"Fear of the Lord is the beginning of wisdom"

Dedicated to our Beloved Parents

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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 Industrial Biotechnology of Anna University was confirmed to be the report of the work done by the above students and then evaluated.

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VIJAY JOSHUA.B

PREMNATH.M

Abstract

ABSTRACT

Reactive oxygen species (ROS) are species of oxygen which are more reactive than molecular oxygen, wherein, the oxygen moiety is reduced to varying degrees. ROS are produced in the plant cells as a result of respiration and photosynthesis which if unchecked can be deleterious to the plant system because they can damage DNA and other macromolecules. Major ROS scavenging mechanisms of plants include superoxide dismutase, ascorbate peroxidase (APX), and catalase. Superoxide is converted into H_2O_2 by superoxide dismutase, an ubiquitous enzyme. Since H_2O_2 is also a ROS, this does not solve the problem and H_2O_2 must also be detoxified. Consequently, the evolution of all aerobic organisms has been dependent upon the development of efficient ROS scavenging mechanisms. APX exists as isoenzymes and plays an important role in the metabolism of H_2O_2 in higher plants. APX utilizes ascorbic acid (AsA) as its substrate. AsA is an essential molecule for higher plant cells. It is known that AsA is involved in several defense mechanisms against oxidative stress, as well as in the regulation of cell proliferation and elongation. APX isoenzymes play an important role in eliminating H_2O_2 and are distributed in at least four distinct cell compartments, the stroma and thylakoid membrane in chloroplasts, the microbody, and the cytosol. In the present study. APX was isolated and purified from *Vigna radiata* an important legume which is widely used as food supplement in the Indian subcontinent on account of its medicinal proprerties. The effects are attributed to its high concentrations of antioxidants and antioxidant scavenging macromolecules. The present study attempts to isolate the APX cytosolic isoenzyme, purify and characterize it.

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LIST OF ABBREVATIONS AND SYMBOLS

APX	- Ascorbate peroxidase
AsA	- Ascorbate (ascorbic acid)
cAPX	- Cytosolic ascorbate peroxidase
CCP	- Cytochrome c peroxidase
chlAPX	- Chloroplastic ascorbate peroxidase
DAsA	- Dehydro ascorbate
ECM	- Extra cellular matrix
GSH	- Glutathione (reduced)
GSSG	- Glutathione (oxidized)
H_2O_2	- Hydrogen peroxide
HNE	- 4-hydroxy-2-nonenal
mAPX	- Microsomal ascorbate peroxidase
MDAsA	- Mono dehydro ascorbate
MitAPX	- Mitochondrial ascorbate peroxidase
NADH	- Nicotinamide adenine dinucleotide
$NO^{\cdot}$	- Nitric oxide
$O_2^{-\cdot}$	- Superoxide
$OH^{\cdot}$	- Hydroxyl radicals
PS I	- Photo system I
ROS	- Reactive oxygen species
sAPX	- Stromal ascorbate peroxidase
tAPX	- Thylakoid ascorbate peroxidase
SOD	- Superoxide dismutase

Introduction

1. INTRODUCTION

Reactive oxygen species (ROS) have been of interest to biologists for many years. Originally ROS were recognized as being instrumental for mammalian host defence, and early work led to the characterization of the respiratory burst of neutrophills and finally the NADPH oxidase complex, which is now recognized as a primary source of ROS in mammals. Hydrogen peroxide (H_2O_2) is produced predominantly in plant cells during photosynthesis and photorespiration, and to a lesser extent, in respiration processes. It is the most stable of the reactive oxygen species (ROS), and therefore plays a crucial role as a signalling molecule in various physiological processes. Intra and intercellular levels of H_2O_2 increase during environmental stresses. Hydrogen peroxide interacts with thiol-containing proteins and activates different signalling 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 signalling 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.

1.1. REACTIVE OXYGEN SPECIES

ROS are species of oxygen which are in a more reactive state than molecular oxygen, and in which, therefore, the oxygen is reduced to varying degrees. A radical (also called a "free radical") is a clusters 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. This is the reaction catalysed by NADPH oxidase, with electrons supplied by NADPH:

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

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

Further reduction of oxygen produces hydrogen peroxide. This can arise from the dismutation of superoxide, which can occur spontaneously, especially at low pH:

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

However, this reaction can also be catalysed by a family of enzymes known as superoxide dismutases (SOD).

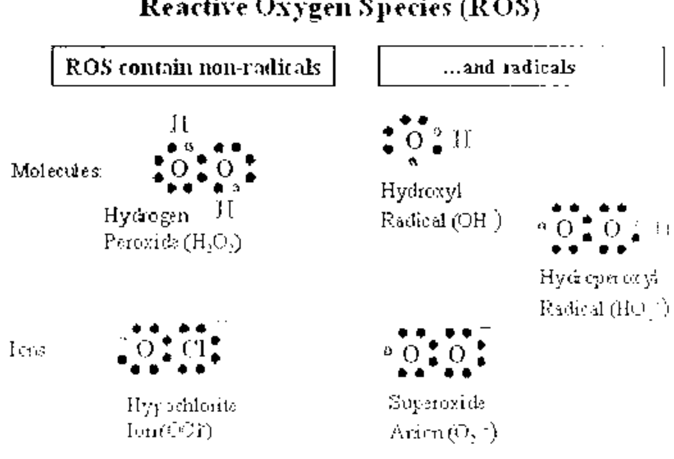


Figure 1.1: Important reactive oxygen species (ROS)

Therefore, under physiological conditions, once superoxide is formed the presence of hydrogen peroxide becomes almost inevitable. Further reactions may lead to the formation of hydroxyl radicals ($OH^{\cdot}$), especially in the presence of metal ions, Hydroxyl radicals are 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 superoxide may also react with nitric oxide ($NO^{\cdot}$) to form another relatively reactive molecule, peroxynitrite.

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

Thus appears that, following the formation of superoxide anions, a cascade of ROS production is likely. 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.

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. H_2O_2 as a molecule is weakly reactive, but the single bond between the two oxygen atoms is easily broken, so that it readily fragments into a hydrogen 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 reactive oxygen species), 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 (Figure 1.2) that indiscriminately react with a

wide variety of organic substrates causing peroxidation of lipids, cross-linking and inactivation of proteins, and mutations in DNA. Hydroxyl radicals form when H_2O_2 is exposed to ultraviolet light or when it comes in contact with a range of transition metal ions, of which the most important is iron. 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 into 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), 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. FORMATION OF REACTIVE OXYGEN SPECIES

Reactive oxygen species are formed by several different mechanisms:

- The interaction of ionizing radiation with biological molecules
- As an unavoidable by-products of cellular respiration. Some electrons passing "down" the electron transport chain leak away from the main path (especially as they pass through ubiquinone) and go directly to reduce oxygen molecules to the superoxide anion.

- Synthesized by dedicated enzymes in phagocytic cells like neutrophils and macrophages
    - NADPH oxidase (in both type of phagocytes)
    - myeloperoxidase (in neutrophils only)

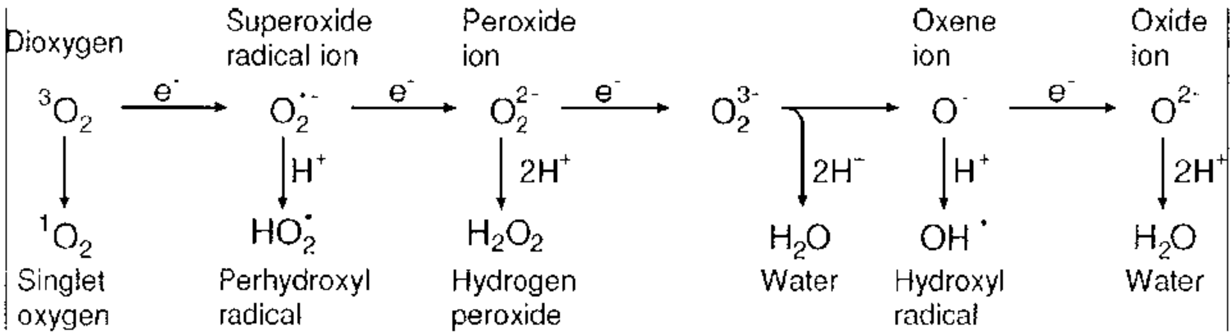


Figure 1.2: Generation of different ROS by energy transfer or sequential univalent reduction of ground state triplet oxygen

1.2.1. BIOTIC STRATEGIES TO GENERATE ROS

One of the most rapid defense reactions to pathogen attack is the so-called oxidative burst, which constitutes the production of ROS, primarily superoxide and H_2O_2, at the site of attempted invasion. Several different enzymes have been implicated in the generation of ROS.The NADPH-dependent oxidase system, similar to that present in mammalian neutrophils, has received the most attention. In animals the NADPH-oxidase is found in phagocytes and B lymphocytes.

1.2.1.1. ENZYMIC GENERATION OF ROS BY NADPH OXIDASE

NADPH oxidase was first discovered in neutrophils, which on stimulation undergo the respiratory burst, with the release of superoxide into the phagosome. Although the reaction that the enzyme catalyses is apparently simple, the enzyme itself is surprisingly complex. It catalyzes the production of superoxide by the

one-electron reduction of oxygen using NADPH as the electron donor. The $O_2^{-\bullet}$ generated by this enzyme serves as a starting material for the production of a large variety of reactive oxidants, including oxidized halogens, free radicals, and singlet oxygen. These oxidants are used by phagocytes to kill invading microorganisms, but at the same time they may also damage surrounding cells of the host.

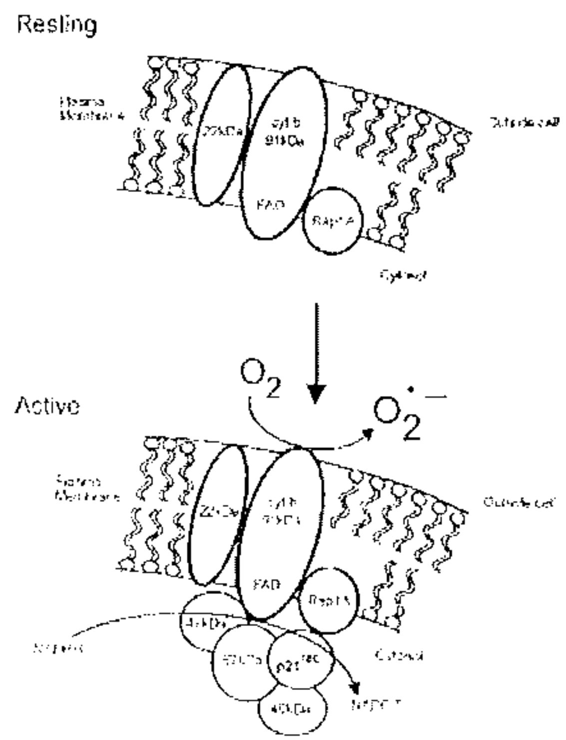


Figure 1.3: Schematic representation of NADPH oxidase and its activation

1.2.1.2. ROS GENERATED BY OTHER PEROXIDASES

Many peroxidases are localized in the apoplastic space and are ionically or covalently bound to cell wall polymers. Peroxidases can act in two different catalytic modes. In the presence of H_2O_2 and phenolic substrates they operate in the peroxidatic cycle and are engaged in the synthesis of lignin and other phenolic polymers. Horseradish peroxidase can reduce hydrogen peroxide to hydroxyl radicals.

1.2.2. ABIOTIC STRATEGIES TO GENERATE ROS

In plants, ROS are continuously produced predominantly in chloroplasts, mitochondria, and peroxisomes. Production and removal of ROS must be strictly controlled. However, the equilibrium between productions and scavenging of ROS may be perturbed by a number of adverse abiotic stress factors such as high light, drought, low temperature, high temperature, and mechanical stress.

1.2.2.1. ROS PRODUCTION IN CHLOROPLASTS

Oxygen is continuously produced during light-driven photosynthetic electron transport and simultaneously removed from chloroplasts by reduction and assimilation. There are three types of oxygen-consuming processes closely associated with photosynthesis: (a) the oxygenase reaction of ribulose-1, 5 bisphosphate carboxylase-oxygenase (Rubisco), (b) direct reduction of molecular oxygen by photosystem I (PSI) electron transport, and (c) chlororespiration. The two primary processes involved in formation of ROS during photosynthesis are the direct photoreduction of O_2 to the superoxide radical by reduced electron transport components associated with PSI and reactions linked to the photorespiratory cycle, including Rubisco in the chloroplast and glycolate-oxidase and CAT-peroxidase reactions in the peroxisome.

1.2.2.2. ROS PRODUCTION IN MITOCHONDRIA

Mitochondria are a major source of ROS in cells. However, the relative contribution of mitochondria to ROS production in green tissues is very low. One reason that plant mitochondria do not produce more ROS could be the presence of the alternative oxidase (AOX) that catalyzes the tetravalent reduction of O_2 by ubiquinone. The AOX competes with the cytochrome bc1 complex for electrons

and thus may help to reduce ROS production in mitochondria. This suggestion is supported by findings that H_2O_2 induces the expression of AOX, and overproduction of AOX in transgenic cell lines reduces ROS production, whereas antisense cells with reduced levels of the AOX accumulate five times more ROS than control cells.

1.3. ROS AND MACROMOLECULAR DAMAGE

Peroxidation of polyunsaturated fatty acids by a 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, and the membrane phospholipid diphosphatidylglycerol is damaged presumably via peroxidation of the polyunsaturated fatty acids, 18:2 and 18:3, which are the main fatty acids in this lipid. The damage to diphosphatidylglycerol causes an inhibition of cytochrome c oxidase, which appears to require this phospholipid for activity (Paradies *et al.*, 2000).

Proteins can be damaged by ROS either through direct chemical interaction or indirectly, involving end products of lipid peroxidation. 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. Such modifications can affect the function of proteins. 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.

One of the theories of ageing is that oxidatively modified proteins accumulate over time. 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 mtDNA. During aging in mammals, mutations in mtDNA accumulate faster than in nuclear DNA, possibly because mtDNA is closer to the site of ROS synthesis. However, plant mtDNA does not have a particularly high rate of mutation; in fact, rearrangements are more common. Little is known about ROS-induced DNA modifications in plant mitochondria.

1.4. SCAVENGING OF ROS

Superoxide is converted into hydrogen peroxide by superoxide dismutase, an enzyme found in all mitochondria. Since hydrogen peroxide is also a ROS, this does not solve the problem and hydrogen peroxide must also be detoxified. There are five potential enzymes (or enzyme systems) for removing hydrogen peroxide in plants.

- 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.

- The ascorbate/glutathione cycle comprises four enzymes (APX, MDAsAR, DAsAR, GR) and two low-molecular-weight compounds—ascorbate (vitamin C) and glutathione—and 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, 1998; Chew *et al.*, 2004).

- 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 also have the ability to reduce hydrogen peroxide.

- 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.

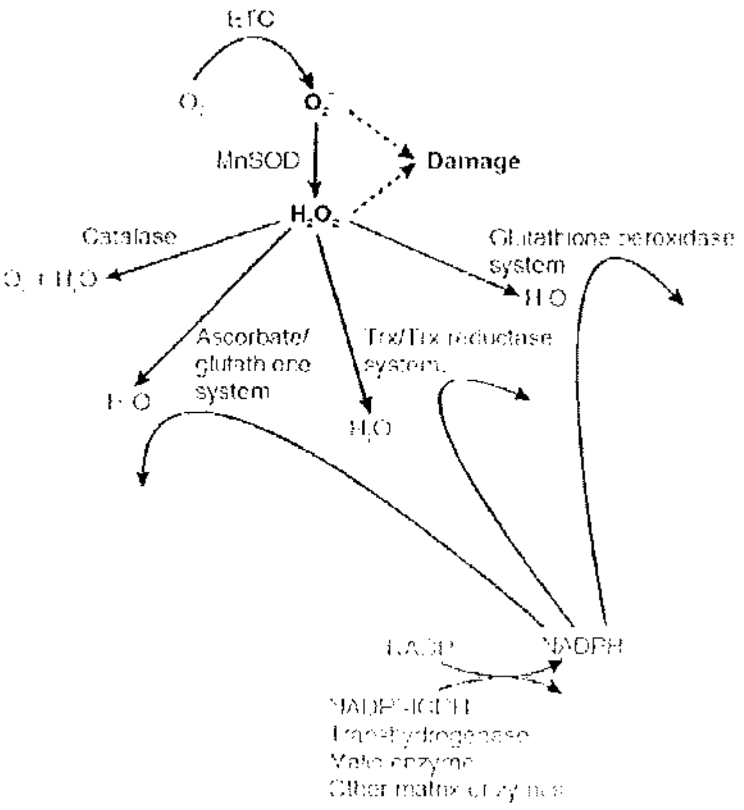


Figure 1.4: Summary of the enzyme systems involved in ROS detoxification in plant mitochondria. ETC, electron transport chain; ICDH, isocitrate dehydrogenase; MnSOD, Mn-superoxide dismutase; Trx, thioredoxin.

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).

The present study focuses on one such scavenging enzyme, Ascorbate peroxidase that uses ascorbic acid as the electron donor to scavenge the hydrogen peroxide.

The relevance of oxidative stress response under various environmental conditions has been extensively addressed in mammalian systems. Such a detailed knowledge is not available for majority of plants species particularly leguminous plants.

The model system-the germinating seedlings of *Vigna radiata*, was chosen for the present study on account of its high metabolic and short generation time. The present study was carried out to address

- The Optimal growth conditions and growth parameters in germinating seeds of *Vigna radiata*.
- Standardization of the assay procedure for ascorbate peroxidase activity.
- Extraction and purification of the enzyme using various down processing processes.
- Characterization of the catalytic properties of the enzyme.

Literature review

2. LITERATURE REVIEW

2.1. PEROXIDASES

Peroxidase is a ubiquitous enzyme found in plants, mammals, fungi and prokaryotes. It catalyses 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:

(a) peroxidases, where the oxidation products of the electron donors have physiological roles, and

(b) peroxidases whose function lies in the scavenging of hydrogen peroxide or organic hydroperoxides

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 door fro 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 over produced.

2.2. ASCORBATE PEROXIDASE

APX is a class 1, non animal haem peroxidase. 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.

2.2.1. CLASSIFICATION

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

1 Oxidoreductases

1.11 Acting on a peroxide as acceptor

1.11.1 Peroxidases

1.11.1.11 L-ascorbate peroxidase

2.2.2. REACTION CATALYSED

Ascorbate Peroxidase isoenzymes have high specificity for Ascorbate (AsA) as the electron donor. Ascorbate peroxidase catalyses the following reaction

HO — OH

L-Ascorbate + Hydrogen Peroxide → Dehydroascorbate

2.2.3. PHYSIOLOGICAL ROLE

- Ascorbate Peroxidase utilizes ascorbate (AsA) as its specific electron donor to reduce H_2O_2 to water with the concomitant generation of monodehydroascorbate (MDAsA), a univalent oxidant of AsA.
- MDAsA is spontaneously disproportionated to AsA and dehydroascorbate (DAsA). MDAsA is also directly reduced to AsA by the action of NAD (P) H-dependent MDAsA reductase.
- DAsA reductase utilizes glutathione (GSH) to reduce DAsA and thereby regenerate AsA. The oxidized GSH is then regenerated by GSH reductase, utilizing reducing equivalents from NAD (P) H.

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).

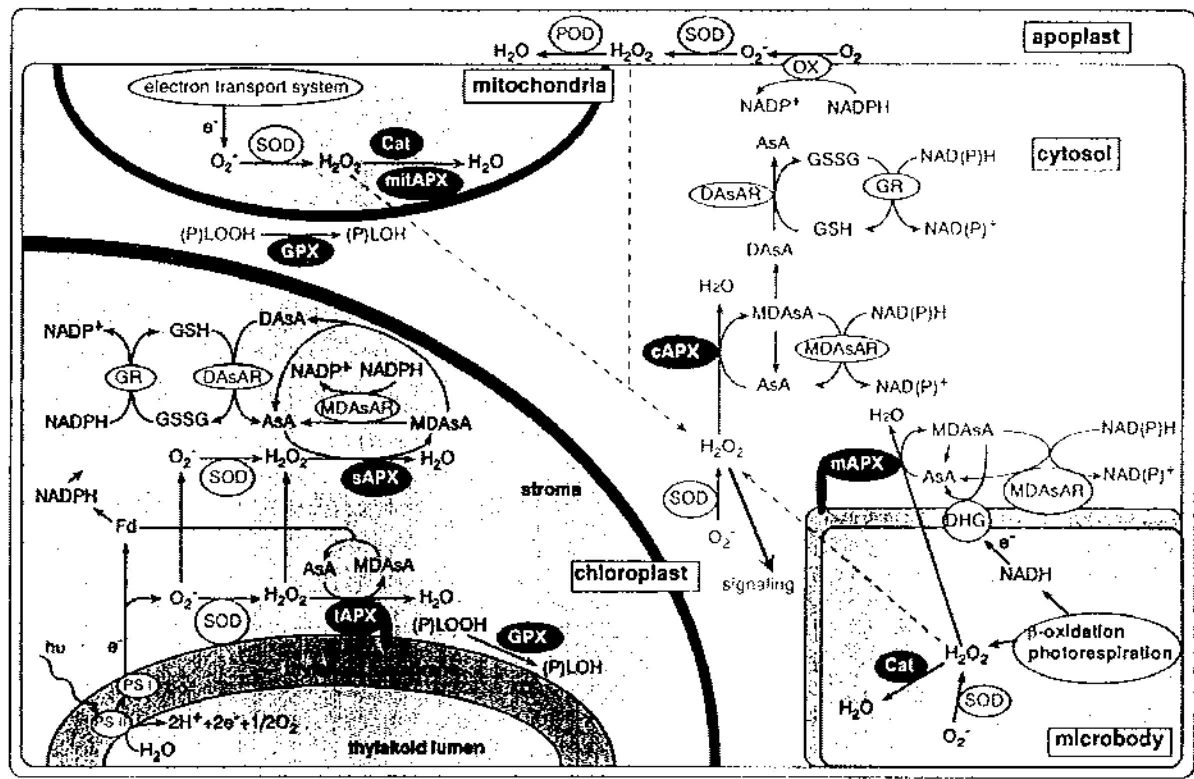


Figure 2.1: ROS scavenging system of the active oxygen species in higher plants.

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.

2.2.4. PHYLOGENIC DISTRIBUTION OF APX

Ascorbate peroxidase has been found in the angiosperms so far surveyed: leaves of pea (Gerbling *et al*., 1984; Mittler *et al*., 1991b), spinach (Nakano and Asada, 1981, Tanaka *et al*., 1991), duckweed and sycamore (Drotar *et al*., 1985), tea (Chen and Asada, 1989), maize (Nakano and Asada, 1987) and White broom

(Mittler *et al.*, 1991), as well as root nodules of legumes (Dalton *et al.*, 1987), soybean embryonic axes (Puntarulo *et al.*, 1988) and endosperms of castor bean (Klapheck *et al.*, 1990).The ascorbate peroxidase has so far mainly been detected in a soluble form, but Groden and Beck (1979) have shown the existence of a thylakoid-bound enzyme. Ascorbate peroxidase has also been detected in eukaryotic algae including *Euglena* (Shigeoka *et al.*, 1980), *Chlamydomonas* (Yokota *et al.*, 1988; Miyake *et al.*, 1991) and *Zooxanthella* (Lesser and Shiek. 1989).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. 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 $H_2{}^{18}O_2$ 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 *Trypanosome cruzi* (Boveris *et al.*, 1980), but not in the fungi and mammals. Only in the bovine eye tissue has ascorbate peroxidase been found (Kaul *et al.*, 1988), but its enzymatic properties are very different from those of the plant enzymes.

2.2.5. APX 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; Jimenez *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):

(a) As compared with the cytosolic isozyme, the chloroplastic isozyme has a very short life time in the ascorbate-depleted medium.

(b) The sensitivities of the chloroplastic isozyme to thiol reagents and to suicide inhibitors are higher than those of the cytosolic isozyme.

(c) The chloroplastic isozyme is more specific to ascorbate as the electron donor than the cytosolic isozyme - for example, the cytosolic isozyme can oxidize pyrogallol at an appreciable rate.

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 Tel-Or, 1991c) 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).

2.2.6. EVOLUTION OF APX ISOENZYMES

On the basis of the amino acid sequences, plant peroxidases can be classified into three well-separated classes (Welinder, 1992). Class I includes the intracellular peroxidases of prokaryotic origin, Class II the fungal peroxidases, and Class III the classical plant peroxidases. APX, yeast cytochrome c peroxidase (CCP) and cyanobacterium CPX belong to the Class I family (Welinder, 1992; Mutsuda *et al.*, 1996; Zámock'y *et al.*, 2000). On the other hand, guaiacol peroxidase (GP) belongs to the Class III family, which constitutes a separate lineage from APX. A novel Class III peroxidase in tea leaves showed high specificity for AsA as an electron donor (Kavratskhelia *et al.*, 1997).

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 2.2). 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.

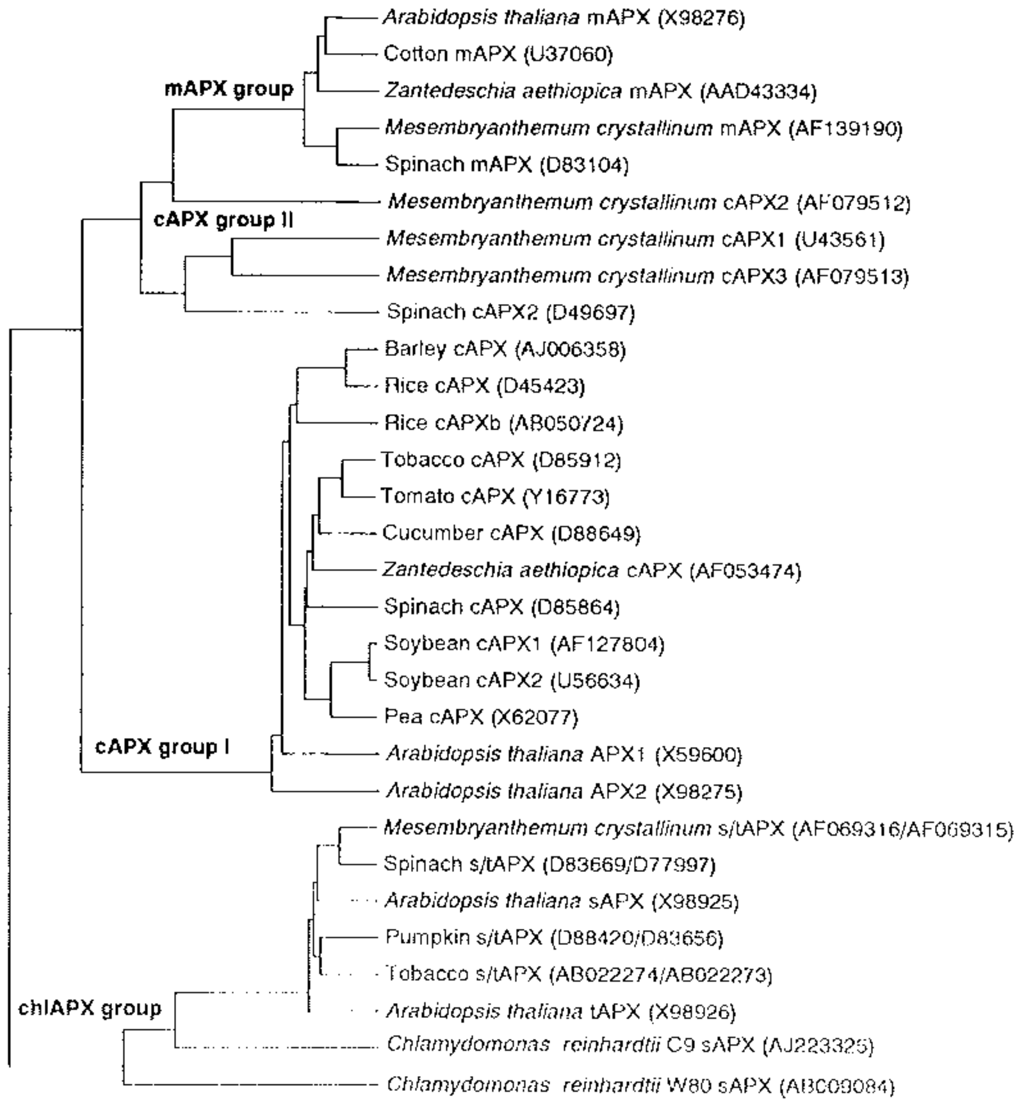


Figure 2.2: Phylogenic tree for APX isoenzymes.

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). 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.

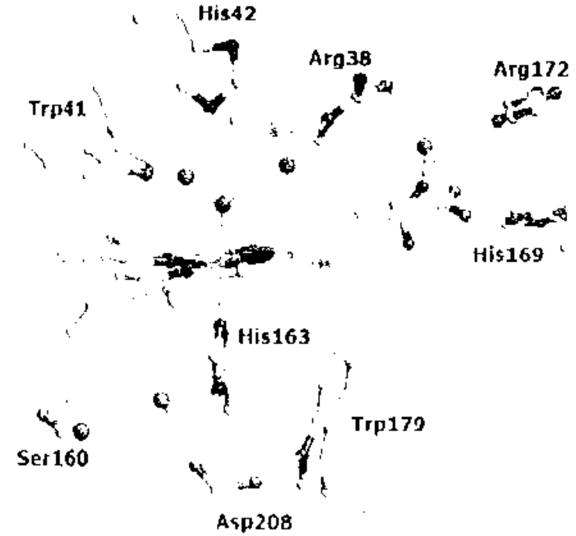


Figure 2.3: X-ray crystal structure of the active site residues of recombinant ascorbate peroxidase

2.2.7. ENZYMATIC PROPERTIES OF APX 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 Zillinskas, 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).

2.2.8. ARTIFICIAL SUBUSTRATES OF APX

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, 1996 b; 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).

2.2.9. INHIBITORS OF APX

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.

2.2.10. cDNAs ENCODING APX ISOENZYMES

The cDNAs encoding APX isoenzymes from plant species have been isolated and characterized by several research groups (Mittler *et al.*, 1991b; Ishikawa *et al.*, 1995, 1996a; Bunkelmann and Terlease, 1996). The cDNA encoding mitAPX has not been isolated yet. Higher plants contain two or more putative cAPXs, indicating that cAPX isoenzymes are encoded by a multigene family (Santos *et al.*, 1996; Jespersen *et al.*, 1997). In spinach, cAPX2 (SAP1), which appears to belong to a second family of cAPX-related enzymes, has been cloned and characterized (Ishikawa *et al.*, 1995); however, neither the protein nor the activity of cAPX2 has been detected in crude homogenates of spinach leaves, although its mRNA is constitutively expressed (Yoshimura *et al.*, 2000). Thus, the expression of cAPX2 seems to be suppressed by translational regulation. In *Mesembryanthemum crystalinum*, cDNAs encoding three types of putative cAPXs have been isolated; however, their corresponding proteins have not been identified yet. cDNA clones encoding APXs localized in the stroma of *crystalinum* C9 and *crystalinum* W80 were recently isolated (Takeda *et al.*, 2000).

Interestingly, the N-terminal 364 amino acids of spinach sAPX are completely identical with those of tAPX, whereas the C-terminal 50 amino acids differ (Ishikawa *et al.*, 1996 a). A similar finding was also made in pumpkin

(Mano *et al.*, 1997), *M. crystalinum*, and tobacco. These chlAPX isoenzymes are encoded by only one gene (APXII), and their mRNAs are generated by alternative splicing of the gene's two 3'-terminal exons as described below. By contrast, in *Arabidopsis thaliana*, the nucleotide sequences of the cDNAs for sAPX and tAPX share only 66.1% identity and these two isoenzymes are encoded by different genes (Jespersen *et al.*, 1997).

chlAPX isoenzymes have a transit peptide consisting of approximately 70 residues in their N-terminus. This peptide contains few acidic residues and is rich in Ser and Thr, thus resulting in a net positive charge (Ishikawa *et al.*, 1996 a; Yamaguchi *et al.*, 1996; Jespersen *et al.*, 1997). In all sequences of transit peptides for import across the chloroplast envelopes, position–2 relative to the cleavage site is occupied by a Lys residue; however the functional significance of this is unknown (Jespersen *et al.*, 1997). tAPX has one major hydrophobic domain which is responsible for spanning to the stroma-exposed thylakoid membranes in chloroplasts. A similar membrane-spanning region in the C-terminal region is present in mAPX and is bound to the external side of the membrane of glyoxysomes (Yamaguchi *et al.*, 1995b; Bunkelmann and Trelease, 1996; Ishikawa *et al.* 1998).

This fact indicates that the physiological function of mAPX is to scavenge H_2O_2 generated both in microbodies and the cytosol in combination with catalase and cAPX (Yamaguchi *et al.*, 1995 b; del Rio *et al.*, 1998; Ishikawa *et al.*, 1998). The transmembrane domain within mAPX functions as an overlapping peroxisomal endoplasmic reticulum-sorting signal and a peroxisomal membrane-targeting signal type 2 (Mullen and Trelease, 2000).

2.2.11. ANTIOXIDANT DEFENSE SYSTEM INVOLVING APX ISOENZYMES

The rate of O_2^{2-} and H_2O_2 formation within intact chloroplasts under optimal conditions is estimated to be 240 and 120 µM s^{-1}, respectively (Asada and Takakhashi, 1987). SOD isoenzymes and H_2O_2-sacavenging enzymes are preferentially located near the site of O_2^{2-} production (Miyake and Asada, 1992; Ogawa *et al.*, 1995). As a consequence, it is estimated that the H_2O_2 concentration is less than 8 x 10^{-7} M under normal conditions (Asada, 1999). Under photo-oxidative conditions, the actual level of H_2O_2 must increase several orders of magnitude with respect to that estimated for non-stressed chloroplasts, especially within the sites of localization of antioxidant enzymes. When the ROS production far exceeds the endogenous ROS-scavenging capacity, the regulated balance between the generation system and the scavenging system of ROS may be upset, leading to the inactivation of defense enzymes. Thus, O_2^{2-} and H_2O_2 must be completely scavenged to preserve photosynthetic activity.

A characteristic property of APX isoforms is their labiality in the absence of their electron donor, AsA (Nakano and Asada, 1981; Chen and Asada, 1989). In the presence of AsA, the reaction intermediate of APX Compound I successively oxidizes AsA to produce two molecules of MDAsA in the catalytic cycle. However, unless Compound I of APX is reduced by AsA, it is oxidized and degraded to the inactivated form by 10 nM levels of H_2O_2 within 2 min, levels which are generated via auto-oxidation of AsA at micromolar levels. Cu/Zn-SOD is inactivated by H_2O_2 with an apparent rate constant of 0.8 M^{-1} s^{-1}; the half-time for the inactivation is about 30 min in 0.4 mM H_2O_2 at neutral pH (Asada *et al.*, 1975; Casano *et al.*, 1997). Stromal Fe-SOD is also inactivated by H_2O_2 at a similar rate of 0.6 M^{-1} s^{-1}. H_2O_2 reduces the Cu (II) and then reacts with

the Cu (I) to give a ·OH at the reaction centre of the enzyme. The ·OH oxidizes His-118 of the active site of the enzyme to 2-oxo-His, resulting in the inactivation and fragmentation of the radical (Casano *et al.*, 1997). DAsA reductase is labile in the absence of thiols and inactivated by 0.5 mM H_2O_2 (Hossain and Asada, 1984). GSH reductase is inactivated by NADPH in the absence of GSSG (Lascano *et al.*, 1998). Thus, based on the data reported so far, it seems likely that the AOS-scavenging enzymes themselves show sensitivity to oxidative stress.

Manipulation of the expression of enzymes involved in the AOS-scavenging systems has focused on SOD, APX, and GR isoenzymes that are targeted into the cytosol or plastids (Sen Gupta *et al.*, 1993; Pitcher *et al.*, 1994; Aono *et al.*, 1995; Foyer *et al.*, 1995; Slooten *et al.*, 1995; Webb and Allen, 1996). The modification of ROS-scavenging systems can lead to considerable changes in oxidative stress tolerance (Allen, 1997). To evaluate the potential of the ROS-scavenging system of chloroplasts to respond to photo-oxidative stress imposed under high light and drought conditions (1600 $\mu mol\ m^{-2}\ s^{-1}$ without watering), the degradation of chlorophyll in the leaves of wild-type plants was detectable after 24 hrs, and severe chlorosis occurred at 72 hrs. However, transgenic plants did not show any chlorosis for at least 96 hrs under the stress conditions. Furthermore, when sprayed with 50 μM paraquat and exposed to high light intensity (300 $\mu mol\ m^{-2}\ s^{-1}$ or 1600 $\mu mol\ m^{-2}\ s^{-1}$), wild-type plants developed visible severe leaf injury after 24 hrs, while the transgenic plants did not exhibit any signs of chlorosis. These results demonstrate that transgenic tobacco plants have increased tolerance to photo-oxidative damage imposed by ROS. Interestingly, the chlAPX isoenzymes in the wild-type and transgenic plants were completely inactivated under the stress conditions, while PRK remained active. The total SOD activity in the control plants dramatically

decreased, while, in the transgenic plants, the initial activity of SOD was retained. These data suggest that ROS-scavenging enzymes, especially chlAPX isoenzymes, are much more strongly inactivated by oxidative stress than PRK, which is believed to be one of the thiol-modulated enzymes that is most sensitive to H_2O_2. chlAPX isoenzymes have been found to be the primary targets among the H_2O_2-sensitive enzymes under paraquat-induced photo-oxidative stress in spinach leaves (Mano *et al.*, 2001).

2.3. 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; Gonzalez-Reyes *et al.*, 1994; Cordoba-Pedregosa *et al.*, 1996). In addition, AsA has an important role in excess energy dissipation in thylakoids (Muller-Moule *et al.*, 2003).

Figure 2.4: 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.

2.4. *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 either 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.

Materials and Methods

3. MATERIALS AND METHODS

3.1. 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* were obtained from a local market.

3.2. METHODS

3.2.1. GERMINATION OF SEEDLINGS

The seeds were surface sterilized with 0.1% mercuric chloride for few seconds and were layered on a cotton and cheese cloth substrate spread over a Petri plate and were frequently moistured with Formulated Mineral Salts (FMS) media. The FMS media was formulated by comparing 7 different media namely White's, Heller's , Murashige Skoog , Eriksson , Gamborg , Nitsch and Nagata and Takebe .

The FMS media contained the following:

Table 3.1: 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

The control seedlings were germinated using distilled water. The seedlings were allowed to grow for 48 hrs and then used for the experimental procedures.

3.2.2. TISSUE HOMOGENIZATION

The homogenization was carried out at 4 $^{\circ}$C using a pestle and mortar. The seedlings were ground well in the presence of homogenization media (Appendix I) that contained the following components namely 50 mM potassium phosphate buffer (pH 7) / 0.3 M mannitol / 10 mM mercaptoethanol / 10 mM magnesium sulphate / 1 mM calcium chloride / 0.1% BSA and 1 mM ascorbate. The ideal homogenization medium should be capable of maintaining the morphological and functional integrity of organelles. 0.3 M mannitol was used so as to be isotonic with the cytosol.

The homogenate was centrifuged at 4000 rpm for 3 min and the supernatant was taken in a new tube. This supernatant was once again centrifuged at 6000 rpm for 5 min to pellet the nuclear fractions. The supernatant was then centrifuged at 10000 rpm for 20 min to pellet the mitochondria. The supernatant so obtained contained the crude enzyme which was taken in a new tube and used

for further purification processes. All the above centrifugation process was carried out at 4 °C.

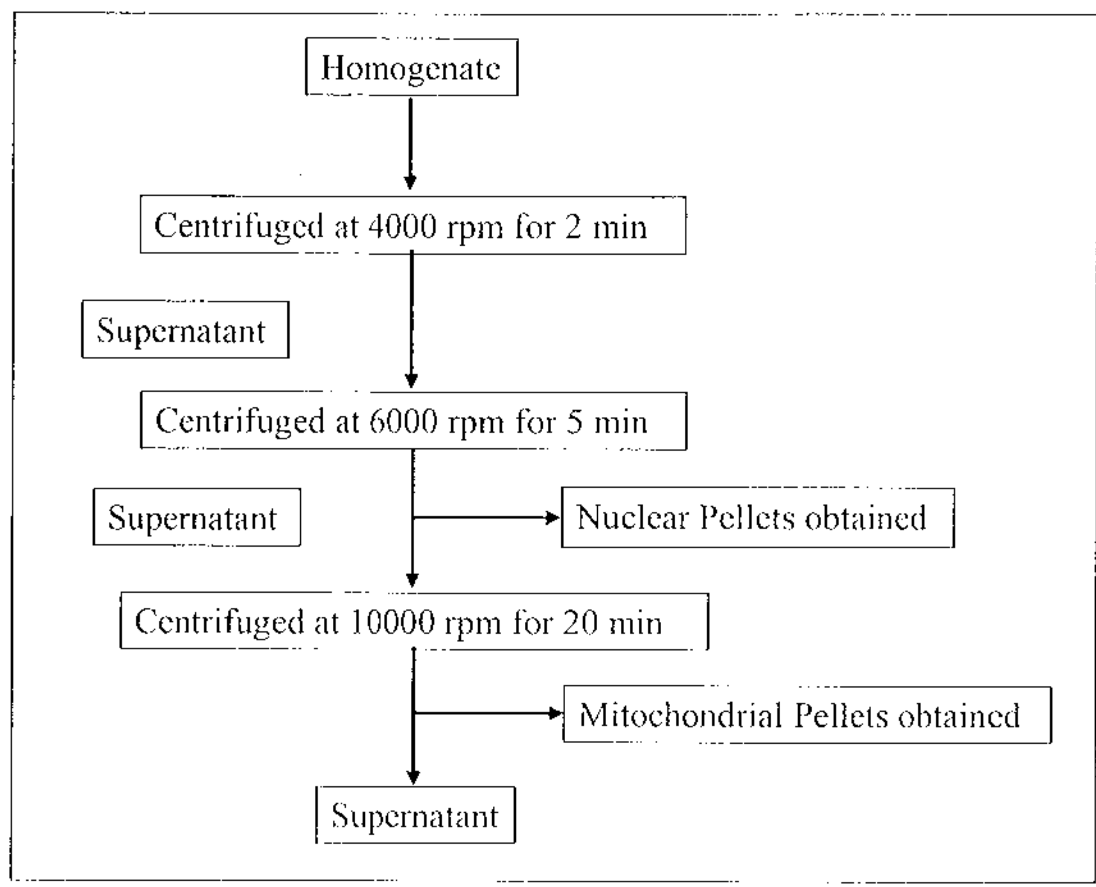


Figure 3.1: Differential centrifugation of sub-cellular organelles

3.2.3. ASCORBATE PEROXIDASE ASSAY

The ascorbate peroxidase assay was performed based upon the method suggested by Chen and Asada (1989).

3.2.3.1. PRINCIPLE

Ascorbate peroxidase assay is based upon the reduction of H_2O_2 by ascorbate to water. The reaction catalyzed by ascorbate peroxidase is as follows:

$$\text{Ascorbate} + H_2O_2 \longrightarrow \text{dehydroascorbate} + \text{water}$$

The reaction was started by the addition of hydrogen peroxide. Oxidation of ascorbate was followed by the decrease in the absorbance at 290nm between 1.5 s and 15 s after the start of the reaction. The total and specific activity of the enzyme was calculated The absorption coefficient of ascorbate at 290nm was $2.8mM^{-1}cm^{-1}$.

One unit of APX activity is defined as the amount of enzyme that oxidizes 1μmol of ascorbate per min at room temperature under the above conditions.

3.2.3.2. PROCEDURE

The reaction volume of 2 ml contained 50 mM potassium phosphate buffer (pH 7) / 0.5 mM ascorbate / 0.1 mM hydrogen peroxide and enzyme extract. The reaction was started by adding hydrogen peroxide. The absorbance was measured at 290nm and the enzyme activity was calculated.

3.2.4. PROTEIN ESTIMATION

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

3.2.4.1. PRINCIPLE

Protein reacted with Folin-Ciocalteau reagent to give a blue coloured complex. The blue coloured complex so formed was due to the reaction of alkaline copper reagent with the protein and the reduction of phosphomolybdate by tyrosine and typtophan present in the protein. The intensity of colour depends

on the amount of aromatic amino acids present and thus varied for different proteins. The blue colour developed was colorimetrically measured at 660 nm.

3.2.4.2. PROCEDURE

The reagents required for the estimation of the protein are described in appendix II. 0.2 ml-1.0 ml of working standard solution was pipetted out, concentrations varying from 20µg-100 µg into a series of test tubes. The given unknown sample was then diluted with distilled water and 0.4 ml of this was pipetted out into a test tube. To all the test tubes distilled water was added and made upto 1ml. distilled water served as blank. To all the tubes 2.1 ml of alkaline copper reagent was added and incubated at room temperature for 10 minutes. Then 0.2 ml of Folin's reagent was added and kept at room temperature for 20 minutes in dark. The blue colour developed was colorimetrically 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.

3.2.5. PARTIAL PURIFICATION

The seedlings were homogenized in the presence of a homogenizing media (appendix I) at 4 ^{0} C using a mortar and pestle. The homogenate was then centrifuged at 10000 rpm for about 10 min and the supernatant (soluble fractions) and the pellet (membrane fractions) were collected separately (Amako K. *et al* 1994). The supernatant was then subjected to ammonium sulphate precipitation which was followed by dialysis overnight.

3.2.5.1. AMMONIUM SULPHATE PRECIPITATION

The ammonium sulphate precipitation was carried out as described by Sadasivam and Manickam, 2004.

3.2.5.1.1. PRINCIPLE

The solubility of proteins is markedly affected by the ionic strength of the medium. As the ionic strength is increased, protein solubility increases. This is referred to as 'salting in'. However, beyond a certain point the solubility begins to decrease and this is known as 'salting out'. 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.

At high ionic strengths much water becomes bound by the added ions that not enough remains to properly hydrate the proteins. As a result, protein-protein interactions exceed protein-water interactions and the solubility decreases. Because of differences in structure and amino acid sequence, proteins differ in their salting in and salting out behaviour. This forms the basis for the fractional precipitation of proteins by means of salt.

Ammonium sulphate is a particularly useful salt for the fractional precipitation of proteins. It is available in highly purified form, has great solubility allowing for significant changes in the ionic strength and is inexpensive. 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.

3.2.5.1.2. 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 required amount of ammonium sulphate was added to the supernatant and stirred steadily to dissolve it completely. Then the supernatant was centrifuged at 7000 rpm for 10 min at 4 °C. If the protein of interest is found in the pellets, the supernatant was discarded and the pellets were suspended in a suitable buffer and further purification was carried on. If the protein of interest was found in the supernatant, it was then allowed for further purification steps or subjected to precipitations at higher concentrations to screen the protein of interest.

3.2.5.2. DIALYSIS

The protein solution after the ammonium sulphate precipitation should undergo dialysis to remove the salts. This was carried out by filling the protein solution in dialysis bags and dialysing at 4 °C overnight against a solution of 50 mM potassium phosphate buffer (pH 7) and 2 mM Ascorbate with the intermittent change in buffer every 8 hrs.

3.2.6. ULTRAFILTRATION

The Ultrafiltration equipment used to separate the proteins was manufactured by PALL India Pvt. Limited, Mumbai (Model number 77200-52).

3.2.6.1. PRINCIPLE

Ultrafiltration is based upon the principle of separation of proteins with the aid of a porous membrane under pressure. The membranes of varying cutoff values are available. With respect to the cut of value of the membrane the proteins are separated based on their size as filtrate and retenate.

3.2.6.2. PROCEDURE

The dialyzed ammonium sulphate precipitated enzyme was filtered with a 50KDa Omega ultra filtration membrane in a PALL ultra filtration unit. The samples were run for approximately about 2 hours. The retenate and filtrate was collected separately and was checked for APX activity.

3.2.7. SODIUM DODECYL SULPHATE -POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) OF PROTEINS

SDS-PAGE of proteins was performed by the method of Lamelli (1970).

3.2.7.1. PRINCIPLE

SDS is an anionic detergent which binds strongly to, and denatures, proteins. The number of SDS molecules bound to a polypeptide chain is approximately half the number of amino acid residues in that chain. The protein-SDS complex carries net negative charges, hence move towards the anode and the separation is based on the size of the protein.

3.2.7.2. PROCEDURE

The glass plates and spacers were thoroughly cleaned and dried and assembled properly. The assembly was held firmly together with clips and clamped in an upright position. Petroleum jelly was then applied around the

edges of the spacers to hold them in places and to seal the chamber between the glass plates.

The separating gel (10%) was prepared using the composition given in appendix page. The mixture was poured into the chamber between the glass plates, distilled was layered on top of the gel and was left to set for 30-60 min. The stacking gel (4%) was prepared using the composition given in appendix page.

The layered water was removed from the top of the gel and washed with a little stacking gel solution. The stacking gel was poured into the slab spacer and the comb was positioned appropriately in the stacking gel. The gel was allowed to set for a period of 30-60 min. After the stacking gel has polymerized, the comb was removed without distorting the wells. The gel was carefully installed in the electrophoresis apparatus. The apparatus was filled with electrode buffer and trapped air bubbles were removed at the bottom of the gel. The cathode was connected at the top and the power was turned on briefly to check the electrical circuit.

The samples were prepared for electrophoresis, following suitable extraction procedures. The protein concentration of each sample was adjusted using the 5-strength sample buffer and water in such a way that the same amount of protein is present per unit volume. The concentration should be such as to give a sufficient amount of protein (50-200μl) in a volume (25-50 μl) not greater than the size of the sample well. The required volume of sample solutions containing the marker dye bromophenol blue was taken in a microsyringe and carefully injected into a sample well through the electrode buffer. The current was

maintained at 10-15 mA for initial 10-15 min until the samples travel through the stacking gel. The stacking gel helps to concentrate the protein in the samples. The run was continued at 30 mA until the bromophenol blue reaches the bottom of the gel.

After the run was complete, the gel was carefully removed and immersed in staining solution (coomassie brilliant blue) for atleast three hours or overnight with uniform shaking. The proteins are stained by coomassie brilliant blue. The gel was transferred to a suitable container with atleast 200-300 ml of destaining solution and shaked gently and continuously. Dye that is not bound to the protein was removed. The destainer was changed frequently, particularly during initial periods, until the background of the gel is colorless. The proteins fractionated into bands were seen colored blue. The gel was photographed using gel documentation system.

Results and Discussion

4. RESULTS AND DISCUSSION

4.1. WEIGHT GAIN OF SEEDLINGS DURING GROWTH

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 the seedlings were allowed to grow for a period of 72 h by irrigating with distilled water. The growth was monitored every 24 h and the wet weight and dry weight were measured. The percentage increases in wet and dry weights were calculated and the results are shown in Table 4.1. The seedlings were then homogenized and centrifuged and protein content in the soluble cell fraction was estimated as indicated in Table 4.2.

Table 4.1: Weight gain of seedlings during germination using distilled water

Growth Period	Weight(g/ 100 seedling)		Percentage increase	
(h)	Wet weight	Dry weight	Wet weight	Dry weight
24	7.09	4.98	---	----
48	13.46	8.23	89.84	65.26
72	17.34	10.24	144.56	105.62

From the table it was observed that the seedlings showed about 90% increase in wet weight and 65% increase in dry weight after 48 hours. This increase is remarkable in comparison to the increase at 24 h and 72 h growth.

Table 4.2: Protein content of the seedlings during germination using distilled water

Growth Period (h)	Protein concentration (mg/ g of seedlings)	Percentage increase
24	7.31	-----
48	11.84	62
72	13.82	89

The seedlings were found to be healthier around 48 h germination period compared to 72 h. This is evident from the rapid increase of protein in the 48 h when compared to the 72 h.

Figure 4.1: Germinating seedlings after 48 hours

From the above results it can be concluded that rapid growth and therefore rapid rate of metabolism is maximum around the 48 h. Thus the seedlings grown upto 48 h was preferred for subsequent studies.

4.2. MONITORING THE GERMINATION OF THE SEEDLINGS IN DIFFERENT CONCENTRATIONS OF FMS MEDIA

The seedlings were grown in the varying concentrations of the FMS media. The seedlings were grown with 1x and 2x FMS media concentrations for 72 growth periods. The seedlings grown for the same duration in distilled water served as the control.

The wet weight and dry weight of the seedlings were determined for every 24 h (Table 4.3). The increases in wet and dry weight of the seedlings were calculated and the percentage change was determined.

Table 4.3: Comparison of wet weight and dry weight of seedlings

Growth period	24 h		48 h		72 h	
	Weight (g per 100 seedlings)					
Seedlings	Wet weight	Dry weight	Wet weight	Dry weight	Wet weight	Dry weight
Control	7.09	4.98	13.46	8.23	17.34	10.24
1x FMS media	7.98	5.03	14.84	9.02	19.87	11.67
2x FMS media	7.46	5.01	14.57	8.98	19.47	11.45

It can be seen that the seedlings germinated with 1x FMS media showed more increase in wet and dry weight when compared to those grown with 2x FMS media. This can be attributed to the inhibitory effects of certain salts at higher concentrations in the FMS media. Thus FMS media at 1x concentration will provide an optimum concentration of the nutrients for the germination of the seedlings.

Table 4.4: Percentage increase in weight of seedlings

Growth period	24 h		48 h		72 h	
	Weight percentage increase					
Samples	Wet weight	Dry weight	Wet weight	Dry weight	Wet weight	Dry weight
1x FMS media	12.55	1.01	10.25	9.6	14.59	13.96
2x FMS media	5.21	0.61	8.25	9.11	12.28	11.82

In a set of further experiments the seedlings were germinated for 48 hours with a wide range of concentrations of FMS media. The seedlings were homogenized and the protein content per gram of seedlings was estimated. The percentage increase in the weight and protein content was calculated (Table 4.5).

Table 4.5: Percentage increase in weight and protein content of the seedlings.

FMS Medium	Percentage increase	
	Weight of the seedlings (48 h)	Total protein / gram of seedlings
Control	---	---
0.25 x	4.14	11.89
0.5 x	7.89	10.72
1 x	19.38	19.29
1.5 x	22.11	18.23
2 x	9.66	18.32

In 1x concentrations of the FMS media the seedlings were markedly healthy and showed more significant increase in weight and protein content compared to the seedlings grown under other concentrations of FMS media. It was concluded from the above observations that the seedlings could be grown with inorganic salts supplemented as FMS media at 1x concentrations to obtain optimal growth yields.

4.3. OPTIMIZATION OF APX ASSAY

The optimization of the substrate concentration for the assay of APX was carried out by comparing the concentrations of the substrates (Ascorbate and H_2O_2) in the reaction (Chen and Asada 1989, Hiner *et al.* 2000, Kavratskhelia *et al.* 1997).

The APX activity under varying substrate concentrations is tabulated in Table 4.6.

Table 4.6: APX activity of cytosolic fraction under varying substrate concentration

Condition		APX activity
AsA Concentration	H_2O_2 concentration	(units/g of seedling)
0.5 mM [1]	0.1 mM [1]	101.66
0.5 mM [2]	10 mM [2]	85.33
1 mM [3]	0.5 mM [3]	92.54

1. Chen and Asada 1989, 2. Hiner *et al.*, 2000, 3. Kavratskhelia *et al.*, 1997

The activity of APX varied along with the change in substrate concentration. It was found that the maximum activity of APX occurred when the substrate concentrations were 0.5 mM and 0.1 mM for ascorbate and hydrogen

peroxide respectively. Subsequent experiments were carried out at these optimal substrate concentrations.

4.4. TOTAL ACTIVITY AND SPECIFIC ACTIVITY IN DIFFERENT CONCENTRATIONS OF FMS MEDIA

The seedlings were germinated under the different concentrations of FMS media and seedlings germinated with distilled water were used as control. The total enzyme activity and the specific activity were determined and tabulated (Table 4.7).

Table 4.7: Total activity and specific activity in different concentrations of FMS media

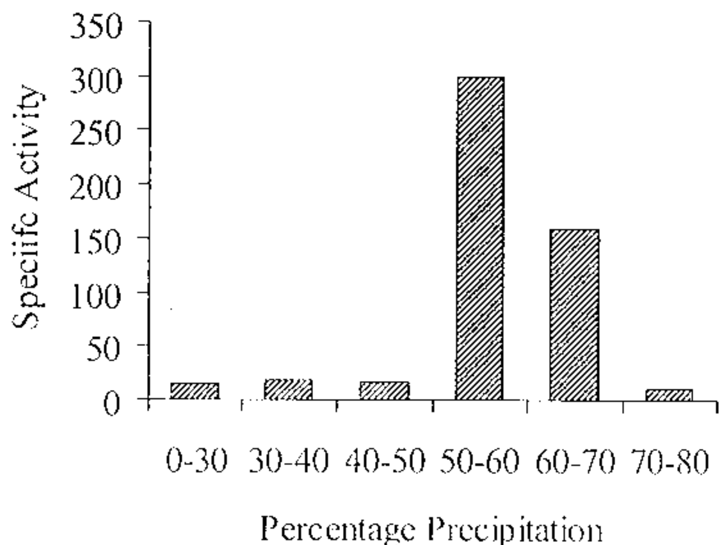
Growth conditions	Total activity (units / g of seedlings)	Specific activity (units / mg of protein)
Control	1093.60	106.39
0.25x	1057.00	100.37
0.5x	1175.08	109.72
1x	1312.24	135.56
1.5x	1213.59	131.64
2x	1383.25	113.94

Seedlings germinating in 1x concentration of FMS media showed maximum specific activity. This can be attributed to the optimum concentrations of growth media and is in accordance with the earlier results.

4.5. AMMONIUM SULPHATE PRECIPITATION AND DIALYSIS

The supernatant obtained from homogenization was then subjected to ammonium sulphate precipitation at different percent saturations and the samples were subsequently dialysis for 24 hrs. The dialyzed samples were then assayed for APX activity. The specific activity of the enzyme obtained in different fractions showed maximum activity in the 50%-60% fraction (Figure 4.2).

Figure 4.2: Specific activity of enzyme at different concentrations of ammonium sulphate



Further purification of the enzyme was carried out at 50%-60% saturation of ammonium sulphate in order to eliminate unwanted proteins and obtain a partially purified preparation of APX.

4.6. ULTRAFILTRATION

The dialyzed samples were then ultrafiltered using a 50kDa membrane and the filtrate and retenate were collected separately and assayed for APX activity. The protein content of the samples was simultaneously estimated and the specific activity was calculated (Table 4.8).

Table 4.8: Ultrafiltration of partially purified APX enzyme

Sample	Protein (mg/g of seedlings)	Activity (units/g of seedlings)	Specific Activity (units/mg of protein)
Before UF	3.26	971	298
Filtrate	1.03	533	549
Retenate	2.23	45	20

It was observed that the specific activity of the enzyme increased two folds after ultrafilteration.

4.7. SDS-PAGE OF PARTIALLY PURIFIED APX

The ultrafiltered APX sample was loaded in a SDS-PAGE gel along with the protein marker PSW-L. The samples were run for about 4 hours and the gel was stained by commassie brilliant blue for about a hour. Then the gel was washed with the destaining solution and photographed in the gel documentation section.

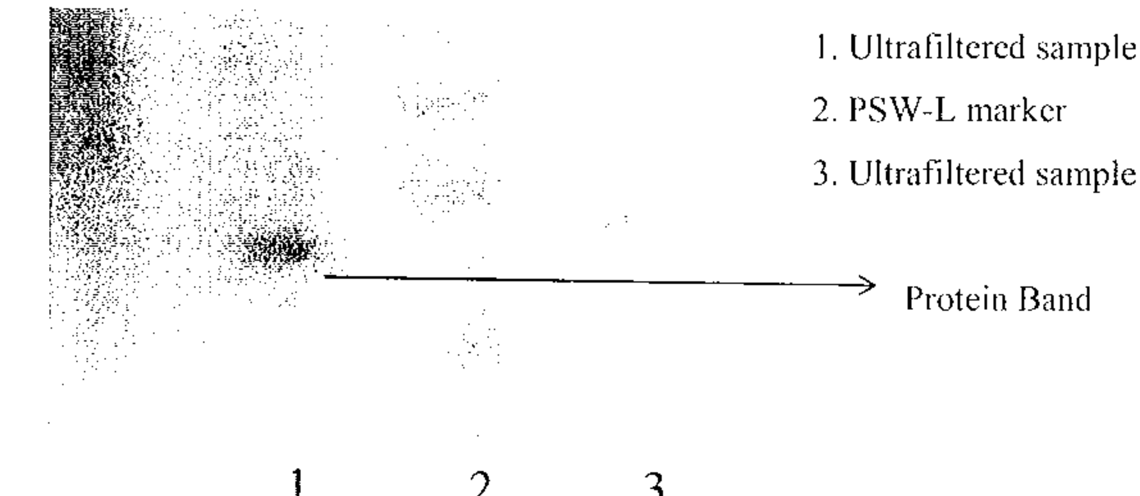


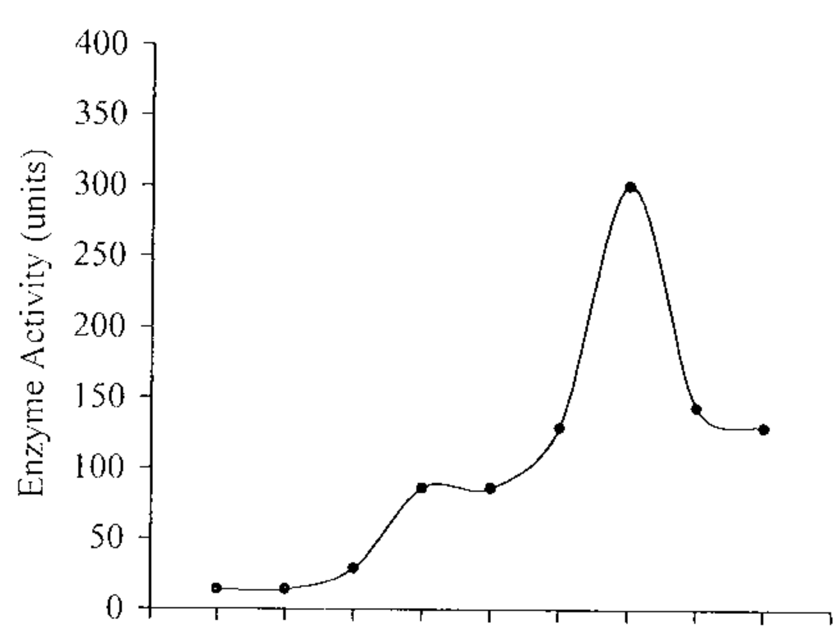
Figure 4.3: SDS-PAGE of partially purified APX

It can be observed from the gel that a single band appears near the 31kDa mark. This shows that the cytosolic APX of *Vigna radiata* has a molecular weight of approximately 31kDa.

4.8. EFFECT OF pH ON ENZYME ACTIVITY

The optimum pH required for the enzyme activity was determined by varying the pH of the reaction mixture using a standard buffer. The pH range selected was from 4 to 8 at 0.5 pH unit intervals. The assay was performed by the usual method and the total activity was calculated and tabulated. The activity was plotted (Figure 4.4).

Figure 4.4: Effect Of pH on enzyme activity

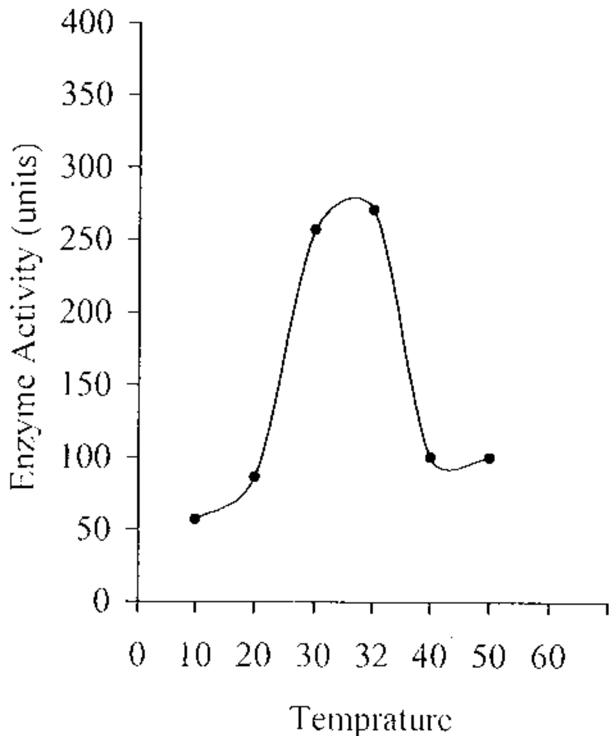


Maximal enzyme activity was observed at pH 7.

4.9. EFFECT OF TEMPERATURE ON ENZYME ACTIVITY

The optimum activity for enzyme was determined by incubating the enzymes at different temperatures viz 10 ° C, 20 ° C, 30 ° C, 40 ° C, 50 ° C and also at room temperature. The assay was then performed and the enzyme activity was calculated (Figure 4.5).

Figure 4.5: Effect of temperature on enzyme activity



The maximum enzyme activity occurred at room temperature (32 °C) which is the optimum temperature for the enzyme activity.

4.10. EFFECT OF INHIBITORS ON APX ACTIVITY

The APX was preincubated with 50 mM potassium phosphate buffer (pH 7) / 0.5 mM Ascorbate / 0.1 mM hydrogen peroxide and a known concentration of inhibitors like hydroxylamine hydrochloride, resorcinol, p-chloromercuric benzoic acid, potassium thiocyanide and urea. The enzyme activity was then assayed and the percentage change in total activity was calculated (Table 4.9).

Table 4.9: Effect of inhibitors on enzyme activity

Inhibitor	Concentration (mM)	APX activity (In %)
None	---	100
Hydroxylamine hydrochloride	5	10
	10	0
Resorcinol	5	100
	10	100
p-Chloromercuric benzoic acid	5	44
	10	26
Potassium thiocyanide	5	23
	10	6
Urea	5	100
	10	100

Different compounds inhibited ascorbate peroxidase to various extents. Urea and resorcinol did not inhibit APX activity. This is in accordance with the observation by Chen and Asada (1990).

Conclusion

CONCLUSION

It has emerged from the present study that the properties of cytosolic APX of *Vigna radiata* are similar to those found in peas and spinach. Also the APX was found to be unstable in the absence of ascorbate in the homogenizing medium. In the addition to the distribution of activity in various sub cellular fractions, variation in the total activity of ascorbate peroxidase during growth was maximal in the soluble fractions. The enzyme was purified by various downstream processing techniques and the molecular weight was found to be around 31kDa using SDS-PAGE. The optimum temperature was found to be 32°C and the optimum pH was 7. Supplementation with various artificial inhibitors showed remarkable decrease in activity of the enzyme. Further studies on cytosolic ascorbate peroxidase from *Vigna radiata* will shed more light on its molecular properties.

Appendix

APPENDIX

APPENDIX 1

TISSUE HOMOGENIZATION

1. 50 mM potassium phosphate buffer (pH 7)

Solution A:

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

Solution B:

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

35 ml of solution A and 65 ml of solution B was mixed together and made upto 200 ml.

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

APPENDIX II

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

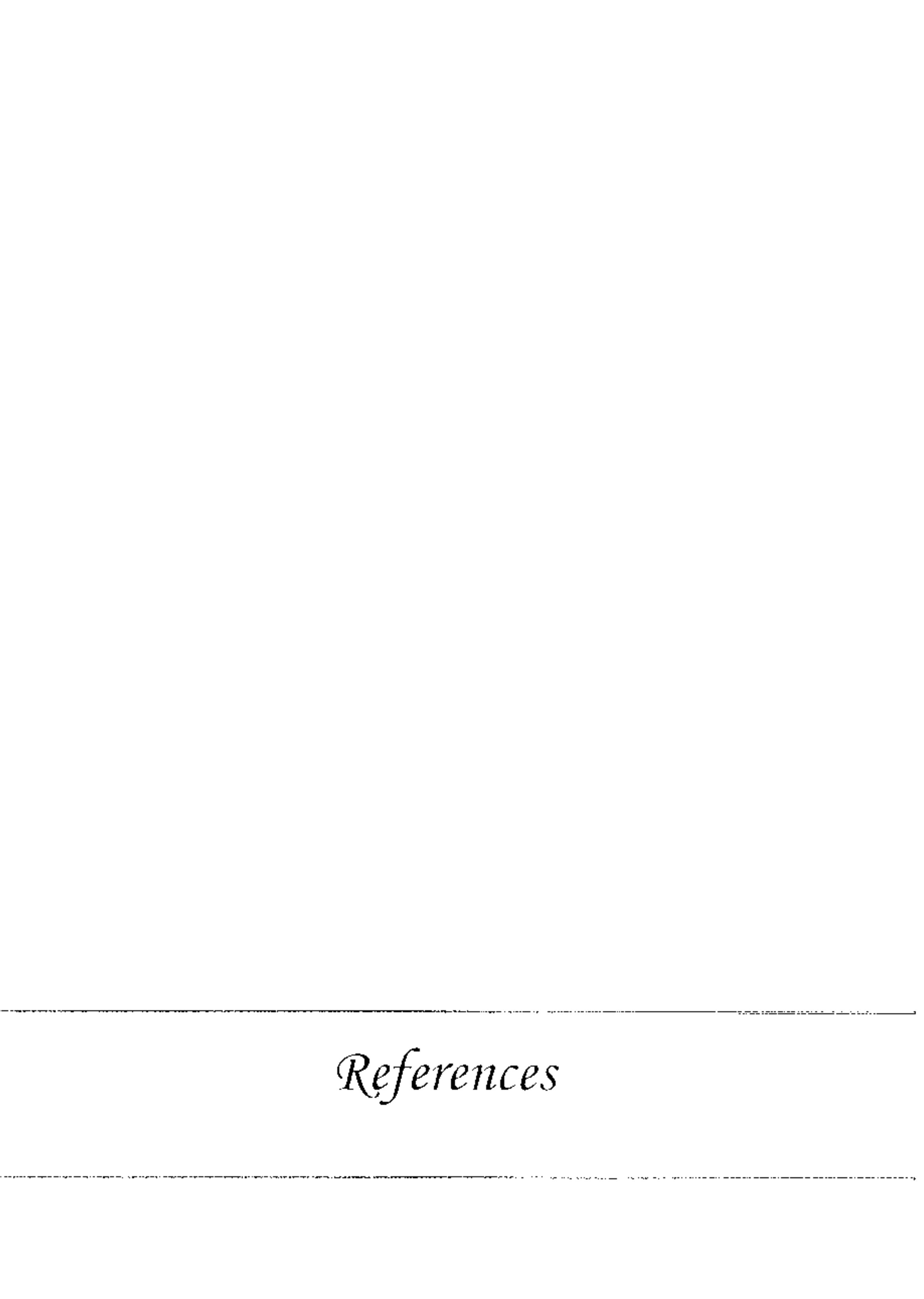
    Mix 50 ml of solution A and 1 of solution B prior to use

4. Folin-Ciocalteau reagent

APPENDIX III

AMMONIUM SULPHATE PRECIPITATION

Initial concentration of ammonium sulphate (g of ammonium sulphate to ad to 100 ml of solution)	Final concentration of ammonium sulphate (% saturation at $0^{o}C$)						
		30	40	50	60	70	80
	0	16.6	22.9	29.5	36.6	44.2	52.3
	30	-	5.7	11.9	18.4	25.3	32.8
	40	-	-	5.9	12.2	19.0	26.2
	50	-	-	-	6.1	12.7	19.7
	60	-	-	-	-	6.3	13.1
	70	-	-	-	-	-	6.6



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