Japanese radish (Ohya *et al.*, 1997), but one exception to this generality is the APX from the salivary gland of *Helicoverpa zea*, an insect studied by Mathews *et al.*, (1997) who has reported that this enzyme may not necessarily be an haem-peroxidase. The presence of haem moiety in APX has also been reported in *Chlorella vulgaris* (Takeda *et al.*, 1998), *Gladieria partita* (Sano *et al.*, 2001) and *Trypanosoma cruzi*, a parasite (Wilkinson *et al.*, 2002).

3.2.12. STABILITY OF ASCORBATE PEROXIDASE

Shigeoka *et al.*, (1980) investigated the stability of APX in *Euglena gracilis Z* cytosol and found that its maximum activity was maintained up to 37°C, which when incubated for 5 min. at 40°C got inactivated. This loss in activity at 40°C was reduced by pre-incubation with 30% sucrose and 0.05 mM Ferrous Sulfate which ultimately retained 88% of the original activity after 5 minutes but complete inactivation was observed at 52°C. Gerbling *et al.*, (1984) reported that there was loss of activity of ascorbate peroxidase after incubation of crude extracts from *Pisum sativum* shoots with either pronase or chymotrypsin.

Dalton *et al.*, (1987) investigated the temperature stability of APX from the root nodules of a variety of legumes such as *Arachis hypogea, Glycine max, Pisum sativum, Vicia faba, Vicia sativa, Vigna unguiculata,*

etc., and found that at 4°C the enzymes had a half-life of 10 hours. The APX of *Pisum sativum*, gets completely inactivated at 70°C when incubated for 5 min, while loss of activity has been reported when incubated for 10 minutes at 100°C.

In *Glycine max*, they monitored the melting temperatures of the Ferric and Ferric-Cyanide derivatives of the enzyme by circular dichroism spectroscopy and found it to be 49°C and 57°C respectively. They also studied the storage stability of APX from *Glycine max* and observed that the crude extracts of root nodules were stable when stored in 50 mM potassium phosphate buffer (pH 7.0) at -20°C or -80°C for several months. It was also found that the stability of intact soybean nodules could be maintained for 3 years at -80°C. The crude extracts of root nodules at 4°C under aerobic conditions were stable for 6 days but a 25% loss in activity was observed under the same conditions when the duration of storage was prolonged to 15 days.

Nakano and Asada (1987) analyzed the enzyme from *Spinacia oleracea* chloroplast and stated that the enzyme lost its stability under aerobic conditions as it was prone for oxidation but stabilization could be achieved by incorporation of ascorbate and sorbitol. Ishikawa *et al.*, (1996b) stated that the enzyme from the leaves of *Brassica rapa* is quite stable between a pH of 6.5 and 7.5 but completely loses its activity at a temperature of 55°C.

Ohya et al., (1997) purified the APX from roots of Japanese radish (Raphanus sativus) and reported the stability of the enzyme in the absence of ascorbate. Investigations revealed that dialysis of crude enzyme preparations for 24h against a buffer depleted of ascorbate or sorbitol results in 90% and

20% loss in enzyme activity, respectively. In air saturated solutions, salting-out results in a significant loss in enzyme activity, therefore exchange to N_2 gas was performed prior to salting-out of the root enzyme.

The activity of APX from *Chlorella vulgaris* was observed to be fully retained up to 50°C between a pH of 6.0 and 7.5 but at a temperature of 60°C the enzyme completely lost its activity. The half inactivation time of the enzyme from *Chlorella vulgaris* has been found to be 15 min when incubated in an ascorbate-depleted medium (Takeda *et al.*, 1998).

The enzyme from the mitochondria of potato (*Solanum tuberosum*) was investigated and found to be completely inactivated within 30s in ascorbate-depleted medium under anaerobic conditions (Leonardis *et al.*, 2000). In contrast, it has been reported by Takeda *et al.*, (2000) that the enzyme from a halotolerant *Chlamydomonas sp. W80* strain is relatively stable in an ascorbate-depleted medium. Sano *et al.*, (2001) stated that the recombinant enzyme from *Galdieria partita* remained active for at least 180 min after depletion of ascorbate.

Sharma and Dubey, (2004) investigated the stability of two isoenzymes, APX 1 and APX 2 from the leaves of *Oryza sativa* in the presence of polyethylene glycol (PEG 6000) after thier purifiaction and reported that 40% of PEG in assay medium resulted in 50% inhibition of APX 1 and 65% inhibition of APX 2 with increase in enzyme Km values but the enzyme activity was found to be restored by the incorporation of 1 M proline, glycine betaine or sucrose in presence of PEG in the assay medium.

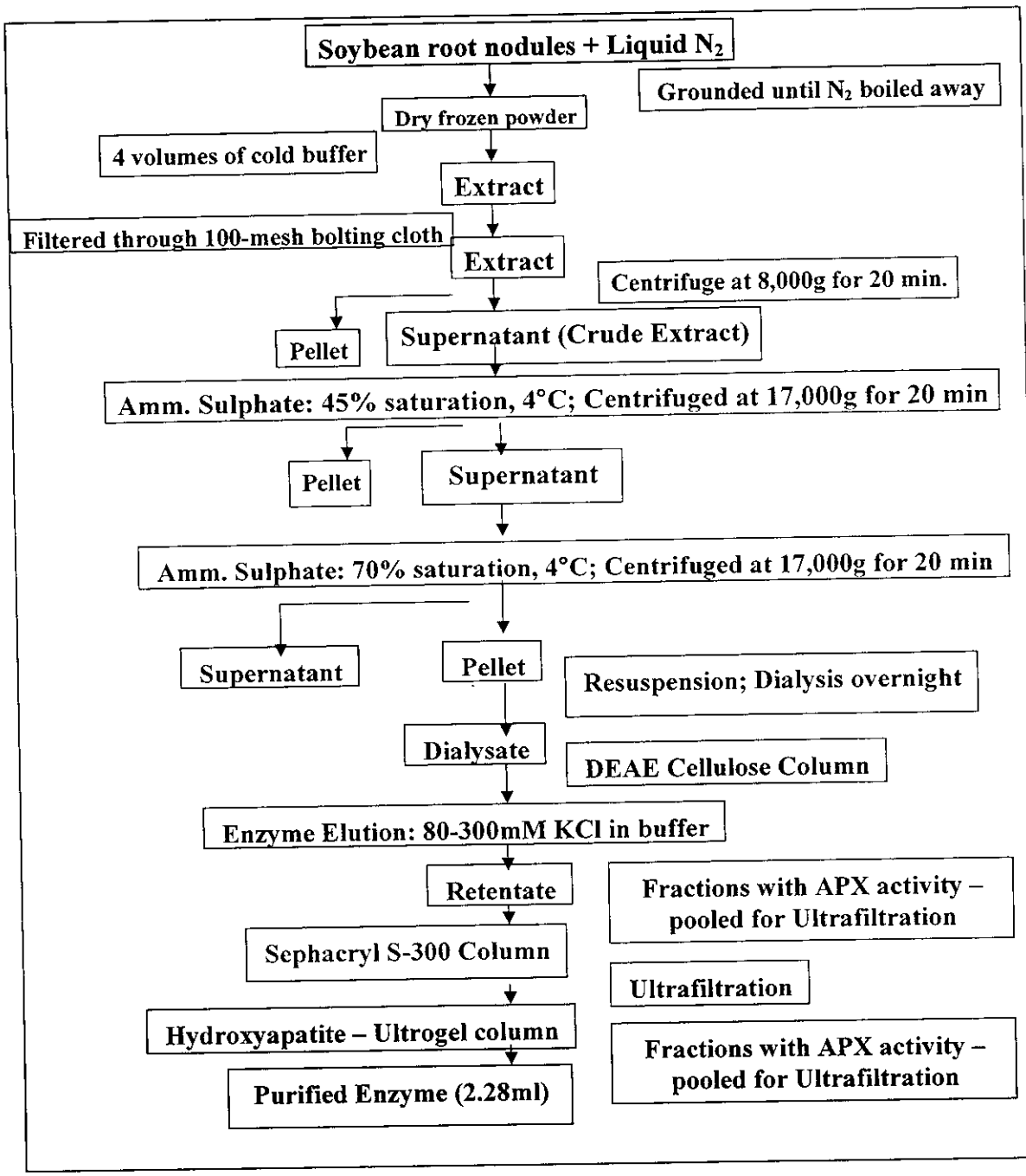
Tsai *et al.*, (2005) investigated the activity of ascorbate peroxidase in the roots of rice seedlings in response to NaCl and H_2O_2 levels and reported an increase in enzyme activity.

3.2.13. PURIFICATION OF ASCORBATE PEROXIDASE

During purification of APX, all or most of the steps have been reported to be carried out between 0-4°C. The general purification strategy adopted for the enzyme's purification involves ammonium sulphate fractionation, followed by dialysis or ultrafiltration. The sample after concentration is loaded onto an anion exchanger for further purification which is followed by hydrophobic interaction chromatography. Dialysis or ultrafiltration is carried out in between the chromatographic steps of purification.

Shigeoka *et al.*, (1980) purified the enzyme ascorbate peroxidase from *Euglena gracilis* Z and reported some of its properties. Gerbling *et al.*, (1984) reported the partial purification of soluble ascorbate peroxidases from the leaves of *Pisum sativum* and investigated their properties.

Dalton *et al.*, (1987) purified ascorbate peroxidase from soybean root nodules and investigated its properties and distribution in several other legume root nodules including *Glycine max* (Soy bean), *Arachis hypogea*, *Pisum sativum*, *Vicia faba*, *Vicia sativa*, *Vigna unguiculata* etc., The purification of APX from soybean was carried out by the following method (*Figure 3.2.13.1.)*



igure 3.2.13.1. Purification of Ascorbate Peroxidase from soybean (Glycine max) root nod

Chen & Asada (1989) reported the purification of two APX isoenzymes from tea leaves (*Figure 3.2.13.2.*). They homogenized fresh tea leaves with homogenization medium and filtered through 8 layers of cheese cloth. After filtration, the homogenate was centrifuged for 10min at 10,000g which was followed by ammonium sulphate fractionation at 35% saturation and centrifugation. The supernatant was loaded onto a Butyl - Toyopearl column and eluted with a linear gradient (25-0% saturation) of ammonium sulphate solution. The fraction with maximum activity was pooled and dialyzed against 10mM potassium phosphate buffer (pH 7.6), 1mM Asa and 1mM EDTA for 10 hours with four changes of this buffer; this was then followed by the addition of sorbitol to give a final concentration of 20%(v/v) and then the enzyme was loaded onto DEAE – Sephacel column. The elution of the two different isoenzymes APX1 and APX 2 was achieved by elution at 110-130mM and 170-190mM NaCl respectively.

The active fractions of the two isoenzymes were separately pooled and concentrated by ultrafiltration. The two isoenzymes were separately loaded onto a gel filtration column, Sephadex G-100 followed by ultafiltration; these two steps were repeated until the phosphate concentration fell well below 0.1mM. The sample was then applied to a column of Hydroxylapatite. Isoenzyme I was not absorbed while isoenzyme II got absorbed and eluted. The active fractions were collected and stored at -84°C which was stable for two months.

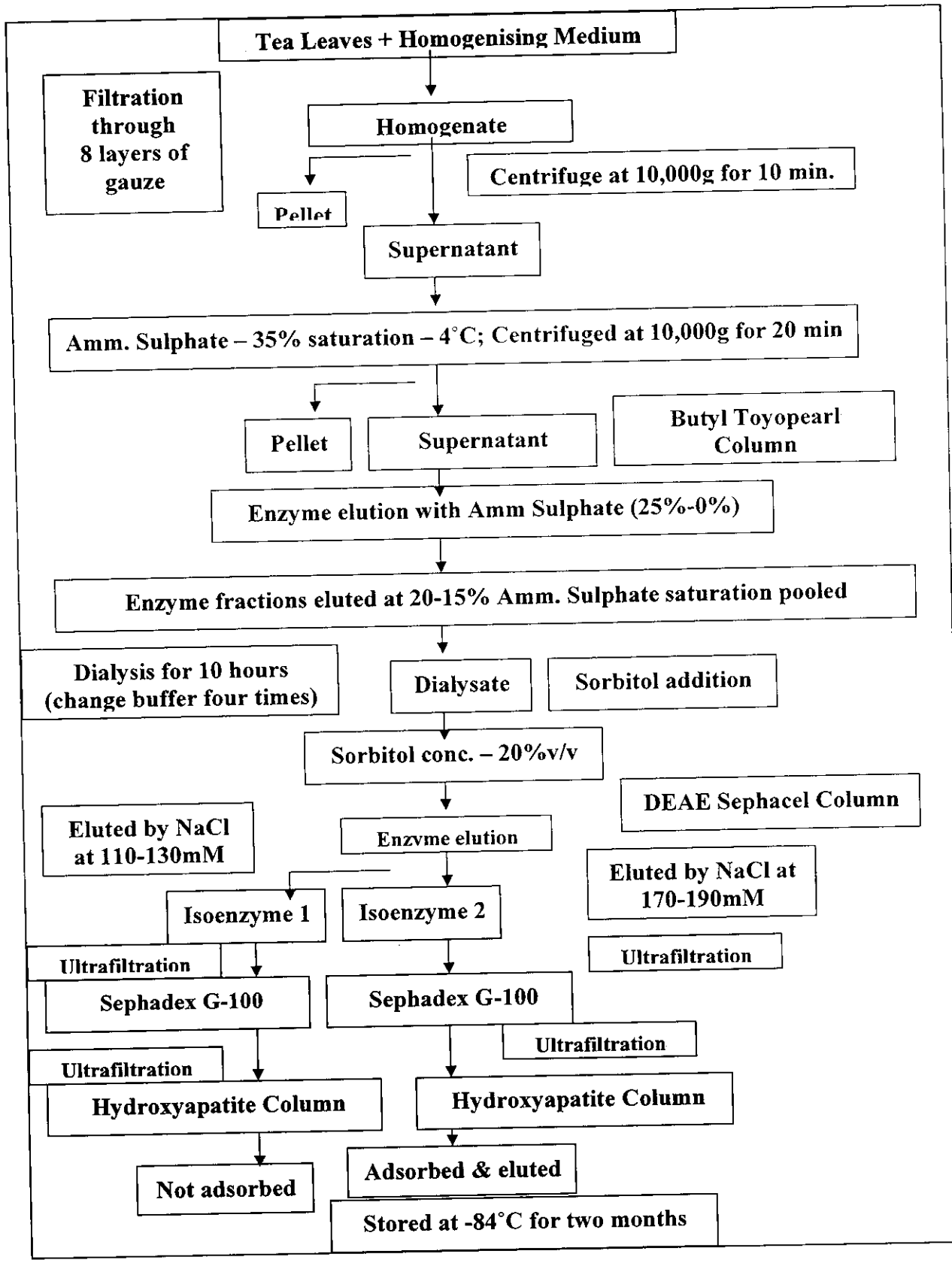


Figure 3.2.13.2. Purification of Ascorbate Peroxidase isoenzymes from tea leaves

Mittler and Zilinskas, (1991a) purified cytosolic ascorbate peroxidase from pea shoots (*Figure 3.2.13.3.*). Tissue homogenization, ammonium sulphate fractionation and dialysis were performed at 4°C, while liquid chromatography steps were carried out at room temperature. The pea shoots were homogenized with ice-cold homogenization medium and the crude extract was filtered through four layers of Miracloth. The homogenate was centrifuged for 30 minutes at 40,000g and the supernatant thus obtained was subjected to ammonium sulphate precipitation at 50% saturation, followed by centrifugation at 10,000g for 20 minutes. The supernatant obtained was again subjected to ammonium sulphate precipitation by bringing its concentration to 90% and centrifuged at 10,000g for 20 minutes. The pellet thus obtained was resuspended in potassium phosphate buffer (pH 7.8) and 1mM EDTA and dialyzed for 16 hours against the same buffer changed twice.

The dialysate was applied to a fast flow ion exchange column (FFQ) and eluted with KCl and the fractions with highest APX activity were pooled and dialyzed again. The dialysate obtained was again loaded onto MonoQ column and eluted with the same salt gradient and the fractions with APX activity were pooled and dialyzed; the resulting dialysate was applied to hydroxyapatite column and after equilibration, washing with a step gradient of potassium phosphate buffer was carried out which resulted in the elution of the enzyme in 15mM buffer fraction. The active fractions were concentrated using ultrafiltration and then applied to Gel filtration column (Superose 12) which resulted in 750µl of purified enzyme which was stored in -70°C

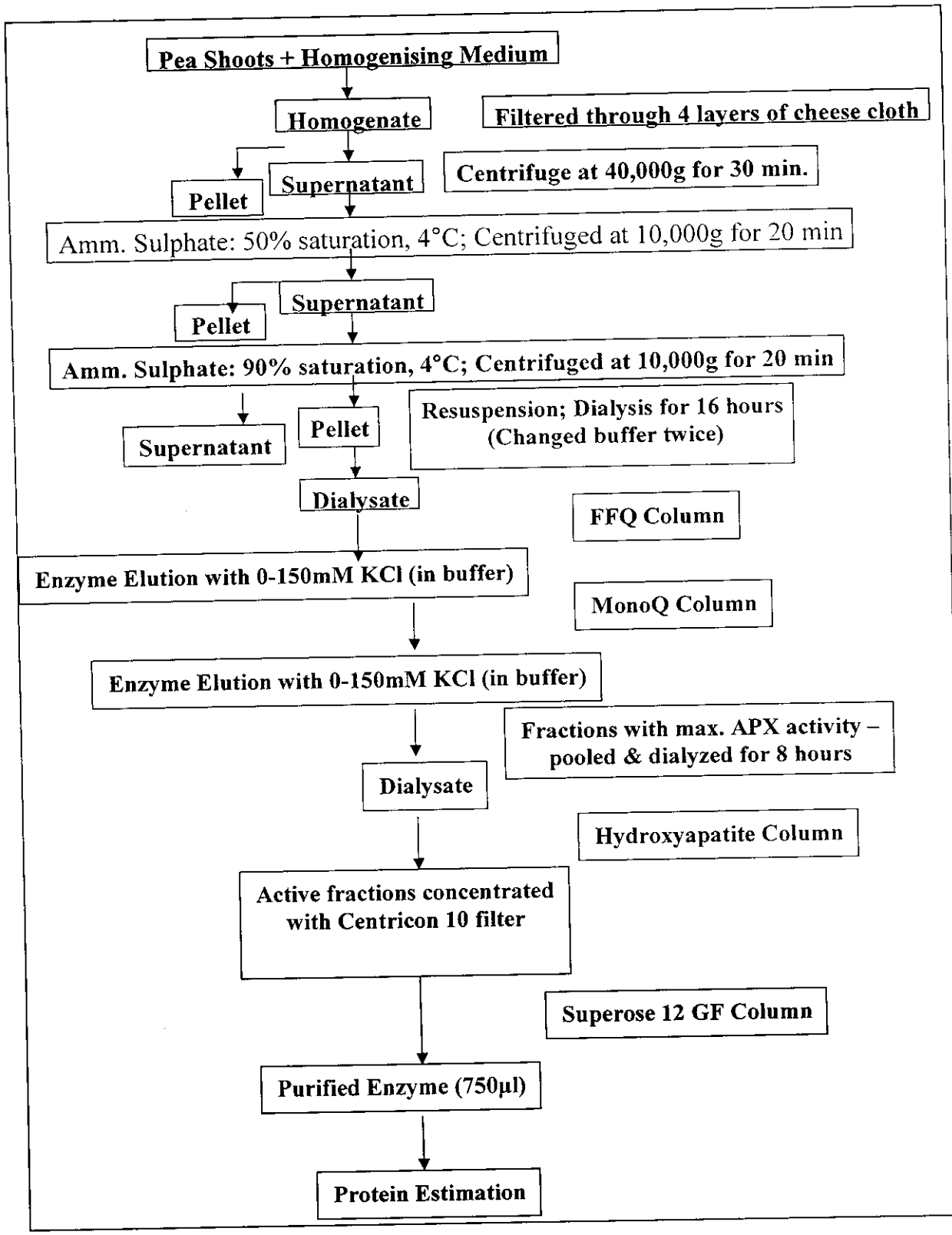


Figure 3.2.13.3. Purification of Ascorbate Peroxidase from Pisum sativum *(pea) shoot*

Ishikawa *et al.,* (1996a) reported the purification of cytosolic ascorbate peroxidase from *Brassica rapa* (komatsuna) using DEAE Sephacel column chromatography, ammonium sulphate treatment followed by hydrophobic interaction chromatography and gel filtration chromatography using butyl toyopearl and TSK G3000SW columns respectively. The purification was carried out to electrophoretic homogeneity and the enzyme was found to be a monomer with a molecular weight of 28kDa. Takeda *et al.,* (1998) purified and characterized APX from *Chlorella vulgaris* to homogeneity and found that it was a monomer with a molecular mass of 32kDa.

The purification and characterization of ascorbate peroxidase from the mitochondria of *Solanum tuberosum* L. (potato) was carried using column chromatography on DEAE-Sephacel, followed by Sephadex G-75, DE-52 and hydroxylapatite and its molecular weight was found to be 31kDa (Leonardis *et al.,* 2000). The isoenzymes (APX-A & APX-B) of *Galdieria partita*, a unicellular red alga were separated from each other by the hydrophobic chromatography step of purification and characterized following individual purification. The enzymes were found to be monomers with molecular weights of 28kDa and the isoenzyme, APX-B was found to have 85% of the total enzymatic activity (Sano *et al.,* 2001). Sharma and Dubey, (2004) have reported the purification of two ascorbate peroxidases – APX 1 and APX 2 to homogeneity from leaves of *Oryza sativa.* Karyotou *et al.,* (2005) have purified and characterized ascorbate peroxidase from the glyoxysomal membrane of *Ricinis communis* and have found the molecular weight of the enzyme to be 34kDa.

3.3. ASCORBIC ACID

Ascorbic Acid (ascorbate, AsA) is an essential molecule for higher plant cells. It is known that AsA is involved in several defense mechanisms against oxidative stress (Noctor and Foyer, 1998), as well as in the regulation of cell proliferation (Arrigoni and De Tulliio, 2002) and elongation (Hidalgo *et al.*, 1989; Cordoba-Pedregosa *et al.*, 1996). In addition, AsA has an important role in excess energy dissipation in thylakoids (Muller-Moule *et al.*, 2003).

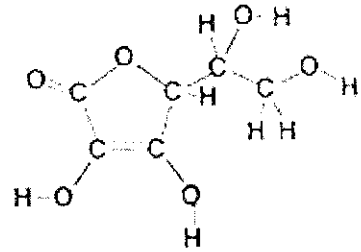


Figure 3.3.1. Structure of Ascorbic acid (Vitamin C)

Ascorbic Acid (Vitamin C), a water soluble vitamin, is a potent antioxidant and studies suggest that this nutrient may prevent premature death from heart disease and cancer. Ascorbic acid has an antibacterial effect against tubercle bacilli (Boissevain and Spillane, 1937; Sirsi, 1952) and many other microorganisms. Vitamin C was originally revealed for its anti-scurvy properties but the focus nowadays is more on its potential cell protection properties. An interesting note is that most animals synthesize their own Vitamin C from glucose, but man must rely upon dietary sources. Smokers and older people have a greater need for this vitamin. Ascorbic acid plays a vital role in the protection against cancer and heart disease, strengthens immunity and may prevent colds or minimize them through its mild antihistamine effects, potent antioxidant, helps prevent cataracts,

increases iron absorption, assists in lowering blood cholesterol and helps treat asthma.

3.4. *Vigna radiata*

Vigna radiata has been grown in India since ancient times. It is still widely grown in Southeast Asia, Africa, South America and Australia. It was apparently grown in the United States as early as 1835 as the Chickasaw pea (Oplinger *et al.*, 1990). It is also referred to as green gram, mung been, golden gram and chop suey bean. Green gram is an erect or sub-erect herb, 0.5-1.3m tall (Purseglove, 1974). Flower is pale yellow. The seed color exhibits a wide range of variations from yellow, greenish yellow, light green, shiny green, dark green, dull green, black, brown, and green mottled with black. Pod color is black, brown or pale gray when mature. 100 seeds usually weigh 3-7g. Green grams are grown widely for use as a human food (as dry beans or fresh sprouts), but can be used as a green manure crop and as forage for livestock. Sprouts are high in protein (21%–28%), calcium, phosphorus and certain vitamins. Because they are easily digested they replace scarce animal protein in human diets in tropical areas of the world.

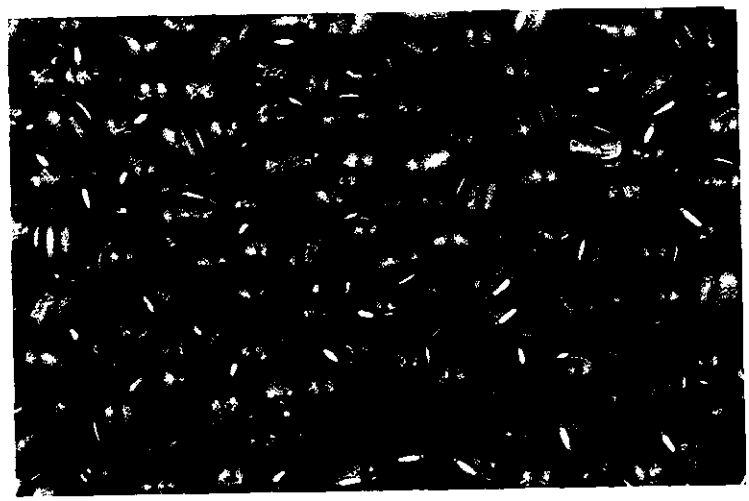


Figure 3.4.1. Seeds of Vigna radiata

Materials and Methods

4. MATERIALS AND METHODS

4.1. MATERIALS

All chemicals used for the study were analytical grade, unless specified otherwise. Double distilled water was used for the experiments, unless specified otherwise. The seeds of *Vigna radiata* (L.) R. Wilczek, *Macrotyloma uniflorum* (Lam.) Verdc., *Glycine max* (L.) Merr., *Cicer arietinum* L., *Trigonella foenum-graecum* L. and *Brassica nigra* L. were obtained from a local market.

4.2. METHODS

4.2.1. SCREENING FOR ASCORBATE PEROXIDASE ACTIVITY

Six seeds were selected and germinated which includes five legumes namely *Vigna radiata*, *Macrotyloma uniflorum*, *Glycine max*, *Cicer arietinum* and *Trigonella foenum-graecum*, and one spice namely *Brassica nigra* to screen for ascorbate peroxidase activity in the cytosol of the respective seedlings.

4.2.2. GERMINATION OF SEEDLINGS

The seeds were surface sterilized with 0.1% mercuric chloride for few seconds and were layered on a cheese cloth substratum spread over a Petri plate and the surface was kept moist with Formulated Mineral Salts (FMS) media (Appendix I).The seedlings were allowed to grow for 48h and then used for the experimental procedures.

4.2.3. TISSUE HOMOGENIZATION

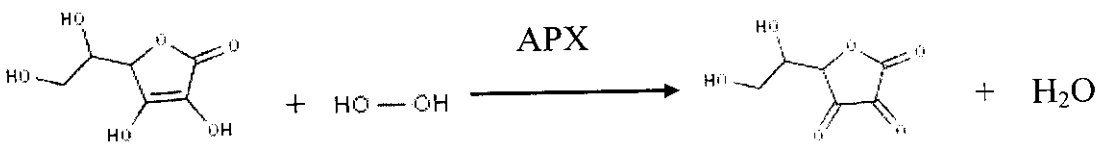
The different seedlings (10g) were homogenized separately using a pestle and mortar with 100ml of ice-cold homogenization media whose

composition is given in Appendix I. An ideal homogenization medium should be capable of maintaining the morphological and functional integrity of organelles. 0.3M mannitol was used so as to be isotonic with the cytosol. After filtration through six layers of cheese cloth, the homogenates of different seedlings were centrifuged at 4000rpm for 5min at 4°C and the supernatants were taken in fresh tubes. The supernatants were once again centrifuged at 6000rpm for 5min to pellet the nuclear fractions. The supernatants were again centrifuged at 10000rpm for 20min to pellet the mitochondria. The resulting supernatants i.e. the cytosol of the various seedlings were screened for ascorbate peroxidase activity.

4.2.4. ASCORBATE PEROXIDASE ASSAY

The ascorbate peroxidase assay was determined as described by Chen and Asada (1989).

4.2.4.1. PRINCIPLE: Ascorbate peroxidase assay is based on the measure of oxidation of ascorbate to dehydroascorbate with the concomitant reduction of hydrogen peroxide to water. The reaction catalyzed by ascorbate peroxidase is as follows:



The reaction was initiated by the addition of hydrogen peroxide, and oxidation of ascorbate was followed by the decrease in absorbance at 290nm. One unit of APX activity is defined as the amount of enzyme that

oxidizes 1μmol of ascorbate per minute at room temperature under the above conditions.

4.2.4.2. PROCEDURE: APX activity was determined in a 2.5mL reaction mixture containing 50mM potassium phosphate buffer (pH 7), 0.5mM ascorbate, 0.1mM hydrogen peroxide and enzyme extract. The activity of ascorbate peroxidase was determined from the decrease in absorbance at 290nm during the time from 1.5-11.5s after the reaction was initiated by the addition of hydrogen peroxide using an absorptivity coefficient of $2.8mM^{-1}$ cm^{-1}(Chen and Asada,1989).

4.2.5. PROTEIN ESTIMATION

The protein was estimated by the method described by Lowry *et al* (1951).

4.2.5.1. PRINCIPLE: Protein reacted with Folin-Ciocalteau reagent to give a blue colored complex. The blue colored complex so formed was due to the reaction of alkaline copper reagent with the protein and the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein. The intensity of color depends on the amount of aromatic amino acids present and thus varied for different proteins. The blue color developed was colorimetrically measured at 660nm.

4.2.5.2. PROCEDURE: The reagents required for the estimation of the protein are described in appendix II. 0.2ml-1.0ml of working standard solution was pipetted out, concentrations varying from 40-200μg/ml into a series of test tubes. The 0.1ml of the given unknown sample was pipetted out

into a test tube. To all the test tubes distilled water was added and the volume was made up to 1ml. Distilled water served as the blank. To all the tubes 5ml of alkaline copper reagent was added and incubated at room temperature for 10min. Then 0.5ml of Folin's reagent was added and kept at room temperature for 20min in the dark. The blue color developed was colorimetrically measured at 660nm and a standard graph was plotted between concentrations of protein and absorbance. The concentrations of the unknowns were found from the standard graph and the protein content in the samples was calculated.

4.2.6. ENZYME PURIFICATION

Tissue homogenization, ammonium sulphate fractionation and dialysis were carried out at 4°C. Chromatography with DEAE-Cellulose and Sepharose 6B were carried out at room temperature.

4.2.6.1 TISSUE HOMOGENISATION

V. radiata seedlings (10g) were homogenized using a pestle and mortar with 100ml of ice-cold homogenization media. After filtration through six layers of cheese cloth, the homogenate was centrifuged at 4000rpm for 5min at 4°C. The homogenate was subjected to ammonium sulphate fractionation followed by dialysis overnight.

4.2.6.2. AMMONIUM SULPHATE FRACTIONATION

The ammonium sulphate fractionation was carried out for the cytosol as well as the homogenate but the subsequent purification steps were carried out for the homogenate of the *V. radiata* seedlings as described by Simpson R.J. (2004).

4.2.6.2.1. PRINCIPLE: Proteins in general, are soluble in dilute aqueous salts but are insoluble or sparingly soluble in water, mostly because the electrostatic charges on the protein surface induce conformations that are not sufficiently solvated to prevent aggregation. The solubility of such proteins can be increased by the addition of small amounts of salt (0.1M-0.2M) that would neutralize these surface electrostatic forces and this phenomenon is called the salting-in or the Debye electrostatic effect. Salting-in works by shielding charges on the protein surface. At low ionic strengths the activity coefficients of the ionizable groups of the proteins are decreased so that their effective concentration is decreased. This is because the ionizable groups become surrounded by counter ions which prevent the interaction between the ionizable groups. Thus protein-protein interactions are decreased and the solubility is increased.

On the other hand, salts at higher concentrations, not only neutralize the electrostatic forces on the protein surface, but also attract water molecules away from the protein. Hence, the salts compete for water molecules that are otherwise available for protein salvation. When the ionic strength is sufficiently increased, proteins are driven to neutralize their surface charges by interacting with one another resulting in fractional precipitation of proteins called as salting-out or the hydrophobic effect. Because of differences in structure and amino acid sequence, proteins differ in their salting in and salting out behaviour. In addition to the salting-in and salting-out effects, neutral salts like Ammonium Sulphate also exert striking and specific effects on the conformational stability and association-dissociation behaviour of proteins.

Ammonium sulphate is a particularly useful salt for the fractional precipitation of proteins. It is available in highly purified form, has great solubility in water allowing for significant changes in the ionic strength, low heat of solution and is inexpensive. Ammonium Sulphate also exerts a stabilizing action on most proteins, and hence it is usually not necessary to carry out precipitation at low temperatures. Furthermore, concentrated Ammonium Sulphate solutions have a low viscosity and density which is an important consideration given that recovery of precipitated protein is typically by centrifugation. Changes in the ammonium sulphate concentration of a solution can be brought about either by adding solid substance or by adding a solution of known saturation, generally, a fully saturated (100%) solution.

4.2.6.2.2. PROCEDURE: The volume of the supernatant obtained after homogenization was measured and the amount of ammonium sulphate required to bring about the desired saturation was calculated from the table found in appendix III. The maximum enzyme activity was found in the 40-60% ammonium sulphate fraction by an initial pilot experiment. The required amount of ammonium sulphate was added to the supernatant slowly and stirred steadily to dissolve it completely at 4°C to give a saturation of 40%. Then the supernatant was centrifuged at 10,000rpm for 15min at 4°C. Then ammonium sulphate was again added to the supernatant thus obtained to bring it to a saturation of 60% and centrifuged at 10,000rpm for 15 min at 4°C. The pellet thus obtained was resuspended in a medium which is similar to the homogenizing medium except for BSA. The resuspended sample was subjected to dialysis.

4.2.6.3. DIALYSIS

The protein solution after the ammonium sulphate precipitation was dialysed to remove the salts. This was carried out by filling the protein solution in dialysis bags and dialysing against a solution of 10 mM potassium phosphate buffer (pH 7), 1mM Ascorbate and 1mM EDTA at 4°C overnight.

4.2.6.4. ION EXCHANGE CHROMATOGRAPHY

Ion exchange chromatography (IEC) using DEAE cellulose was performed as described by Bengt Westerlund (2004).

4.2.6.4.1. PRINCIPLE: Ion exchange chromatography, also called adsorption chromatography is based on the reversible interaction between a charged molecule in solution and an oppositely charged group on the matrix. IEC is a useful and popular method because of its high capacity, high resolving power, mild separation conditions, tendency to concentrate the sample and relatively low cost. The interactions in IEC are due to the electrostatic force between ions in the solute and oppositely charged ligands on the matrix and these interactions are called Coulomb's forces. In addition to the Coulomb's forces, other forces also contribute to the interactions between the protein and the ion exchange medium, notably, Van der Waals forces. The isoelectric point (pI) of the protein to be separated is very essential to perform ion exchange chromatography

Proteins have numerous functional groups that can have both positive and negative charges. Ion exchange chromatography separates proteins according to their net charge, which is dependent on the composition of the

mobile phase. By adjusting the pH or the ionic concentration of the mobile phase, various protein molecules can be separated and eluted. Elution by changing the ionic strength of the mobile phase is a more subtle effect in which the ion from the mobile phase interacts with the immobilized ion in preference over those on the stationary phase. This interaction enables the shielding of the stationary phase away from the protein (and vice versa) and allows the protein to elute.

4.2.6.4.2. PROCEDURE: Slurry of DEAE Cellulose was prepared with twice the volume of 10mM potassium phosphate buffer and centrifuged at 4000rpm for 5min. The bed height that the slurry needs to reach in the column and the volume of sedimented medium were calculated to determine the volume of slurry that should be added to the column. The column media was allowed to settle and then the sample that was obtained after dialysis was added to the column by means of a Pasteur's pipette without disturbing the settled column material. Then equilibriation was carried out with five column volumes of equilibration buffer (10mM phosphate buffer (pH 7.6), 1mM ascorbate, 1mM EDTA, and 0.3M mannitol). Equilibration was followed by elution of the target enzyme at a concentration of 60mM NaCl in the equilibration buffer. The eluted fractions having maximum ascorbate peroxidase activity were pooled for further purification.

4.2.6.5. GEL FILTRATION CHROMATOGRAPHY

Gel filtration chromatography was carried out with Sepharose 6B as described by Helena Hudlund (2004)

4.2.6.5.1. PRINCIPLE: Gel filtration or Size-exclusion chromatography (SEC) is a method in which particles are separated based on their size, or in more technical terms, their hydrodynamic volume. The underlying principle of SEC is that particles of different sizes will elute through a stationary phase at different rates resulting in the separation of a solution of particles based on size. In SEC, the particles of the same size should elute together provided that all the particles are loaded simultaneously or near simultaneously.

In gel filtration, the column is packed with extremely small porous polymer beads designed to have pores of different sizes which are usually made up of cross-linked polymers like polyacrylamide, dextran or agarose called the stationary phase. These pores may be depressions on the surface or channels through the bead. As the solution travels down the column some particles enter into the pores while larger particles cannot enter into as many pores. The larger is the particle; lower will be the overall volume to traverse over the length of the column, and the faster the elution.

SEC is a widely used technique for the purification and analysis of proteins, polysaccharides and nucleic acids. Biologists and biochemists typically use a gel medium called the stationary phase — usually polyacrylamide, dextran or agarose — and filter under low pressure. The advantage of this method is that the various solutions can be applied without interfering with the filtration process, while preserving the biological activity of the particles to be separated. This is one of the most common methods used in protein purification next only to ion-exchange chromatography and is complementary to it.

4.2.6.5.2. PROCEDURE: The Sepharose 6B was stored in 20% ethanol and hence it was washed with five volumes of water after loading onto the column. The fractions 4 to 10 obtained from ion exchange chromatography elution which amounted to 9mL was applied to the column. The column was equilibrated with equilibration buffer containing 10mM phosphate buffer, 1mM Asa, 1mM EDTA, 0.3M Mannitol and 150mM NaCl. NaCl is included in the equilibration buffer to suppress the ionic interactions with the SEC media. The equilibration was followed by elution with the same buffer. The eluted fractions which showed maximum ascorbate peroxidase activity was used for the kinetic study.

Results and Discussion

5. RESULTS AND DISCUSSION

5.1. SCREENING FOR ASCORBATE PEROXIDASE ACTIVITY

Seed development and maturation is a highly orchestered multi-step process during which embryos are formed and supplied with the carbohydrates, proteins and lipids needed for the subsequent germination. During seed development there is an increase in size is due to cell division and expansion, followed by a progressive accumulation of storage compounds.

In the present study, six different seeds including five legumes namely *Vigna radiata*, *Macrotyloma uniflorum*, *Glycine max*, *Cicer arietinum* and *Trigonella foenum-graecum*, and one spice namely *Brassica nigra* were allowed to germinate for 48 hours. The germinated seedlings were screened for ascorbate peroxidase activity in their respective cytosols. The total enzyme activity and specific activity of the respective seedlings were calculated and tabulated as in Table 5.1.1.

Table 5.1.1.: Total Activity and Specific Activity in different seedling samples

Seedling Samples	Enzyme Activity	
	Total Activity x 10^3 (U*/g of seedling)	Specific Activity x 10^3 (U/mg of protein)
Vigna radiata	22.3	0.73
Macrotyloma uniflorum	5.1	0.16
Glycine max	6.9	0.37
Cicer arietinum	12.9	0.44
Trigonella foenum-graecum	11.1	0.73
Brassica nigra	16.3	0.50

* One Unit corresponds to 1µmol of ascorbate oxidized per minute.

The maximum total activity was observed in the case of *Vigna radiata* followed by *Trigonella foenum-graecum, Cicer arietinum, Brassica nigra, Glycine max* and *Macrotyloma uniflorum* while the specific activity was highest in the case of *Trigonella foenum-graecum* followed by *Vigna radiata* and others. Hence *Vigna radiata* was used for further analysis of the enzyme.

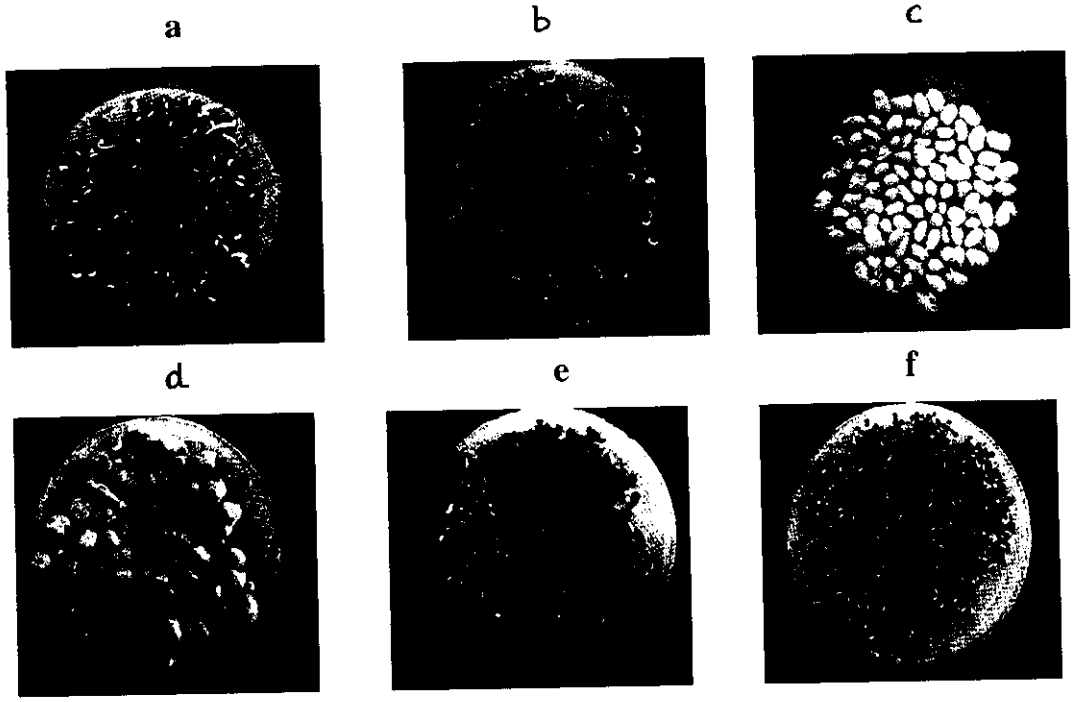


*Figure 5.1.1. Germinating Seedlings after 48 hours – **a**) Vigna radiata, **b**) Macrotyloma uniflorum, **c**) Glycine max, **d**) Cicer arietinum, **e**) Trigonella foenum-graecum, **f**) Brassica nigra*

5.2. AMMONIUM SULPHATE PRECIPITATION STUDIES

The homogenate was subjected to ammonium sulphate precipitation to determine the saturation at which the enzyme gets precipitated. Towards this the fractionation was done at different levels of saturations in the cytosol as well as the homogenate.

5.2.1. AMMONIUM SULPHATE FRACTIONATION OF CYTOSOL

As a pilot experiment, the supernatant (soluble fraction) was separated from the pellet (membrane fraction) after centrifugation of the homogenate. The soluble fraction was subjected to ammonium sulphate fractionation and the pellets thus obtained corresponding to the ammonium sulphate saturation were resuspended separately followed by overnight dialysis. The dialyzed samples were then assayed for APX activity. The total enzyme activity and specific activity obtained in different fractions were calculated and tabulated as below. The maximum ascorbate peroxidase activity was present in the 60%-80% fraction (*Figure 5.2.1.1. & Figure 5.2.1.2.*). The total activity at 40-60% fraction was 6.75% compared to the levels at 60-80% in the cytosol. However, the specific activity of the cytosol in the 20-40% fraction was 22.9% compared to that at 60-80% fraction.

Table 5.2.1.1: Total Activity and Specific Activity in cytosol in different fractions of ammonium sulphate saturations

Ammonium Sulphate Saturation (%)	Enzyme Activity	
	Total Activity x 10^3 (U/g of seedling)	Specific Activity x 10^3 (U/mg of protein)
0-20	-	-
20-40	13.71	1.82
40-60	23.14	0.28
60-80	342.86	7.94
80-100	2.57	0.28

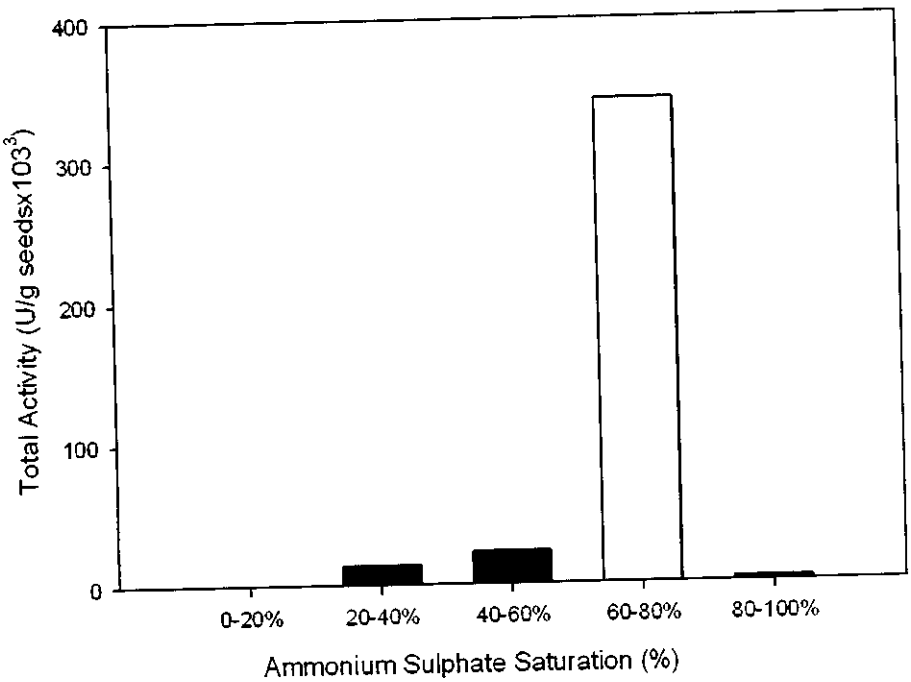


Figure 5.2.1.1: Ascorbate peroxidase total activity in ammonium sulphate fractions of cytosol

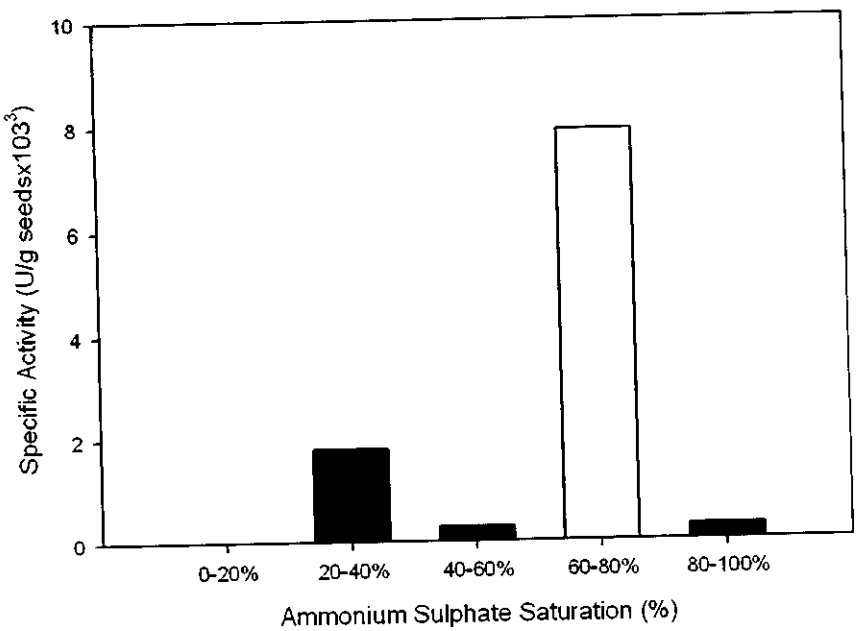


Figure 5.2.1.2. Ascorbate peroxidase specific activity in ammonium sulphate fractions of cytosol

5.2.2. AMMONIUM SULPHATE FRACTIONATION OF HOMOGENATE

In order to include any isoenzyme that might be present in organelle fractions, subsequent purification was carried out using the whole homogenate. The crude extract was subjected to Ammonium Sulphate fractionation after centrifugation at 4000rpm for 5min. The total enzyme activity and specific activity obtained in different fractions were calculated and tabulated as below. The maximum ascorbate peroxidase activity was present in the 40%-60% fraction (*Figure 5.2.2.1.*). Lesser activities were found in the 20-40% and 60-80% fractions and were 33.33% and 48.77% when compared to the major fraction. The specific activities were somewhat higher in other fractions (*Figure 5.2.2.2.*). However, since we were interested in the total concentrations of ascorbate peroxidase the 40-60% fraction was chosen for further purification as the total activities were maximal in that fraction.

Table 5.2.2.1.: Total Activity and Specific Activity in homogenate in different fractions of Ammonium Sulphate saturations

Ammonium Sulphate Saturation (%)	Enzyme Activity	
	Total Activity x 10^3 (U/g of seedling)	Specific Activity x 10^3 (U/mg of protein)
0-20	4.29	0.39
20-40	3.86	0.12
40-60	11.57	0.23
60-80	5.64	0.27
80-100	1.29	0.32

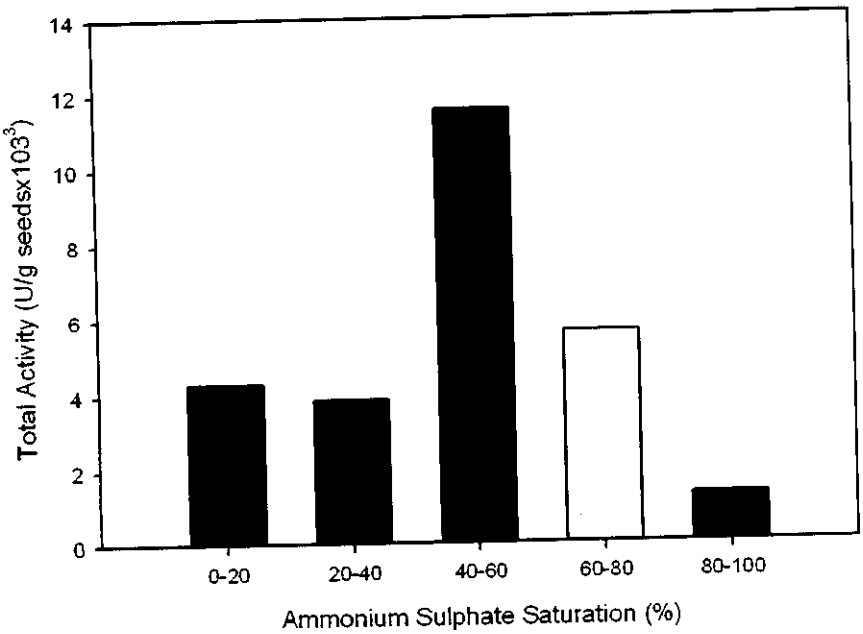


Figure 5.2.2.1. Ascorbate peroxidase total activity in ammonium sulphate fractions of homogenate

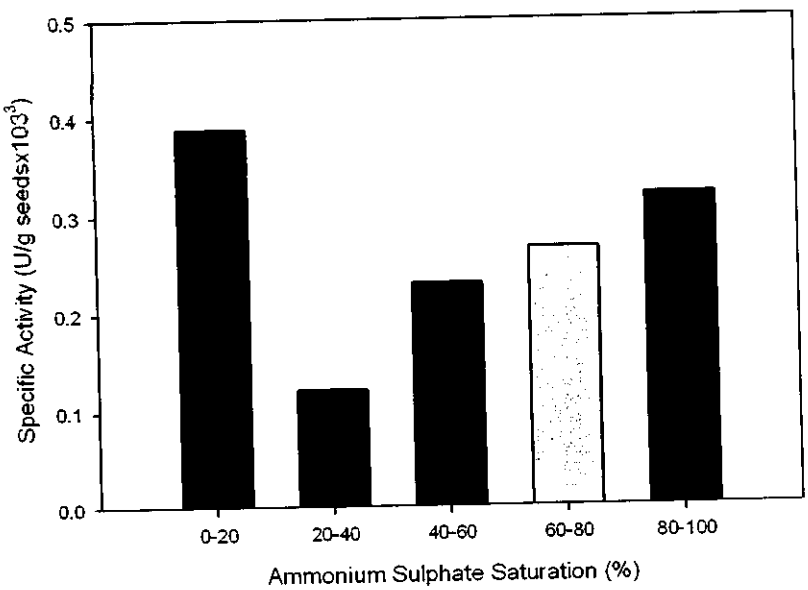


Figure 5.2.2.2. Ascorbate peroxidase specific activity in ammonium sulphate fractions of homogenate

5.3 ION EXCHANGE CHROMATOGRAPHY

Ion exchange chromatography is the most widely used method for protein purification. Ascobate peroxidase was found to be an anionic protein with a pI of 5.55 in the cytosol of *Pisum sativum.* (Mittler & Zilinskas, 1991a) while it was 5.32 in the case of *Ipomoea batatas* (Park *et al.*, 2004). Dalton *et al.*, (1987) employed DEAE-Cellulose as the column material for purifying ascorbate peroxidase from root nodules of *Glycine max* for the first step of purification while Chen & Asada (1989) used DEAE-Sephacel Column for purifying the enzyme from tea leaves during the initial purification steps which resulted in the separation of two isoenzymes. Hence DEAE-Cellulose was chosen as the column material for purification. The dialysate was loaded onto the DEAE-Cellulose column and the elution was carried out with 60mM NaCl. Elution resulted in fractions from 4 to 9 showed maximal ascorbate peroxidase activity (Table 5.5.1.).

5.4 GEL FILTRATION CHROMATOGRAPHY

Gel filtration chromatography is one of the most important methods in protein purification which is complementary to ion exchange chromatography and has been used for the purification of ascorbate peroxidase. Mittler & Zilinskas (1991a) used Superose 12 while Chen & Asada (1989) employed Sephadex G -100 for the purification of the enzyme from *Pisum sativum* and tea leaves respectively. Dalton *et al.*, (1987) purified the enzyme from soybean root nodules using Sephacryl S-300 gel filtration column material during the final steps of purification. Hence, Sepharose 6B column was chosen for the final purification step.

The eluent from the ion exchange column was loaded onto the Sepharose 6B column and the elution resulted in the purified enzyme with high activity in the 10th fraction (Table 5.5.1.).

5.5 PURIFICATION SCHEME OF ASCORBATE PEROXIDASE FROM *Vigna radiata*

Tissue homogenization, ammonium sulphate fractionation and dialysis were carried out at 4°C. Chromatography with DEAE-Cellulose and Sepharose 6B were carried out at room temperature. *V. radiata* seedlings (10g) were homogenized using a pestle and mortar with 100ml of ice-cold homogenization media. After filtration through six layers of cheese cloth, the homogenate was centrifuged at 4000 rpm for 5 min at 4°C. Ammonium sulphate granules were added onto the homogenate to reach a saturation of 40% and the mixture was continuously stirred for around 15 minutes followed by centrifugation at 10,00rpm for 15 minutes. The pellet obtained following centrifugation was discarded and the concentration of ammonium sulphate in the supernatant was then similarly brought to 60% saturation. The pellet resulting from the centrifugation at 10,000 rpm for 15 minutes was resuspended in media of the same composition as the homogenization media excepting BSA. The resuspended sample was dialysed overnight against 10mM potassium phosphate buffer containing 1mM Asa and 1mM EDTA. The dialysate was loaded onto the DEAE Cellulose Column (1.4cm*15.0cm) and equilibrated with 10mM phosphate buffer containing 1mM Asa, 1mM EDTA and 0.3 M Mannitol (Solution A) . The elution was carried out with the Solution A containing 60mM NaCl, the enzyme got eluted in the fractions 4 to 10 which were pooled and loaded onto the Sepharose 6B Column (1.4cm*10.0cm). The equilibration was carried out

with 10mM phosphate buffer containing 1mM Asa, 1mM EDTA, 0.3M Mannitol and 150mM NaCl. The elution resulted in 1.5mL of purified enzyme in the 10th fraction (*Figure 5.5.1.*).

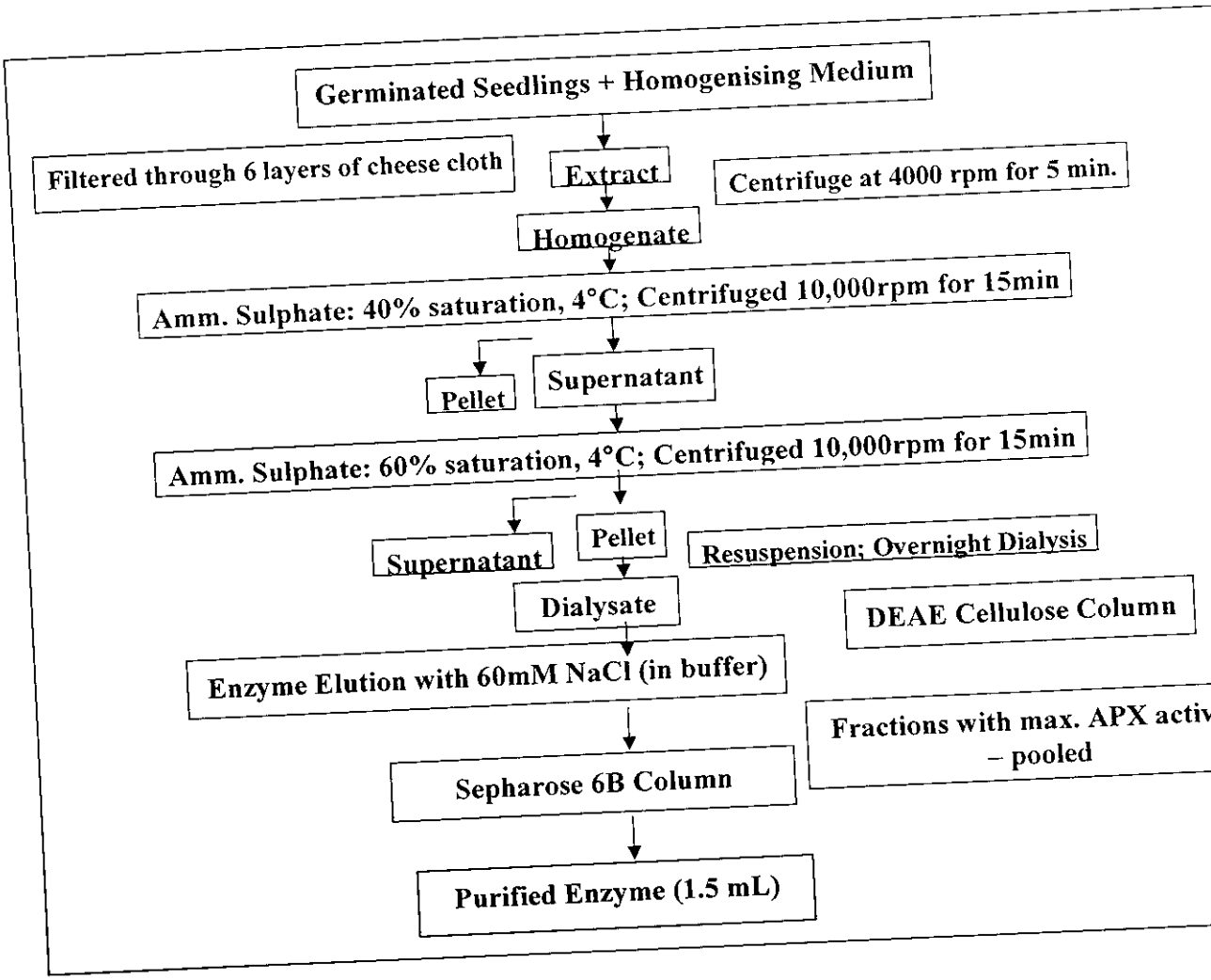


Figure 5.5.1. Purification scheme of Ascorbate Peroxidase from Vigna radiata

Table 5.5.1. Purification chart of Ascorbate Peroxidase from *Vigna radiata*

Purification Step	Enzyme Activity		% Yield	Purification fold
	Total Activity x 10^3 (U/g of seedling)	Specific Activity x 10^3 (U/mg of protein)		
Crude Homogenate	55.71	0.26	100	1
Ammonium Sulphate Fractionation & Dialysis	32.14	1.20	20	4.66
Ion exchange Chromatography	26.48	8.36	9	32.61
Gel Filtration Chromatography	23.57	27.53	1.5	107.39

5.6 KINETIC CHARACTERIZATION OF ASCORBATE PEROXIDASE

The homogeneity of the purified APX was verified by PAGE by the method of Laemmli (1970).The gel showed a single band of protein when stained with coomassie blue. Further studies were carried out to determine the kinetic characteristics of the enzyme.

5.6.1. EFFECT OF ASCORBATE CONCENTRATION ON ENZYME ACTIVITY

The concentration of ascorbate was varied from 100μM to 1000μM and the difference in absorbance for the different concentrations were determined and tabulated as below. The graph is plotted between enzyme activity and the concentration of ascorbate as given in *Figure 5.6.1.1.* The enzyme showed sigmoidal type of curves for substrate dependence. Sigmoidal kinetics is indicative of allosteric interactions. This is further observed in the Lineweaver-Burk plot which deviates from the linearity (*Figure 5.6.1.2.*)

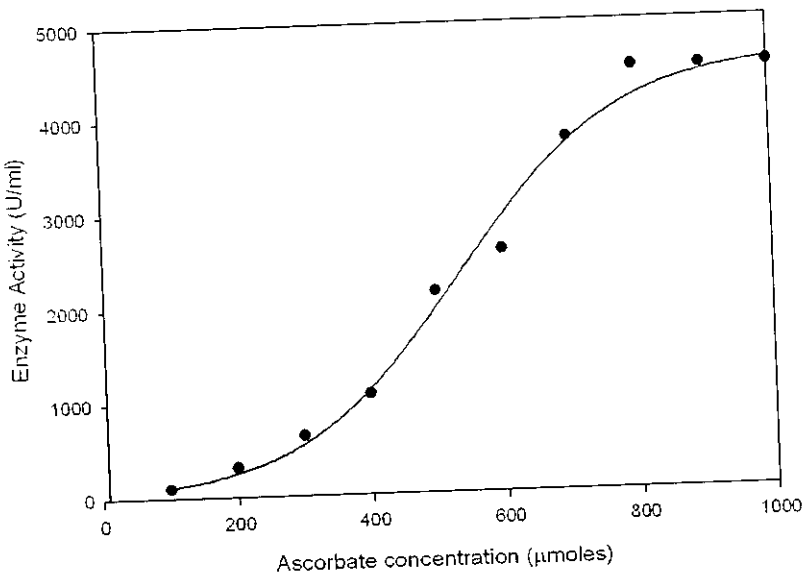


Figure 5.6.1.1. Effect of ascorbate concentration on enzyme activity

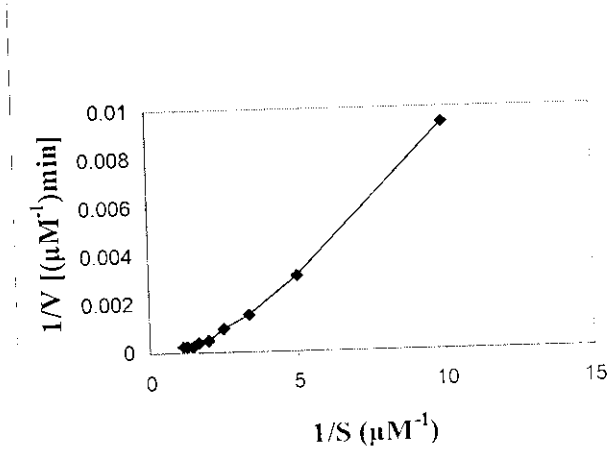


Figure 5.6.1.2. Lineweaver-Burk Plot for Ascorbate dependent enzyme activity

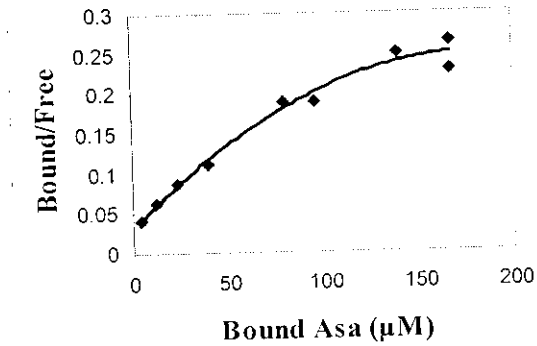


Figure 5.6.1.3. Scatchard Plot for Ascorbate

From the Scatchard plot (*Figure 5.6.1.3.*) it is confirmed that the nature of cooperativity is positive for ascorbate as the curve is convex upwards. From the slope and the intercept, we calculate the K_m to be 417μM and V_{max} as 13.6μM/min respectively for ascorbate as substrate.

5.6.2. EFFECT OF HYDROGEN PEROXIDE CONCENTRATION ON ENZYME ACTIVITY

The concentration of hydrogen peroxide was varied from 10μM to 100μM and the difference in absorbance for the different concentrations were determined and tabulated as below. The graph is plotted between enzyme activity and the concentration of hydrogen peroxide as given in *Figure 5.6.2.1.* The enzyme showed sigmoidal type of curves for substrate dependence. Sigmoidal kinetics is indicative of allosteric interactions. This is further observed in the Lineweaver-Burk plot which deviates from the linearity (*Figure 5.6.2.2.)*

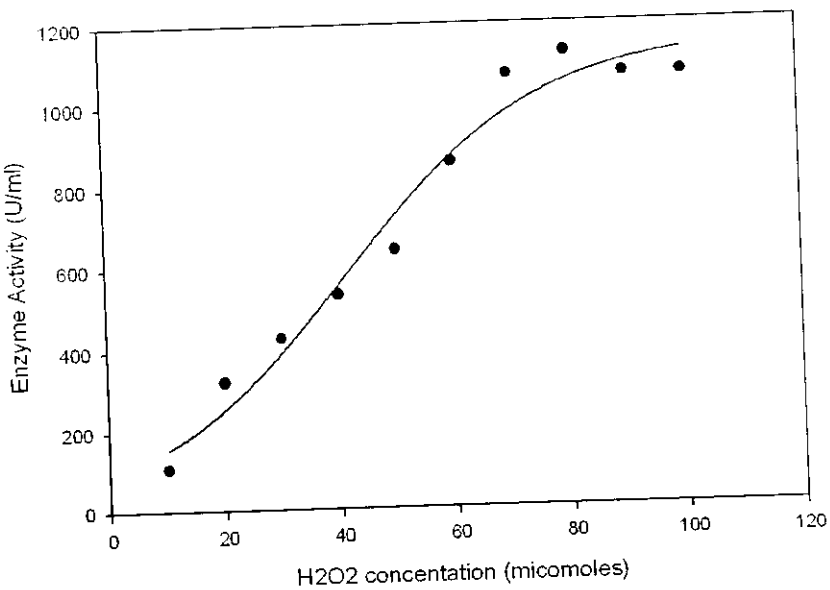


Figure 5.6.2.1. Effect of Hydrogen peroxide on enzyme activity

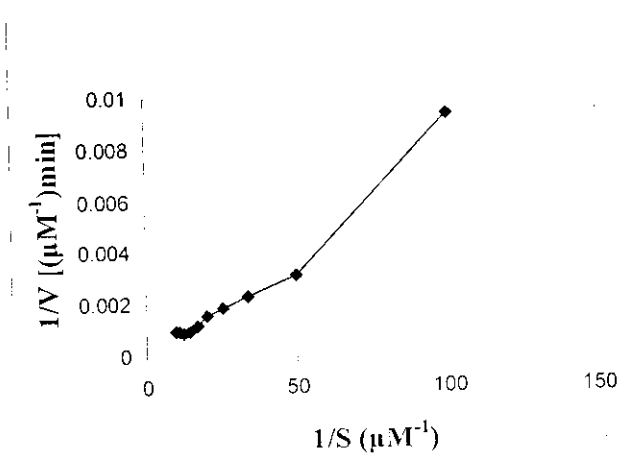


Figure 5.6.2.2. Lineweaver-Burk Plot for Hydrogen peroxide dependent enzyme activity

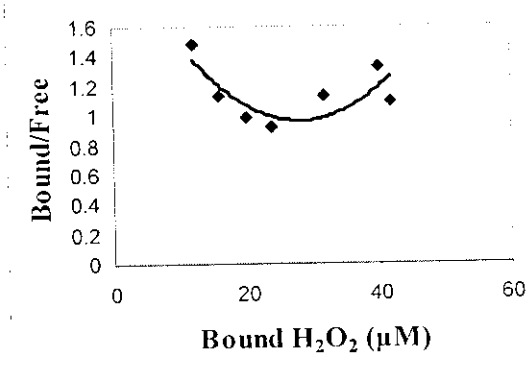


Figure 5.6.2.3. Scatchard Plot for Hydogen peroxide

From the Scatchard plot (*Figure 5.6.2.3.*) it is confirmed that the nature of co-operativity is negative as the curve is concave downwards. From the slope and the intercept, we calculate the K_m to be 11.1μM and V_{max} to be 24.8μM/min respectively for hydrogen peroxide as substrate.

5.6.3. EFFECT OF ENZYME INHIBITORS

The purified enzyme was preincubated with the following inhibitors namely salicylic acid, p-chloromercuribenzoic acid, cysteine and potassium cyanide for 3 min at the given concentrations (Table 5.6.3.1.). The enzyme activity was determined by the assay procedure described in "Materials & Methods" with additions to maintain the concentration of the inhibitor at the same level as in preincubation. The percentage decrease in total activity was calculated and tabulated as below.

Table 5.6.3.1. Effect of Enzyme Inhibitors

Inhibitor	Concentration (mM)	APX activity (In %)
None	---	100
Salicylic acid	5	75
	10	60
p-chloromercuribenzoic acid	5	80
	10	70
Cysteine	5	68
	10	55
Potassium cyanide	5	40
	10	25

It is observed that potassium cyanide being an inhibitor of heme proteins, is a very strong inhibitor of the enzyme, which is in accordance with the studies by Mittler and Zilinskas (1991a) while the sulfhydryl

inhibitors namely p-chloromercuribenzoic acid (p-CMBA) and cysteine have a moderate inhibitory action followed by salicylic acid.

5.6.4. EFFECT OF pH ON ENZYME ACTIVITY

The enzyme activity was determined over a pH range varying from 4 to 10 at 1.0 pH unit intervals by the usual assay procedure. The total activity was plotted against pH (*Figure 5.6.4.1.)* and the optimal pH was found to be 7.0.

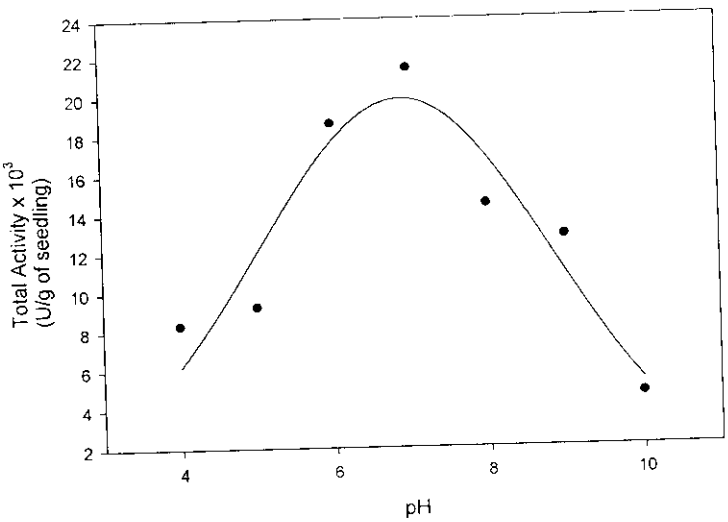


Figure 5.6.4.1. Effect of pH on enzyme activity

5.6.5. EFFECT OF TEMPERATURE ON ENZYME ACTIVITY

The purified enzyme was incubated at different temperatures varying from 10 °C to 70 °C at every 10°C intervals. The enzyme activity was calculated and plotted against different temperatures (*Figure 5.6.5.1.)*.

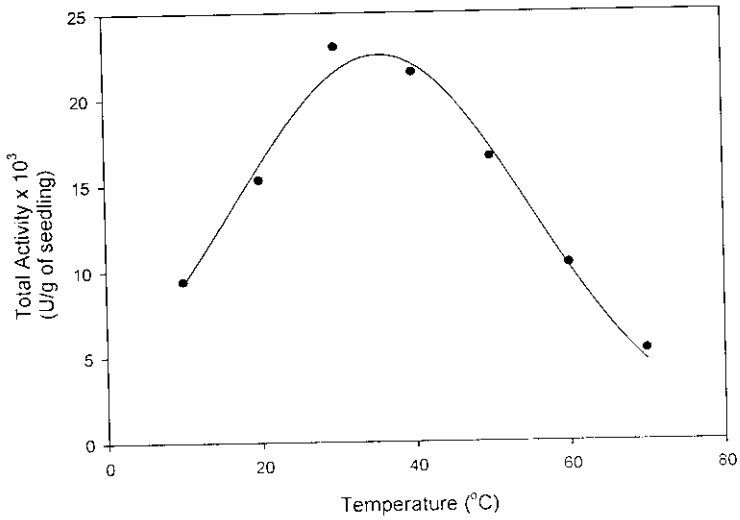


Figure 5.6.4.1. Effect of temperature on enzyme activity

The optimal temperature of the enzyme was found to be 35°C.

Conclusion

CONCLUSION

The present study was carried out to isolate and purify ascorbate peroxidase from germinating seedlings. Seed germination is an energy intensive process that involves generation of reactive oxygen species notably hydrogen peroxide. The latter is ably removed by ascorbate peroxidase and there forms an important defense against oxidative stress. Preliminary screening of ascorbate peroxidase activity from among species of legumes and spices revealed high activities of APX to be found in *Vigna radiata*. Having chosen *Vigna radiata* to purify ascorbate peroxidase, initial studies were carried out to isolate the enzyme from the cytosol. However since much higher activity was found in the homogenate, further purification attempts were on the homogenate. The homogenate was subjected to ammonium sulphate fractionation and maximal activity was found in the 40-60% fraction. Following dialysis, the crude enzyme in the 40-60% fraction was loaded onto a DEAE column. Elution carried out with sodium chloride solution resulted in a 32-fold purification. The enzyme was further loaded onto a Sepharose 6B column and eluted with a phosphate buffer to give pure enzyme with a purification factor of more than hundred. The homogeneity of the purified APX was verified by PAGE. Further studies were carried out to determine the kinetic characteristics of the enzyme. Interestingly, the enzyme showed sigmoidal type of kinetics with respect to substrate concentration. Further analysis by Scatchard plots confirmed cooperativity and showed to be positively cooperative with respect to ascorbate and negatively cooperative with respect to hydrogen peroxide. Potassium cyanide was found to be the most effective inhibitor of the enzyme and the enzyme showed optimal activity at pH 7.0 and 35°C, a characteristic common to earlier published results on APX from other species.

Appendices

APPENDIX I

CONCENTRATION OF DIFFERENT INORGANIC SALTS IN THE FMS MEDIA

Components	Concentration (mg/100 ml)
$MnSO_4.4H_2O$	1.5
$MgSO_4.7H_2O$	30
$CuSO_4.5H_2O$	0.02
$ZnSO_4$	0.6
H_3BO_3	0.2
$CaCl_2.2H_2O$	25
KI	0.01
$NaMoO_4.2H_2O$	0.010
$Na_2EDTA.2H_2O$	3.73
$FeSO_4.7H_2O$	2.78
KH_2PO_4	20
NH_4NO_3	100

APPENDIX II
TISSUE HOMOGENIZATION MEDIA

1. 50 mM potassium phosphate buffer (pH 7)

Solution A:

13.609 g of potassium di-hydrogen phosphate was dissolved in 100 ml of distilled water.

Solution B:

17.418 g of di-potassium hydrogen phosphate was dissolved in 100 ml of distilled water.

38.5 mL of solution A and 61.5 mL of solution B was mixed together to form 100mL of 1M potassium phosphate buffer.

2. 0.3 M mannitol
3. 10 mM magnesium sulphate
4. 1 mM calcium chloride
5. 0.1% BSA
6. 1mM Asa
7. 1mM EDTA

APPENDIX III

PROTEIN ESTIMATION -LOWRY'S METHOD

1. Stock Standard Bovine serum albumin

Dissolve 100 mg of BSA and make up to 100ml with distilled water

2. Working standard solution

Take 10ml of the stock solution and dilute to 100ml with distilled water

3. Alkaline copper solution

Solution A - 2% sodium carbonate in 0.1N sodium hydroxide

Solution B - 0.5% copper sulphate in 1% potassium sodium tartrate

Mix 50 ml of solution A and 1 of solution B prior to use

4. Folin-Ciocalteau reagent

APPENDIX IV

AMMONIUM SULPHATE PRECIPITATION

Initial concentration of ammonium sulphate (g of ammonium sulphate to add to 100 ml of solution)	Final concentration of ammonium sulphate (% saturation at 0°C)					
		20	40	60	80	100
	0	10.7	22.9	36.6	52.3	70.7
	20	-	11.5	24.4	39.2	56.5
	40	-	-	12.2	26.2	42.4
	60	-	-	-	13.1	28.3
	80	-	-	-	-	14.1
	100	-	-	-	-	-

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