FERRITIN IN *VIGNA MUNGO* (L.) HEPPER (BLACK GRAM): SOME FUNCTIONAL ASPECTS

THESIS SUBMITTED TO THE
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by

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Declaration

I hereby declare that the work in this thesis entitled "Ferritin in *Vigna mungo* (L.) Hepper (Black gram): Some functional aspects" has been carried out by me under the supervision of Dr. M.N.V. Prasad, Reader, Department of Plant Sciences, School of Life Sciences, University of Hyderabad and this work has not been submitted for a degree or diploma of any other University or Institute.

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Certificate

This is to certify that, Mr. T. Rama Kumar has carried out the research work embodied in the present thesis entitled "Ferritin in *Vigna mungo* (L.) Hepper (Black gram): Some functional aspects" in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad for the full period prescribed under the ordinance of the University. The thesis is recommended for submission to the University of Hyderabad for the Doctor of Philosophy in Plant Sciences.

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June 5 (World Environment Day), 1998 T. Rama Kumar

ABBREVIATIONS

ABA	Abscisic acid
APS	Ammonium persulphate
APX	Ascorbate peroxidase
ATP	Adenosine 5-triphosphate
BCIP	5-bromo-4-chloro-3-indolphosphate
BSA	Bovine serum albumin
CaM	Calmodulin
CaM	Calcium/calmodulin-dependent kinase
cAMP	Adenosine 3'5'-cyclic monophosphate
cGMP	Guanosine 3'5'-cyclic monophosphate
CAT	Catalase
conc.	Concentration
dASA	Dehydro ascorbic acid
DE-52	Diethyl cellulose
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethyleneglycol-bis-(B-amino-ethyl ether)
FC	Ferric citrate
Fe	Iron
F. Wt	Fresh weight.
GPX	Guaiacol peroxidase
GSH	Glutathione
HEPES	N-(2-Hydroxyethyl)piperiazine-N'-(2-ethane sulphonic acid)
kDa	kilodalton
KN-62	(l-(N,O-bis(5-isoquinolinesulfonyl)-N-^^^
M	mannitol
Min	minute
MW	Molecular weight
NAC	N-Acetyl cysteine
NaCl	Sodium chloride
NET	Nitroblue tetrazolium
PAGE	Polyacrylamide gel electrohoresis
PMSF	Phenylmethylsulfonylfluoride
PKC	Protein kinase C
PVPP	Polyvinyl poly pyrrolidone
SB	Sodium benzoate
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
TEMED	N, N, N^1, N^1,tetramethylene diamine
μCi	Microcurie
L	Litre

1 Ferritins in plants - an overview

1.1 Introduction

Ferritin was first discovered and isolated from horse spleen. Laufberger (1932) was the first to describe ferritin. Ferritin is an iron storage protein, ubiquitous in all organisms (Theil 1987). The protein was also found in fungi (David and Easterbrook 1971) and several bacteria (Chen and Crichton 1982; Rocha *et al* 1992; Steifel and Watt 1979; Wai *et al* 1995). Ferritin is an essential protein for aerobic metabolism. Atmospheric transition and evolution of aerobic atmosphere on earth placed several demands on iron metabolism in view of the extreme insolubility and potential toxicity of free ferric iron. Therefore, organisms have developed a wide variety of strategies to deal with this challenge, including extracellular Fe^{3+} binders (siderophores), Fe^{3+} transport systems (transferrins) and iron storage systems (ferritins) (Grossman *et al* 1992). Many review articles have appeared on ferritin, the most being those on animal ferritin (Aisen and Listowsky 1980; Bezkorvany 1980; Crichton 1982; Munro and Linder 1978). Phytoferritin has been reviewed by several researchers (Andrews *et al* 1992; Bienfait and van der Mark 1983; Briat and Lobreaux 1997; Ragland *et al* 1990; Seckbach 1982; Theil and Hase 1993).

1.2 Cellular localization

Ferritin in plants was discovered in transmission electron microscopic investigations (Hyde *et al* 1962). The vast majority of the articles that appeared in the beginning about plant ferritin were based on electron microscopic observations. Ferritin is generally observed in cells which are inactive in photosynthesis such as in roots or root nodules (Amelunxen *et al* 1970; Bergersen 1963), seeds and young etiolated leaves or hypocotyl

(Hyde *et al* 1963; Whatley 1974), reproductive cells (Sheffield and Bell 1978) and senescing cells (Barton 1970). Deposition of ferritin as a result of physiological stress can be observed in virus infected cells (Craig and Williamsion 1969) or in damaged or diseased tissues (Maramorosch and Hirumi 1973). Large aggregates of ferritin are observed in leaf tissues with high iron content (Seckbach 1968). Ferritin clusters deposited in the plastids are classified in several categories from amorphous granulation to highly ordered arrays (Seckbach 1982).

In contrast to diverse cellular localization of animal ferritin, iron containing plant ferritin is invariably found in etioplasts or chloroplasts. This is a logical place for ferritin to be, when one considers that in green leaves most of the cellular iron is located in the chloroplast, playing an active role in the process of photosynthesis (Seckbach 1972). The appearance and disappearance of ferritin iron cores occurs at specific stages of chloroplast development. Almost all plastids of the outer layers of developing cotyledons of pea seeds contain deposits of ferritin iron cores.

1.3 Characterization

1.3.11ron and phosphorus content

Variations in the iron content occur among phytoferritins. The iron content of ferritins studied in plants (seeds, undifferentiated cells) was found to be relatively insensitive to iron overload (Lescure *et al* 1990; Raguzzi *et al* 1988).

Phosphorus content was considered to be a constant component (Fe:P= 8:1) of ferritin iron cores. In humans lower phosphate content was associated with diseases, for example *Thalassaemia,* in which the Fe:P ratio is 20:1 (Mann *et al* 1986).

Table 1. Iron content (atoms/molecule) of the ferritin from some legume seeds

Taxon	Fe/molecule	Reference
Pea *(Pisum sativum)*	1800-2100	Crichton *et al* 1978
Soybean *(Glycine max)*	2500	Laulhere *et al* 1988
Lentil *(Lens esculenta)*	2100	Laulhere *et al* 1988
Jackbean *(Canavalia ensiformis)*	900	Briat *et al* 1990
Black gram *(Vigna mungo)*	1100	Present investigation

1.3.2 Mechanisms of iron core formation and methods of measurement

Oxidation and hydrolysis are required to form the iron core of ferritin from Fe (II), which is the only form of iron that can be used to reconstitute ferritin from protein coats *in vitro* and probably *in vivo*. Formation of the bulk iron phase of ferritin replicates a purely inorganic process, but protein-Fe interactions are observed at the early stages of iron core formation (Bauminger *et al* 1989; Yang *et al* 1987). Early events in iron core formation involve an Fe(II)-tyrosinate complex in ferritin iron core formation (Waldo *et al* 1993).

A variety of physical analyses have been used to characterize the different forms of mature ferritin cores, as well as those of intermediate stages. X-ray diffraction and high resolution electron microscopy have been used to determine the long range order of iron cores. X-ray absorption spectroscopy, extended X-ray fine structure determination, and X-ray absorption near-edge structure (XANES) analyses have been used to monitor the oxidation of ferrous to ferric ions continuously and to determine the nature of the average

short range structure in different variants of ferritin iron cores and in different intermediates during core formation (Theil and Sayers 1990). Mossbauer spectroscopy is yet another technique which provides information about the type and strength of iron interactions and can be used to examine both the early and the intermediate stages of core formation as well as the final core (Bauminger *et al* 1989; Rohrer *et al* 1989; Yang *et al* 1987).

1.4 Conserved properties

Three dimensional structure studies of the crystallized ferritin protein coat shows that the polypeptide subunits are folded into bundles of four alpha helices (Banyard *et al* 1978; Harrison and Liley 1990). Structure predictions indicate that the same polypeptide folding pattern occurs in ferritin from all vertebrates belonging to mammals, birds and amphibia (Crichton 1990; Theil 1987, 1990) and plants viz. soybean, pea (Lescure *et al* 1991; Ragland *et al* 1990). The primary structure of ferritin in prokaryotes is at the first sight conserved poorly but sophisticated sequence analysis has recently shown conservation of key residues in ferritin among prokaryotes and eukaryotes (Grossman *et al* 1992). In contrast, conservation of sequences (nucleotides and amino acids is 55%) among plants and animals (Lescure *et al* 1991; Ragland *et al* 1990). For the sequences cloned in higher plants, the identity between pea, soybean, bean, and maize ferritin is very high (78%) (Lobreaux *et al* 1992; Spence *et al* 1991). The sequence similarity among ferritins in higher eukaryotes clearly points to a common ancient progenitor. Ferritin in contemporary prokaryotes has significant homology to eukaryotic ferritins but displays considerable divergence from the progenitor line that produced eukaryotes (Grossman *et al* 1992).

1.5 Specific features of ferritin from plants

Plant ferritin specific antigenic determinants are shared among the ferritins from the seeds of pea *(Pisum sativwri),* soybean *(Glycine max),* jackbean *(Canavalia ensiformis),* maize *(Zea mays),* lentil *(Lens esculentum)* and black gram *(V. mungo),* but are absent in ferritin from animals (Laulhere *et al* 1988; Lobreaux *et al* 1992). The common epitopes shared among seed ferritins also are found in leaves of bean, pea, maize (Laulhere *et al* 1988; van der Mark and van den Briel 1985), root and flower of pea (Laulhere *et al* 1988) and nodule of soybean (Ragland and Theil 1990). The N-terminal leader sequence is a distinctive feature of the primary structure of plant ferritin subunits that is used for the transport of ferritin synthesized in the cytoplasm to plastids. The subunits of plant ferritins as determined by electrophoresis in SDS-gels range from 30 kDa to 22 kDa. In plants (seeds and *in vitro* translated mRNA transcripts) the subunit precursor migrates with a mobility equivalent to 30 kDa-32 kDa (van der Mark *et al* 1983a) which accounts for the predicted mass of the transit peptide (Ragland *et al* 1990). Mature protein from seed, leaves or meristem dissociates into subunits of 28 kDa or 26 kDa (Laulhere *et al* 1988; Sczekan and Joshi 1987; Spence *et al* 1991; van der Mark and van den Briel 1985). Heterogeneity of subunits observed is related at least in part to posttranslational modification of the extension peptide during germination and isolation (Laulhere *et al* 1989; Sczekan and Joshi 1987).

In bean and soybean ferritin subunits, a precursor of mature subunit has been observed and shown to be posttranslationally cleaved during transport across the chloroplast membrane (Proudhan *et al* 1989; van der Mark *et al* 1983b). The soybean transit peptide contains 54 amino acids and shares many features with transit peptides of other nuclear encoded chloroplast proteins. Intact prokaryotic ferritin sequence is not observed so far in

chloroplast genomes (Ragland *et al* 1990) confirming that all plant ferritin is derived from the nuclear encoded higher eukaryotic type. Tryptic peptide maps of ferritin from soybean, pea and lentil seeds are similar (Crichton *et al* 1978; Sczekan and Joshi 1987). Amino acid analysis of ferritin from seeds for lentil, pea, jackbean and soybean also share extensive similarities (Crichton *et al* 1978; Sczekan and Joshi 1987). Until recently, the occurrence of multiple forms of ferritin isolated from plants such as peas and beans have been attributed to partial degradation of the N-terminal region of the subunits by peptidases (Lobreaux and Briat 1991). An early, but apparently neglected report of van der Mark *et al* (1983) presented evidence from electrophoresis for two propeptide subunits of ferritin in beans. Wicks and Entsch (1993) have shown functional genes for three different plant ferritin subunits in *Vigna unguiculata* (legume) in which they have presented the evidence that molecular forms of ferritin in plant tissues can also arise through differential expression of a family of genes, rather than differential processing of a single translational product alone.

1.6 Ferritin regulation and gene expression

Iron is the best characterized signal for regulating ferritin in plants. In order to determine the specificity of the response to iron overload, de-rooted maize plantlets when incubated for 6 hrs in solutions (10 mM) containing different transition or heavy metals, failed to accumulate ferritin mRNA as confirmed by a northern blot, even with copper, cadmium etc. (Lobreaux *et al* 1995). In plants the effect of iron on the accumulation of ferritin protein is similar to that in animals. Induction of ferritin has been observed in leaves and cultured soybean cells (Lescure *et al* 1991) and *Arabidopsis* plantlets (Gaymard *et al* 1996). Induction of ferritin by iron is entirely at the level of mRNA synthesis in

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contrast to animals where it is at the translational level. The iron induced ferritin is the same as that synthesized in uninduced tissue, the protein is distributed in the same fashion as in normal cells (Lescure *et al* 1991). To date there is no evidence for iron induced changes in translation of ferritin mRNA in plants. In order to determine whether features of soybean ferritin mRNA itself inhibit specific translational regulation, the animal iron responsive element (IRE) was fused to soybean ferritin cDNA and the translation of the *in vitro* transcripts analyzed in the rabbit reticulocyte lysates containing endogenous iron responsive element binding protein (IRE-BP) which is a IRE-specific negative regulator (Dickey *et al* 1988; Walden *et al* 1988). The IRE conferred essentially no specific translational regulation on soybean ferritin mRNA in rabbit reticulocyte lysates, neither positive nor negative control with or without IRE (Dix *et al* 1992). The isolated IRE and the IRE in animal ferritin mRNA were used to predict structure in the 5'-untranslated region of the chimera of the frog ferritin IRE and soybean ferritin mRNA with the help of computer programs that could predict the hairpin loop (Jaeger *et al* 1990). The investigations indicated that the IRE hairpin loop did not form. The altered structure of the IRE sequence in the chimeric ferritin mRNA, atleast in the computer prediction, can explain the absence of translational regulation (Kimata and Theil 1994). Inspite of the high level of sequence conservation among the coding sequences of plants and animal ferritins (Ragland *et al* 1990), sequences specific to plant ferritin mRNA appear to prevent ERE function. Thus, it is premature to conclude the non-existence of iron induced translational control of ferritin in plants as the studies have been done only in a few cell types and 'issues. Moreover the information about the mechanism of iron induced transcription of Ferritin genes is rather scanty.

1.7 Developmental regulation of ferritin

Developmental regulation of ferritin in plants has been investigated at the molecular level only in the recent past. However, changes in iron-rich ferritin were reported by Seckbach (1968) and Whatley (1977) during chloroplast and nodule development using electron microscopy. In each case, iron-rich ferritin appeared to decrease as chloroplasts matured or nitrogen fixation increased. As both processes require the synthesis of iron-rich proteins, it was suggested that iron in ferritin of leaves or nodules was the iron precursor (Seckbach 1968; Whatley 1977).

In *Pisum sativum* (pea) ferritin was detected in total protein extracts from flowers, pods and accumulated in seeds. In an immunological study of pea ferritin, ferritin concentration decreased in cotyledons during the first week of germination. In the embryo axis, ferritin disappeared in radicles and epicotyls within 4 days of germination, whereas in cotyledons it is still present on day 7, but at a much lower concentration than on day 1 (Lobreaux and Briat 1991). In maize which has determinate nature of leaf development, it was observed that senescent part of the leaf contained higher levels of ferritin when compared to younger green sections of the leaf. The central part of the leaf had no ferritins correlating with diferentiating stage of chloroplasts (Theil and Hase 1993).

In developing soybean nodules, it was shown that ferritin mRNA and protein are detected early in nodulation before the accumulation of nitrogenase and leghemoglobin. However, ferritin decreased later in nodule development before the mRNA could decline. Although the mechanism of asynchrony between the regulation of concentration of nodule ferritin in RNA and protein is not yet known, it is clear that soybean ferritin mRNA can be regulated translationally in chimeric mRNA with the animal regulatory sequences (Dix

et al 1992).

1.8 Storage functions of ferritin

Ferritin is invariably found in etioplasts or chloroplasts playing an active role in the process of photosynthesis. Several authors have reported the presence of ferritin iron cores in leaf etioplasts and their disappearance upon illumination (Sprey *et al* 1978). The appearance and disappearance of ferritin iron cores seems to occur at specific stages of chloroplast development. Regulation of leaf ferritin protein concentration is clearly influenced by the signals of chloroplast maturation (Ragland *et al* 1990). Function of ferritin in plants is storage of iron for short or long periods to protect the cell against the toxic effects of unbound iron. Ferritin readily meets the demand for iron of the developing chloroplast. Ferritin is induced in nodules under diverse physiological conditions. The remarkably high negative correlation (0.92) between the concentrations of ferritin and heme, especially during early stages of nodule development, indicate that phytoferritin may play an important role in early nodule development (Dix *et al* 1992; Mann *et al* 1987).

1.9 Detoxification properties of ferritin

Iron forms insoluble complexes in presence of oxygen (hydrous ferric oxide). Secondly, free ionic iron can be extremely toxic, catalyzing the production of highly reactive free radical species leading to cellular damage. Ferritins sequester free iron thereby preventing the cellular damage from toxic free radicals. Ferritin is found to protect the *in vitro* translational system from harmful effects of heavy metal ions. Ferritin, thus plays a crucial role in protecting the biosynthetic machinery at the translational level (Fobis-Loisy *et al* 1996). Apart from sequestering iron, ferritin plays a major role in chelating other

divalent cations, viz. Cd^{2+} Zn^{2+} Co^{2+} Cu^{2+} etc. *in vitro* (Sczekan and Joshi 1989). In fact, among other metals bound to ferritin as isolated *in vivo,* only aluminium was found in measurable quantities. Be^{2+}, Cd^{2+}, Zn^{2+} were all present at less than 3-4 mol/mol protein. Ferritin thus plays a critical role in plants by not only acting in buffering iron but also in protecting the cell from heavy metal toxicity.

1.10 Summary

Plants and animals have ferritin which evolved from an ancient progenitor to store iron in a large core (0.6 - 0.8 nm diameter) of hydrated ferric oxide which is used for house keeping proteins such as the cytochromes, ferredoxins, ribonucleotide reductases as well as hemoglobin, leghemoglobin, myoglobin and nitrogenases. Similarities between plant and animal ferritins extend from the primary structure of the polypeptides that form the protein coat to the dense core observed in the electron microscope, the developmental expression in a variety of specialized cell types, and the induction by excess iron. The conservation of structure, function and expression reveal genetic signals as well. The distinctive location of ferritin in plastids is an important feature in plant ferritins. Ferritin that is targeted for plastids is encoded in an mRNA with a transit peptide that is cleaved on plastid entry but leaves a specific N-terminus. In conclusion the structure and regulation of ferritin in plants is not entirely through transcriptional mechanisms as the reports are scanty. Furthermore, the asynchrony between ferritin mRNA and protein regulation observed in leaf maturation suggests a complex interplay between transcriptional and posttranslational regulation of ferritin. Ferritin thus, is one of the few proteins which perform the function of reverse phase transition of metal. Apart from sequestering iron, ferritin also sequesters other divalent cations viz. Cd^{2+}, Co^{2+}, Cu^{2+}, $A1^{2+}$ etc. protecting the

cell from toxicity, thereby allowing the plant to survive under heavy metal stress. Thus, ferritin forms the front line of defense against heavy metal toxicity.

2 Objectives and scope of the study

2.1 Introduction

Vigna mungo (L.) Hepper (Black gram) (Papilionaceae) is an important pulse crop (Fig. 1). It is grown in about 5.316 x 10^5 ha in the province of Andhra Pradesh, India. The production is about 3.97 x 10^5 tonnes. Andhra Pradesh produces highest quantity in the country and the yield of black gram is about 805 Kg/ha. Blackgram is mainly grown in Krishna and Srikakulam districts. In command areas black gram is cultivated largely in rice fallows of Guntur, Nalgonda, Karimnagar and Nizamabad districts. It is mainly a rabi season (September to October) crop grown particularly in rice fallows. The seasons are as follows. Kharif crop is grown in the months of June to July. The duration of the crop is between 70-75 days.

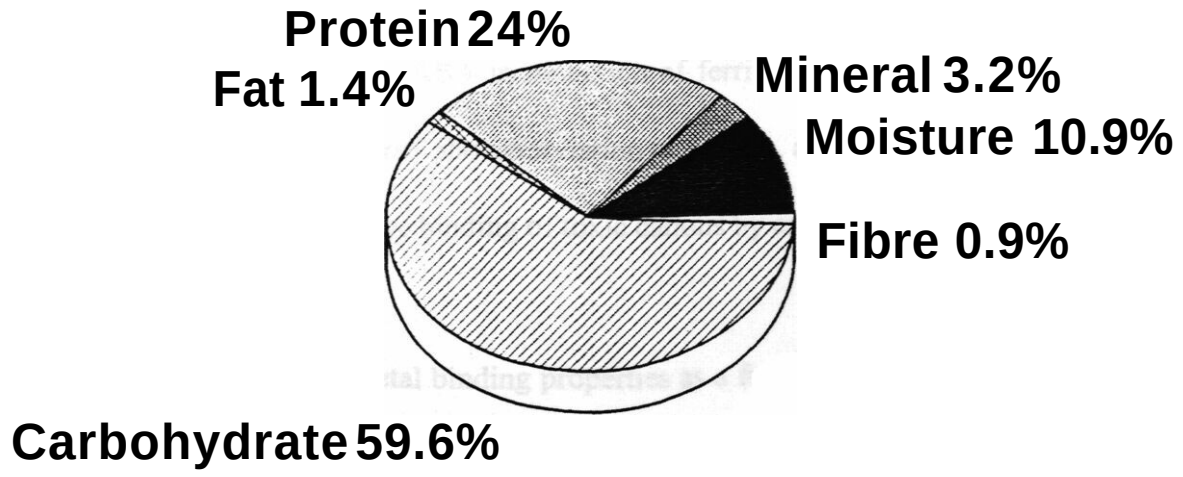


Fig. 1: Food value of black gram

Black gram can be grown on almost all types of soils except alkaline ones and soils with poor drainage. Well drained, clayey and black cotton soils give higher yields. Different varieties that are cultivated are T-9, Krishnayya (LBG-17), Teja (LBG-20), Prabha (LBG-402), PDU 3, and Butta minumu. In the present study, T-9 variety was used. The yield ranges from 10-15 tonnes/ha.

2.2 Objectives of the present study

1. Purification and characterization of ferritin from seeds of *V. mungo*.
2. To examine the role of iron mediated oxidative stress as a function of antioxidative enzymes viz., ascorbate peroxidase, catalase, guaiacol peroxidase and superoxide dismutase in the presence of exogenous free radical scavengers and antioxidants.
3. To examine whether ferritin induction occurs in the presence of exogenous free radical scavengers and antioxidants upon iron overloading so as to ascertain whether oxidative stress induces ferritin.
4. To delineate the role of ABA in induction of ferritin.
5. To understand the *in vitro* phosphorylation of ferritin, as increased phosphorylation of ferritin would enhance binding of toxic cations by creating putative ionic binding sites.
6. To study its *in vitro* metal binding properties as a function of metal detoxification.

2.3 Scope for future work

The promise of future is to determine the variation in regulation of ferritin gene in plant and animal biology. Anaemia induced by iron deficiency is a serious health problem

in various human populations (Yipp 1994). Therefore, suitable strategies need to be developed which would enhance the iron content of edible plant products. The role of ferritin in alleviating various stresses viz. heat shock, heavy metal toxicity, oxidative stress offers immense scope.

Further, the manipulations of genes coding for ferritin could be done to alter the levels of ferritin mRNA and protein. Manipulation of ferritin genes in plants has a beneficial role in soils deficient in iron, as ferritins would help the plant in effectively utilizing the scarce iron that is transported ultimately to the cell, which results in better economy of iron.

3 Materials and methods

3.1 Chemicals

Phenylmethylsulphonyl fluoride (PMSF) was obtained from Boehringer Mannheim, Germany. Nitrocellulose membrane (HAHY 304 FO) was purchased from Millipore corporation, Whatman filter papers (3 mm) were purchased from Whatman Biosys.Ltd., England. Bathophenanthroline disulphonic acid, Bromophenolblue, Calmodulin (CaM), N-acetyl cysteine, EGTA, Glutathione (reduced), Guaiacol, HEPES KN-62, Mol.wt markers, Polyvinyl-polypyrrolidone (PVPP), Riboflavin, Staurosporine, Syntide, Triton X-100, Tween-20, were obtained from Sigma Chemical Co., St.Loius, MO, U.S.A. Cellophane sheets were from Bio-Rad laboratories (Richmond, USA). $^{109}CdCl_2$ (Specific activity 2.61 mci/mg) was purchased from E.I Du Pont de Nemours & Co Inc, USA. Soldex X-Ray developer was obtained from May and Baker Photochem, and the fixer was the acid fixing salt with hardener purchased from Kodak India photographic Co.Ltd., India. Adenosine 5' (^{32}P) triphosphate (specific activity - 3000 Ci/mmol) was obtained from Board of Radiation and Isotope Technology (BRIT), Bhabha Atomic Research Center, Bombay. Tris, acrylamide, N-N-methylene bis-acrylamide, 2-mercaptoethanol and glycine were purchased from Spectrochem (Bombay, India). Silver nitrate, TEMED (N, N, N^1, N^1 tetramethylethylene diamine), Glycerol and Hydrogen peroxide (30%) were obtained from E. Merck, India. All other chemicals were of analytical grade and procured from local commercial establishments.

3.2 Purification of ferritin from seeds of *V. mungo*

3.2.1 Preparation of the extract

V. mungo seeds (Fig. 2) of T-9 variety purchased from local commercial

establishment were powdered (50 g) and homogenized in 300 ml of 50 mM Tris-HCl pH 7.5 containing 1% PVPP. PMSF (I mM) was added as antiprotease. The homogenate was centrifuged at 10,000 x g for 25 min. The supernatant was made 50 mM magnesium chloride with solid $MgCl_2$ and immediately centrifuged for 5 min at 6000 x g at 4℃. The supernatant was used as source of ferritin and for further purification.

Fig. 2: Seeds of *Vigna mungo* (L.) Hepper

3.2.2

Ammonium sulphate fractionation

As bulk of the legume ferritins precipitate at 30% ammonium sulphate concentration (Agrawal and Goldstein 1967), the crude extract was fractionated with 30% ammonium sulphate. The fractionated proteins were redissolved in extraction buffer and dialyzed against the same. The dialysate was recentrifuged and the supernatant was used for anion exchange chromatography.

3.2.3 *Anion exchange chromatography*

DE-52 (20 g) was suspended in 50 mM Tris-HCl, pH 7.5 and the matrix was allowed to settle. The supernatant from the ion exchanger was decanted carefully without disturbing the matrix. The matrix was further washed for three more times with the same buffer until the supernatant was free of particles in the ionic media. The slurry was then packed in glass column (6 x 2.5 cms), the column was equilibrated with the same buffer as mentioned above at 4° C.

The particulate free dailysate after ammonium sulphate cut was passed through the ion exchange column at a flow rate of 1 ml/min. After completion of loading on to ionexchange column, the column was washed with 5 volumes of buffer until the O.D of the flow through was almost 0.05 at 280 nm. The column was then eluted with a linear gradient of 0-1 M NaCl in extraction buffer. Absorbance at 280 and 420 nm was monitored and peak fractions were pooled and lyophilized. The concentrated protein was loaded on to a gel filtration column.

3.2.4 *Gel filtration*

The lyophilized protein from the ion exchange chromatography was solubulized in

Tris-HCl, pH 7.5 and loaded onto a gel filtration column which was packed with 15 g of Sephadex G-200 swollen in Tris-HCl, pH 7.5. The swollen beads were carefiilly packed into a slender column of 50 x 1 cm dimensions. The flow through (1 ml/6 min) was collected in 2 ml fractions. The fractions were monitored at 280 and 420 nm. The peak fractions were pooled, dialyzed and lyophilized. The protein was used for further studies.

3.2.5 *Protein estimation*

Protein was estimated following Lowry *et al* (1951) with a minor modification.

Reagent A: 4% sodium carbonate in 0.2 N sodium hydroxide

Reagent B: 1% cupric sulphate

Reagent C: 2% sodium potassium tartarate and

Reagent D: 1 N Folins reagent.

The working reagent was a mixture of A, B, C in a ratio of 23:1:1. One ml of working reagent was added to 1 ml of the protein sample, allowed to stand for 15 min. Then 0.2 ml of reagent D was added rapidly with vortexing. After 30 min, the absorbance was recorded at 750 nm. Protein content of the samples was calculated from a standard curve prepared using bovine serum albumin (fraction-v) as a standard.

3.2.6 Detection of Ferritin

Ferritin was detected in polyaerylamide gel by separating the proteins by 5% non-denaturing PAGE according to Laemmli (1970). Ferritin was selectively stained in PAGE by soaking the gels in 2% (w/v) potassium ferricyanide in 50% ethanol and 7% acetic acid for four hours.

SDS-Polyacrylamide gel electrophoresis

SDS-PAGE was performed according to Sigma technical bulletin No. MWS-877L based on Laemmli (1970). The following solutions were prepared to polymerize the gels and also to perform the SDS-PAGE.

A. Acrylamide Solution: 28 g acrylamide and 0.74 g methylene bis-acrylamide were dissolved in double distilled water and the volume was made up to 100 ml.

B. Separating gel buffer: 36.3 g Tris in 100 ml distilled water. The pH was adjusted to 8.9 with cone. HC1.

C. Stacking gel buffer: 5.98 g Tris in 100 ml distilled water. The pH was adjusted to 6.8 with cone. HC1,

D. SDS solution: 1% SDS in distilled water.

E. APS solution: 10% ammonium persulphate solution (prepared fresh).

Reservoir buffer: 6.025 g Tris, 14.4 g glycine and 600 mg SDS in 600 ml distilled water.

Sample buffer: 0.062 M Tris-HCl (pH 6.7), 10% glycerol, 2% SDS, 5% mercaptoethanol and 0.001% bromophenol blue.

Separating gels (20 ml) were prepared by mixing 8.92 ml of A, 2 ml of D and 6.07 ml of distilled water and polymerized by adding 20 µl of TEMED and 120 µl of E. Stacking gels (5 ml) were prepared by mixing 0.9 ml of A, 2 ml of C, 2 ml of D and 1.5 ml of distilled water and polymerized by adding 6 µl of TEMED and 60 µl of E. After completion of the electrophoresis, the gels were fixed in 50% methanol, 12% glacial acetic acid and 0.5 ml of 37% formaldehyde/L for silver staining.

Silver staining for the detection of proteins separated by electrophoresis (Blum *el al* 1987)

The gels, following electrophoresis, were fixed for one hour with 50% methanol containing 12% acetic acid and 0.5 ml formaldehyde per liter. The fixed gels were washed thrice, each

time with 200 ml of 50% ethanol for 20 min and treated with 0.02% sodium thiosulphate for one minute. Excess thiosulphate was removed from the gel surface by rinsing thrice with distilled water for 20 sec. Silver nitrate impregnation was carried out in 200 ml of 0.2% silver nitrate solution containing 150 µl of 37% formaldehyde for 20 min. Upon washing with excess of water to remove unimpregnated silver nitrate, the gels were developed with 6% sodium carbonate solution containing 0.5 ml of 37% formaldehyde and 4 mg sodium thiosulphate/L. Following washing with double distilled water the gels were stored in 50% methanol containing 12% glacial acetic acid. After silver staining, the molecular weight of the polypeptide of interest was calculated from the standard molecular wt. markers which were run simultaneously on the gel using UVP-2000 gel documentation software program.

3.2.7 *Raising of antibodies against seed ferritin*

Ferritin protein (100 µg) was injected subcutaneously initially with complete adjuvant, after a gap of 10 days three booster doses with incomplete adjuvant containing 100 mg each were given to rabbit. One week after the final booster, rabbit was bled from the marginal ear vein. The blood was allowed to clot at room temperature for one hour followed by overnight storage at 4°C and then spun at 5,000 x g for 10 min and serum was stored at -20° C in aliquots.

3.2.8 Immunological Methods

The cross reactivity of pea seed ferritin antibody with K *mungo* seed ferritin was checked using Ouchterlony's double immunodiffusion.

3.2.9
Western blot analysis

Western blotting was carried out on nitrocellulose membranes according to Towbin *el al* (1979). After separating the proteins on SDS-PAGE or the native PAGE, wet blotting was done using Biorad trans blot unit, the gels were initially soaked in Buffer A consisting of 0.045 M Tris, 0.192 M glycine and 20% methanol. The separated proteins were transferred on to a nitrocellulose membrane with a current limit of 0.25 Amps and 70 volts for 3 hrs. The membrane was then air dried and was rinsed with solution B consisting of 10 mM Tris, 150 mM Nacl, 0.05% Tween 20. Further, the membrane was blocked with 5% skimmed milk in solution B for 120 min. The primary antibody was then added to 5% skimmed milk in solution B and allowed to react for 120 min or more depending on the titre of the antibody which was (1:1000) and size of the blot. The membrane was then washed thoroughly (10 min x 6) with solution B, so as to avoid non-specific binding of the antibody with the membrane. The secondary antibody (commercial alkaline phosphatase-coupled to anti-rabbit IgG) was allowed to bind to the primary antibody in 5% skimmed milk in solution B. The nitro cellulose membrane was washed with solution B (10 min x 6) and finally transferred into solution C consisting of 100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$. Incubation for 5 min was followed by addition of the substrate, Nitroblue tetrazolium 0.033% to 10 ml of solution C, mixed, added BCIP (5-bromo-4-chloro-3-indoyl phosphate) 0.0165 % substrate and mixed again. Protected from strong light, reactive areas turned purple, usually within 1-15 min. Colour development continued for 30 more minutes. When the colour has developed to the desired intensity the reaction was stopped by rinsing the membrane in deionized water for several minutes. Later the membrane was dried between filter papers and photographed for a permanent record.

3.3.10
Absorption spectrum of ferritin

The absorbance spectrum of ferritin at a concentration of 500 μg/3 ml in 50 mM Tris-HCl buffer was recorded in Shimadzu UV-VIS Spectrophotometer (model UV-160A, Japan).

3.3.11
Fluorescence spectrum of ferritin

Fluorescence emission spectrum of purified ferritin was recorded at room temperature using Hitachi Fluorescence Spectrophotometer (model 4010, Japan) with an excitation wavelength of 286 nm using 5 nm band pass, 5 nm emission pass, and response of 2. Fluorescence excitation spectrum was recorded at 360 nm emission wavelength.

3.4
Culture of *V. mungo* seedlings

Seeds of *V. mungo* (L.) Hepper T-9 variety were purchased from local commercial establishments and were surface sterilized with sodium hypochlorite for 10 min. The seeds after thorough washing were soaked overnight and kept for germination on wet filter paper in polypropylene boxes. *V. mungo* seedlings (Fig. 3) were made iron-deficient by growing them in Hoagland's solution devoid of iron for 10 days (diurnal cycle was maintained with light intensity of 80 μE m^{-2} s^{-1}). These 10 day old seedlings of *V. mungo* were taken and used as an experimental system. Stems of 8 cms length which had primary leaves were cut above the roots (de-rooted) and were used for experiments. Five such stems with primary leaves were used for each treatment.

Fig. 3: Seedlings of *V. mungo* (10 days old)

3.5 Free radical scavengers and antioxidant treatment

One volume of a 100 mM $FeSO_4$ stock solution in 0.06 M HC1 was mixed with one volume 200 mM tri-sodium citrate prior to dilution in water to a final concentration of 500 µ*M* $FeS0_4$/l mM tri-sodium citrate. This freshly prepared solution was used to induce ferritin synthesis in derooted *V. mungo* seedlings. De-rooted seedlings were pretreated for

six hours with antioxidants: GSH (reduced) 1 mM and N-acetyl cysteine 10 mM and free radical scavengers: 10 mM each of sodium benzoate and mannitol, prior to adding iron (500 μM ferric citrate) for a further six hours iron-loading treatment. GSH (reduced) was solubilized immediately before use in deaerated water under vaccum. N-acetyl cysteine was used by dilution in distilled, sterile, deaerated water. Control experiments were performed by incubating demoted seedlings in distilled water which formed the negative control. Ferric citrate treatment (6 hrs) alone formed the positive control. Leaf samples after treatment were processed immediately for enzyme assays and for western analysis.

3.6 Assay and acitivity staining of oxidative enzymes

5.6.1 *Ascorbate peroxidase (APX 1.11.1.11)*

APX activity was measured according to Nakano and Asada (1981). 2 ml of the reaction mixture contained 50 μM phosphate buffer, pH 7.0, 0.2 mM EDTA, 0.5 mM ascorbic acid, 250 μM H_2O_2 and 50 mg of protein. The activity was recorded as the decrease in absorbance at 290 nm for 1 min and the amount of ascorbate oxidized was calculated from the extinction coefficient 2.6/mM/cm.

Activity staining was done according to the published procedure of Mittler and Zilinskas (1993). Total proteins (75 μg) were run on 10% native PAGE with 2 mM ascorbate in the electrode buffer at 4° C. On completion of electrophoresis, the gel was soaked in 50 mM PO_4 buffer, pH 7.0 containing 2 mM ascorbate for 30 min. The gel was then incubated in 50 mM PO_4 buffer, pH 7.0 containing 4 mM ascorbate and 2 mM H_2O_2 for 20 min. This was followed by rinsing with 50 mM PO_4 buffer, pH 7.0 for 1 min. Finally the gel was incubated with shaking in 50 mM PO_4 buffer, pH 7.8 containing 28

mM TEMED and 2.45 mM NET in dark until achromatic bands appeared on blue background. Incubation was stopped before the achromatic bands became cloudy and the background turned too dark. After a brief rinse in distilled water for 3 min the gel was stored in 10% acetic acid and stored at 4° C.

3.6.2
Catalase (CAT 1.11.1.6)

Catalase activity was determined according to Chance and Maehly (1955). The reaction mixture contained 100 mM phosphate buffer, pH 7.0 and 19 mM H_2O_2 in a final volume of 3 ml. The activity was determined by the change in absorbance for 1 min at 270 nm. The change of 1 O.D represents 25 µM H_2O_2 degraded/ml.

Activity staining was done according to modified procedure of Clare *et al* (1984). Total proteins (75 mg) were loaded on a 7.5% native PAGE. After completion of electrophoresis, the gels were soaked in 50 mM potassium phosphate buffer, pH 7.5 containing horseradish peroxidase (0.1 g/L) for 20 min. This was followed by the addition of 5 mM H_2O_2 and incubation continued for another 10 min. The gels were rapidly rinsed with water and placed in phosphate buffer containing 4-chloro-l-naphthol (0.5 g/L). The negatively stained gels were rinsed with water and photographed.

3.6.3
Superoxide dismutase (SOD 1.15. 1.1)

Activity was measured according to Beauchamp and Fridovich (1971). Cocktail was prepared by mixing 27 ml of $NaPO_4$ buffer, pH 7.8, 1.5 ml of methionine (300 mg/ml), 1 ml of NET (14.4 mg/10 ml), 0.75 ml of Triton X-100 and 1.5 ml of 2 mM EDTA. To 1 ml of cocktail, 10 ul of riboflavin and 50 µg of protein were added. The cuvettes were illuminated for 7 min using 3 comptalux bulbs in a water bath. The absorbance was

measured at 560 nm. SOD activity was a measure of the difference between NET reduction with protein and without protein. One unit of activity was the amount of protein required to inhibit 50% of the initial photoreduction of NET.

Activity staining was done according to the published procedure of Beauchamp and Fridovich (1971). Total proteins (75 µg) were run on 10 % native PAGE at 4° C. After completion of electrophoresis the gel was soaked in staining solution containing 50 mM KH_2PO_4, pH 7.8 containing 0.03 mM riboflavin, 0.326% TEMED and 1.25 mM NBT for 30 min. The stain was then removed and illuminated under light for 30 min. Achromatic bands appeared on a dark blue background. The gel was briefly rinsed with distilled water followed by storage in 10% acetic acid.

3.6.4 Guaiacol Peroxidase (GPX 1.11.1.7)

The reaction mixture contained 2 ml of 50 mM PO_4 buffer, pH 7.0, 0.2 ml of 0.2 mM guaiacol, 0.2 ml of 2 mM H_2O_2 and 0.6 ml of distilled water in a total volume of 3 ml. The reaction was initiated by adding 50 µg of the protein. The change in absorbance of 1 unit in 1 min at 470 nm gave the activity of guaiacol peroxidase according to Shinshi and Noguchi (1975).

Activity staining was done according to Dendsay and Sachar (1978). Total proteins (75µg) were run on 10% native PAGE at 4° C. After completion of electrophoreis the gel was stained by soaking in 25% acetic acid containing 1% benzidine for 10 min. Then the gel was incubated in 3% H_2O_2 until blue colour bands appeared. The gel was photographed immediately before the bands could fade away.

3.6.5
Ascorbic acid estimation

Ascorbic acid (ASA) was determined according to the method of Okamura (1980) with modifications for the leaf tissue. Frozen leaf material 150 mg was ground to a fine powder in a prechilled mortor and 0.8 ml of 6% (w/v) trichloroacetic acid (TCA) was added. The frozen sample was reduced to fine powder in liquid nitrogen. The mixture was continuously homogenized until completely thawed, allowed to stand for 15 min on ice and centrifuged for 5 min at 15,600 g (4° C). The clear supernatant was immediately assayed. The reaction mixture for ascorbic acid contained 0.2 ml of the supernatant, 0.6 ml of 0.2 M Phosphate buffer, pH 7.4 and 0.2 ml of double distilled water. The color was developed after addition of 1 ml of 10% (w/v) TCA, 0.8 ml of 42% (v/v) o-phosphoric acid, 0.8 ml of 4% (w/v) 2,2'-bipyridyl dissolved in 70% ethanol and 0.4 ml of 3% $FeCl_3$. Mixtures were incubated in a water bath at 42°C for 40 min and absorbance was read at 525 nm. Ascorbic acid dissolved in 6% (w/v) TCA was used for calibration.

3.7
Iron estimation

Leaf tissue weighing 150 mg was dried to constant weight in oven at 65° C. The dried tissue was digested on a sand bath with 300 µl of cone. HC1, followed by 300 µl of cone. HNO_3, heated till acid evaporated. After cooling, the residue was digested with 60% perchloric acid (400 µl) until colourless. Boiling and splattering of the sample was avoided. The extract was cooled and 1 ml of water was added. Followed by the addition of 750 µl of 1% thioglycolic acid. The extract was neutralized with 1.5 ml of saturated solution of sodium acetate. Iron present in the extract was then estimated according to bathophenathroline disulphonic acid method. Principle: The iron present in Perform is

converted to Fe^{2+} which then reacts with bathophenanthroline disulphonic acid to form a pink coloured complex whose intensity can be measured at 540 nm.

Reagents used:

1. Protein precipitant: Prepared by dissolving 10 ml of 100% TCA, 3 ml of thioglycolic acid and 200 µl of HC1, made upto 100 ml with glass distilled water.

2. Chromogen solution: 100 ml of sodium acetate (2 M) containing 25 mg of bathophenanthroline disulphonic acid.

3. Iron standard solution: 70.2 mg of ferrous ammonium sulphate was dissolved in distilled water containing 0.2 ml of 2 N H_2SO_4 and made upto 1 ml. The stock thus prepared contained 1 mg/100 ml of iron.

Working Standard: 40 ml of the above stock was diluted to 100 ml with glass distilled water. The standard thus prepared contained 400 µg iron/100 ml

Procedure: 1 ml of the extract was taken in acid-washed test tubes and 1 ml of water was added, followed by the addition of 2 ml protein precipitant solution. The solution was mixed thoroughly and allowed to stand for 5 min and centrifuged to get an optically clear supernatant solution (2000 x g for 15 min). The clear supernatant solution of 2 ml was transferred to clean test tube and 2 ml of the chromogen solution added, mixed and allowed to stand for at least 5 min. The blank contained distilled water and carried out through the same procedure.

Standard curve: A standard curve was constructed using 0.1, 0.5, 1.0, 1.5 and 2.0 ml of working standard and the treatments done similar to test sample except for digestion.

The amount of the iron present was calculated from the average of the slopes calculated by the formula slope of the standard divided by cone. The iron concentration of the

unknown was calculated by dividing the O.D of the unknown with the average slope.

3.8 Ferritin status in oxidative stress

Total proteins from the leaves of treatments as described in plant cultures, were used and 12.5% SDS-PAGE was run followed by western analysis with *V. mungo* ferritin antibody to evaluate the induction of ferritin during oxidative stress (500 μM ferric citrate treatment) and to probe for ferritin induction in the presence of antioxidants and free radical scavengers.

Pro-oxidative treatment

De-rooted seedlings were treated with 50-200 μM H_2O_2 as a pro-oxidant (with 50 μM interval) in the presence of non-saturating concentration (50 μM) of iron as ferric citrate. The duration of the treatment was 6 hrs. Light intensity was 80 μE M^{-2} S^{-1}. Total proteins from the treatments were isolated and 12.5% SDS-PAGE (mini gel) was run and western analysis was done using the *V. mungo* ferritin antibody.

3.9 Specificity of ferritin induction

De-rooted iron-deficient seedlings (10 days old) (details in materials and methods) were treated with 500 (μM concentration of each of the following salts of heavy metals viz, cadmium chloride, zinc sulphate, copper sulphate, silver nitrate, cobalt chloride in distilled water. De-rooted seedlings (5 in number) were used for each treatment. Ferric citrate (500 μM) formed the control Light intensity was 80 μE M^{-2} S^{-1}. Duration of the experiment was 6 hrs. Total proteins from the treatments were isolated and 12.5% SDS-PAGE (mini gel) was run and western analysis was done using the *V. mungo* ferritin antibody.

3.10
Influence of ABA treatment on ferritin induction

ABA (50-150 μM) was used to treat 10 day old iron-deficient de-rooted seedlings. Ferric citrate (500 (μM) formed the control. Light conditions and duration of the treatment were similar to that of pro-oxidative treatments. Total proteins from the treatments were isolated and 12.5% SDS-PAGE (mini gel) was run and western analysis was done using the *V. mungo* ferritin antibody.

3.11
Phosphorylation of ferritin

Protein phosphorylation was done according to Combest and Gilbert (1986). Seedlings of *V. mungo* were grown for 8 days in etiolated condition with Hoagland's as nutrient source devoid of iron. These seedlings were treated with Hoagland's solution devoid of iron for 48 hrs for control set and treated seedlings were grown in 200 mM ferric citrate in Hoagland's for 48 hrs. Leaves were used as a source of the total proteins.

3.77.7
Calcium-dependent phosphorylation

Total proteins isolated from iron-deficient and iron treated seedlings were extracted by homogenizing the leaf tissue in 10 mM Tris-HCl pH 7.4. Equal proteins (20 μg) was were incubated in 10 ml of medium containing 10 mM Tris, 10 mM $MgCl_2$, 1 mM DTT and 5 mM $CaCl_2$ for 30 seconds. The reaction was started by the addition of 2 μCi of $\gamma^{32}P$ in 10 μ1 (3000 Ci/mmol) and incubated for 30 seconds. The reaction was stopped by the addition of 20 μl of SDS sample buffer. Sample was boiled at 100 °C for 3 min. Equal protein (10 μg) was taken and 12.5% SDS-PAGE was done followed by silver staining. The gels were then dried under vaccum between cellophane sheets using a Hoeffer gel drier and exposed for 1-3 days to Kodak X-ray film at -70 °C.

3.11.2
Calcium-independent phosphorylation

Total proteins isolated from iron-deficient and iron treated seedlings were extracted by homogenizing the leaf tissue in 10 mM Tris-HCl pH 7.4. Equal protein (20 μg) in 20 μl was taken and incubated in 10 μl of medium described as above except for 5 mM $CaCl_2$ replaced by 1 mM EGTA to chelate calcium and reaction was performed.

The phosphorylated protein was judged to be ferritin by immunoprecipitation of phoshphorylated protein followed by autoradiography. Phosphorylation was also done in the presence of KN-62 (10 μM), CaM (2 μM), KN-62, Staurosporine, cAMP (10 μM), cGMP(10 μM), in phosphorylation medium with the total proteins isolated from leaves of 500 μM ferric citrate treatment. In CaM treatment Ca^{2+} was included in the phosphorylation medium.

3.11.3
Assay of CaM kinase

CaM kinase activity was assayed by the phosphorylation of syntide-2 according to the method of Fukunaga *et al* (1989). Reaction mixture contained 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 0.1 mM (^{32}p) ATP (2000-4000 cpm), 30 μM syntide-2, 2 mM calmodulin, 1 mM $CaCl_2$ 0.1 mM EGTA and suitable amounts of total proteins isolated from leaves of 200 μM ferric citrate treated *V. mungo* seedlings in a total volume of 50 μl Control reaction was carried out in the presence of 1 mM EGTA. After incubation at 30°C for 1 mm, the reaction was terminated by adding 10 μl of 0.4 M EDTA and a 50 μl aliquot was spotted on phosphocellulose paper squares. Radioactivity was determined as described by Roskoski (1983)

3.11.3.1
Developing and fixing of X-ray films

X-ray developer 90 gms, along with 16 gms of the hardener was dissolved in 1 liter of distilled water in order to prepare the developer. The fixer was prepared by dissolving 264 gms of X-ray acid fixing salt with hardener (Kodak) in 1 liter of double distilled water. One week after exposure to radioactivity, the X-ray films were developed until the bands were visible and washed in water to remove any traces of developer. The developed films were fixed for 10 min before washing them under running water. The washed films were dried and photographed.

3.12
In vitro binding of cadmium (^{109}Cd) to ferritin

Ferritin (50 µg) was incubated with 3 µl of $^{109}CdCl_2$ (0.1 µCi) in the presence of cold cadmium for 20 min. The labelling was stopped with addition of native sample buffer. Native gel (5%) was run at 70V, dried and the autoradiogram was developed after 48 his.

3.13
Phosphorylation of ferritin on mimosine treatment

Mimosine (100 ppm) was used to treat the 8 day old seedlings grown in iron-deficient condition (Hoagland's devoid of iron) were treated for 72 hrs, with 500 µM ferric citrate and mimosine (100 ppm) together. Total proteins were isolated from the leaves of the treatments and phosphorylation was done as described earlier both in presence of calcium and absence of calcium. Total proteins phosphorylated from iron-deficient leaves was used as control.

3.14
Ultraviolet difference spectroscopy

Ultraviolet difference spectral recordings were done according to Tan and

Woodworth (1969). Ultraviolet difference spectrum was recorded on Hitachi double beam Spectrophotometer (Hitachi 557 Japan). Ferritin (50 mg) was taken in 2 ml of 50 mM Tris-HC1 (pH 7.5) buffer in both sample as well as reference cuvettes. The base line correction was done with the protein versus protein in both cuvettes from 230 to 340 run. Protein was incubated for 4 hrs at 25°C with various concentrations of cadmium added as cadmium chloride. The ratio of protein to cadmium varied from 1:2 to 1:5, Copper was added as copper sulphate and the concentration varied from 20 to 50 mg copper for 50 mg of ferritin.

3.15
Fluorescence spectroscopy

Metal binding as a function of fluorescence was done by incubating cadmium chloride (200 μM) with ferritin (200 μg) for 4 hrs followed by recording the fluorescence emission spectrum at 15 min intervals within an hour with an excitation wavelength of 286 nm and EX bandpass of 5 run and EM bandpass of 5 nm with scan speed of 120 nm/min and response of 2 sec.

3.16
Laser scanning densitometry

Quantification of immunoblots was done with a computed laser scanning densitometer (Molecular Devices, USA) using the Image Quant software programme.

4 Purification of ferritin from seeds of *Vigna mungo* (L.) Hepper

4.1 Introduction

Iron is an essential element for all living organisms. It is required by many functional molecules involved in a great variety of metabolic pathways, such as nitrogen fixation, ribonucleotide reduction, electron transfer, activation and transport of oxygen, as well as inactivation of reduced forms of oxygen. However, the presence of oxygen has two negative consequences for iron utilization by living organisms. Firstly, ferrous iron can be oxidized, and at a concentration greater than 10^{-18} M, ferric iron forms insoluble hydrous ferric oxides. Secondly, free iron can be extremely toxic, catalyzing the production of free radical species leading to cellular damage. In plants iron deficiency causes chlorosis, and it has been known for over a century that addition of iron causes recovery of iron chlorotic plants (Clarkson and Hanson 1980). This phenomenon can be explained by the fact that iron is involved in many reactions of photosynthesis and chlorophyll synthesis (Crichton and Ward 1992). Thus the tendency to form insoluble salts in aqueous solutions and its potential to generate toxic free radicals as a result of redox reactions in the presence of oxygen has led to the evolution of specific genetic systems which control iron homeostasis in cells (Aisen and Listowsky 1980). Hence, plants have evolved ferritins to protect themselves from toxic free radicals generated by excess free iron which otherwise would have been deleterious to plant cell and in turn to plant system. Ferritins are a class of proteins ubiquitous in living organisms. These are known to sequester and detoxify iron that is taken up by cells but not utilized for metabolic requirements. On the other hand, under conditions of iron need, ferritin iron can be released for cellular utilization. Therefore ferritins are key proteins acting as a buffer for iron, protecting cells from harmful dose of

free iron (Aisen and Listowsky 1980; Theil 1987). Ferritins are multimeric proteins. These molecules consist of protein shells each with a capacity to store upto 4500 atoms of the metal (Fe^{3-}) as ferric oxyhydroxy phosphate complex in its central cavity. Legumes in particular are rich source of ferritins. *V. mungo* was chosen for our study due to its economic importance as an important leguminous crop and also due to its nutritive value (Gopalan *el al* 1987) (Please see page 12). Since purified protein is required in large amounts for conducting different studies, seeds were taken to purify ferritin. In view of the existing literature on ferritin in different plants, ferritin from *V. mungo* seeds was purified so as use the purified protein for conducting studies on functional aspects.

4.2 Results

Ferritin was purified from *V. mungo* seeds to apparent homogeneity using ammonium sulphate fractionation, DE-52 column chromatography and gel filtration. The ammonium sulphate concentrated protein was loaded on to DE-52 column after dialysis. This protein eluted at 0.549 M NaCl (Fig. 4). The protein peaks stained positively with ferricyanide indicating the presence of iron (Fig. 5). The pooled fractions of DE-52 eluent were loaded onto Sephadex G-200 gel filtration column. The protein eluted as a single peak (Fig. 6) and showed a single band at 540 kDa on native gradient gels (Fig. 7). The protein peak coincided with iron stained band. Under denaturing conditions ferritin gave single band with a mol. wt. of 28 kDa (Fig. 8).

4.3 Characterization of the purified ferritin

Spectroscopic and immunological studies were done to characterize the purified protein as ferritin. The purified protein has shown a characteristic UV spectrum of ferritin

having two peaks with maximum absorbance at 225 rim and minimum at 286 nm (Fig. 9) which correlated with fluorescence excitation spectra, the maximum absorbance being at 286 nm and minimum at 225 nm (Fig. 10). Further excitation of protein at 286 nm gave a peak with emission at 340 nm (Fig. 11).

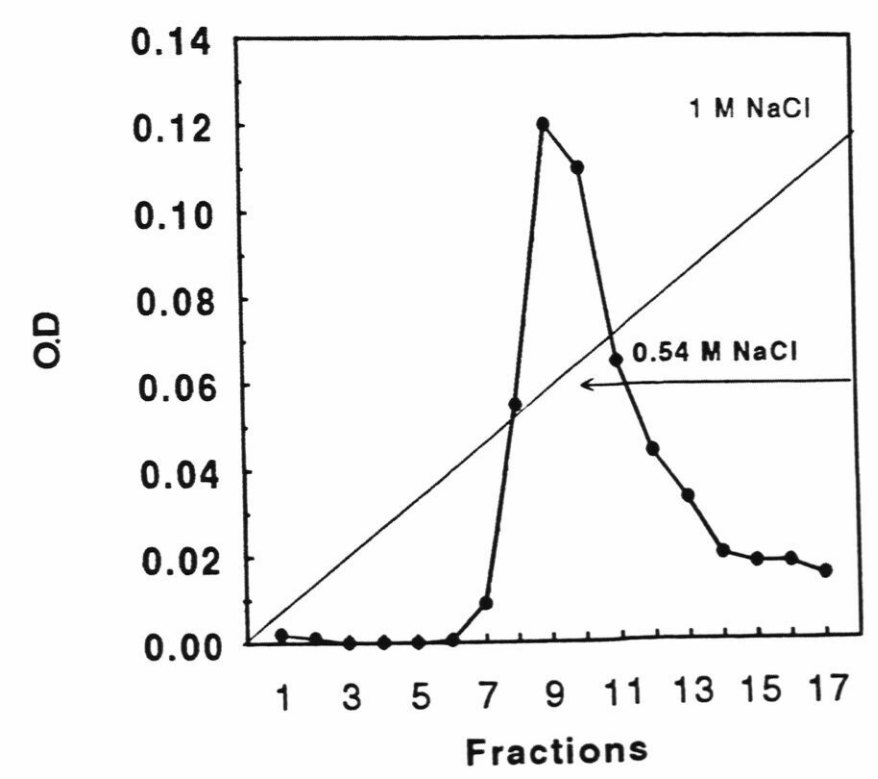


Fig. 4: Anion exchange chromatography of *V.mungo* ferritin on DE-52 column. 30% ammonium sulphate fractionated proteins were loaded on 50 mM Tris-HCl pH 7.5 equilibrated column and after washing with the equilibration buffer, the protein was eluted with linear salt gradient from 0-1 M NaCl. Absorbance of the fractions was recorded at 280 nm. Ferritin eluted at 0.54 M NaCl concentration. Fraction size 4 ml/mm.

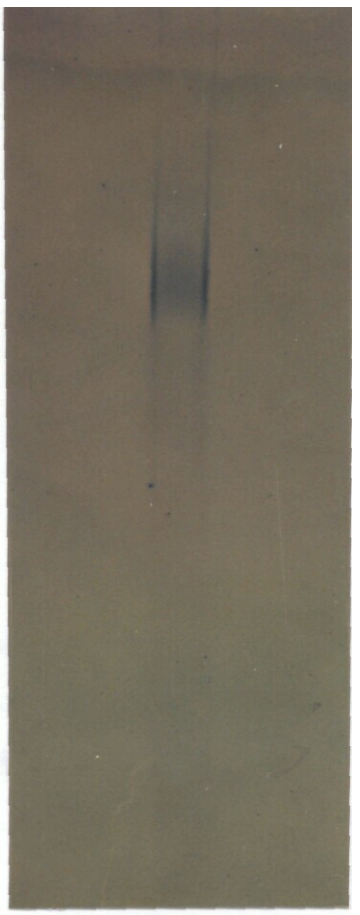


Fig. 5: Iron specific staining of ferritin was carried out by soaking the 5% Native PAGE in 2% potassium ferricyanide in 50% ethanol and 7% acetic acid for 4 hrs. Note the Prussian blue colour of ferritin.

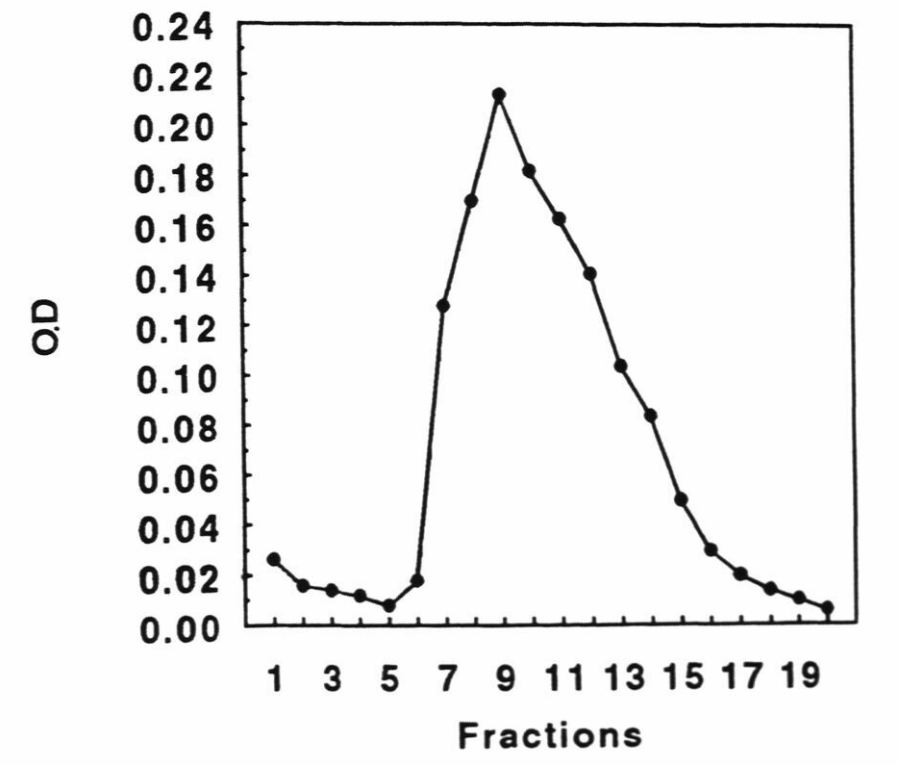


Fig. 6: Separation of ferritin on gel filtration (Sephadex G-200) using open column. Ferritin obtained from peak fractions after ion exchange chromatography was desalted, concentrated and loaded on to Sephadex G-200 column. The protein was eluted with a flow rate of 2 ml/12 min. The absorbance of the fractions was recorded at 420 nm.

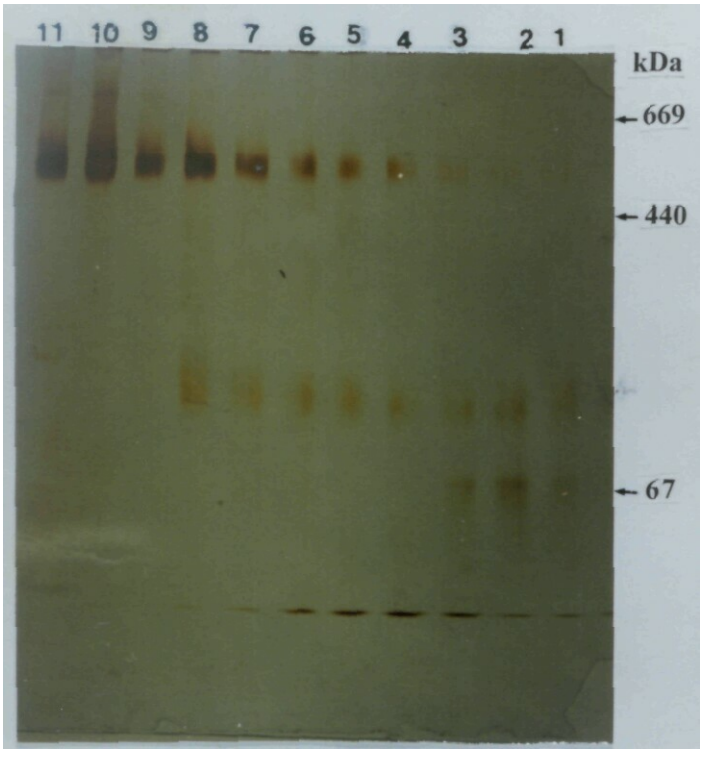


Fig. 7: Native gradient PAGE (5-10%) of fractions from Sephadex G-200 and purified ferritin was observed as single band at 540 kDa in peak fractions. Equal protein (10 µg) was loaded in each lane.

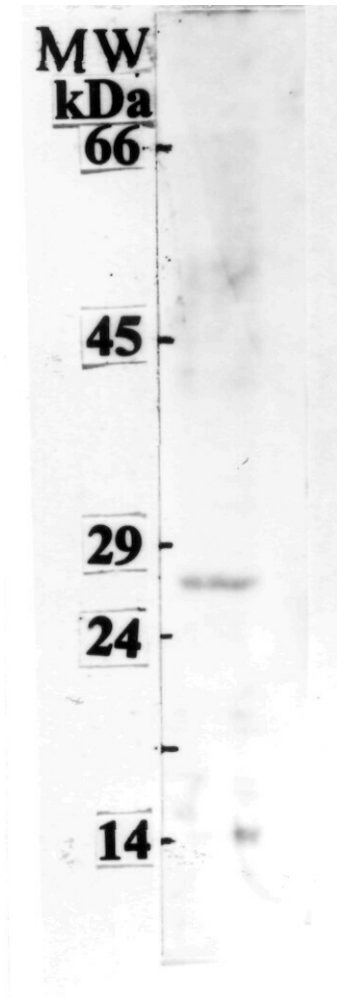


Fig. 8: Silver staining of SDS-PAGE of purified ferritin on 12.5% gel showing a single band of 28 kDa.

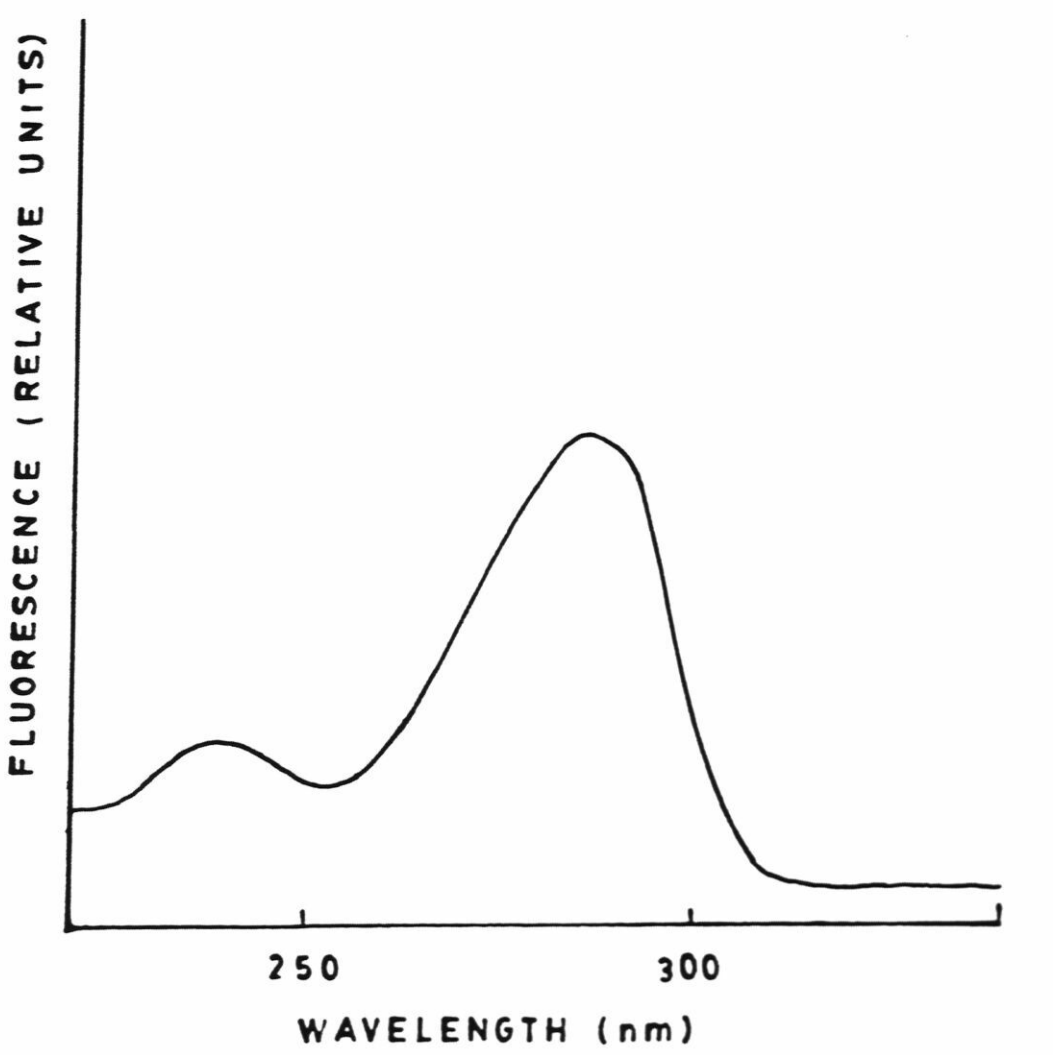


Fig. 10: Fluorescence excitation spectrum of ferntin at room temperature with maxima at 280 nm and minima at 240 nm. Emission wavelength used was 360 nm.

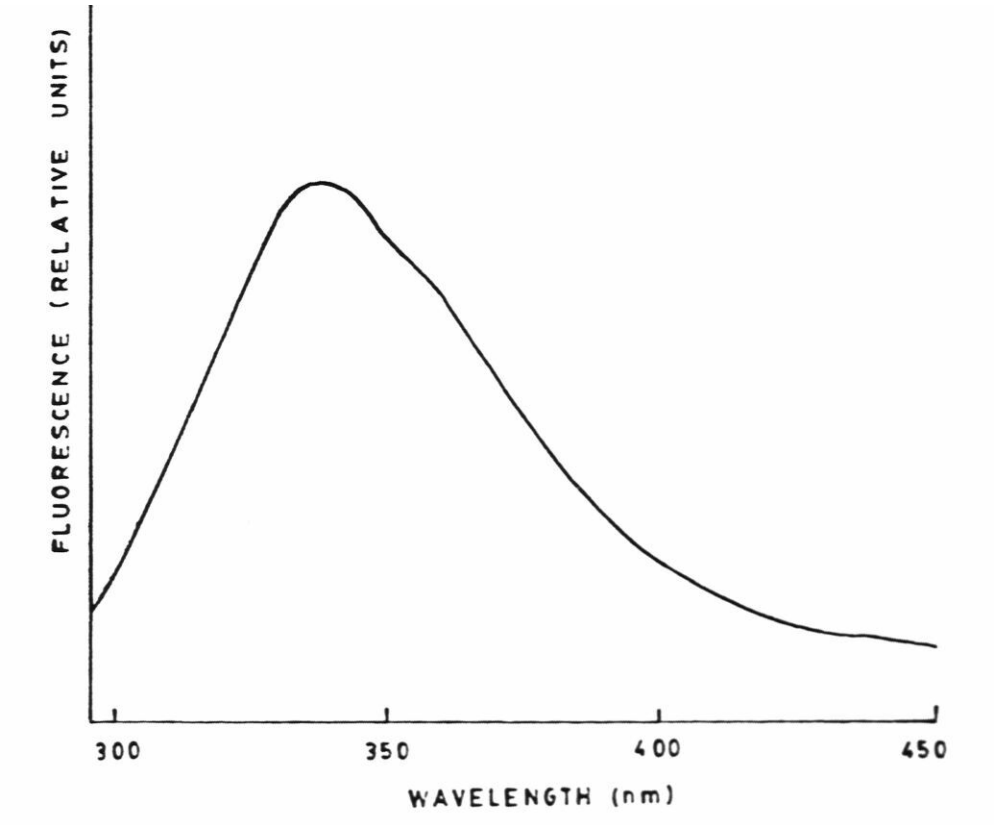


Fig. 11: Fluorescence emission spectrum of ferritin at room temperature showing peak at 340 nm. Excitation wavelength used was 286 nm, 5 nm band pass, 5 nm emission pass and instrument response of 2.

4.4
Immunological Identification

The identification of purified protein as ferritin was done by Ouchterlony's double immunodiffusion based on the cross reactivity of *V. mungo* ferritin with pea seed *(Pisum sativum)* ferritin antibody which gave a clear precipitin line without spur. The purified protein cross reacted with pea seed ferritin antibodies both on Ouchterlony (Fig. 12) and western blot of native gel indicating it as phytoferritin (Fig. 13). SDS-PAGE of total proteins isolated from seeds when subjected to western analysis with *V. mungo* seed ferritin antibody, reacted with 28 kDa peptide. Partial cross reaction with other proteins was also observed (Fig. 14). The amount of ferritin obtained was nearly 3 mg for 100 g of seed powder.

4.5
Discussion

Ferritin has been isolated from animals and plants. Animal ferritins have been extensively studied and a wealth of information is now available concerning their structure, synthesis, function and regulation (Theil 1987). The information pertaining to plant ferritin is scanty although ferritin has been purified from a few taxa of angiosperms (Crichton *et al* 1978; Korcz and Twardowski 1992; Laulhere *et al* 1987; Sczeken and Joshi 1987; van der Mark *et al* 1983). In the present study ferritin was purified from *V. mungo* which eluted at 0.549 M NaCl and the relative molecular weight was determined to be of 540 kDa on 5-10 % gradient native PAGE which is similar to previous reports of pea seed ferritin (Laulhere *et al* 1987). In denaturing conditions ferritin gave a single band at 28 kDa. Ferritin from *V. mungo* showed characteristic absorbance spectrum with peaks at 225 run and 286 nm which correlated with fluorescence excitation spectrum. Further, when excited

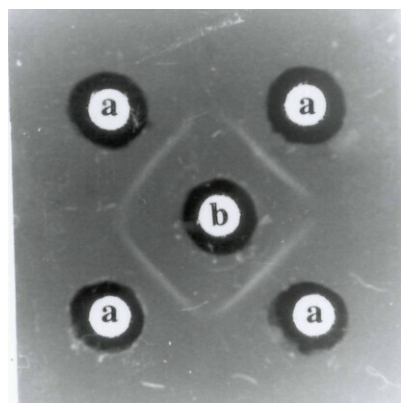


Fig. 12: Ouchterlony's double immunodiffusion showing cross reactivity of pea seed ferritin antibody with *V. mungo* seed ferritin. Central well contains pea seed ferritin antibody, peripheral wells are loaded with *V. mungo* purified ferritin.

Fig. 13: Western analysis of ferritin in native condition (5%) probed with *Pisum sativum* seed ferritin antibodies.

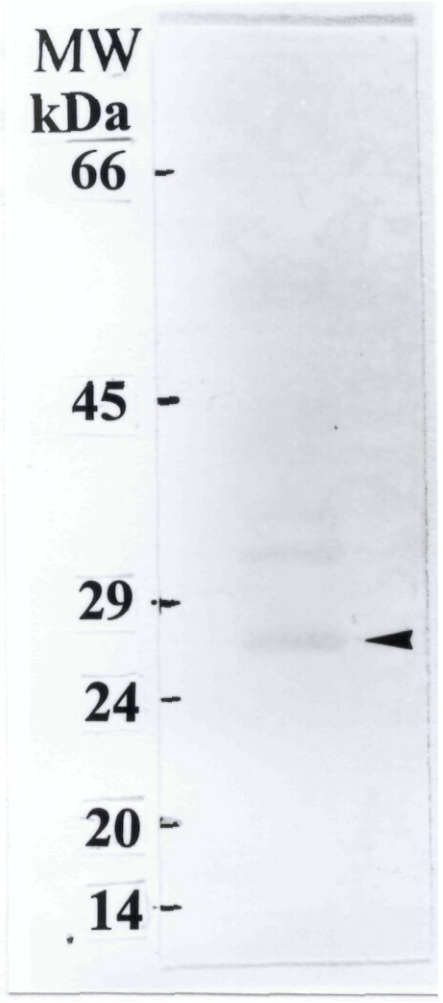


Fig. 14: Western analysis of SDS-PAGE of total proteins isolated from seeds probed with *V. mungo* seed ferritin antibodies. Partial cross reaction with another polypeptide was observed.

at 286 nm, *V. mungo* seed ferritin gave an emission spectrum characteristic of tryptophan emission of ferritin at 340 nm indicating this protein as ferritin (Fareeda *et al* 1992; Wuytswinkel and Briat 1995). The antibodies of pea seed ferritin cross reacted with *V. mungo* ferritin both in Ouchterlony and western blot analysis suggesting the immunological similarity of the two proteins and confirming it as ferritin. The amount of ferritin obtained was 3 mg/100 g seed powder which is low compared to earlier purifications from pea *(Pisum satiwm),* nevertheless sufficient for conducting various studies with purified protein.

5 The role of iron induced oxidative stress and ABA in induction of ferritin in de-rooted *V. mungo* seedlings in the presence of antioxidants and free radical scavengers

5.1 Introduction

Iron is an essential element for many proteins involved in cellular processes of higher plants, most notably photosynthesis and respiration. The principle of the uptake route has been known for more than 20 yrs, namely that soil Fe^{3+} chelates are reduced at the root surface to Fe^{2+} which is the actual uptake form (Chaney *et al* 1972). Iron metabolism should be adapted to cellular demands. Iron deficiency results in chlorosis, whereas iron excess causes oxidative stress. Low oxygen concentrations are a feature of many soils, particularly in waterlogged or wetland conditions (Armstrong 1967). Such soils are often associated with iron concentrations in excess of 1 mM as a result of reducing conditions and the solubilization of ferrous ions (Ponnampemma *et al* 1972). The major form of iron available to plants in well-aerated soils is the relatively insoluble ferric (Fe^{3+}) form (Lindsay 1972).

Excess of iron causes oxidative stress. Being a transition metal, iron reacts with reduced forms of oxygen through the Haber-Weiss and Fenton reactions to generate free radicals (Halliwell and Gutteridge 1984). Aerobic organisms eliminate the less reactive oxygen species as efficiently as possible. In plants, the ascorbate /glutathione cycle is also an important component of the defense against oxidative damage (Halliwell 1987). H_2O_2 is removed by catalases, ascorbate peroxidase and glutathione peroxidases. Besides enzymatic reactions, free radicals are scavenged by small molecules such as carotenoids and

thiol compounds viz. reduced GSH, cysteine (Elstner 1987).

Besides enzymatic scavenging of reactive oxygen intermediates, control of the concentrations of free transition metals known for their prime role in oxygen activation could form an important complementary way in the prevention of oxidative damage. Among transition metals, iron is the most abundant in biological systems, and iron generates hydroxyl radicals through Fenton reaction (Halliwell and Gutteridge 1988). The potential toxicity and insolubility of iron in presence of oxygen is handled well by ferritins. These are a class of multimeric proteins involved as buffer molecules for storage and release of iron for various needs within the cell (Theil 1987). Animal ferritins are cytosolic proteins whereas plant ferritins are localized in plastids (Andrews *et al* 1992). The synthesis of ferritins in animals and plants is induced upon iron overload. However, the regulation of induction is not similar. Induction of ferritin is controlled translationally in animal cells (Klausner *et al* 1993) whereas in plants it is regulated transcriptionally (Lescure *et al* 1991; Lobreaux *et al* 1992; van der Mark *et al* 1983). Recent evidence suggests that ferritin accumulation is also regulated at posttranscriptional level (Fobis-Loisy *et al* 1996).

In hydroponically raised maize seedlings, ferritin mRNA and protein accumulation in root and leaf tissues is mediated via two pathways (Lobreaux *et al* 1993): one dependent on the plant hormone ABA as demonstrated by inhibition of ferritin synthesis in response to iron in the *vp2* ABA deficient maize mutant, another mediated by oxidative stress as reported in maize (Lobreaux *et al* 1995). In contrast, only oxidative pathway was found to operate in *Arabidopsis* (Gaymard *et al* 19%). It has also been reported that in animals ferritin plays a role in prevention of oxidative stress by detoxification of free iron excess. Endothelial cells treated by H_2O_2 are protected against cytolysis by injection of H-type

ferritin into their cytoplasm (Balla *et al* 1992).

It has been shown at mRNA level that oxidative stress and ABA play an important role in ferritin induction in maize (Lobreaux *et al* 1995; Briat and Lobreaux 1997). This has been proved at both mRNA and protein level in *Arabidopsis* (Lobreaux *et al* 1993) in oxidative pathway. Further, it has been reported that pro-oxidant treatment using H_2O_2 induces ferritin synthesis. Hence, the activities of antioxidant enzymes viz. catalase, superoxide dismutase, guaiacol peroxidase and ascorbate peroxidase were measured in treatments with free radical scavengers and antioxidants viz. sodium benzoate, mannitol, GSH (reduced) and N-acetyl cysteine in *V. mungo*. Further, inhibition of ferritin induction was studied during aforesaid treatments so as to ascertain whether oxidative stress mediates ferritin induction.

Pro-oxidant (H_2O_2) treatment was also done to evaluate its ability in induction of ferritin. Further, experiments were also conducted with ABA to discern its role in ferritin induction in iron-deficient de-rooted seedlings. Since lowering of levels of endogenous antioxidants like ascorbate has been shown to be an evidence of oxidative stress in partially de-rooted seedlings of *Nicotiana plumbaginifolia* (Kaempfenkel *et al* 1995), levels of ascorbic acid were estimated in different treatments. Here advantage was taken of plant growth in hydroponic culture and the very rapid infiltration of any kind of water soluble compound upon de-rooting via the transpiration stream as demonstrated in literature (Kaempfenkel *et al* 1995; Lobreaux *et al* 1995).

5.2
Results

5.2.1
Activities of ox/dative stress responsive enzymes

5.2.1.1
Ascorbate peroxidase (APX 1.11.1.11)

The ascorbate peroxidase activity increased from 176 ± 21.68 in control to 235 ± 13.07 μmoles/mg protein/min in ferric citrate-treated seedlings. The specific activities in mannitol, NAC and sodium benzoate treatments were almost similar to FC treatment. In GSH (reduced) treatment the activity increased to a considerable extent (Fig. 15). Different isozymes of APX were visualized on 10% native PAGE (Fig. 16).

5.2.1.2
Guaiacol peroxidase (GPX 1.11.1.7)

The activity of Guaiacol peroxidase increased by about 30% from 0.566 ± 0.80 in control to 0.74 ±. 0.142 units/mg protein/min in FC treatment. The increase in activity was controlled more by mannitol followed by NAC and sodium benzoate (Fig. 17). Three different isozymes were resolved on 10% native PAGE of which there was increased expression of 1st and 2nd isozyme and suppression of 3rd isozyme in GSH treatment (Fig. 18)

5.2.1.3
Catalase(CAT 1.11.1.6)

The specific activities of catalase increased from 0.433 ± 0.32 in control to 1.25 ± 0.25 μmoles/mg protein/min in FC treated seedlings (Fig. 19). Mannitol was efficient in reducing the activity followed by GSH and NAC. Sodium benzoate was least effective in suppressing the increase in activity (Fig. 19). Activities of catalase were visualized on 10% native PAGE (Fig. 20).

5.2.1.4

Superoxide Dismutase (SOD 1.15. 1.1)

The specific activity of superoxide dismutase increased from 0.882 units/mg/min in control to 0.946 units/mg/min in FC treatment. NAC was most effective in suppressing the activity followed by sodium benzoate and mannitol (Fig. 21). Two isozymes were seen on 10% native PAGE, showing increased expression in GSH and NAC treatments (Fig. 22).

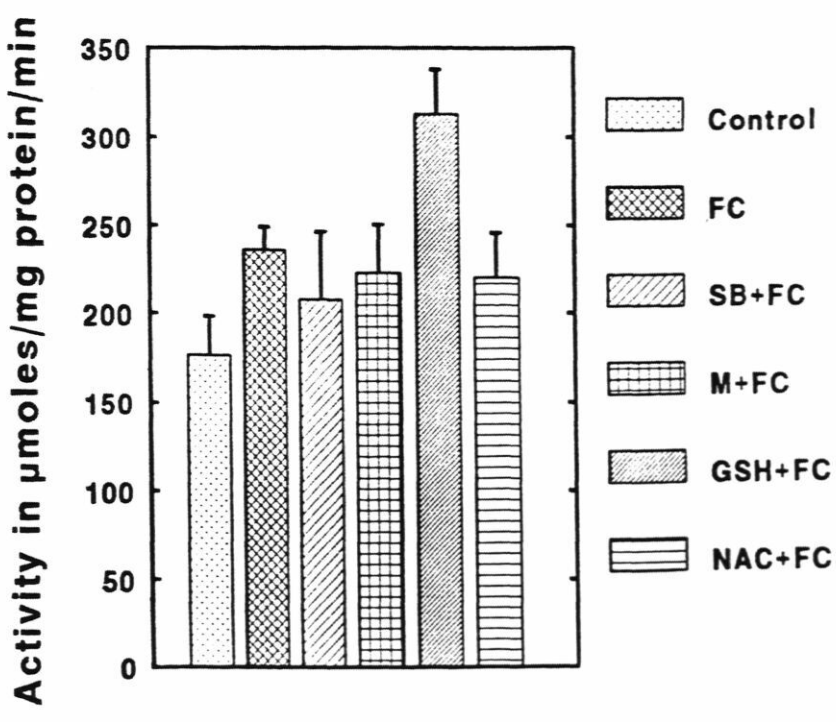


Fig. 15: Activity of APX in iron-deficient, de-rooted, 10 day old seedlings of *V. mungo* pretreated for six hours by incubating with SB = Sodium benzoate (10 µM), M = Mannitol (10 µM), GSH = Glutathione (1 µM), NAC = N-acetyl cysteine (10 µM) individually, followed by co-treatment with FC (500 µM) for further six hours. Seedlings incubated in distilled water formed negative control. FC (500 µM) alone for 6 hrs was taken as positive control. GSH treatment enhanced the activity. Results are the means $\pm$ SD (n=3 with two replicates each).

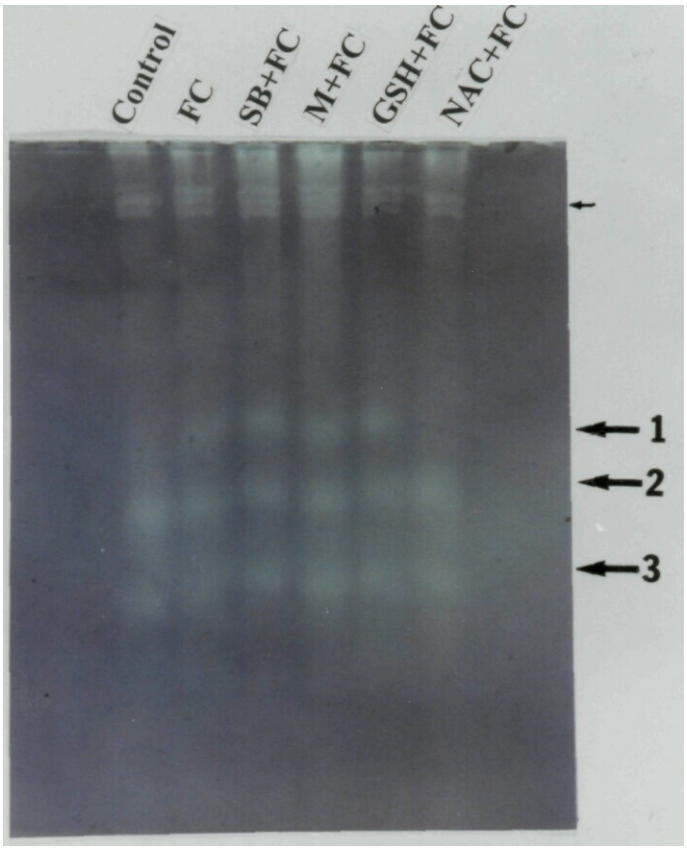


Fig. 16: 10% native PAGE of APX (ascorbate peroxidase) showing the activities of three isozymes under different treatments with free radical scavengers and antioxidants (For details see legend for Fig. 15). The activities are predominant under glutathione treatment viz. 1, 2 and 3 (<--) indicated the position of three slow moving high mol.wt. isozymes. APX 1 isozyme activity was suppressed in NAC treatment. Equal protein (75 µg) was loaded from each treatment.

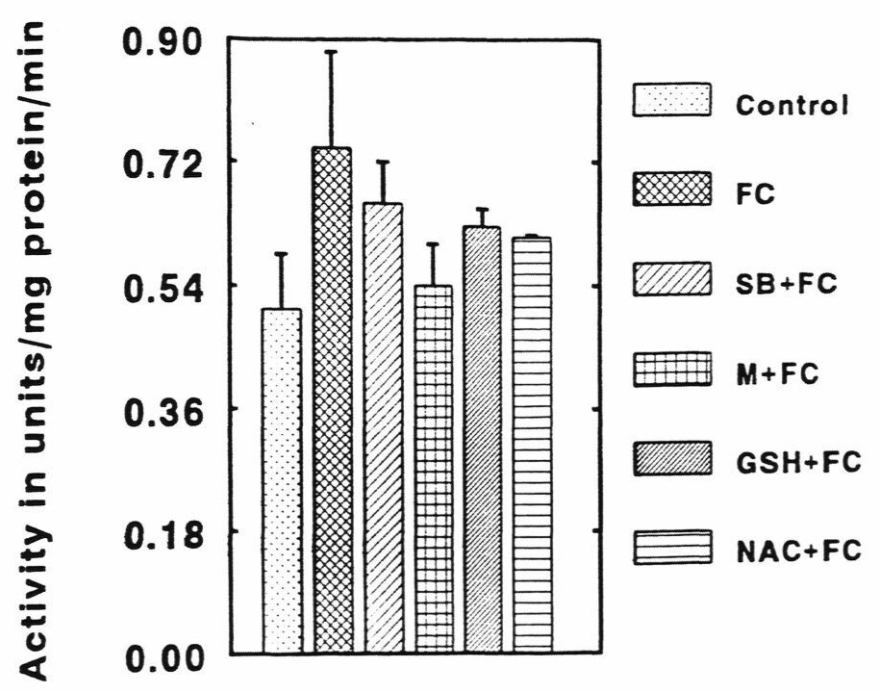


Fig. 17: Activity of GPX in de-rooted, iron-deficient, 10 day old seedlings, treated with FC, SB, M, GSH and NAC (treatment conditions as stated in the legend for Fig. 15). Note mannitol was more effective in controlling the increase in activity, followed by NAC and SB. Results are the means $\pm$ SD (n=3 with two replicates each)

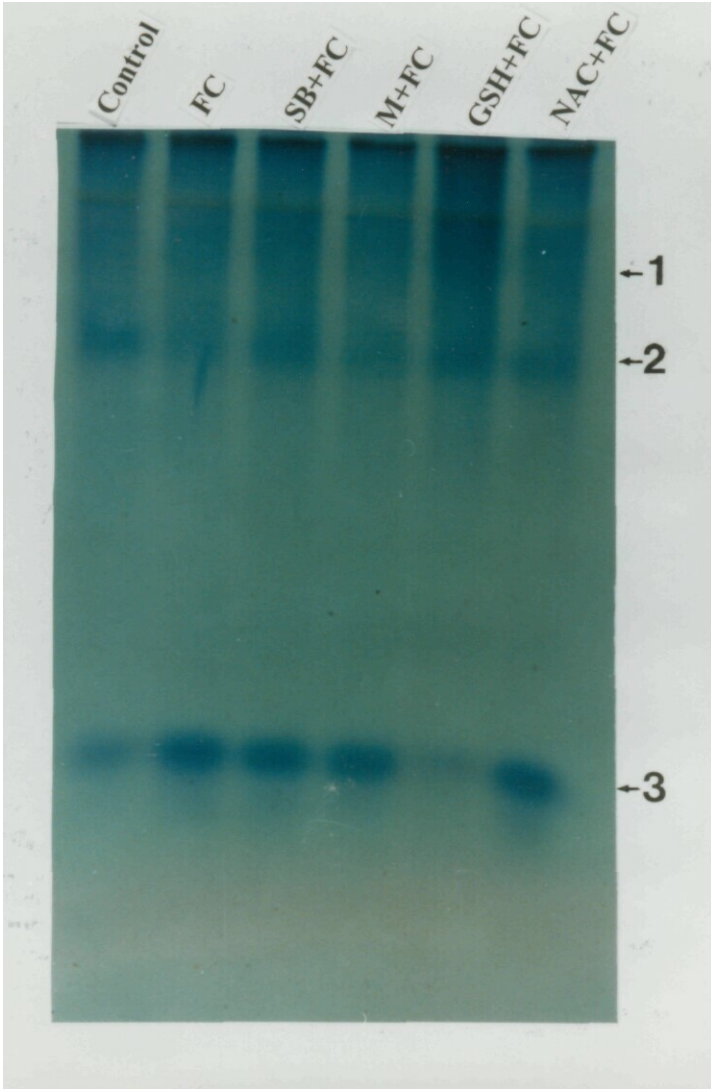


Fig. 18: 10% native PAGE of GPX showing the activities of isozymes under different treatments with free radical scavengers and antioxidants (for details of treatment, please see the legend for Fig. 15). Increased expression of 1st and 2nd isozyme and inhibition of 3rd isozyme in GSH treatment. Equal protein (75 μ.g) was loaded from each treatment.

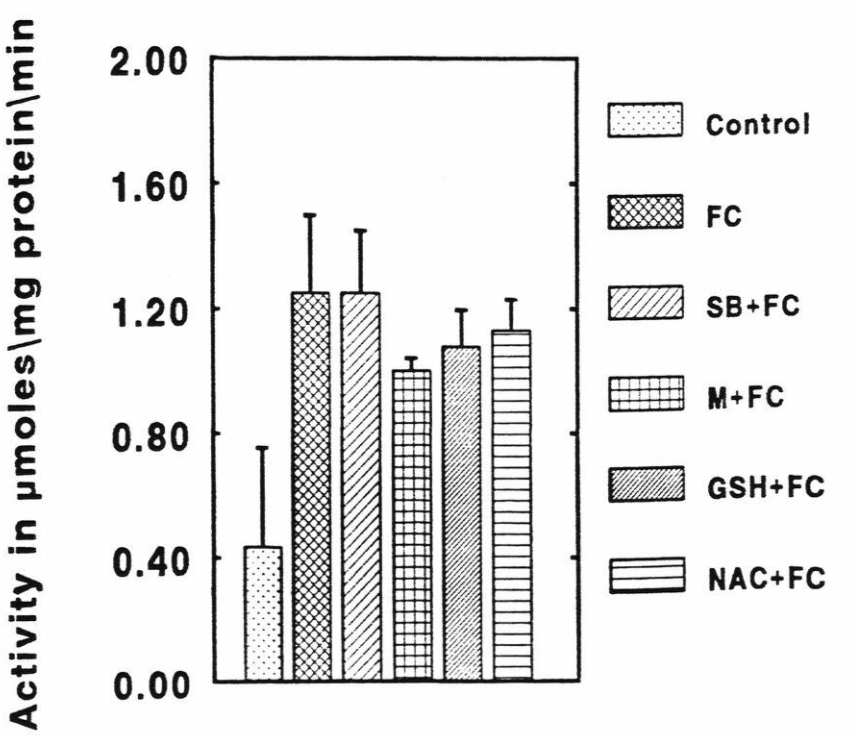


Fig. 19: Activity of CAT in de-rooted, iron-deficient, 10 day old seedlings treated with FC, SB, GSH and NAC (treatment conditions as stated in the legend for Fig. 15). Note that mannitol was efficient in reducing the activity followed by GSH and NAC. Results are the means $\pm$ SD (n= 3 with two replicates each)

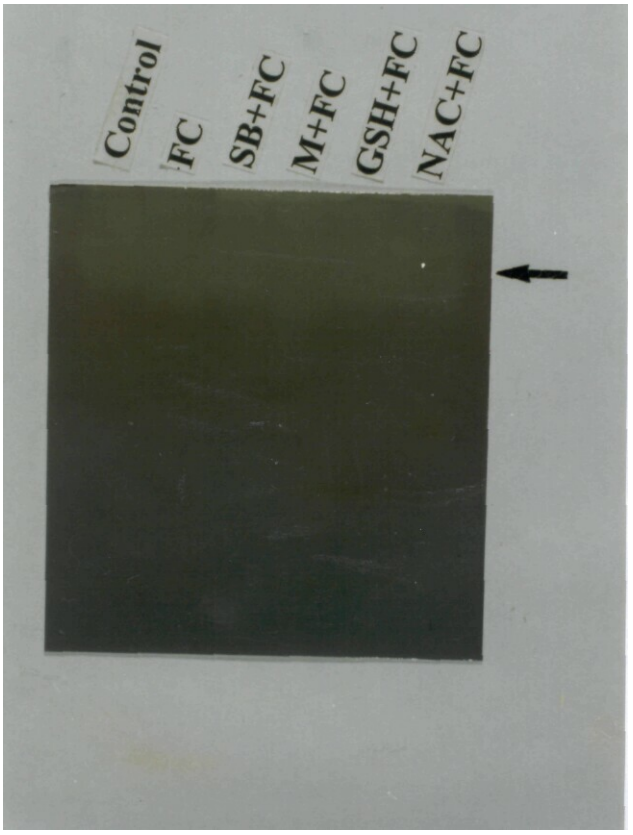


Fig. 20: 7.5% native PAGE of CAT showing the activities of isozymes under different treatments with free radical scavengers and antioxidants (for treatment details see legend for Fig. 15). Equal protein (75 μg) was loaded from each treatment.

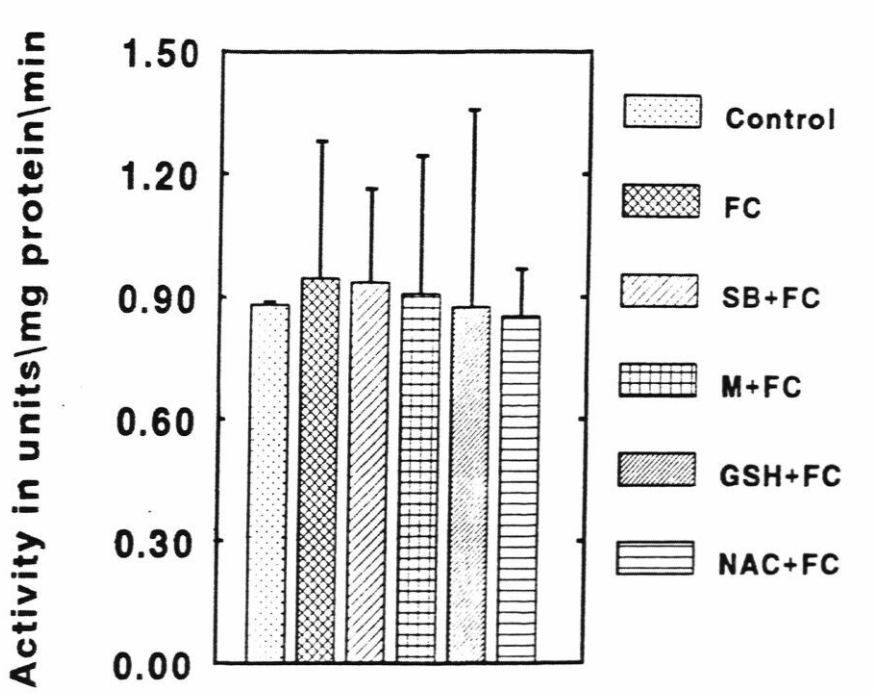


Fig. 21: Activity of SOD in iron-deficient, de-rooted, 10 day old seedlings treated with free radical scavengers and antioxidants (treatment conditions as shown in legend for Fig. 15). NAC was most effective in inhibiting the activity followed by SB and mannitol. Results are the means ± SD (n=3 with two replicates each).

5.3
Influence of ferric citrate on ascorbic acid (antioxidant) levels

Metabolite levels were compared in control and iron-deficient, de-rooted seedlings and in antioxidant and free radical treatments. Endogenous levels of ASA decreased from 0.438 in control to 0.294 μmoles/g.f.wt. in FC treatment (Fig. 23). Mannitol was more effective in restoring the ascorbate content to control level compared to sodium benzoate and GSH. Ascorbate levels showed two fold increase in NAC treatment (Fig. 23).

5.4
Estimation of total iron

The iron content of FC treated seedlings was 42.97 μg/g.f.wt compared to 32.13 μg/ g f.wt in control seedlings. The iron contents in antioxidant and free radical treatments were almost similar and were slightly lower in FC-treatment (Fig. 24).

5.5
Induction of ferritin

There was dose-dependent increase of ferritin induction from 0 to 250 μM ferric citrate in western analysis of native gel probed with *V. mungo* seed ferritin antibodies in leaves. In roots the increase was only upto 200 μM (Fig. 25). Ferritin induction was observed in FC-treated, de-rooted seedlings. Sodium benzoate treatment failed to inhibit ferritin induction. Inhibition of ferritin induction was observed in mannitol, GSH and NAC treatments (Fig. 26).

There was dose-dependent increase in ferritin induction upon pro-oxidant (H_2O_2) treatment with 0.5, 1 and 2 mM H_2O_2 along with 50 μM FC (non saturating cone.). (H_2O_2) (0.5 mM) was used as control (Fig. 27). Specificity of ferritin induction by iron was proved upon treatment with different heavy metal ions viz. Cd^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+} Ag^{2+} (Fig. 28).

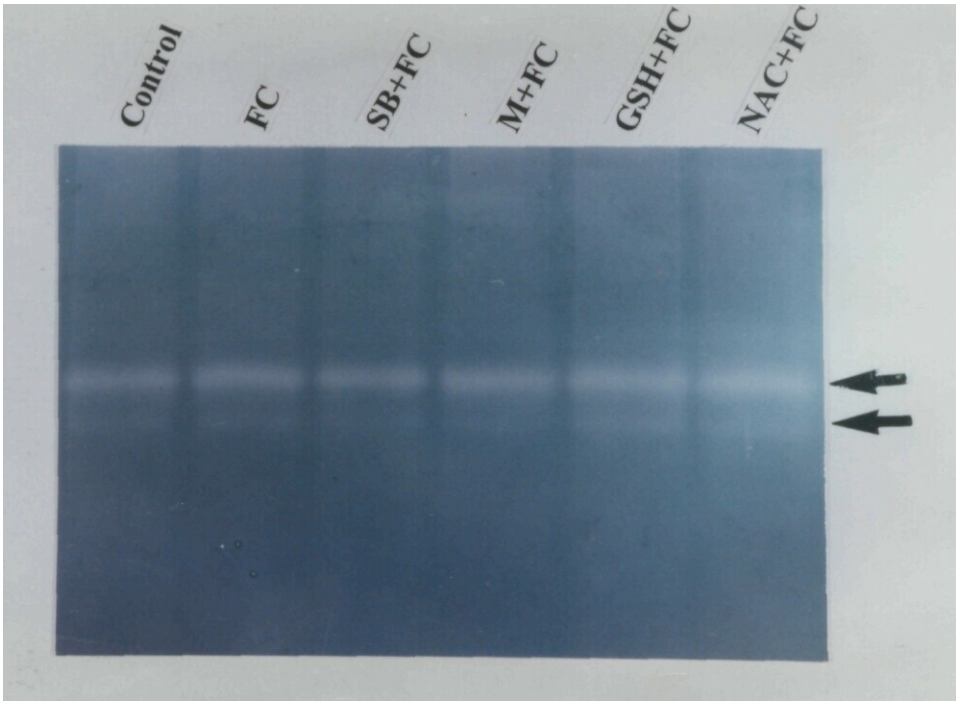


Fig. 22: 7.5% native PAGE of SOD showing the activities of isozymes under different treatments with free radical scavengers and antioxidants (for treatment details see the legend for Fig. 15). Expression of both the isozymes increased in GSH and NAC treatments. Equal protein (75 μg) was loaded from each treatment.

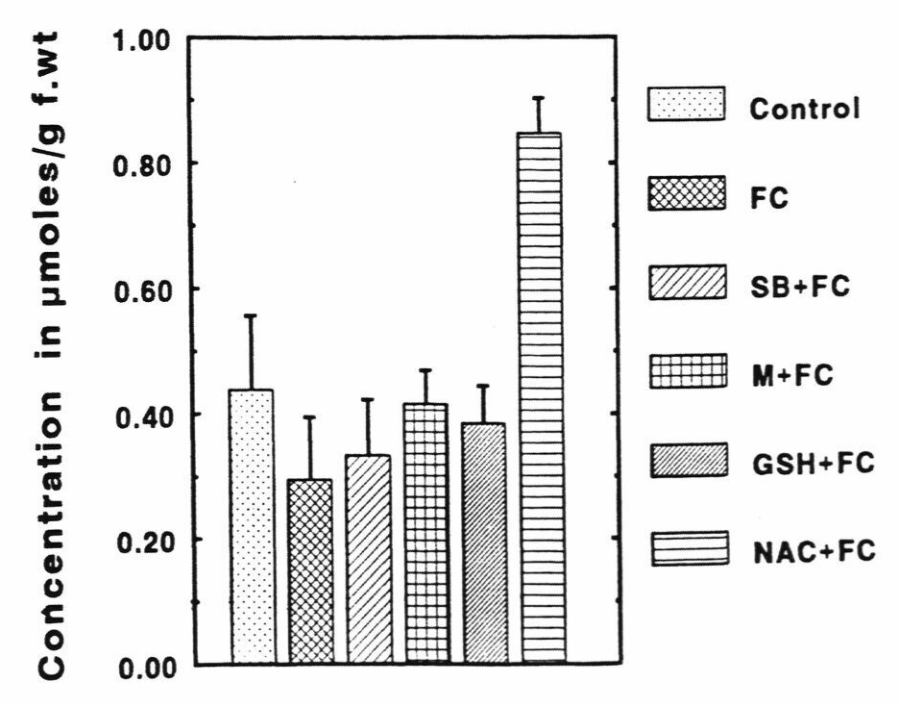


Fig. 23: Influence of pretreatment with free radical scavengers and antioxidants (treatment conditions are same as shown in legend for Fig. 15, Control= Distilled water, FC = Ferric citrate, SB = Sodium benzoate, M = Mannitol, NAC = N- Acetyl cysteine) on endogenous levels of ascorbic acid. Note the decrease in ascorbic acid content in FC. Higher level of ascorbic acid was observed in NAC.

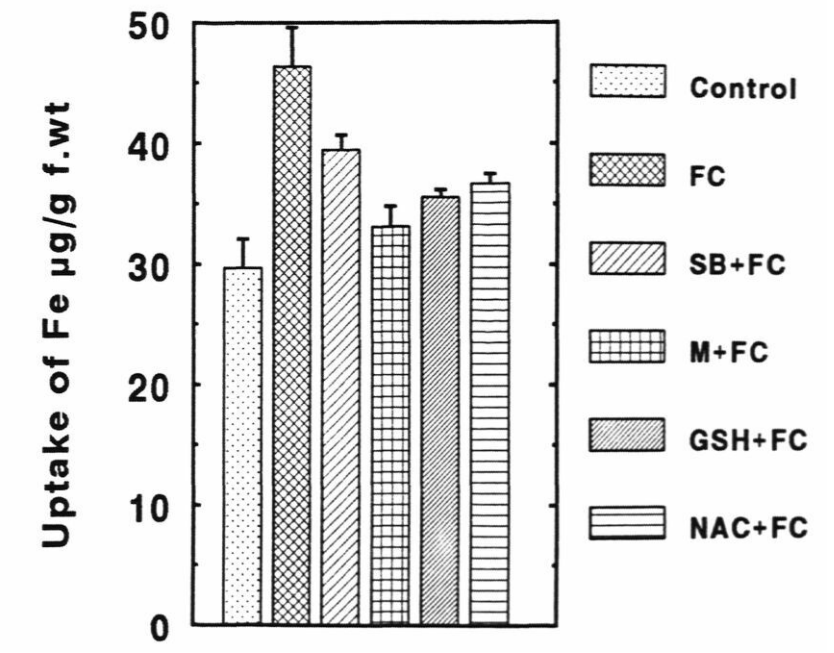


Fig. 24: Influence of pretreatment with free radical scavengers and antioxidants (treatment conditions as shown in legend for Fig. 15) on the iron uptake by iron-deficient, de-rooted, 10 day old seedlings of *V. mungo*. Note that the iron uptake is not largely modified in the presence of antioxidants and free radical scavengers. Results are the means $\pm$ SD (n=3 with two replicates each).

Fig. 25: Western analysis of the total proteins isolated from seedlings grown for 7 days in etiolated condition followed by treatments mentioned as below: Control (std. Hoagland's), 150 μM, 200 μM, 250 μM FC treatments, probed with antibodies raised against *V. mungo* seed ferritin. Note the dose-dependent increase of ferritin in leaves and roots. Equal protein (20 μg) was loaded from each treatment.

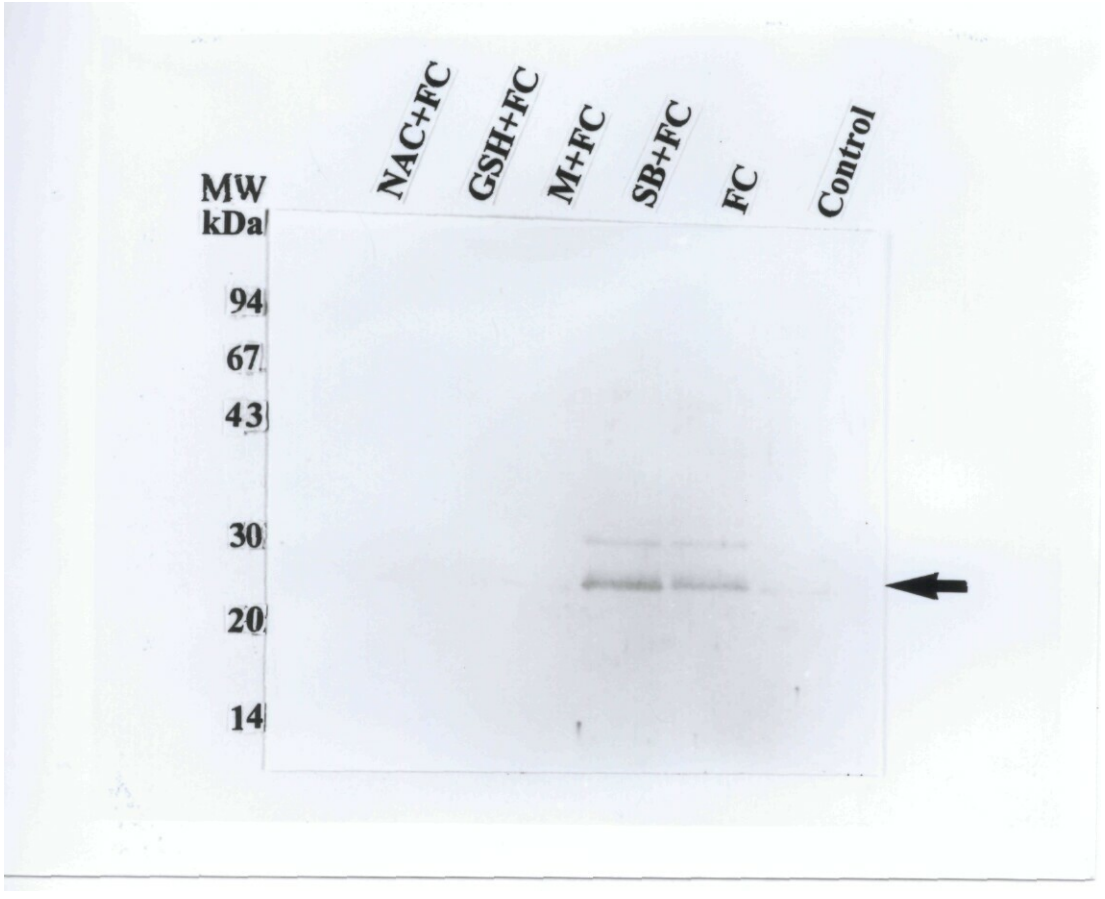


Fig. 26: Western analysis of SDS-PAGE of total proteins isolated from iron-deficient, de-rooted seedlings of *V. mungo* treated with free radical scavengers and antioxidants (treatment conditions as stated in the legend for Fig. 15) probed with antibodies raised against *V. mungo* seed ferritin. Note the induction of ferritin in FC. SB treatment did not inhibit ferritin induction. Inhibition of ferritin induction was observed in other treatments. Eaual protein (20 μg) was loaded from each treatment.

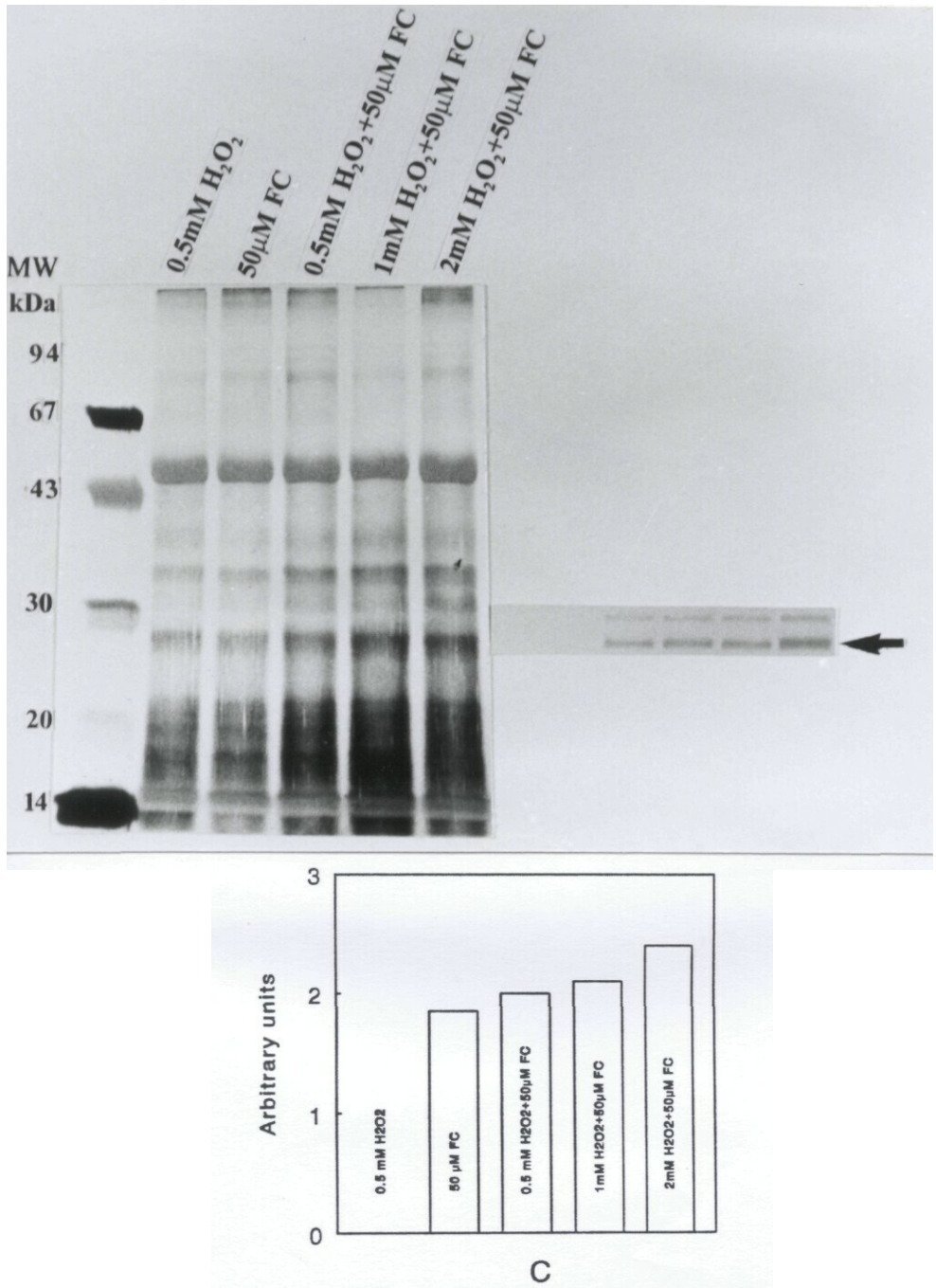


Fig. 27a: SDS-PAGE showing the effect of pro-oxidant (H_2O_2) treatment on ferritin levels in iron-deficient, de-rooted, 10 day old seedlings of *V. mungo*, with 0.5 mM H_2O_2, 50 µM FC, 1 mM H_2O_2 + 50 µM FC, 2 mM H_2O_2 + 50 µM FC, 3 mM H_2O_2 + 50 µM FC for 6 hrs. Equal protein (20 µg) was loaded from each treatment, b. Western analysis with *V.mungo* seed ferritin antibody showing the effect of pro-oxidant treatment (H_2O_2) as in 27 a on ferritin levels. Dose-dependent increase of ferritin is depicted, c. Densitometric scanning of the blot depicting dose-dependent increase of ferritin induction.

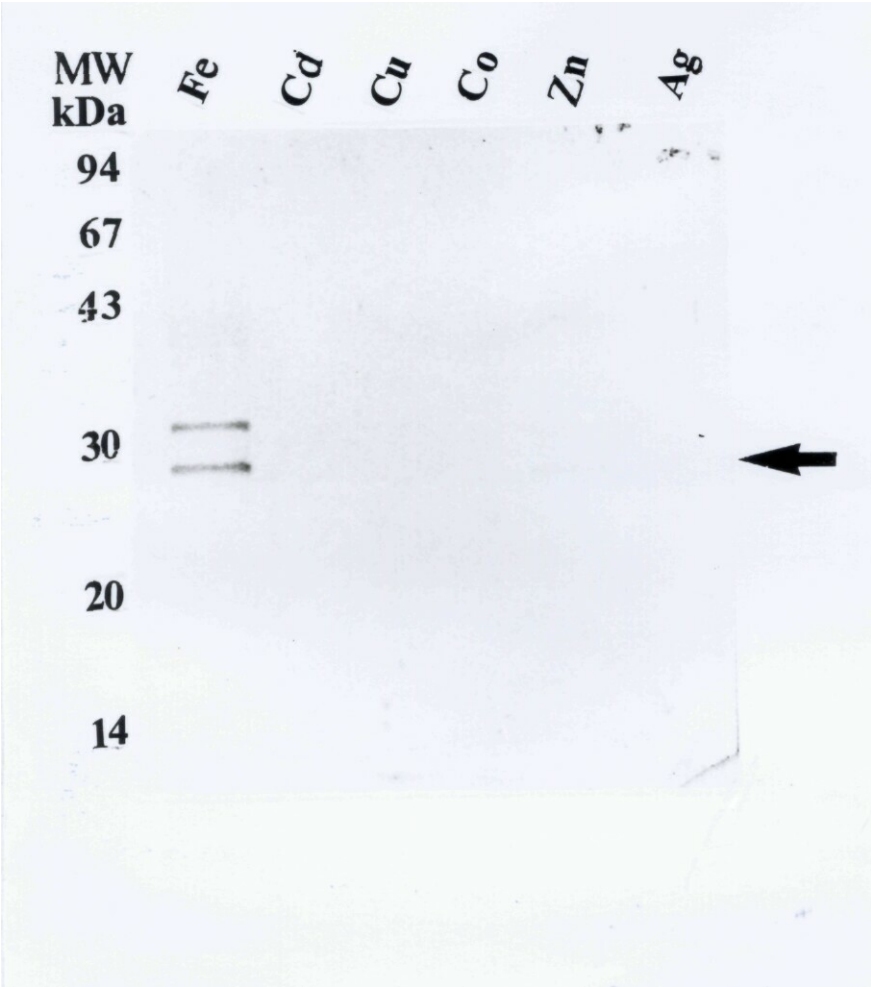


Fig. 28: Western blot of SDS-PAGE probed with polyclonal antibodies against *V. mungo* seed ferritin. Please note that iron induced ferritin (lower band) and partial cross reaction with another polypeptide (upper band). There is no induction of ferritin with Cd, Cu, Co, Zn and Ag. Equal protein (20 µg) was loaded from each treatment.

Increase in ferritin induction was observed upto a concentration of 100 µM ABA from 50 µM,beyond which (upto 200 µM) there was no further increase in ferritin (Fig. 29). 100 µM concentration of ABA could be saturating concentration.

5.6 Discussion

Iron loading of the leaves is limited by the root barrier in maize as observed by Lobreaux *et al* (1992). To circumvent the obstacle, Lobreaux *et al* (1992) followed the procedure of Kaempfenkel *et al* (1995) by using de-rooted seedlings. A similar procedure was followed in this study. Ferric citrate being the main chemical form in which iron is transported in xylem sap, was used in the study (Cataldo *et al* 1988). Iron toxicity can be a natural problem for plants. Low oxygen concentrations are a feature of many soils, particularly in waterlogged or wetlands (Armstrong 1967). Such soils are often associated with high iron concentrations (in excess of 1 mM) as a result of reducing conditions and the solubilization of ferrous ions (Martin 1968). In rice for example, anaerobic conditions and acidic pH favour reduction of iron which facilitate easy uptake of iron to an abnormal levels in leaves (Fitter and Hay 1989). This situation is best referred to as bronzing phenotype of rice which results in necrotic spots on the leaves (Ponnamperuma *et al* 1955). Iron overload results in oxidative stress mediated both by Fenton and Haber-Weiss reactions (Halliwell and Gutteridge 1988). The aim was to study the iron-induced oxidative stress and ferritin synthesis and also the inhibitory effect of free radical scavengers and antioxidants (for details refer the section materials and methods) on ferritin induction.

The increased activities of different antioxidant enzymes have long been established as a sign of oxidative stress along with decreased levels of endogenous antioxidants (Burke *et al* 1985; Gamble and Burke 1984; Smirnoff and Colombe 1988). Hence, the activities

of APX, GPX, CAT, and SOD were studied. The isozyme profiles of antioxidant enzymes were also investigated to ascertain whether new isozymes that might provide additional protection are synthesized in response to stress.

The activity of ascorbate peroxidase increased by 33.52% in FC treatment compared to control. The activity levels were almost similar in SB, Mannitol and NAC treatments. There was almost two fold increase in APX activity in GSH treatment compared to control. This may be due to the fact that GSH being a substrate for dehydroascorbate reductase (DHAR), helps in converting the oxidized dASA back to its reduced form ASA, which is the principal electron donor for APX. Increased availability of the substrate results in enhanced activity of the enzyme (Cakmak and Marschner 1992; Foyer 1993; Iturbe-Ormaetxe *et al* 1998). These results are consistent with previous findings (Kaempfenkel *el al* 1995). In the isozyme profile three distinct isozymes were observed with additonal three slow moving high mol. wt. isozymes. One of the isozymes APX 1 activity was distinctly suppressed in NAC treatment (Fig. 16).

The activity of GPX increased by 30.74% in FC treatment compared to control. The activity was reduced more with mannitol and NAC treatments compared to GSH and SB (Fig. 17). Rapid uptake of iron stimulates the production of hydroxyl radicals (Price and Hendry 1991). Mannitol being a potent scavenger of hydroxyl radicals (Shen *et al* 1997; Elstner 1982) prevents the reaction of OH radicals with OH ions to form H_2O_2. Reduced formation of H_2O_2 results in decreased activity of GPX in mannitol treatment. In the isozyme profile three different isozymes were noticed of which, GPX 2 and 3 activities were suppressed in mannitol and GSH treatments respectively (Fig. 18). Targetting mannitol biosynthesis to tobacco chloroplasts enhanced the hydroxyl radical scavenging

capacity of the plants and increased resistance to oxidative stress (Shen *et al* 1997; Tarczynski *et al* 1993).

The specific activitiy of catalase increased three fold in FC compared to control. Mannitol was most effective followed by GSH and NAC whereas SB had no protective effect (Fig. 19). Despite the important role of catalase in controlling H_2O_2 levels within the cell, few studies were done on the role of catalase under oxidative stress (Cakmak and Horst 1991; Luna *et al* 1994; Zhang and Kirkham 1994, 1996) at activity level. This is due to its peroxisomal location, because most studies were performed at molecular level with respect to their roles during environmental stress (Scandalios 1994; Sharma and Davis 1994; Willekens 1994). Activity staining for catalase did not reveal any distinct differences in various treatments (Fig. 20).

The actvity of SOD increased by only 7% in FC compared to control. NAC was better in reducing the increase in activity whereas SB, mannitol and GSH did not have any control upon reduction of activity (Fig. 21). As mannitol and SB are scavengers of hydroxyl radicals they have little effect on levels of O_2" radicals, thus offering no protection from O_2 mediated increase in SOD activity. The activity staining revealed SOD 1 and SOD 2 which showed increased activities in GSH and NAC treatments (Fig. 22).

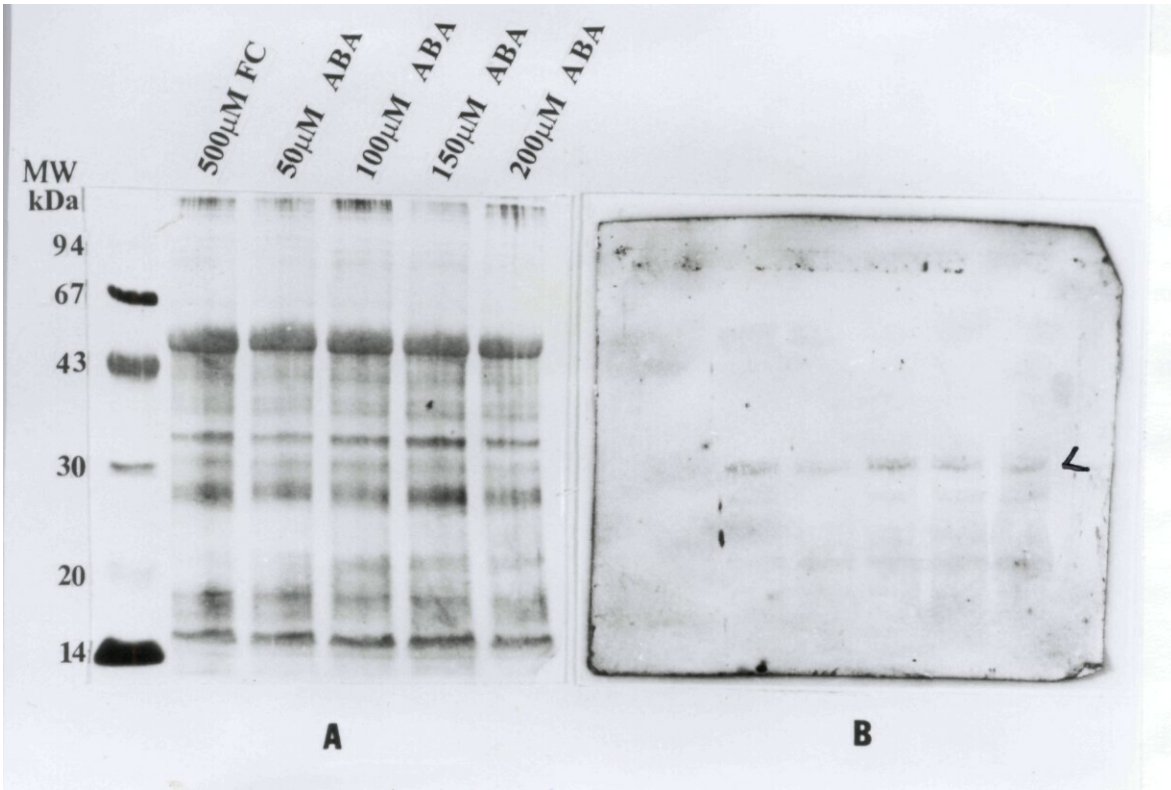
Endogenous levels of ascorbic acid as cellular antioxidant were estimated in various treatments (for details see materials and methods). Ascorbic acid is a key component of ascorbate/glutathione cycle (De vos and Schat 1991). It is a scavenger of O_2" and OH radicals. Its utilization under stress conditions viz. H_2O_2, SO_2, drought, water stress, ozone, and iron, in both enzymic and non enzymic reactions leads to its depletion in the cell (Guri 1983; Kaempfenkel *et al* 1995; Law *et al* 1983; Mukherjee and Choudhury 1983; Ranieri

et al 1997; Zhang and Kirkham 1996). Ascorbic acid content decreased from 0.438 in control to 0.294 μmoles/g.f.wt in FC treatment. Pretreatment with antioxidants and free radical scavengers followed by co-treatment with FC restored the ascorbic acid content to control level. NAC and mannitol restored the ascorbic acid levels to that of control. Particularly NAC significantly increased ascorbic acid content in comparison to control (Fig. 23). This could be due to its lower oxidation or a result of conversion of oxidized ascorbic acid to its reduced form since it was reported by Fruton and Simmonds (1965) that dehydroascorbic acid can be reduced to ascorbic acid by reduced GSH or cysteine. Similar results were obtained by Mukherjee and Choudhury (1983) in *V. mungo* seedlings under water stress. The decrease in ascorbate concentration is interesting in that concentration lower than 2.5 mM favour iron uptake *in vitro* while higher concentrations promote iron release (Laulhere 1990; Laulhere and Briat 1993). Estimation of total iron in the leaves has shown that the iron concentration is almost similar to that of FC treatment. Antioxidant treatment did not modify iron loading of the leaves (Fig. 24).

Ferritin induction occurred in dose-dependent manner from control (Hoagland's with iron) to 250 μM FC in Hoagland's in leaves. In roots 200 μM FC was found to be apparently saturating concentration (Fig. 25). To verify if oxidative stress does induce ferritin, western analysis of the total proteins isolated from various treatments (as mentioned in materials and methods) was probed with *V. mungo* seed ferritin antibodies. Excepting SB and FC treatments, the ferritin induction was inhibited (Fig. 26). Thus, antioxidant treatment inhibited ferritin induction except in SB giving credence to the fact that oxidative stress generated by iron does induce ferritiiL Moreover, the decrease in ascorbic acid concentration also generates favourable redox conditions for iron uptake by

ferritin. Therefore, oxidative stress due to iron toxicity leads to ferritin accumulation (Fig. 26). Similar results were reported in maize at mRNA level (Lobreaux *et al* 1995). Hence, this result is concurrent with the hypothesis that induction of ferritin synthesis requires the generation of reactive oxygen intermediates. Our results conform to the above mentioned hypothesis. Since free radicals mediate ferritin induction as was evident above, we focussed on the ability of pro-oxidant (H_2O_2) treatment (generator of free radicals) of de-rooted seedlings in combination with non saturating concentration of iron to induce ferritin. Non-saturating concentration of iron is required for initiation of free radical cascade. The maximum concentration of H_2O_2 used in the experiment was 2 mM in the medium which is consistent with the concentrations used by others (Bradley *et al* 1992; Lobreaux 1995). There was dose-dependent increase in ferritin induction upto 2 mM H_2O_2. H_2O_2 alone did not induce ferritin thereby indicating the requirement of free radicals to induce ferritin. Seedlings used in this experiment were starved of iron for 10 days before these treatments. As a consequence the concentration of free iron could be too low to activate H_2O_2 (Fig. 27). Similar results were obtained although it was at the level of transcript abundance by Lobreaux *et al* (1995). Further, the specificity of iron in induction of ferritin was evaluated by western analysis of the total proteins isolated from leaves of various treatments with other heavy metals. The results drive home the point that ferritin induction is indeed specific for iron (Fig. 28). Control of expression of genes in response to stress is often mediated by a transduction pathway which involves the plant hormone ABA (Skriver and Mundy 1990). An attempt was made to demonstrate the involvement of ABA in iron-stress response leading to ferritin induction even in *V. mungo*. There was a dose-dependen increase in ferritin level from 0 to 100 µM ABA. Treatments upto 150 µM ABA revealed

that 100 µM ABA is probably the saturating concentration, as there was no further increase in the induction of ferritin protein from 100 to 150 µM ABA (Fig. 29). Exogenous application of ABA induced ferritin in iron-starved *V. mungo* de-rooted seedlings. The results clearly indicate that ABA may be involved as hormonal relay during iron-induced ferritin biosynthesis (Lobreaux *el al* 1993). The results presented here indicate that iron loading of leaves of de-rooted *V. mungo* plantlets generates an oxidative stress that is responsible for ferritin protein accumulation. Further, ABA also induces ferritin. It is important to note that environmental and developmental controls are not necessarily mediated by a single pathway (Pena-Cortez *et al* 1991). Thus, in these investigations ferritin emerges as an important component of the oxidative stress response in plants and it probably participates in the protection of plastids, where it is localized, from oxidative stress by storing excess of free iron.



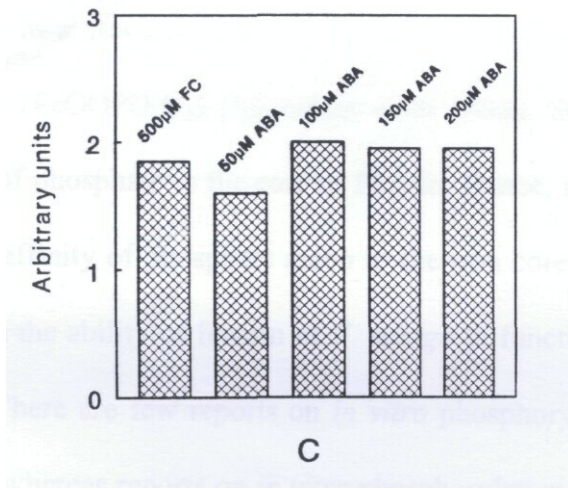


Fig. 29: Effect of ABA treatment on ferritin induction in de-rooted, iron-deficient seedlings, a. SDS PAGE (12.5%) of total proteins isolated from following treatments. Control: 500 mM FC, 50 μM, 100 μM and 150 μM ABA. b. Western analysis of the SDS-PAGE as in fig. 29 a probed with *V. mungo* seed ferritin antibodies showing 100 μM ABA as saturating cone. c. Densitometric scanning of the blot.

6 Kinases involved in phosphorylation of ferritin

6.1 Introduction

Phosphorylation is a form of posttranslational modification of proteins that provides a mechanism by which organisms can respond rapidly to changes in their internal and external environments over time scales that preclude a transcriptional response (Reddy and Prasad 1995). The iron storage protein, ferritin, ubiquitous in bacteria, plants and animals, has a high capacity for iron which is stored in a micellar form in the centre of the protein shell (Harrison *et al* 1977). Upto 4500 iron atoms may be stored, but there is seldom a saturation of this capacity. Polynuclear iron-phosphate interactions occur in ferritin, a complex of protein, hydrous ferric oxides and phosphate with the iron core inside the hollow protein. Ferritin provides an intracellular reservoir of iron, overcoming the extreme insolubility of Fe(III) 10^{-18} M at physiological pH. The iron core of ferritin has an average composition of $(FeOOH)_8$ $(FeOOP0_3H_2)$ (Michaeles *et al* 1943). Several authors have investigated the existence of phosphate in the core of ferritin. Hence, in view of the above mentioned features of the affinity of phosphate anion to the iron core both *in vivo* and *in vitro*, we chose to focus on the ability of ferritin of *V. mungo* to function as a substrate of phosphorylation *in vitro.* There are few reports on *in vitro* phosphorylation of ferritin in animals (Ihara *et al* 1984) whereas reports on *in vitro* phosphorylation of ferritin in plants are almost non-existent. Hence, we made an attempt to study the phosphorylation of ferritin and also to delineate the kinases involved in the process.

6.2
Results

Changes were observed in the phosphorylation of ferritin subunit, a 28 kDa polypeptide, which was identified by immunoprecipitation with *V. mungo* seed ferritin antibody followed by autoradiography. The extent of phosphorylation of the 28 kDapeptide is more pronounced in ferritin from iron-treated seedlings compared to ferritin from iron-deficient seedlings (Fig. 30). In iron-treated seedlings, there would be higher induction of ferritin and the percentage of ferritin in total protein would be more, which presents a larger surface area for the phosphate anion to localize. Also, the extent of phosphorylation was equal both in media containing calcium or devoid of calcium. In the phosphorylation assay, in the presence of CaM medium highest phosphorylation of ferritin was observed (Fig. 31). Inhibition of phosphorylation did not occur in the presence of KN-62 and staurosporine (Fig. 32).

In the presence of syntide-2 (a synthetic peptide) which is a substrate for CaM kinase, results showed increased phosphorylation. The amount of ^{32}P incorporated into syntide-2 (cpm) increased from 1802 $\pm$ 75.13 in the medium containing both EGTA and substrate to 2379 $\pm$ 108.39; an increase of 32% indicating that phosphorylation of ferritin may be mediated by CaM kinase. Incorporation of ^{32}P into syntide-2 occurred even in the presence of EGTA, which hints at the possibility of involvement of calcium-independent kinases in the phosphorylation of ferritin (Fig. 33).

Phosphorylation of total proteins isolated from leaves of iron-deficient seedlings treated with mimosine (iron chelator) showed significant inhibition of ferritin phosphorylation in comparison to ferritin from iron-deficient seedlings. Seedlings treated with iron (as ferric citrate) followed by mimosine, restored the phosphorylation of ferritin

to similar levels as in iron-deficient seedlings without mimosine treatment (Fig. 34).

6.3 Discussion

In the present investigation ferritin was found to act as a substrate for phosphorylation by internal kinases. This is the first report of phosphorylation of ferritin *in vitro* in plants. According to Mann *et al* (1986), the phosphate content of iron cores from microorganisms is higher than that in vertebrates, approaching an Fe:P ratio of 1.4:1. Similarly, plant cells also have high phosphate concentrations (Briat *et al* 1990). Ferritin acquires metal ions inside the plastid. The iron core of pea seed ferritin has high phosphate content characteristic of ferritin mineralized in bacteria (Wade *et al* 1993), which indicates that bacteria share with plastids a high phosphate content compared to the cytoplasm of plants and animals. Ferritin can thus be added to the group of nuclear encoded and plastid located metalloproteins such as plastocyanins and ferredoxin (Li *et al* 1990; Merchant and Bogarad 1986) which share the property of assembly of the protein-metal complex in the plastid. In many cases signal transduction has been shown to involve a transient increase in calcium concentration and changes in Ca^{2+}/calmodulin-dependent phosphorylation (Friedmann and Pooviah 1991). The increased phosphorylation in iron-treated seedlings could be due to the fact that the percentage of ferritin in total protein was more compared to that of iron-deficient seedlings. This resulted in increased phosphorylation due to better availability of ferritin (Fig. 30). The enhanced phosphorylation observed in iron-treated seedlings in the presence of CaM may be mediated by Ca^{2}/calmodulin (Fig. 31), since as reported in *Pisum sativum,* calmodulin is located in the chloroplast, which is also the site of location of ferritin (Jarret *et al* 1982). Calmodulin (CaM) is a 16 kDa acidic calcium-binding protein which helps in transducing the signals from external stimuli in all

eukaryotes (Babu *el al* 1998). Calcium binding triggers a conformational change in the protein, which in turn facilitates specific interaction with Ca^{2+}/CaM dependent enzymes, kinases etc. (Ling and Zieleinski 1989). cAMP and cGMP failed to stimulate or inhibit the phosphorylation of ferritin (Fig. 32). Staurosporine (an inhibitor of protein kinase C) also failed to inhibit the phosphorylation, indicating that ferritin phosphorylation is not PKC mediated (Fig. 32). CaM kinase assay with syntide-2 (substrate for CaM kinase) showed an increase of 32% in phosphorylation, suggesting that phosphorylation of ferritin may be mediated by CaM kinase. Moreover, phosphorylation occurred even in the presence of EGTA, indicating the possible mediation of phosphorylation by calcium-independent kinases (Fig. 33). Soybean cells contain a calcium-binding protein which possesses protein kinase activity (Harmon *et al* 1987). Further, two calmodulin genes have been reported to be induced in response to physical and chemical stimuli in *V. radiata* (Botella and Arteca 1994), lending credence to the fact that calmodulin genes are present in *Vigna* species. Calmodulin isoforms were detected in *Arabidopsis* as reported by Gawienowski *et al* (1993). Braam (1992), Braam and Davis (1990) have shown that rain, wind, touch, high external calcium and heat shock could lead to a strongly and rapidly up-regulated expression of four calmodulin-related TCH genes in *Arabidopsis.* The phosphorylation of ferritin may be mediated by Ca^{2+}/CaM-dependent kinases and calcium-independent kinases as was evident in CaM kinase assay (Fig. 33). A recent report of Pandey and Sopory (1998) showed biochemical evidence for a novel calcium-dependent, calmodulin-stimulated protein kinase from etiolated maize coleoptiles, which substantiates the present observation of ferritin phosphorylation being mediated by Ca^{2+}/CaM-dependent kinase. The enhanced phosphorylation being observed in iron-treated seedlings is of special significance in metal

detoxification. In *vitro* studies of Sczekan and Joshi (1989) using model iron cores have shown that iron cores have high affinity for phosphate anion. Apart from phosphorylation of the residues lining the channels of iron deposition in the protein shell of ferritin, the phosphate anion is also deposited in the iron cores. In iron cores devoid of phosphate anion there was a marked reduction in metal binding. The binding of phosphate groups to the core could result in the neutralization of the large surface charge, resulting in marked anionic character on the surface. This in turn would facilitate the creation of a large number of putative ionic binding sites for non-ferrous metal cations. Thus enhanced phosphorylation of ferritin in the iron-treated seedlings of *V. mungo* may have a role in heavy metal detoxification by enabling binding of non-ferrous metals to ferritin. Mimosine is a non-protein aminoacid which chelates metals, especially iron (Smith and Fowden 1966). The reduction in phosphorylation of ferritin, seen in iron-deficient seedlings treated with mimosine, may be due to non-saturation of mimosine with the low amount of iron available, leaving enough capacity for the complexation of phosphate (Fig. 34). Whereas in iron-treated seedlings upon mimosine treatment, there was restoration of phosphorylation, which can be explained by the fact that mimosine has higher affinity towards iron compared to phosphate, leaving the phosphate free to phosphorylate ferritin. Thus, the phosphorylation of ferritin may be mediated by both Ca^{2+}/CaM-dependent kinases and calcium-independent kinases and it probably has a pivotal role in chelation of non-ferrous metal cations leading to metal detoxification.

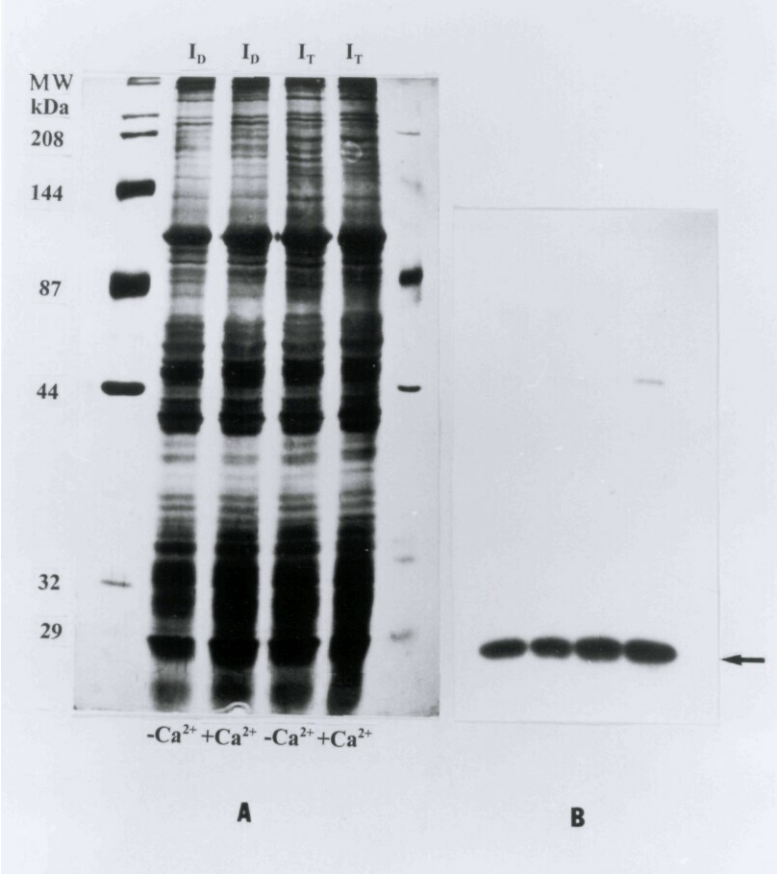


Fig. 30 a: SDS-PAGE of the total proteins phosphorylated in the presence and absence of Ca^{2+} in iron-deficient leaves as well as in iron-treated leaves. Equal protein (20 μg) was loaded in each well. b: Autoradiogram of the SDS-PAGE as in fig. 30a. Enhanced phosphorylation of the 28 kDa polypeptide (subunit of ferritin) observed in FC treatment.

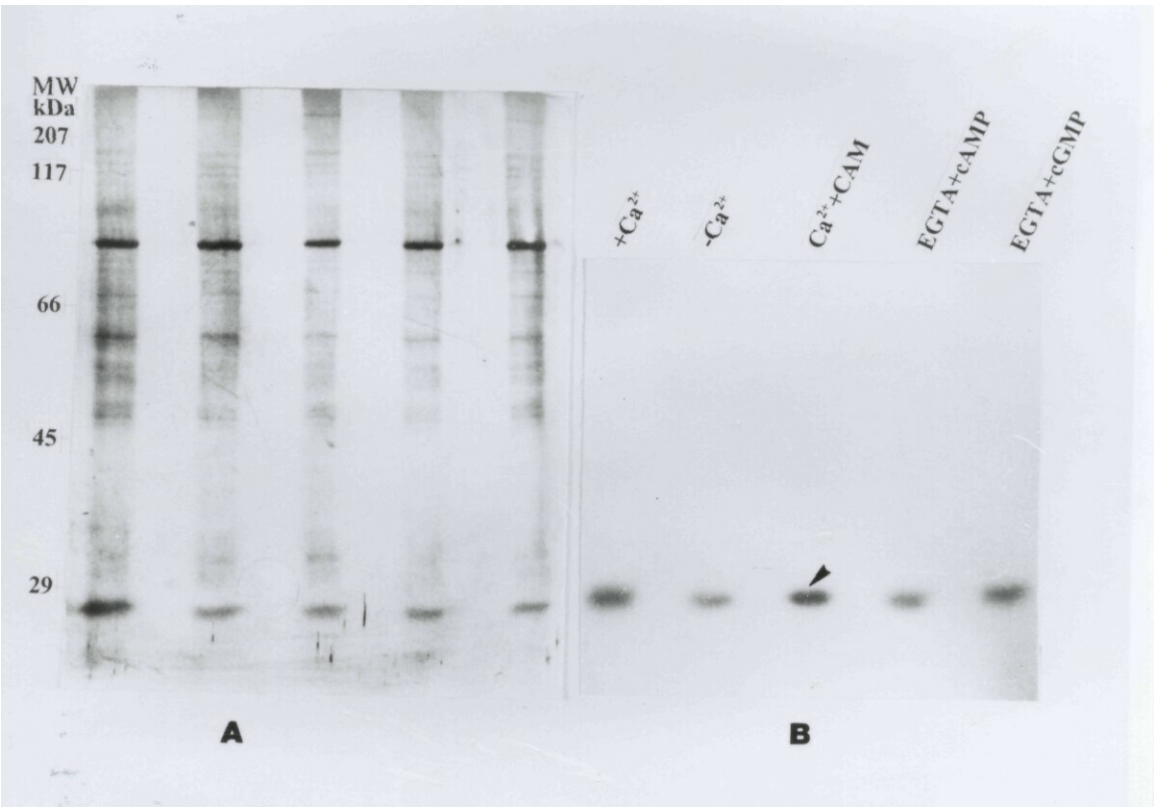


Fig. 31 a: SDS-PAGE of total proteins (20 µg) isolated from iron-treated (500 µM FC) leaves and phosphorylation in the presence of EGTA, Ca^{2+}, Ca^{2+}+CaM, EGTA+cAMP, EGTA+cGMP. Equal protein (10 µg) was loaded in each lane. b: Autoradiogram of the SDS-PAGE as in fig. 3la. Note the enhanced phosphorylation of the 28 kDa polypeptide in presence of Ca^{2+} and CaM.

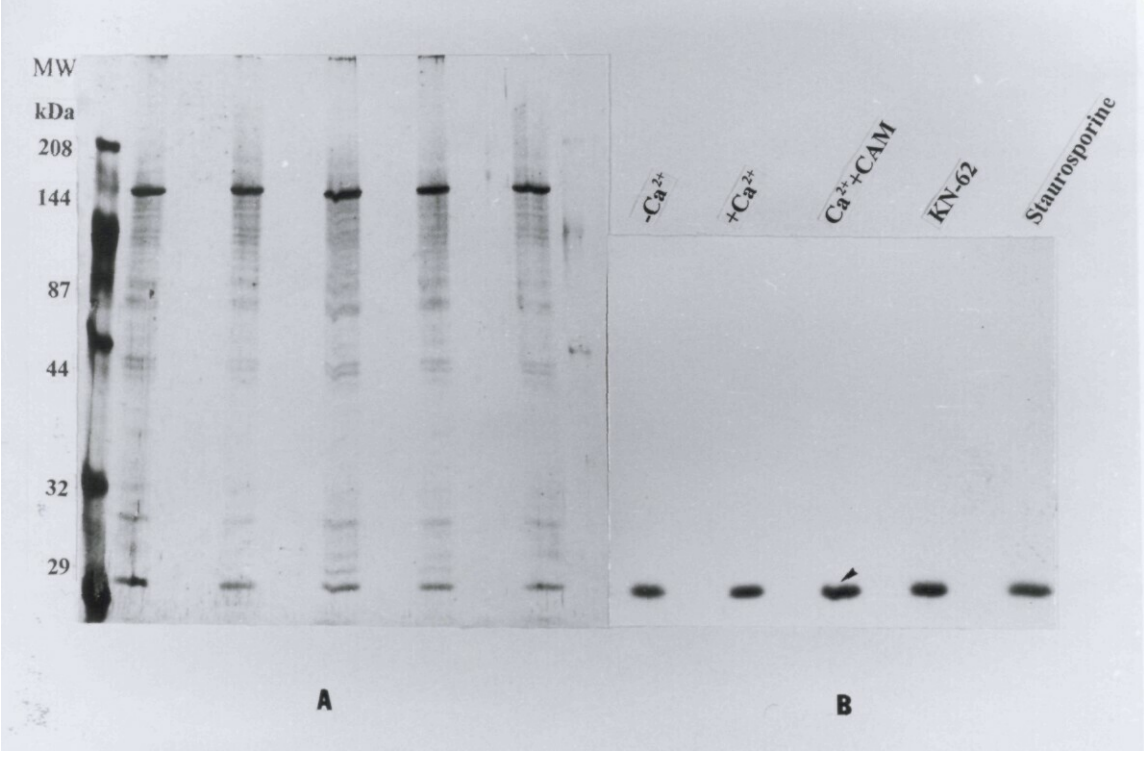


Fig. 32 a: SDS-PAGE of total proteins (20 µg) isolated from iron-treated (500 µM) and phosphorylated in the presence of Ca^{2+}, in the absence of Ca^{2+}, Ca^{2+}/CaM, KN-62 and Staurosporine. b. Autoradiogram of the SDS-PAGE as in fig. 32a showing enhanced phosphorylation of the 28 kDa polypeptide (subunit of ferritin) in the presence of Ca^{2+}/CaM.

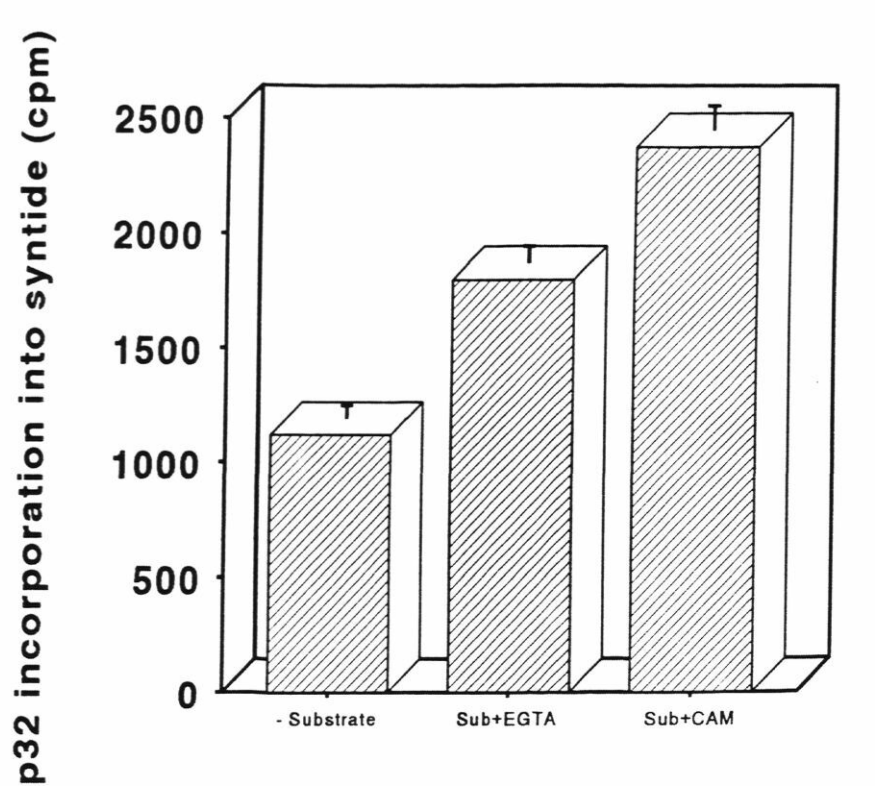


Fig. 33: Assay of CaM kinase activity in crude extract in the absence of syntide-2 (substrate), substrate+EGTA, substrate+Ca^{2+}/calmodulin depicting the enhanced phosphorylation in presence of Ca^{2+}/CaM.

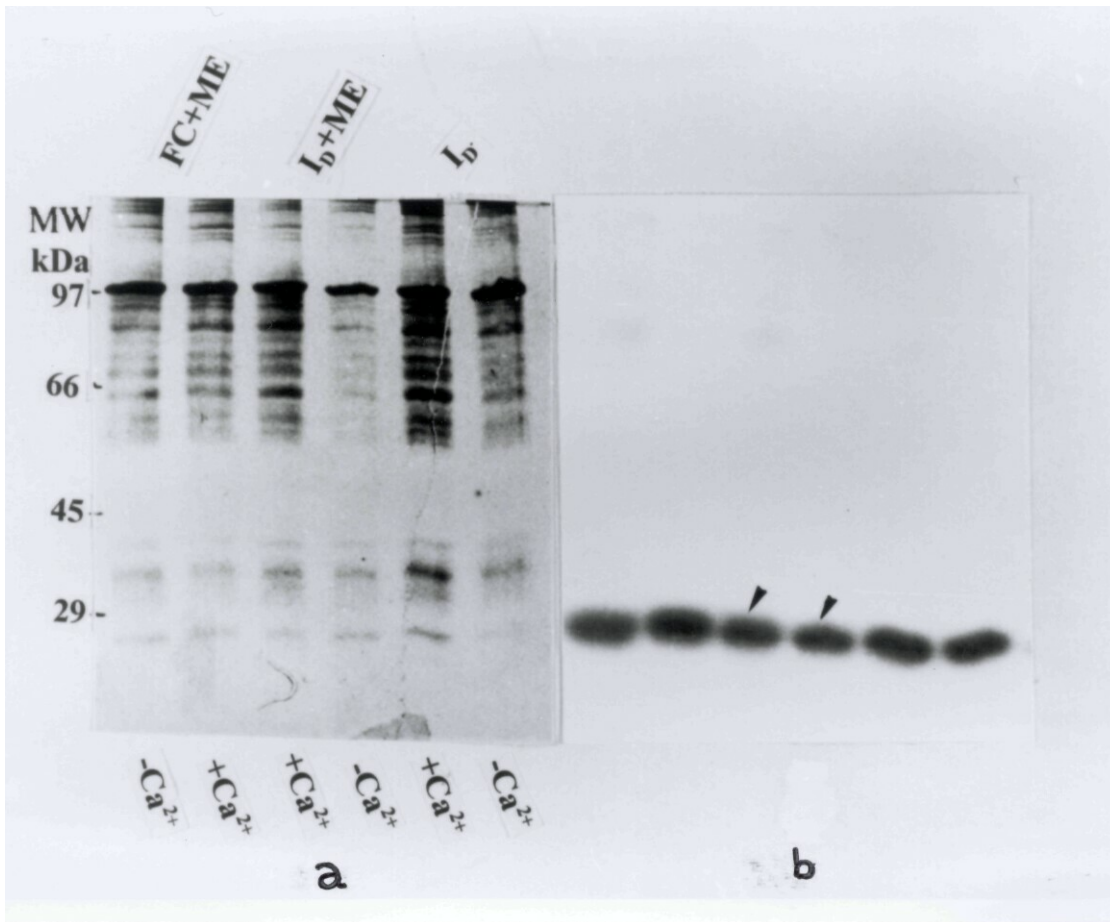


Fig. 34 a: SDS-PAGE of the phosphorylated total proteins isolated from I_D, I_D+100 ppm mimosine, 500 μM FC+mimosine treatments. Equal protein (10 μg) was loaded in each lane. b. Autoradiogram of the SDS-PAGE as in 34a depicting the significant inhibition of phosphorylation of 28 kDa polypeptide (subunit of ferritin) in presence of mimosine, restoration of phosphorylation in the presence of 500 μM FC+mimosine.

7 Non-ferrous metal-binding properties of ferritin in *V. mungo:* Possible role in heavy metal detoxification

7.1 Introduction

Iron storage protein ferritin is ubiquitous from bacteria and plants to animals (Harrison *et al* 1975). Ferritin molecule can store upto 4500 iron atoms as a polymeric hydrous ferric oxide core encased in a hollow, spherical protein shell (Aisen and Listowsky 1980). The major physiological role of ferritin is to sequester and thus detoxify iron that is taken up by cells but not utilized for metabolic requirements. Therefore, ferritins are key proteins acting as a buffer for iron, protecting cells from a harmful overdose of free iron, and tuning properly their immediate need for iron (Aisen and Listowsky 1980; Theil 1987). Seldom does ferritin get completely saturated with iron, leaving enough space for other metals to be accommodated. While iron is the predominant metal stored, other divalent and trivalent cations may also be bound, although in lesser numbers. Initial studies by Price and Joshi (1982) indicated that ferritin could bind large amounts of beryllium. Infact it was shown that the affinity of horse spleen ferritin for beryllium was in the order of 6.8×10^{-6} M which was able to remove Be^{2+} from phosphoglucomutase affording partial protection to the enzyme from inactivation. Studies of Sczekan and Joshi (1989) have shown that ferritin from *Glycine max* was capable of binding metal ions viz. Cd^{2+}, Zn^{2+}, Be^{2+} and Al^{2+}. Thus, in view of existing reports on ferritin binding to non-ferrous metals, we have chosen to study if similar non-ferrous metal-binding properties occur in *V. mungo* ferritin. This helps in assigning detoxification of heavy metals as an additional function of ferritin in addition to storage of iron in *V. mungo*.

7.2
Metal-binding studies

Metal-binding studies were done to characterize the functional properties of the purified *V. mungo* seed ferritin. The purified protein showed significant binding with cadmium and copper as revealed by UV difference spectroscopy. UV difference spectrum of the protein with cadmium has shown peaks at 242 and 295 nm (Fig. 35).

UV difference spectrum of ferritin with copper resulted in peaks at 242 and 275 nm (Fig. 36). Binding studies with labelled cadmium (^{109}Cd) were done to further confirm the metal-binding ability of the protein. Ferritin was incubated with ^{109}Cd and electrophoresis was performed according to Abrahamson *et al* (1992). The autoradiogram showed an intense radio active band at 540 kDa indicating the binding of ferritin with cadmium (Fig. 37).

7.3
Fluorescence experiments

Quenching of fluorescence emission spectra was observed at 340 nm at different time points after initial incubation with $CdCl_2$ for four hours. There was significant quenching of fluorescence from control (Fig. 38).

7.4
Discussion

Diverse mechanisms exist for the detoxification of heavy metals in living organisms. The protein metallothionein is induced in animals exposed to heavy metals viz. lead, cadmium copper and zinc to sequester heavy metals, thereby rendering protection (Robinson and Jackson 1986). Analogous to metallothionein in animals, phytochelatins are synthesized in plants which are cysteine-rich (Grill *et al* 1987). It was demonstrated by Sczekan and Joshi (1982) both *in vitro* and *in vivo* that ferritin binds non-ferrous metal ions.

Iron is the predominant metal stored in ferritin. However, other divalent and trivalent

cations may also be bound albeit in lesser quantities in animal ferritin (Price and Joshi 1987). Investigations of Sczekan and Joshi (1989) have shown that ferritin from *Glycine max* is capable of binding heavy metals *in vitro.* It is also demonstrated that metal-binding of ferritin in animals results in the appearance of peaks with maximum absorbance at 242 and 295 run in UV difference spectra. These difference spectra are generally considered to be characteristic to deprotonated tyrosine. Similar results were obtained in the present study with *V. mungo* seed ferritin exhibiting peaks with maximum absorbance at 242 and 290 nm in UV difference spectra after binding with cadmium. The ratio of protein to cadmium varied from 1:2 to 1:5 (Fig. 35) and peaks with maximum absorbance at 240 and 275 nm with copper indicating the possibility of involvement of tyrosine residues in the metal-binding ability of the protein (Fig. 36) (Harris and Madisen 1988; Tan and Woodworth 1969). It was reported by Price and Joshi (1983), that Be^{2+} was bound to carboxyl residues of aspartic or glutamic acid or the hydroxyls of tyrosine on the protein shell.

Further, *in vitro* binding assay using labelled ^{109}Cd also showed a band corresponding to ferritin on autoradiogram further confirming the binding ability of the protein with heavy metal cadmium (Fig. 37). Moreover, fluorescence spectra as depicted by the quenching of fluorescence with heavy metals binding to ferritin (Fig. 38), clearly point to the fact that apart from iron storage function, it also chelates heavy metals, leading to multifaceted ftinctioning of ferritin.

Thus, ferritin from F. *mungo* was purified for the first time and it is suggested that ferritin may also function as a chelator for other heavy metal ions as well, thereby leading to an expanded role. Since plants cannot avoid toxic situations and must instead endure them, efficient defence mechanisms are crucial for survival and ferritin may form the front line

defence. Thus, storage of iron is the foremost function of ferritin, besides chelating other metals, thus sparing the plant from expensive way of synthesizing phytochelatin in the presence of toxic levels of metals.

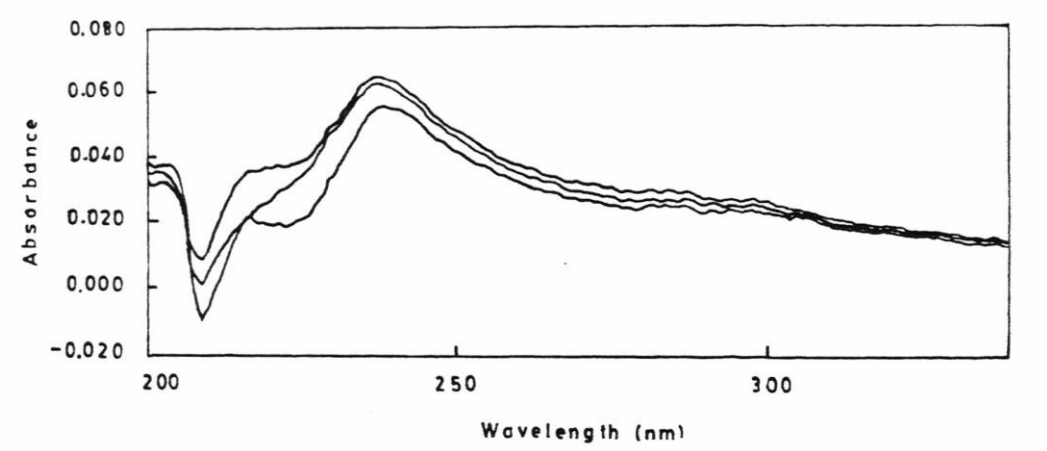


Fig. 35: UV difference spectrum of ferritin binding cadmium resulting in peaks at 242 and 295 nm indicating the deprotonation of tyrosine residues.

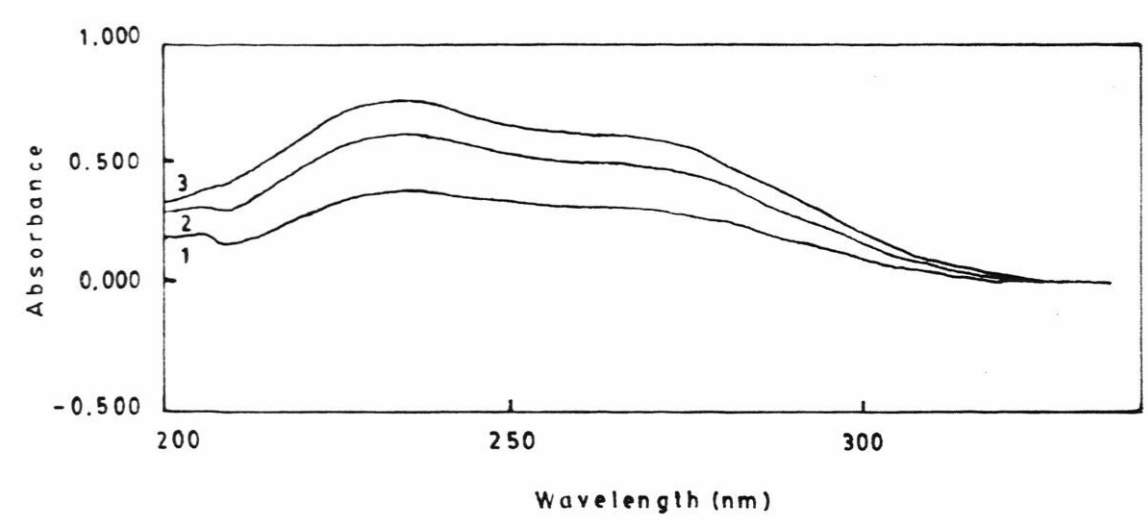


Fig. 36: UV difference spectrum of ferritin binding copper resulting in peaks at 242 and 275 nm indicating the deprotonation of tyrosine residues.

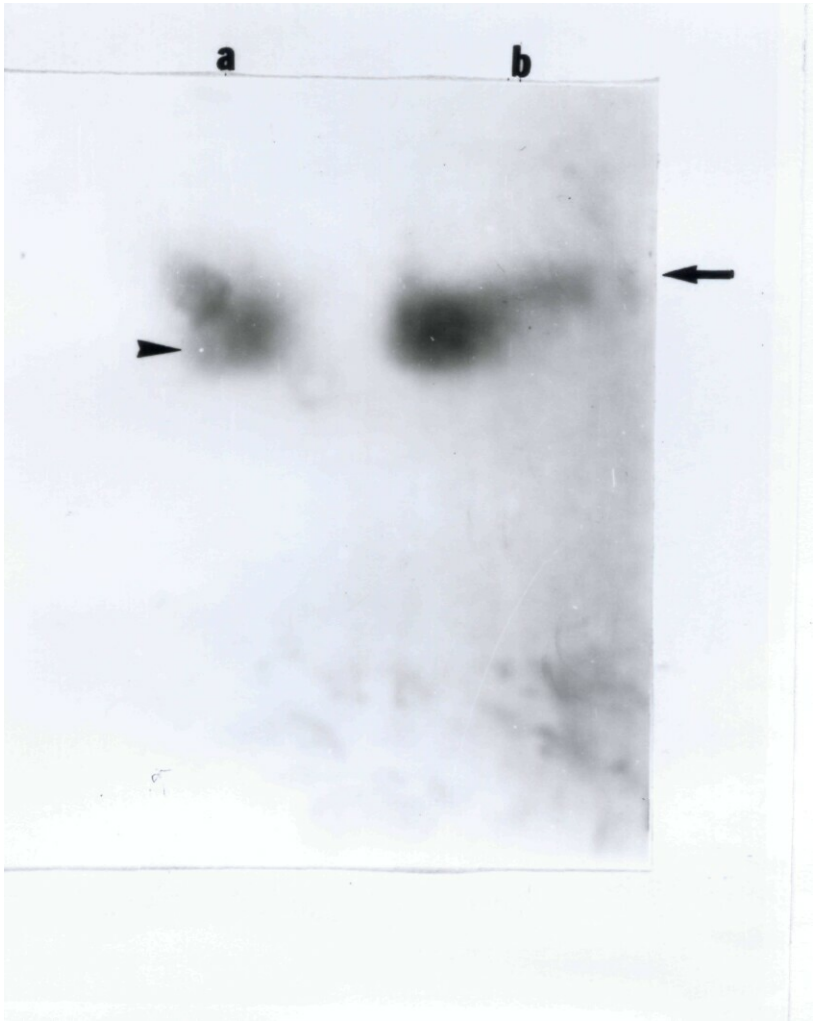


Fig. 37: Autoradiogram of ferritin binding with radioactive ^{109}Cd. (a) *V. mungo* ferritin. (b) Horse spleen ferritin.

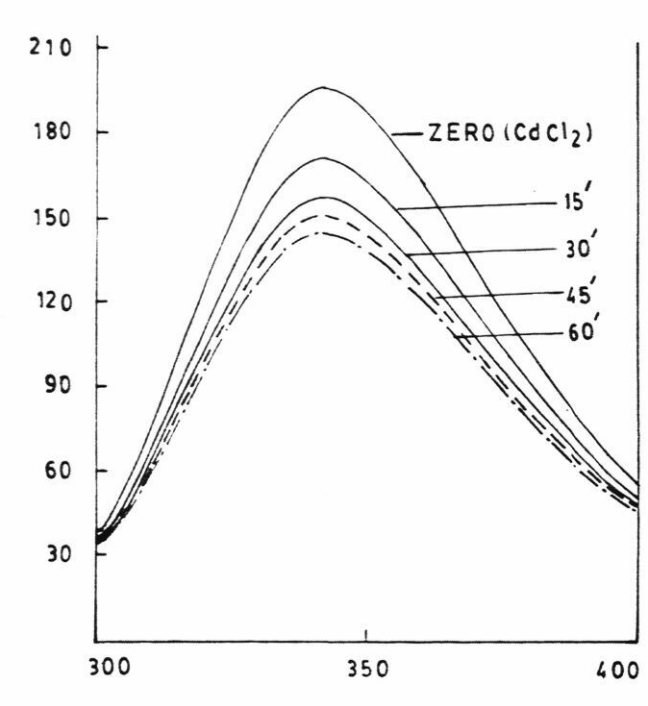


Fig. 38: Fluorescence emission curves of fenitin binding with cadmium. Quenching of fluorescence with time is depicted relative to ferritin without cadmium.

8 Conclusions

1. The purified ferritin from *V. mungo* had a molecular weight of 540 KDa in native form, and 28 kDa in denaturing conditions with a characteristic absorption spectrum with maxima at 240 and 280 nm and excitation spectrum with maxima at 280 and 240 nm, emission peak at 340 nm. The iron content of the ferritin being 1100 atoms/ molecule.
2. Immunologically *V. mungo* ferritin is similar to pea seed ferritin.
3. Iron mediates oxidative stress as revealed by the upregulation of activities of antioxidative enzymes and inhibition of the activities in the presence of antioxidants and free radical scavengers. Ferritin also functions as an antioxidant by sequestering excess iron thereby reducing the availability of free iron.
4. Pretreatment of iron-deficient seedlings (de-rooted) with mannitol, GSH (reduced) and N-acetyl cysteine followed by co-treatment with ferric citrate (500 μM) inhibited ferritin induction suggesting that iron-mediated oxidative stress results in ferritin induction.
5. Endogenous ascorbic acid levels decreased in ferric citrate treated seedlings which was restored in the presence of free radical scavengers and antioxidants. NAC increased ascorbic acid levels to almost three fold compared to control.
6. Hydrogen peroxide (pro-oxidant) treatment with non-saturating concentration of iron showed dose-dependent increase in ferritin indicating that oxidative stress plays a role in ferritin induction.
7. Induction of ferritin is specific for iron and not for other metals Ag, Cu, Cd, and Zn.
8. ABA induced ferritin in a dose-dependent manner upto 100 μM in iron-deficient and de-rooted seedlings beyond which no further increase was observed.

9. In addition to iron storage of iron, ferritin also binds other heavy metal ions *in vitro* viz. Cd^{2+} and Cu^{2+}. Thus, Ferritin plays an important role in heavy metal detoxification.

10. Iron-treated seedlings showed increased phosphorylation of Ferritin. Both calcium-dependent(Ca^{2+}/calmodulin-dependentkinase) as well as calcium-independent kinases mediate phosphorylation of ferritin.

11. In iron-deficient seedlings mimosine (a non-protein aminoacid and an iron chelator) is not saturated with iron. It even complexes phosphate, thus reducing the phosphate availability for phosphorylation of ferritin which results in reduced phosphorylation. In iron-treated seedlings, mimosine strongly competes for divalent cations compared to phosphate. Thus, availability of phosphate enhanced phosphorylation of ferritin.

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