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Metal Binding Properties of Ferritin in *Vigna mungo* (L.) Hepper (Black Gram): Possible Role in Heavy Metal Detoxification

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Iron storage protein "ferritin" is ubiquitous in organisms (Theil 1987). Ferritin molecule can store up to 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 iron 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 chelated. While iron is the predominant metal stored, other divalent and trivalent cations may also be bound, although in lesser numbers. Price and Joshi (1982) indicated that ferritin could bind large amounts of beryllium. It was demonstrated that the affinity of horse spleen ferritin for Be^{2+} 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. Ferritin from *Glycine max* was capable of binding metal ions viz. Cd^{2+}, Zn^{2+}, Be^{2+} and Al^{2+} (Sczekan and Joshi 1989). Hence, we have chosen to study the non-ferrous metal binding properties of *V. mungo* seed ferritin. This helps in assigning detoxification of heavy metals as a supplementary function of ferritin besides storage of iron in *V. mungo*.

MATERIALS AND METHODS

Vigna mungo seed ferritin was isolated, purified and characterized (Kumar 1998). Protein was estimated following Lowry *et al* (1951). For detection of the proteins separated by electrophoresis silver staining was adopted (Blum *et al.* 1987). The molecular weight of the polypeptide of interest was calculated from the standard molecular weight markers which were run simultaneously on the gel using UVP-2000 gel documentation software program.

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

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Ultraviolet difference spectra were recorded on Hitachi double beam Spectrophotometer (Hitachi 557 Japan) (Tan and Woodworth 1969). Ferritin (50 µg) was taken in 2 mL of 50 mM Tris-HCl (pH 7.5) buffer in sample as well as reference cuvettes. The baseline correction was done with the protein versus protein in both cuvettes from 230 to 340 nm. Protein was incubated for 4 hr at 25°C with various concentrations of cadmium added as cadmium chloride in dark. 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 µg copper for 50 µg of ferritin.

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

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 according to Abrahamson *et* al (1992) and electrophoresis was performed according to Laemmli (1970).

RESULTS AND DISCUSSION

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 290 nm (Fig. 1). UV difference spectrum of ferritin with copper resulted in peaks at 242 and 275 nm (Fig. 2).

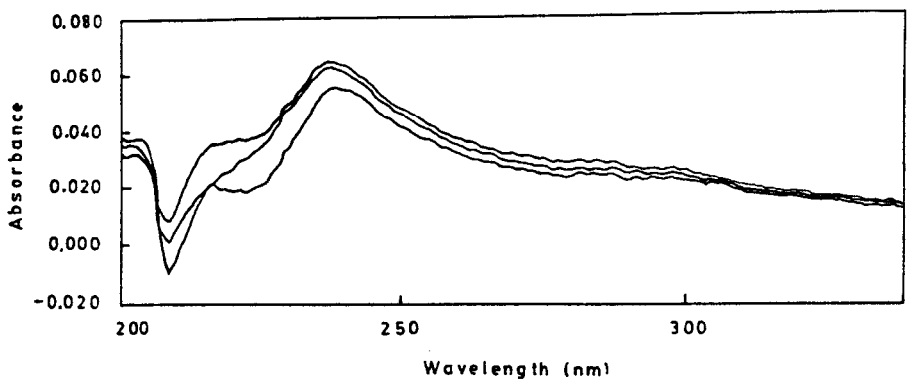

Figure 1. Ultraviolet difference spectra of ferritin binding cadmium resulting in peaks at 242 and 290 nm indicating the deprotonation of tyrosine residues.

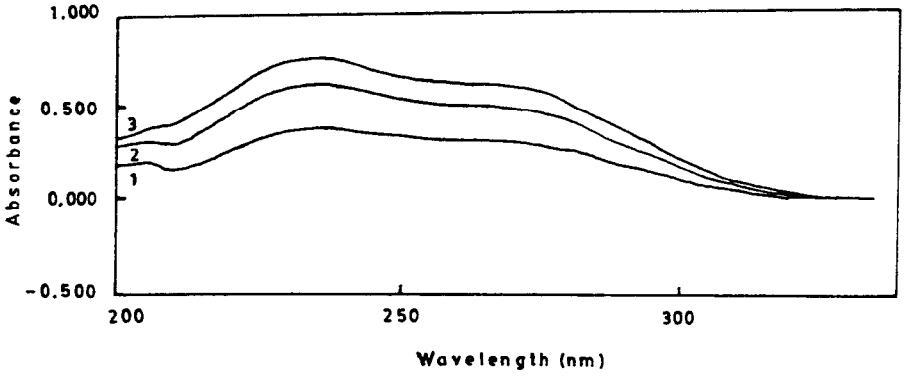

Figure 2. Ultraviolet difference spectra of ferritin binding copper resulting in peaks at 242 and 275 nm indicating the deprotonation of tyrosine residues.

The autoradiogram showed an intense radioactive band at 540 kDa indicating the binding of ferritin with cadmium (Fig. 3). Quenching of fluorescence emission spectra was observed at 340 nm at different time intervals after initial incubation with $CdCl_2$ for 4 hr. There was significant quenching of fluorescence from control (Fig. 4).

Diverse mechanisms exist for the detoxification of heavy metals in plants, animals and fungi. The recent developments in the area of metal-biomolecule interactions and their relevance to environmental toxicology and social aspects was discussed by Lobinski and Potin-Gautier (1998) and Prasad (1998). Metallothioneins are induced in animals exposed to heavy metals viz. lead, cadmium, copper and zinc to sequester heavy metals (Stillman 1995). In plants, phytochelatins are synthesized which induce tolerance to metal toxicity (Grill *et al.* 1987; Rauser 1995). Sczekan and Joshi (1989) illustrated that ferritin binds non-ferrous metal ions both *in vitro* and *in vivo*

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.* Release of protons occurs during metal ion binding to ferritin to provide charge compensation. Proton release was associated with Cobalt(II) and Zinc(II) binding to bacterioferritin (Le Brun *et al.* 1996).

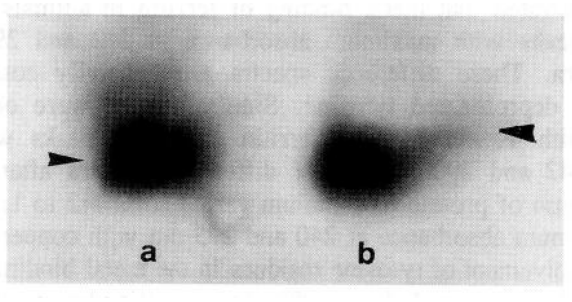

Figure 3. Autoradiogram of ferritin binding with radioactive ^{109}Cd. (a) Horse spleen ferritin. (b) *V. mungo* ferritin.

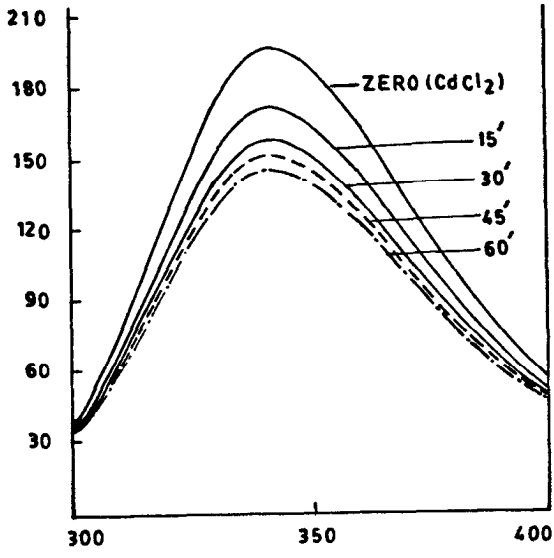

Figure 4. Fluorescence emission curves of ferritin binding with cadmium. Quenching of fluorescence with time is depicted relative to ferritin without cadmium.

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. Wardeska *et al* (1986) have shown that in horse spleen apoferritin Mn(II), VO(IV) , Cd(II) and Tb(III) showed binding within hydrophilic channels.

It is also demonstrated that metal binding of ferritin in animals results in the appearance of peaks with maximum absorbance at 242 and 295 nm 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. 1) 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. 2) (Harris and Madisen 1988; Tan and Woodworth 1969). Further, in vitro binding assay using labelled ^{109}Cd also showed a band corresponding to ferritin on autoradiogram, confirming the binding ability of the protein with heavy metal cadmium (Fig. 3). Moreover, fluorescence spectra as depicted by the quenching of fluorescence with heavy metals binding to ferritin (Fig. 4), clearly point to the fact that apart from iron storage function, it also chelates cadmium and copper, leading to multifaceted functioning of ferritin.

Thus, ferritin from *V. mungo* was purified for the first time and it is suggested that ferritin may also function as a chelator for other heavy metal ions like cadmium and copper as well, thereby leading to an expanded role. Since plants cannot avoid toxic situations and must instead endure them, efficient defense mechanisms are crucial for survival and ferritin may form the front line defense. Thus, storage of iron is the foremost function of ferritin, besides chelating other metals at low toxic levels prior to phytochelatin synthesis.

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