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I am submitting herewith a dissertation written by Steven R. Sczekan entitled "Studies in Phytoferritin." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy with a major in Biochemistry.

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STUDIES IN PHYTOFERRITIN

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Steven R. Sczekan

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ABSTRACT

Phytoferritin obtained from soya bean (Glycine max) was characterized and compared to horse spleen ferritin. The plant protein is present in two distinct forms, the smaller of which appears to be derived from the larger via proteolytic processing. The protein shows considerable sequence homology, as monitored by TLC peptide mapping, with both rat liver and horse spleen ferritin, although no immuno-crossreactivity could be detected. Only the larger (28 kdal.) form is present in 18 hr. soaked seeds, whereas the smaller protein (22 kdal.) becomes apparent after 72 hrs. There is a corresponding appearance of ferritin crystalline arrays after 72 hrs., suggesting the presence of a hemosiderin-like structure. Sucrose gradient centrifugation and native gradient gel electrophoresis indicate a loss of subunits from the small form, with a corresponding decrease in protein stability. Precise reproduction of the 22 kdal. form from the 28 kdal. form may be achieved by treatment with subtilisin, suggesting the plant may mark the ferritin for degradation by minor proteolysis.

Analysis of non-ferrous metal binding to the plant protein shows that the protein can bind Al, Be, Cd and Zn in amounts similar to the mammalian protein. Parallel experiments with synthetic iron polymers (artificial cores) indicate that the iron center of ferritin is responsible for much of this binding.

Large amounts of iron are released from the seed during the initial stages of imbibition. Along with this material is also a low-molecular weight (<1000 dal.) component with properties suggesting a sugar-amino acid conjugate. This partially purified factor is a

potent chemoattractant for Rhizobia bacteria, as measured by a modification of capillary assay analysis. This factor may play an important role in bacterial recruitment for future nodulation.

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INTRODUCTION

Living systems have employed the use of the metal iron in a truly ubiquitous fashion. This metal is found in a plethora of biochemical reactions, including oxygen transport (hemoglobin), electron transport (the cytochromes), DNA synthesis (ribonucleotide reductase) and in oxygen detoxification (superoxide dismutase and catalase). [For a review see Aisen, 1977]. The wide use of iron in these and other reactions is a reflection of basic chemistry of the metal, the most significant feature of which is its redox capability. Iron is able to exist in two stable oxidation states in aqueous solutions, Fe(II) and Fe(III). This one-electron redox couple makes for a very versatile oxidation reaction. In fact, the normal oxidation potential of this redox couple, + 0.77 eV, has been modified by biosystems to encompass the range of +350 mV to -500 mV. It is this wide variety of redox potentials which makes the iron ion so useful in biological systems. However, one potential problem associated with all iron reactions is that of solubility; while the ferrous ion (Fe(II)) is quite soluble at neutral pH, the ferric ion (Fe(III)) possesses one of the lowest solubility products known ($K_{sp} = 10^{-36}$M). Furthermore, at neutral pH, the oxygen tension in biological tissues is sufficient to effect the oxidation of the ferrous ion, further aggravating the problem. As such, biological systems have evolved elaborate mechanisms to sequester iron and maintain the metal in a bio-available form. An additional problem in which the iron may play a role is electrochemical; since the oxidation/reduction reactions in which iron participates are one-electron transfers, there is the persistant

difficulty of free radical generation. These radicals are notorious for eliciting extensive damage to biomolecules, particularly lipids (Girotti, 1985), and as such must be effectively neutralized if the organism is to function.

The importance which many organisms ascribe to the iron atom may be seen in the metabolism of certain procaryotes. These bacterial systems (e.g., Pseudomonas) are well-adapted to scavenging iron from the environment by producing specific chelators (termed siderophores) which have extraordinary affinities for the ferric ion (Neilands, 1982). In many cases, these bacteria are able to out-compete other species, presumably due to the ability to absorb the necessary iron for metabolism. Higher organisms, including plants, have similar mechanisms for the chelation of heavy metals (Grill et al., 1986) which have only recently been investigated.

What all these considerations imply is that while iron is an extremely versatile ion, and as such, one of considerable worth to the organism, efficient control mechanisms are requisite to its incorporation into the metabolic pool.

Storage and transport of iron is effected largely by the ferritins and the transferrins, respectively. These proteins are specialized to tightly bind ferric ions, rendering them soluble and available for metabolic functions (Aisen and Listowsky, 1980). The storage protein ferritin is of particular interest. It is a multimeric protein, composed of 24 subunits of either 18,500 or 21,000 daltons (mammalian ferritins). Depending on the tissue source, the subunit distribution may be predominantly heavy (rat heart) or light

(horse spleen). Intermediate assembleges of various stoichiometries are possible, giving rise to the well-known isoferritins. The physiolgical significance of these various forms remains undetermined.

The subunits of the ferritin molecule are arranged in a spherical distribution, resulting in a hollow protein shell (Harrison et al., 1977). The iron is located in the interior cavity, in an inorganic polymer of approximate composition $[(FeOOH)_8(FeO:OPO_3H_2)]$ (Granick and Hahn, 1944). The capacity of the protein to store iron is unusally large; up to 4500 Fe may be sequestered by a single ferritin molecule (Theil, 1983). This aggregation is present as a large iron micelle, up to 70 Å in diameter. The iron stored in the micelle is available to the organism upon metabolic demand, being released from the core (probably by reduction to Fe(II)), and entering the pool of active iron. In addition, excess iron which would be toxic to the organism may be safely stored in the protein, thus avoiding potential problems.

The metabolism of ferritin is of particular interest. The protein is synthesized in response to iron administration to the organism, through some type of post-transciptional induction. Various studies have indicated that the increase in ferritin is due to increased levels of translation of mRNA, rather than transcription of new mRNA (Drysdale and Munro, 1966; Zahringer et al, 1975, 1976). The latent pool of ferritin mRNA in the cell is activated upon iron administration, resulting in an increased level of ferritin synthesis. There is also evidence which suggests that the ferritin mRNA is a better competitor for the translational machinery (ribosomes, translation factors, etc.), than other mRNA's (Schull and Theil, 1982).

Thus, there appears to be at least two different translational mechanisms in operation. Additional evidence indicates that there may also be transcriptional control which operates in some cell lines (Cairo et al., 1986, Cairo et al., 1985). All of these studies demonstrate the complexity associated with the regulation of ferritin synthesis.

In terms of ferritin degradation, considerably less is known. Ferritin is a relatively stable protein (Schaefer and Theil, 1981), and as such is turned over rather slowly. As the protein contains a large polymer of insoluble ferric iron, simple proteolysis is not sufficient to remove the protein from active metabolism. In this light, the formation of hemosiderin may be considered. This complex is an insoluble heterogeneous mixture of iron polymers and ferritin subunits found principally in hepatocytes in cases of iron overload (Iancu et al., 1977). It is believed to represent the degraded form of ferritin (found principally in lysosomes), and appears to be generated by some mechanism which results in the loss of subunits from the intact ferritin shell (Richter, 1984). This reduction of subunit number decreases the solubility of the protein to the point of precipitation, when even greater loss of protein occurs. These precipitated polymers may be easily seen in electron micrographs of iron-overloaded tissues, where they appear as regular crystalline arrays in the membrane-bound vesicles. It is not known how the cell deals with the subsequent exposure of excess ferric iron.

Recent investigations in this laboratory (Price and Joshi, 1983; Joshi et al., 1984) have revealed additional roles which ferritin may

play in the organism. In addition to the sequestration of large amounts of iron, it appears that ferritin may also be responsible for the binding and detoxification of other, more toxic metals. Early results indicated that the protein is able to bind large quantities of beryllium, one of the most poisonous metals known. This metal is able to effectively inactivate several enzymes, notably alkaline phosphatase (Schubert and White, 1950; Schubert et al., 1952), phosphoglucomutase (Hashimoto et al., 1967; Joshi and Handler, 1969), and $(Na^{+}K^{+})$-ATPase (Toda et al., 1967). Ferritin is able to restore activity to these enzymes by removing the beryllium and storing it itself (Price and Joshi, 1984). Furthermore, ferritin is able to bind a very large number of beryllium ions; in some cases up to 800-1000. These in vitro results may have practical significance, as data also indicate that stimulation of ferritin synthesis by iron injection protects rats against lethal doses of beryllium (Lindenschmidt et al., 1986). As such, it appears that ferritin may possess an additional protective function.

Several investigators have implicated the metal aluminum in various pathological states, including Alzheimer's dementia (Perl, 1985) and dialysis dementia (King et al., 1982). Curiously enough, high levels of the metal are transferred through the plasma bound to transferrin (Cochran et al., 1984), the major iron transport protein of the blood. Furthermore, aluminum has been implicated in various plant pathologies, particularly with regard to calcium and manganese disorders (Foy et al., 1978). The metal has several curious properties, including an inorganic chemistry very similar to

beryllium, and the tendency to form indeterminate polymers at a pH greater than 2 (Baes and Messmer, 1976). In addition, there appear to be very strong interactions between Al(III) and various phospho-compounds, notably adenosine derivatives (Karlik et al., 1983; Mansour et al., 1985), and, as such, may be involved in various perturbations of normal nucleotide metabolism. Since the metal has many properties in common with beryllium, we elected to analyze the interaction of aluminum with ferritin.

Virtually all of the above-mentioned work with regard to ferritin has been performed on animal proteins. Only recently have other sources been analyzed, such as plant (Hyde et al., 1963; Crichton et al., 1978), invertebrates (Mann et al., 1986), and bacteria (Tsugita and Yariv, 1985). In most cases the protein is quite similar, although the bacterial system also incorporates a heme group. Our interest in the detoxification of beryllium by ferritin led us to ask if a similar mechanism occurs in plants. Since metal toxicity, particularly that associated with aluminum, can have severe effects on plant growth (Foy et al., 1978), the organism must be equipped with competent defense mechanisms if it is to survive in an unpredictable environment.

Based on these findings, we chose soya bean (Glycine max) as a source of plant ferritin with which to characterize the metal binding properties of phytoferritin. Since evidence exists suggesting that the iron core may be responsible for considerable non-ferric metal binding (Price and Joshi, 1983), we also investigated the veracity of this hypothesis by analyzing the binding of these metals to artificial

iron polymers, which have been shown to serve as ferritin core models (Spiro and Saltman, 1967).

During the course of the soya ferritin isolation, we observed the release of a large amount of iron from the seed during germination. Because we hypothesized that the iron might be a signal for bacterial recruitment, we tested the seed wash for chemotactic activity against Rhizobium leguminosarum. After finding an active component, attempts were made to partially purify and characterize the factor.

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PART I

ISOLATION AND CHARACTERIZATION OF FERRITIN FROM SOYABEANS (*GLYCINE MAX*)

INTRODUCTION

Ferritin from animal sources is a well-characterized protein. It is a large protein of approximately 450,000 dal., constructed from 24 subunits of 19 - 21 kdal. In mammalian systems, there are at least two different types of subunits, designated heavy and light. Various permutations of the two forms give rise to isoferritins, many of which are associated with specific pathologies or are located in discrete tissues (Drysdale, 1977).

The protein is unique in that it is able to store unusually high amounts of iron, as many as 4500 atoms per protein molecule (Munro and Linder, 1978). This iron sink can serve as a source for iron-requiring metabolic processes in the organism. However, our interest in the molecule originated with the observation that other metals, notably beryllium, may also be sequestered in high numbers. This situation may prove to have detoxification benefits in cases of heavy metal poisoning (Lindenschmidt et al., 1986).

Relatively little is known about ferritin from plant sources. Hyde and co-workers (1963) were able to isolate the protein from bean seeds using $MgCl_2$ to co-precipitate the ferritin and the ribosomes. Crichton et al. (1978) found that the protein obtained from lentil and pea plants was similar in many respects to the mammalian form, was composed of 24 subunits of a discrete size, and was generally larger than its animal counterpart. Van der Mark et al. (1983) investigated the transcription of the ferritin gene in the French bean and found that the mRNA transcript coded for an oversize ferritin precursor which required processing prior to assembly. Ko and co-workers (1985)

have shown that nematode pathology influences the distribution of the so-called "crystalline arrays" of ferritin in soyabean. [For a review, see (Bienfait and Van Der Mark, 1983)].

As our interest in heavy metal detoxification has implicated ferritin in mammalian systems (Price and Joshi, 1983), we elected to characterize the plant protein in order to determine if similar mechanisms occur in botanical systems. Many plants are adversly affected by aluminum and manganese, through mechanisms which are not entirely clear (Foy et al., 1978). Since plants are unable to avoid toxic situations and must instead endure them, efficient defense mechanisms are crucial for survival.

MATERIALS AND METHODS

Soyabeans (Glycine max) were obtained from Mayo Garden Service. Mes and Hepes buffers were obtained from Sigma Chemical Co. Acrylamide, bisacrylamide and ammonium persulfate were obtained from BioRad Laboratories. Phenylmethylsulphonylfloride (PMSF), streptomycin sulfate and $MgCl_2$ were obtained from Sigma Chem Co. and used without further purification.

Purification of the plant protein was carried out by a modification of the procedure of Hyde et al (1963). 500g of seeds were soaked in flat-bottom trays in 3 volumes of double-deionized water. Soaking was carried out for either 18 hrs. or 72 hrs., depending on whether non-germinated or germinated seeds were required. To study the effect of iron induction, some batches of seeds were also soaked in water containing 50 mM ferric ammonium citrate.

Homogenization was performed in a Waring Blender set on high for 60 sec. in 2 volumes of double-deionized water made to 0.1 mg./ml. in PMSF, by dissolving the compound into isopropanol at a concentration of 20 mg/ml, then dissolving the isopropanol in the water immediately before homogenization. The slurry was filtered through 4 layers of cheesecloth and centrifuged at 12000 x g for 15 min. Following the ribosomal/ferritin precipitation by $MgCl_2$, the centrifuged pellet was redissolved in 0.01 M $NaPO_4$/HCl, pH 7.4 (PO_4 buffer), and dialyzed against 3 x 6 Liters of this buffer. The $MgCl_2$ supernatant was concentrated with ammonium sulfate (50%), and dialyzed in the same buffer as well.

Chromatography was performed on DEAE-Sephacel column (5 cm x 25 cm) in 0.01 m PO_4 buffer, pH 7.4. The column was washed with 500 ml of PO_4 buffer, and then a linear gradient of 0-0.5 M NaCl in the buffer was used to elute the protein, with the eluant monitored for iron by atomic absorption spectroscopy. The first major iron peak eluting in the region of 0.15 M NaCl was pooled and concentrated with ammonium sulfate, and chromatographed on a Sephacryl S-300 superfine gel filtration column (2.5 cm x 90 cm). The major iron peak was again concentrated with ammonium sulfate, and stored at $4^{o}C$ in 0.01% NaN_3. Purity of the preparation was assessed by gel electrophoresis in a non-denaturing system (Arnon, 1977), and native molecular weight was estimated by both gel filtration and gradient gel electrophoresis according to Manwell, 1977. Subunit distribution was analyzed by SDS-polyacrylamide electrophoresis (Bury, 1981).

Immunology

Polyclonal antisera against the purified phytoferritin was raised in Balb-C mice according to the procedure of Tung et al., 1976. Antisera was also raised in New Zealand White rabbits by subcutaneous injection (Clausen, 1981). Double diffusion immunoprecipitation was done according to Crowle (1973).

Fingerprinting

Approximately 200 ug. (10 nmole) of phytoferritin in dH_2O was denatured by precipitation with 1 volume of cold 20% trichloroacetic acid, centrifuged at 5000 x g to pellet the protein, and washed with acetone containing 0.025 M α,α dipyridyl and 0.01 M $Na_2S_2O_4$ (sodium dithionite) until all iron was removed, as judged by the absence of a red color, and resuspended in 50 ul of 0.05 M NH_4HCO_3, pH 8.5. An aliquot of TPCK-trypsin (Worthington) was added to a final ratio of 1/20 (w/w), the sample incubated at 37°C for 1 hr, and another aliquot (1/20, w/w) was added. The sample was then digested overnight at 37°C. Horse spleen ferritin and rat liver ferritin were carried through the same procedure.

2-Dimensional peptide analysis (fingerprinting) was performed according to Allen (1981), with thin-layer cellulose plates (0.25 mm) from Kodak. Electrophoresis at pH 4.4 was conducted in pyridine/acetic acid/acetone/water (20:40:150:790, by vol.) for 2 hrs. at 10°C for the first dimension, followed by ascending chromatography in n-butanol/ acetic acid/water/pyridine (15:3:12:10, by vol.) for the second dimension. The peptides were detected by spraying the plates with 0.25% ninhydrin in ethanol/acetic acid (50:1, by vol.).

Iron Uptake

The ability of various preparations of phytoferritin to catalyze the oxidation of ferrous to ferric metal was measured spectrophotometrically in a Beckman DU-7 spectrophotometer at 310 nm. To an aliquot of 0.1 M NaHepes buffer, pH 7.0 in a 1 ml. quartz cuvette (light path = 1 cm.) was added various amounts of soya ferritin which had been dialyzed against the same buffer. 20 ul of 0.05 M ferrous ammonium sulfate (in dH_2O) was then added, bringing the final volume to 1 ml. The sample was immediately mixed and placed in the cell holder of the spectrophotometer, and the rate of ferric formation was followed by measuring the colored product formed at 310 nm (E_{310} = 45 ml/mg Fe/cm). Controls were as described, minus the protein. As the rate of ferric formation became non-linear after approx. 20-30 sec., only initial velocities were used for comparison.

Iron Loading

Purified ferritin was loaded with iron by incubating the protein with sequential aliquots of ferrous ammonium sulfate in 0.1 M Hepes buffer, pH 7.0. The amount of iron used was at a 1/1000 mol/mol ratio of protein to iron, and was added in steps of 200 gram atoms of iron. After the final addition, the protein was dialyzed against the Hepes buffer centrifuged for 2 min. at 5,000 x g, and the iron in the supernatant was quantitated by atomic absorption. Protein was measured by the BioRad Bradford assay (Bradford, 1976).

Electron Microscopy

For micrographic analysis, soyabeans were germinated in three (3)

volumes of deionized water for either 18 or 72 hours at room temperature. The cotyledons were removed from the rest of the plant and subepidermal 1 mm^3 cubes were excised and fixed in 3% glutaraldehyde and 0.1% OsO_4 for 2 hours. After dehydration in an alcohol series, the cubes were embedded in Epon and cured for 2 days. Thin sections were then made and were subsequently stained with 0.25% lead citrate for 1 minute and then with saturated uranyl acetate for 30 minutes. Analysis via microscopy was with a Hitachi H-800 Transmission Electron Microscope equipped for Energy Dispersive X-Ray Analysis.

Sucrose Gradient Centrifugation

In order to compare the relative sizes of the native heavy and light forms of the soya protein, we employed gradient centrifugation. Samples of two proteins (approx. 5 mg.) were deferrated by repeated dialysis against 0.05 M Mes, pH 5.5 containing 0.1% thioglycollic acid and 0.01 M dipyridyl, until no color was observed in the dialysate. The samples were then dialyzed against 0.05 M Hepes. pH 7.5 and subsequently loaded onto 5 ml. 20%-50% sucrose gradients, prepared according to Luthe, 1983. The gradients were spun in an SW 50.1 Beckman rotor at 37.000 rpm for 8 hrs., and fractionated with a peristaltic pump from the bottom. Fractions of 6 drops were collected, and protein monitored by fluorescence (ex. 280 nm, em. 340 nm) on a Perkin-Elmer LS-5 spectrofluorimeter. Standards used to calibrate the gradients were apo-horse spleen ferritin (16.5 S) and thyroglobulin (pig, 19.3 S).

Subtilisin Cleavage

To assess the susceptibility of the 28 kdal. form of soyaferritin to proteolysis, the protein was incubated with subtilisin (Worthington) in 0.05 M PO_4 buffer, pH 7.5 (1/40, w/w), and electrophoresed in SDS as described. The 22 kdal. form was also tested, along with horse spleen ferritin (holo and apo) as controls.

RESULTS

Ferritin can be isolated from soyabean in good yield, with approximately 20-35 mg. of protein per 500 g. of seeds. The protein as isolated contains between 800 and 1200 gram atoms of iron. Electrophoresis on non-denaturing gels indicates that the ferritin is quite pure, with only a single band detected on a Coomassie-stained gel (Figure I-1). The same band in the gel also stains positive for iron with the Prussian Blue, indicating the high level of iron in the protein. Generally, we found between 800 and 1200 gram atoms of iron associated with the protein, although variation occurs with the source of the protein (see below).

Subunit analysis of the protein by SDS-PAGE indicated that while the protein most often was composed of a single subunit of approximately 28,000 dal., occasional preparations were obtained in which lower molecular weight subunits predominated (Figure I-2). These proteins were always of discrete sizes, corresponding to 26 kdal., 23.5 kdal. and 22 kdal. No protein smaller than 22 kdal was obtained. To preclude the possibility of proteolytic breakdown during isolation, 0.1 mg/ml of phenylmethylsulfonylfloride was included in the

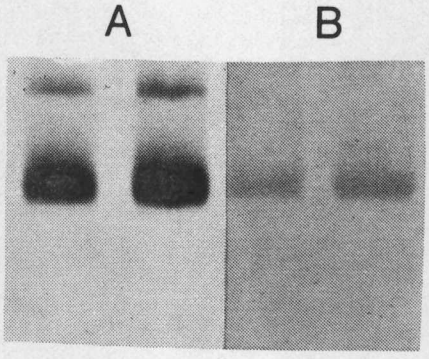
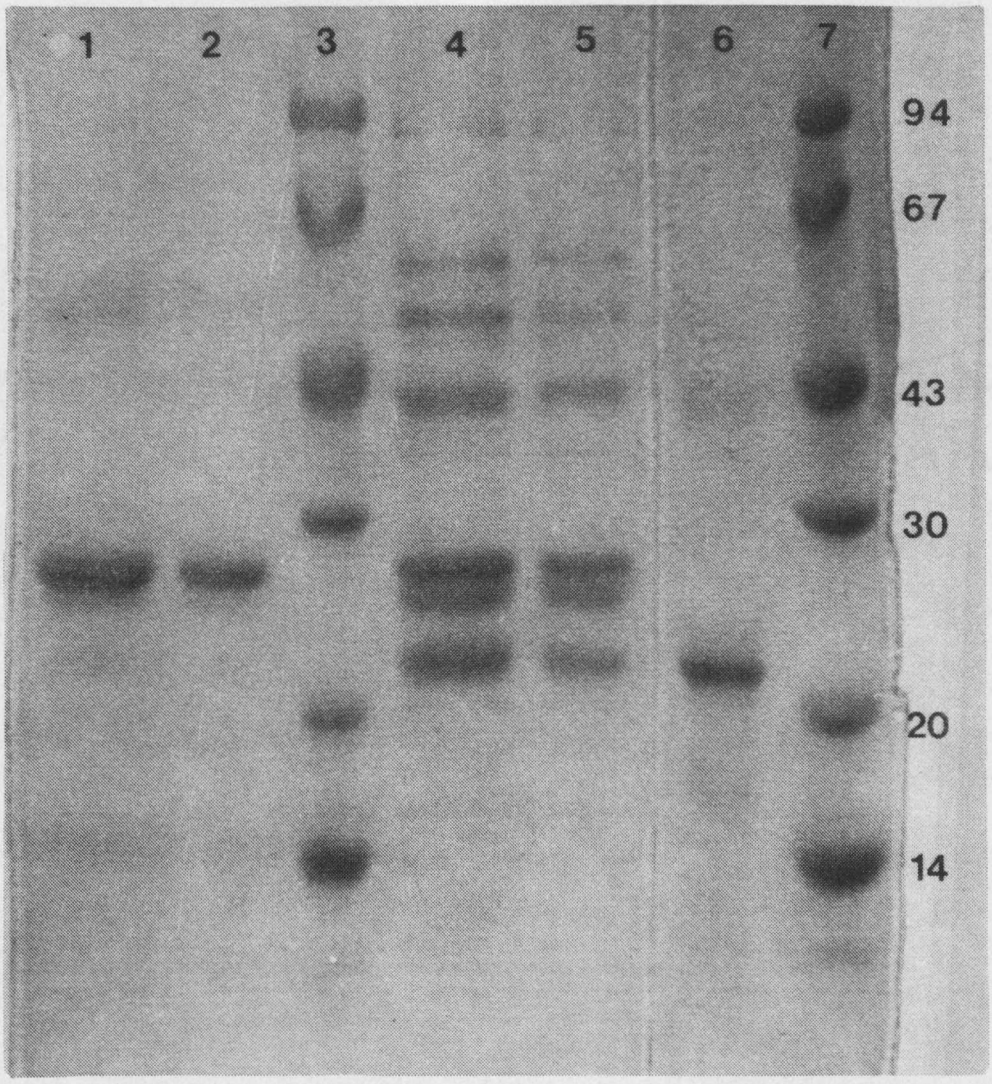


Figure I-1. Polyacrylamide gel electrophoresis of purified native soya ferritin. (A) is stained for protein (Coomassie Blue) and (B) is stained for iron (Prussian Blue, according to Gaal et al., 1980).

Figure I-2. SDS-PAGE of heavy and light forms of soya ferritin. Lanes 1 and 2: 28 kdal. form ($MgCl_2$ pellet), 20 and 10 ug; lanes 4 and 5: intermediate form ($MgCl_2$ supernatant), 20 and 10 ug; lane 6: 22 kdal. form ($MgCl_2$ supernatant), 10 ug; and lanes 3 and 7: molecular weight standards, in kdal.

For denaturing electrophoresis, the ferritin samples were dialyzed against dilute phosphate buffer to remove excess salts. The protein was then denatured and precipitated by addition of an equal volume of ice-cold 20% trichloroacetic acid, centrifuged for 5 min. at 10,000 x g in a clinical centrifuge, and washed 3 X with cold acetone to remove excess acid. The dried pellet was then suspended in 50 ul of 0.025 ammediol buffer, pH 8.5 containing 2% sodium dodecyl sulfate and 1% β-mercaptoethanol. The samples were placed in a boiling water bath for 10 minutes, centrifuged briefly to remove the insoluble iron cores, and loaded onto the SDS gel (13% acrylamide). Electrophoresis was carried out for 4-5 hours at 150 volts, constant voltage, until the tracking dye (phenol red) reached the bottom of the gel. The gel was then fixed in 50% methanol, 10% acetic acid for 30 minutes, and stained in 0.25% Coomassie Blue R-250 in the same solvent overnight. The gel was destained in the same solvent (minus the stain) until good contrast was obtained.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homogenization medium. This had no discernable effect on the subunit distribution. Such heterogeneity was seen when the isolation procedure used streptomycin sulfate to precipitate the nucleic acids prior to ammonium sulfate fractionation. Accordingly, we altered our protocol and employed $MgCl_2$ as the precipitant. When the $MgCl_2$ precipitate was analyzed for ferritin, it was found that essentially all of the protein was of the 28 kdal form, whereas the $MgCl_2$ supernatant contained a considerable amount of ferritin, with much of the protein present as the lower molecular weight form. These results were obtained principally with seeds which had been soaked for 72 hrs, whereas seeds soaked for only 18 hrs. had very low levels of either type of ferritin in the $MgCl_2$ supernatant.

As the phytoferritin purified from the $MgCl_2$ supernatant was comprised of smaller subunits, it seemed reasonable to assume the overall size of the native protein would be correspondingly smaller. Analysis of the molecular dimensions of the proteins on non-denaturing gradient gels for 16 hrs. (molecular sieving) (Figure I-3) indicated that the smaller-subunit form of the protein actually migrated a shorter distance than the 28 kdal form of the protein, suggesting that the 22 kdal. form may have a larger overall size.

Fingerprinting

In order to determine the relationship of the two predominant forms of the protein (28 kdal and 22 kdal) to each other, tryptic fingerprints of the two forms were prepared. The resultant data indicate that there is extensive homology between the two forms, with greater than 80% of the peptide maps showing complete overlap (Figure

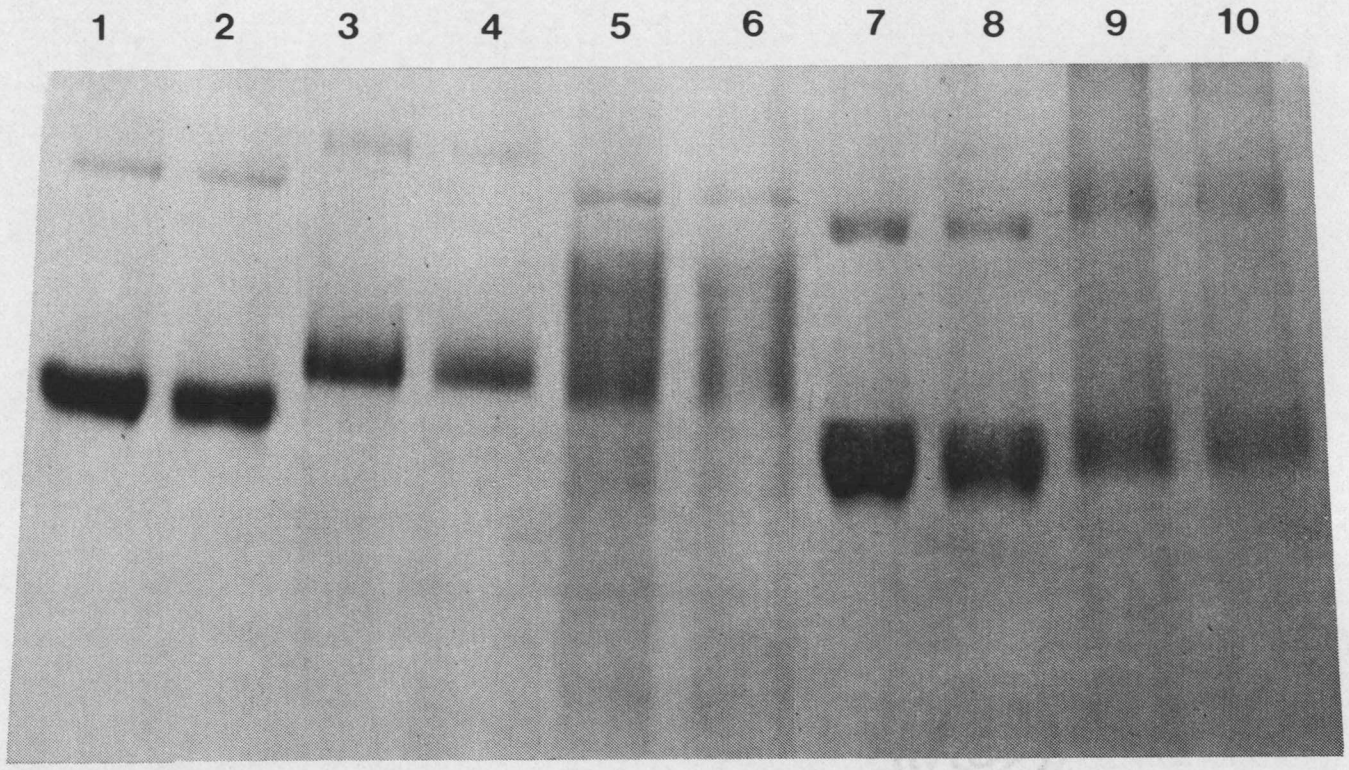

Figure I-3. Non-denaturing gradient gel electrophoresis of phytoferritin and mammalian ferritins. Lanes 1 and 2: horse spleen ferritin, 30 and 15 ug; lanes 3 and 4: heavy soya ferritin, 20 and 10 ug; lanes 5 and 6: light soya ferritin, 30 and 15 ug; lanes 7 and 8: rat liver ferritin, 30 and 15 ug; and lanes 9 and 10: human brain ferritin, 20 and 10 ug.

The gel was a 5% - 25% linear polyacrylamide gel, with a 3% stacking gel. The buffer system was the one described by Bury, 1981, with the exception that there was no SDS in the system. Electrophoresis was conducted at 80 volts, constant voltage, for 18 hours, after which time no movement of the ferritin could be detected. The gel was stained with Coomassie Blue as described.

I-4). With such extensive homology between the two forms, it appears very likely that the 22 kdal. form represents a proteolytic form of the 28 kdal. subunit.

In order to compare the peptide digests to known proteins, the fingerprinting was also carried out on apo-ferritin from horse spleen (Miles) and rat liver ferritin (isolated in this lab). The results indicate that there is also a great deal of homology between the plant ferritin and the mammalian forms.

Effect of Iron on Ferritin Distribution

To assess the subunit distribution of the ferritin as a function of iron overload, seeds were soaked in iron-enriched water prior to ferritin isolation. The results are shown in Table I-1. Surprisingly, the net effect of iron exposure during germination was a reduction in the amount of isolatable ferritin, both in the $MgCl_2$ pellet and in the supernatant. However, the amount of iron bound per ferritin molecule increased by more than 2-fold in the $MgCl_2$ pellet and almost 4-fold in the supernatant. As such, the total amount of ferritin-bound iron was relatively unchanged in the supernatant, but decreased markedly in the pellet.

Immunology

As the tryptic peptide maps indicate that there is a substantial amount of similar sequences in the plant and animal forms of ferritin, the possibility of similar antigentic sites occuring seems reasonable. To investigate this idea, we raised polyclonal antisera to the 28 kdal. form of the plant protein in both Balb-c mice and New Zealand

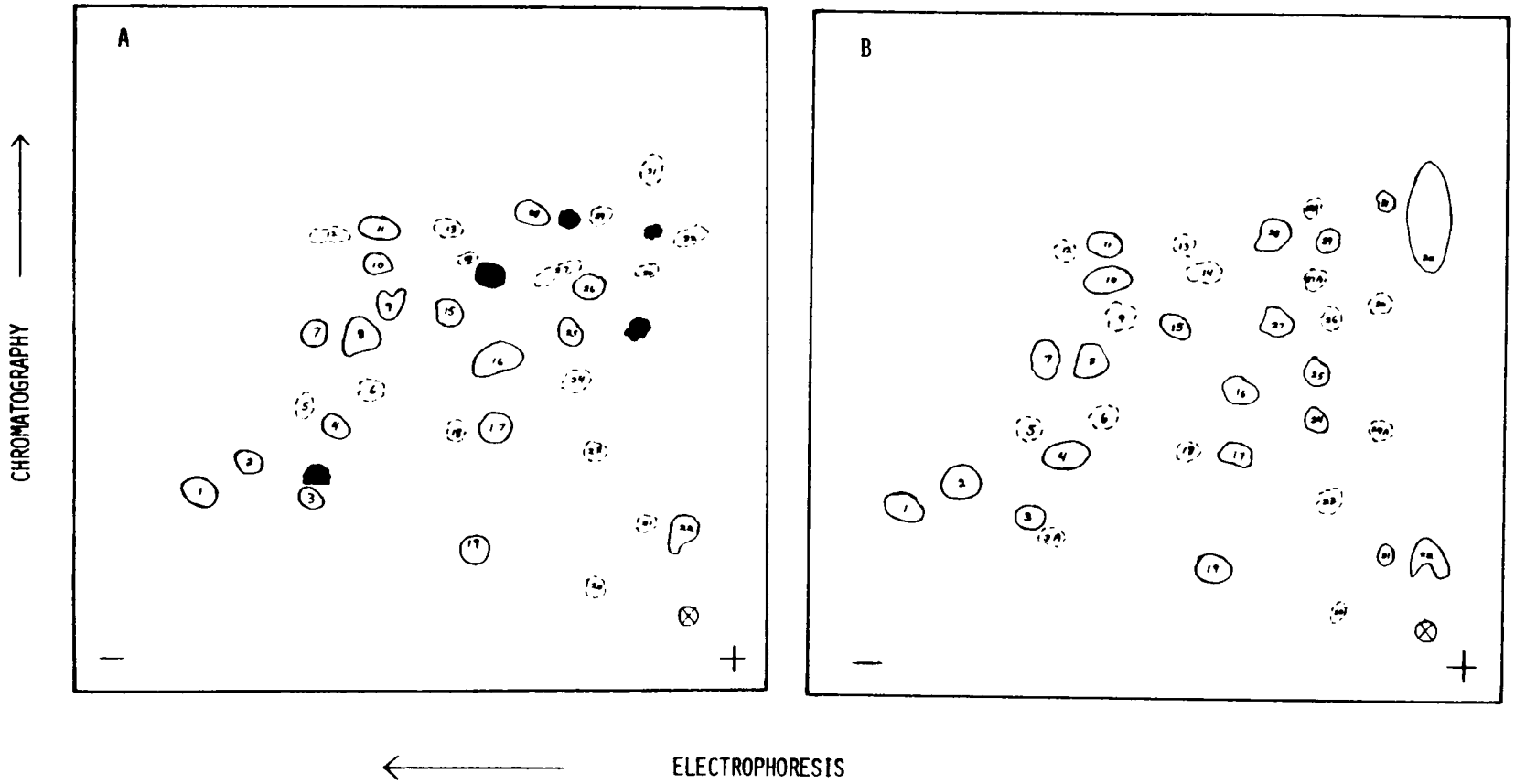

Figure I-4. Tryptic fingerprints of the two forms of soya ferritin compared to mammalian ferritins. (A) 28 kdal soya ferritin; (B) 22 kdal soya ferritin; (C) Horse spleen ferritin and (D) rat liver ferritin. x marks the sample origin.

The solid spots in (A) indicate peptides found in the 28 kdal. form which are absent in the 22 kdal. form (B). The solid spots in (C) and (D) represent spots which are homologous between the specific mammalian ferritin and the 28 kdal. soya ferritin.

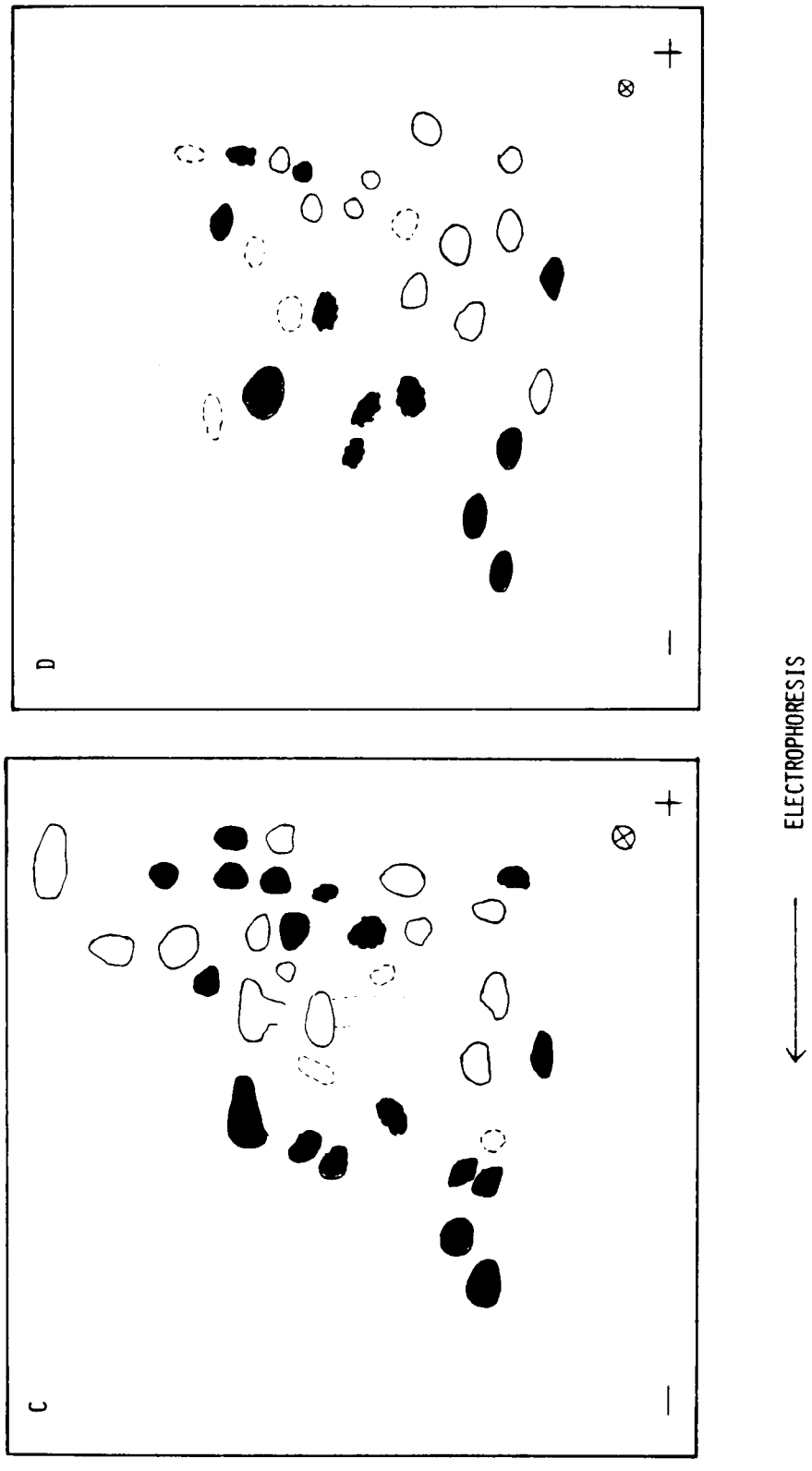
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TABLE I-1

FERRITIN DISTRIBUTION IN NORMAL AND IRON-SOAKED SEEDS

	mg Ferritin/ 500 g Seeds	G Atoms Fe	mg Fe Bound
$MgCl_2$ Pellet	2.88	1045	269
$MgCl_2$ Pellet + Fe	0.40	2278	82
$MgCl_2$ Supn.	47.5	250	1200
$MgCl_2$ Supn. + Fe	12.0	936	1140

White rabbits. These antisera were then tested for crossreactivity against both horse spleen ferritin and rat liver ferritin. The results can be seen in Figure I-5. While both the 28 kdal. and the 22 kdal. phytoferritin reacted well with both antisera, neither the horse or rat proteins gave any detectable reaction. We were also unable to detect any reaction of the plant ferritin with antisera against horse spleen ferritin raised in this lab (data not shown). As such, we could find no detectable shared determinants between the plant and animal forms.

Iron Loading

Since there appears to be at least two different forms of the plant ferritin (28 kdal. and 22 kdal.), we attempted to determine if their capacity to store iron was correspondingly altered. By presenting the proteins with excess ferrous iron and monitoring the amount of iron incorporated into the protein, we found that greater than approximately 1500 gram atoms of iron resulted in an extensive denaturation of both forms of the protein (Table I-2). Both the heavy and the light form accepted from 600-700 additional gram atoms of iron, but at the expense of protein loss on the order of 35 - 65%. Attempts to load the proteins with more than 2000 gram atoms of iron (total) resulted in complete precipitation and denaturation of the ferritin.

Iron Uptake

The rate of ferrous oxidation by ferritin is another measure of the protein's ability to sequester additional iron. The relative

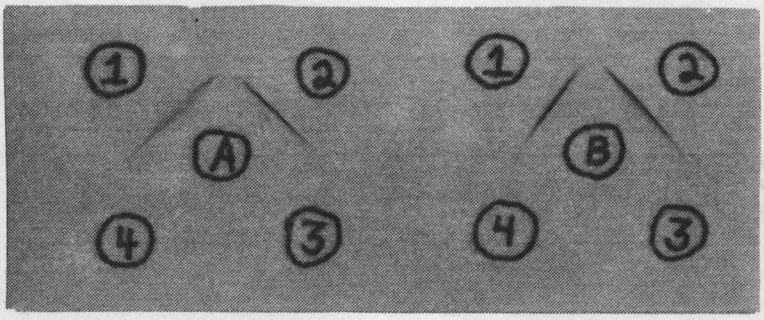


Figure I-5. Immunodiffusion of phytoferritin and mammalian ferritin. (A) Rabbit anti-soya ferritin antiserum, 7 ug: (1) 28 kdal. soya ferritin, 9.5 ug; (2) 22 kdal. soya ferritin, 11 ug; (3) horse spleen ferritin, 10 ug; (4) rat liver ferritin, 10 ug. (B) Mouse anti-soya ferritin antiserum, 8.6 ug: (1) 28 kdal. soya ferritin, 9.5 ug; (2) 22 kdal. soya ferritin, 11 ug; (3) horse spleen ferritin, 10 ug; (4) rat liver ferritin, 10 ug.

Samples were placed in 10 ul wells cut in a 1% agarose plate (1 mm thick), allowed to diffuse for 48 hours, washed in dilute phosphate buffer (pH = 7.3) and stained in Coomassie Blue R-250 (0.25% in acetic acid/methanol/water, 1:5:5).

TABLE I-2

IN VITRO Fe-LOADING OF SOYA FERRITINS

	Initial # Fe	Final # Fe	% Protein Loss
Soya Light	618	1335 (1000)	35%
Soya Heavy	1045	1670 (1000)	63%
Horse	2040	2130 (500)	26%
	2040	2345 (500)	50%

ferroxidase activity of the various phytoferritins is shown in Figure I-6. While both the heavy and the light forms of the protein are able to catalyze the oxidation of the metal, the small form (22 kdal.) does so at a greatly accelerated rate. As such, the smaller form is evidently well suited to function as an active ferritin. The smaller form was initially higher in iron than the larger, and this may be the reason for the advanced rate of oxidation observed.

Electron Microscopy

The distribution of the ferritin in the cotyledon of the germinating soyabean can be seen in Figures I-7 and I-8. Ferritin in plant sources is often arranged in crystalline arrays, usually in the amyloplasts of the cells. We found abundant evidence of such arrays in seeds which were soaked for 72 hours, always localized in the amyloplasts. In contrast, no ferritin arrays were detectable in seeds soaked for 18 hours only, either inside or outside the amyloplasts. The protein may have been present and simply not associated in the array formation.

Sucrose Gradient Centrifugation

The distribution of the two forms of soyaferritin (28 kdal. and 22 kdal.) may be seen in Figure I-9. The heavy form of the protein migrates as a single, sharp peak with an S value of approximately 18. The smaller form of the protein (22 kdal.) moves more slowly than the large form, but moves as a very broad, heterogeneous peak, encompassing a range of 10 S to 16 S. These sedimentation values correspond to molecular weights ranging from 240 kdal. to 460 kdal.

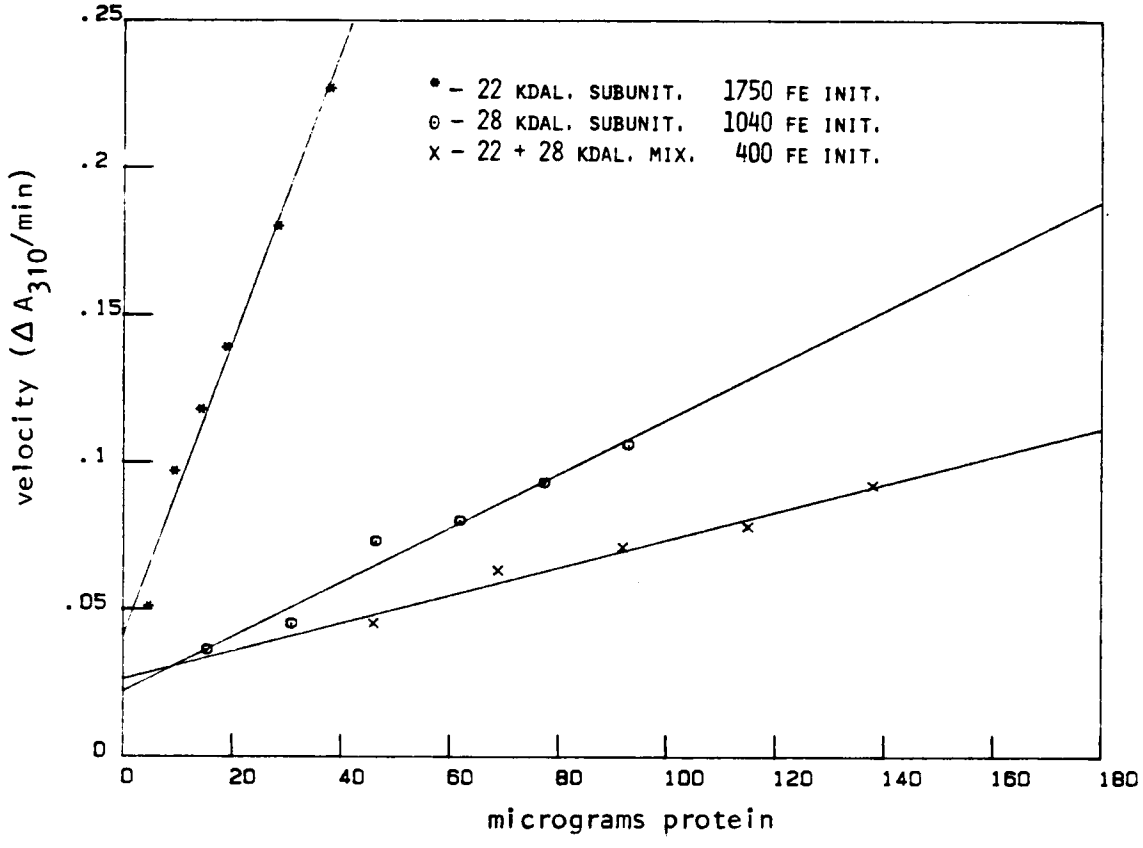


Figure I-6. Ferritin-catalyzed uptake of ferrous iron. Catalysis by (*) 22 kdal soya ferritin subunit; (o) 28 kdal soya ferritin subunit, and (x) 22 and 28 kdal soya ferritin subunit mix.

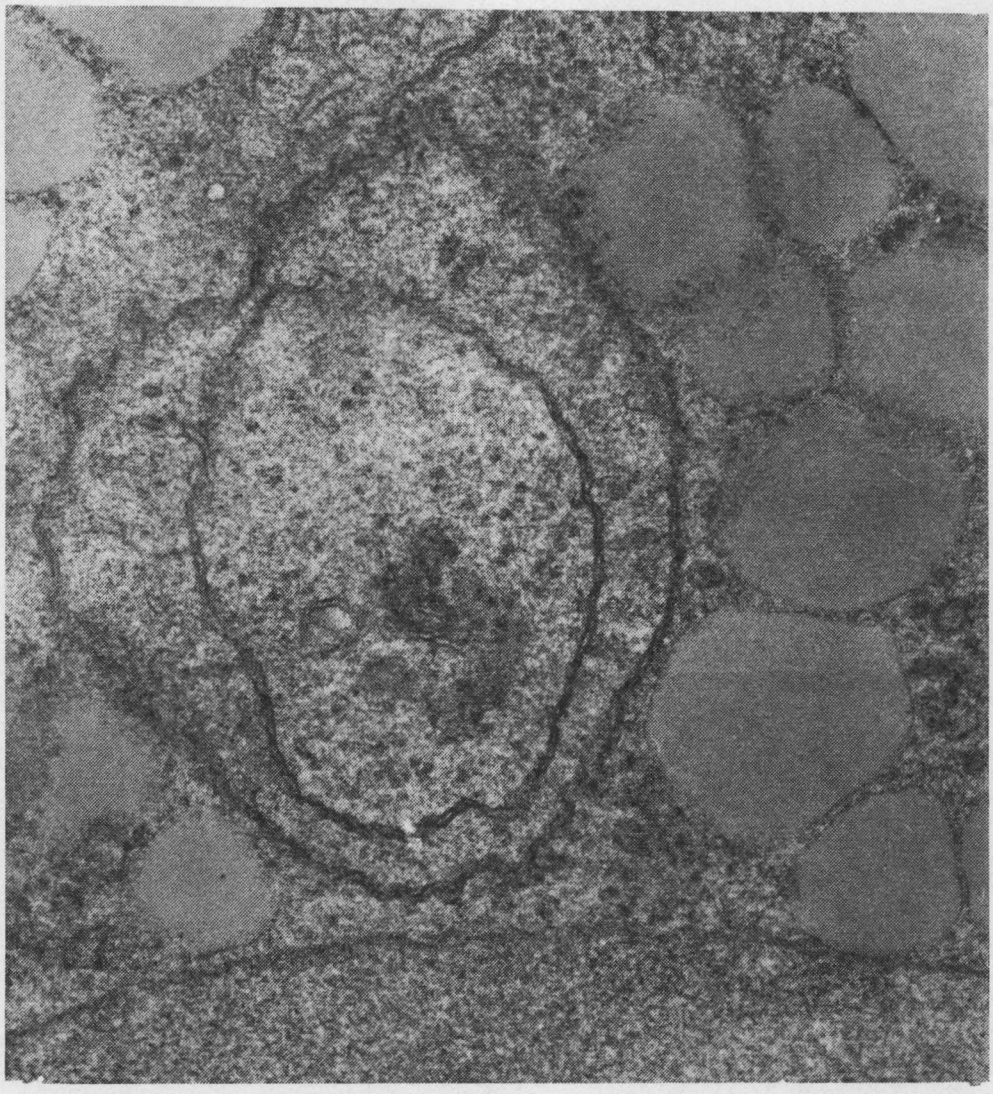


Figure I-7. Electron microscopy of soya beans soaked for 18 hours (30,000x).

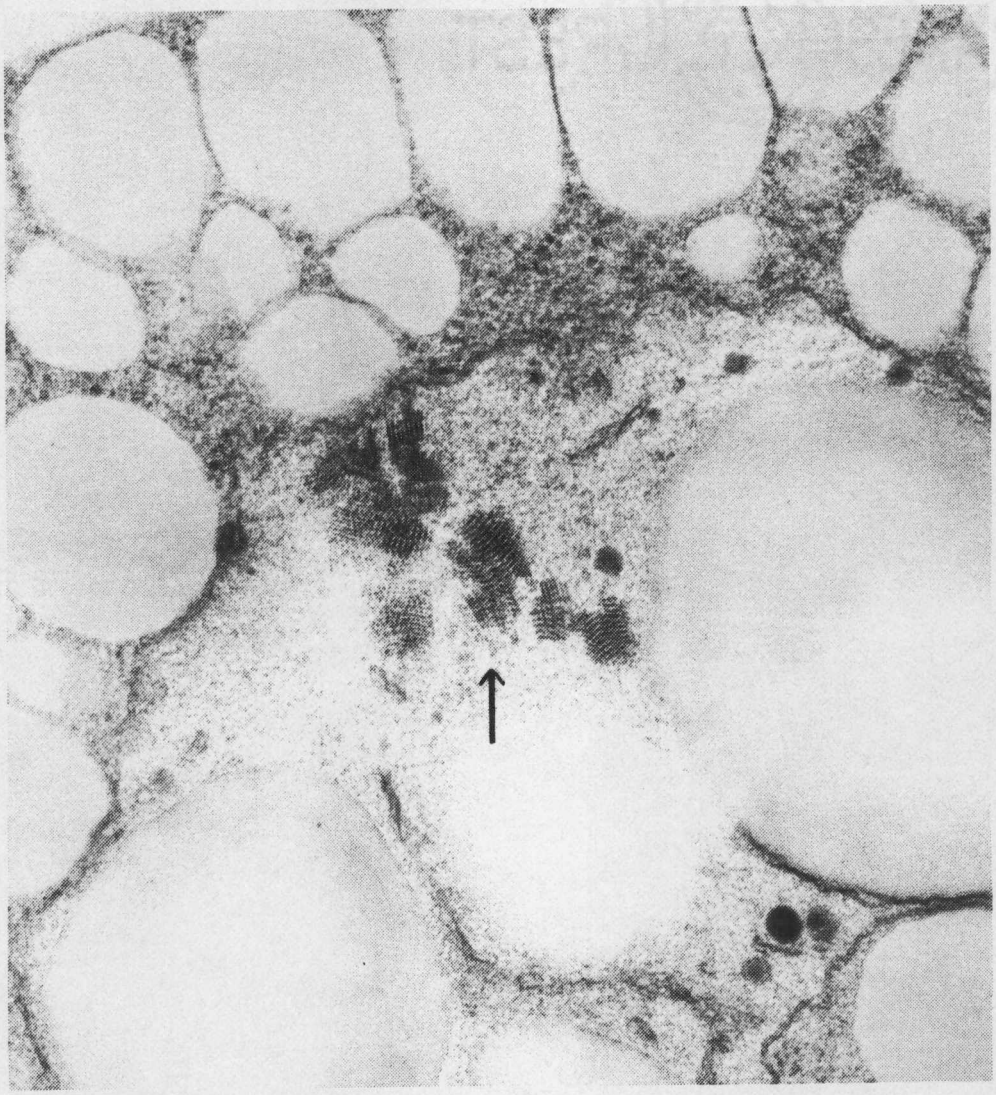


Figure I-8. Electron micrograph of soya beans soaked for 72 hours (30,000x). The arrow marks the location of the ferritin crystalline arrays.

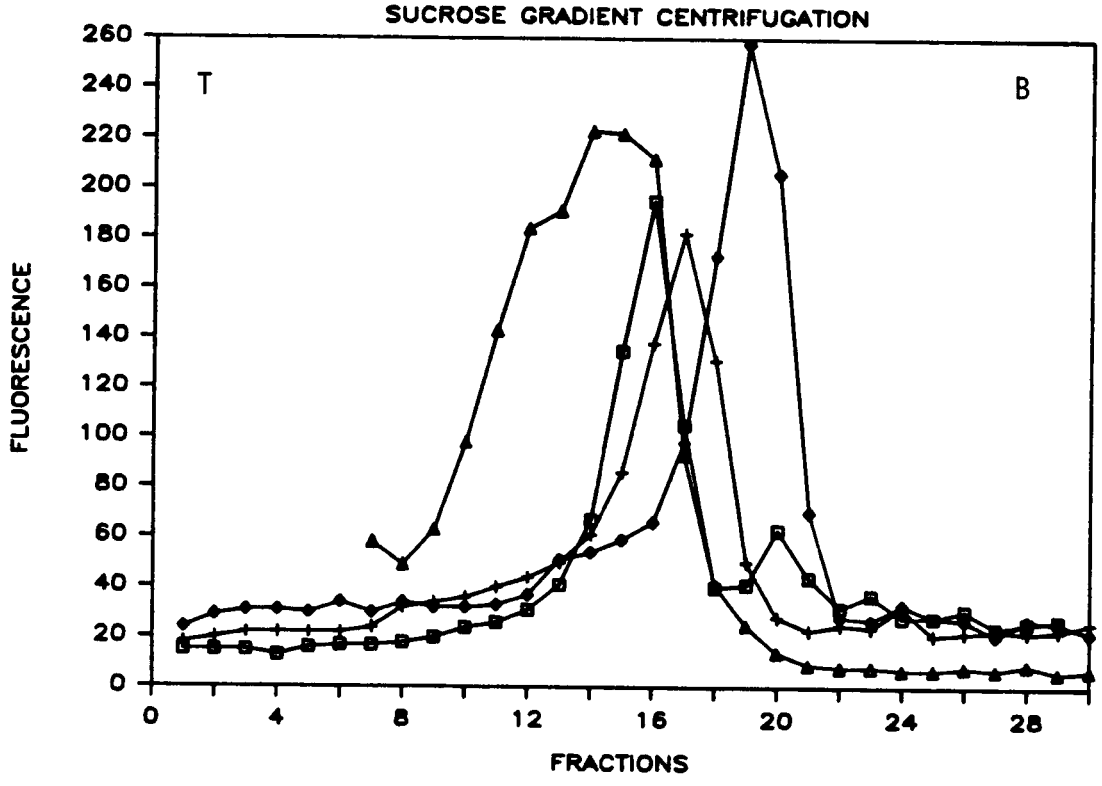


Figure I-9. Sucrose density gradient centrifugation of apo-soya ferritin. (△) light soya ferritin (10-16 S); (◇) thyroglobulin (19 S); (+) heavy soya ferritin (18 S); and (□) horse spleen ferritin (16 S). Based on these values, the heavy soya ferritin has a molecular weight of 572 kdal., and the light soya ferritin has a molecular weight ranging from 240 kdal. to 460 kdal.

This is considerably smaller than horse spleen ferritin, with a molecular weight of approximately 480 kdal. As such, it appears that the smaller form of the soya protein is very heterogeneous with respect to its native composition, probably having a range of subunit numbers associated into ferritin molecules, instead of the normal 24.

Subtilisin Cleavage

Ferritin as a rule is very resistant to proteolysis. However, as can be seen in Figure I-10, the protease subtilisin rapidly cleaves the 28 kdal. form, generating a set of two subunits of approx. 22-23 kdal. in equal amounts. This pattern of cleavage also generates two to three fainter bands on SDS electrophoresis, in the region of 35-40 kdal. These larger proteins are also generated when the 22 kdal. form is incubated with the protease, and again when horse spleen ferritin is treated (Figure I-11). The origin of these bands is not clear.

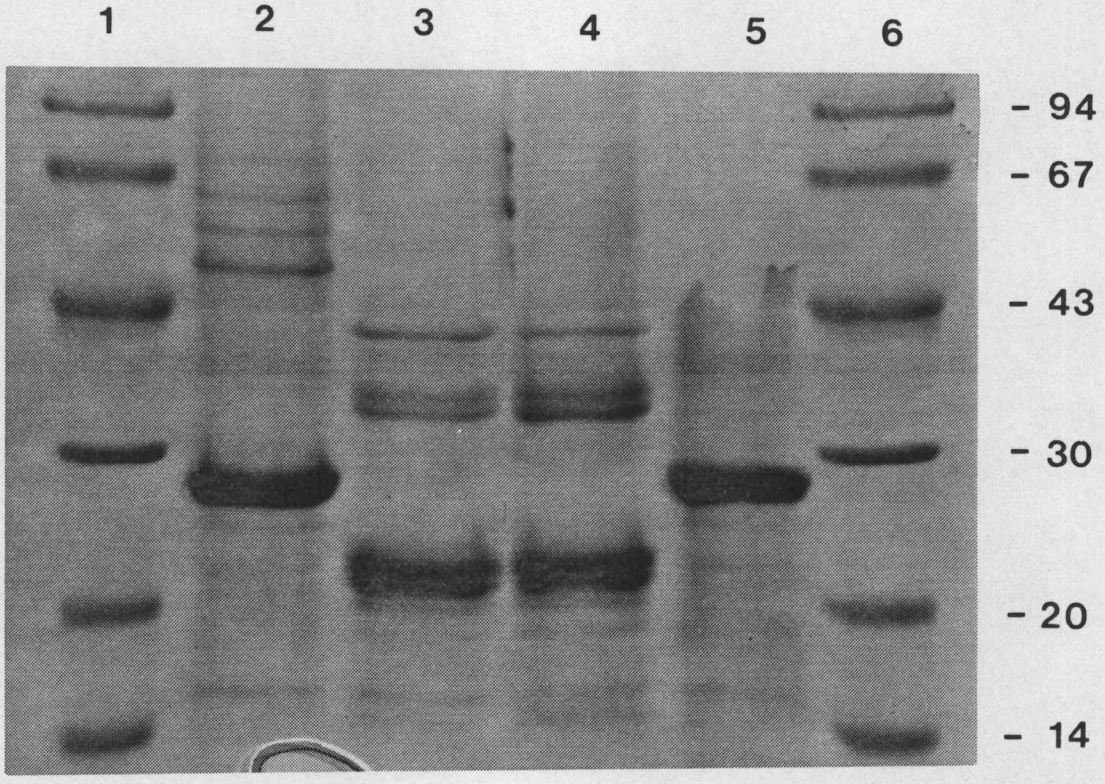
The lower-molecular weight bands (22-23 kdal.) generated by the protease from the 28 kdal. protein are remarkably similar to the native pattern seen with the protein isolated from the $MgCl_2$ supernatant of germinated seeds (22 kdal. form). As such, it seems logical to assume that this form of the protein is generated in the plant by a proteolytic process, resulting in the new form of the protein.

DISCUSSION

In many respects, the ferritin isolated from soyabean is quite similar to its mammalian counterpart. The protein is well-adapted to

Figure I-10. Subtilisin cleavage of heavy soya ferritin. Lanes 1 and 6: molecular weight standards, in kdal; lane 2: heavy soya ferritin; lane 3: heavy soya ferritin with subtilisin at a ratio of 1:40 (w/w); lane 4: heavy soya ferritin with subtilisin at a ratio of 1:5 (w/w); and lane 5: heavy soya ferritin denatured with SDS prior to treatment with subtilisin.

Specifically, 40 ug of ferritin in 80-100 ul of buffer was incubated with 1 ug of subtilisin in the same buffer (10 uL of a 0.1 mg/ml solution) for 30 minutes at 25°C. The protein was then denatured and precipitated by addition of 100 uL of ice-cold 20% trichloroacetic acid, centrifuged for 5 min. at 10,000 x g in a clinical centrifuge, and washed 3 X with cold acetone to remove excess acid. The dried pellet was then suspended in 50 ul of 0.025 ammediol buffer, pH 8.5 containing 2% sodium dodecyl sulfate and 1% β-mercaptoethanol. The samples were placed in a boiling water bath for 10 minutes, centrifuged briefly to remove the insoluble iron cores, and loaded onto the SDS gel (13% acrylamide). Electrophoresis was carried out for 4-5 hours at 150 volts, constant voltage, until the tracking dye (phenol red) reached the bottom of the gel. The gel was then fixed in 50% methanol, 10 % acetic acid for 30 minutes, and stained in 0.25% Coomassie Blue R-250 in the same solvent overnight. The gel was destained in the same solvent (minus the stain) until good contrast was obtained.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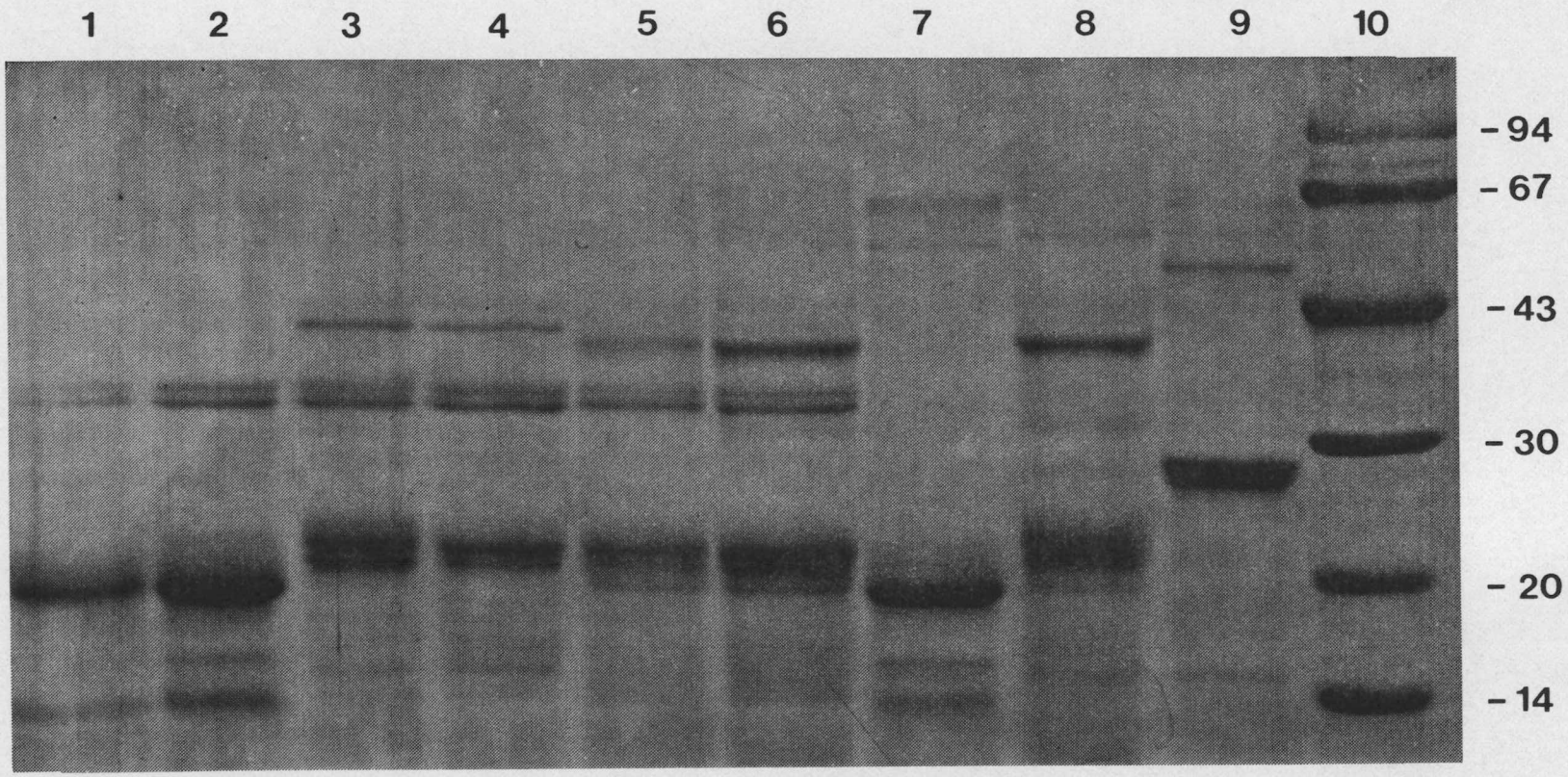

Figure I-11. Subtilisin cleavage of soya ferritins and horse spleen ferritin. Lane 1: holo-horse spleen ferritin; lane 2: apo-horse spleen ferritin; lane 3: heavy holo-soya ferritin; lane 4: heavy apo-soya ferritin; lane 5: light holo-soya ferritin; lane 6: light apo-soya ferritin; lane 7: apo-horse spleen ferritin with no protease; lane 8: light apo-soya ferritin with no protease; lane 9: heavy apo-soya ferritin with no protease; and lane 10: molecular weight standards, in kdal. All lanes contain 40 ug of the respective ferritin.

storing large amounts of iron, and as such can function as an iron reserve during maturation and metabolism.

We elected to study ferritin from soyabean and pumpkin seeds because these seeds are among the highest sources of iron found in plants (Adams, 1975). Curiously, while ferritin from soyabeans is readily isolated, we were unsuccessful in our attemps to purify the protein from pumpkin. The reason for this is not clear, but we confined our future work to soyabean.

Ferritin as isolated from animal sources is generally found as a heteropolymer, with at least two types of subunits present (Stefanini et al., 1982). These types of subunits have been found to represent discrete gene products (Jain et al., 1985), rather than modifications of the protein after translation. The various permutations of subunit assembly give rise to the isoferritins, which may be responsible for differential iron metabolism (Boyd et al., 1985).

Ferritin from soyabean appears to be homogeneous with respect to subunit composition, as long as only the ferritin precipitable with the ribosomes (via $MgCl_2$) is examined. As the present data show, not only is there a considerable amount of ferritin present which is not precipitated by the $MgCl_2$, but this ferritin also contains a heterogeneous subunit population. The subunits present in this population are all smaller than the 28 kdal. found in the $MgCl_2$ precipitate, with the predominate form being about 22 kdal. in size. As such, it seemed likely that the smaller subunits could have been derived from the larger form. The evidence from the tryptic fingerprints indicates a very high level of homology between the two

forms, with no detectable mismatched peptides present in the 22 kdal. form. Thus, the most logical interpretation is that the small subunit represents a processed version of the larger.

An unexpected finding was the apparent high degree of homology found between the plant ferritin and the mammalian protein. Crichton et al. (1978), in a parallel study of pea and lentil ferritin, found no similarity in the peptide maps of either of the plant ferritins and the mammalian forms. Theil (1983) reports that while there are many differences present in ferritins from diverse sources, it is likely that the initial iron binding sites are conserved. Our results would support such a conclusion. We found no cross-reactivity in immunodiffusion experiments between the plant and animal proteins, suggesting that there are few if any conserved sequences on the surface of the molecules.

The fact that the smaller ferritin isolated from the $MgCl_2$ supernatant is fully capable of absorbing additional iron would indicate that the protein is not simply an artifact of the isolation protocol, but is a functional protein. In addition, the smaller component represents by far the majority of the isolatable ferritin in the seed. As such, it constitutes a major fraction of the iron stores of the plant. When the germinating seeds are exposed to excess iron, both the precipitable and non-precipitable ferritins are decreased in amount. However, while the total amount of iron bound to the precipitable ferritin also decreases, the iron bound to the supernatant ferritin remains roughly equivalent.

The fact that the total amount of isolatable ferritin decreases upon incubation of the seeds with iron suggests an overload mechanism may be operating in which the additional iron is stored in a non-ferritin (or a non-isolatable) form. Since our in vitro attempts to load ferritin resulted in a significant amount of the protein being denatured, it is possible that a system similar to the formation of hemosiderin in animals may be occuring. When excess iron is presented to mammalian tissues (principally heptocytes), much of the iron may be found in the hemosideric fraction, which is usually thought to represent overloaded, denatured ferritin. The compound is generally insoluble in aqueous systems, has a protein component that reacts with anti-ferritin antibodies, and contains unusually high amounts of iron (Hoffbrand et al., 1977). As such, hemosiderin is thought to play a role in ferritin degradation, with the protein progressively losing subunits until there is insufficient protein to maintain the iron core in a soluble form.

The electron micrographic analysis of the germinated and non-germinated soyabean reveals that while the 72 hr. seed contained large amounts of the crystalline arrays of ferritin associated in the amyloplasts, we were not able to find any such arrays in the seeds which had been soaked for only 18 hours. This does not mean that no ferritin is present, only that it is not associated in arrays. Furthermore, during the initial imbibition of water by the seed (during the first 3-6 hours), there is a large efflux of iron from the seed (data not shown) into the surrounding water. Most or all of this iron-rich water is reabsorbed by the third day of soaking, which

results in the uptake of the previously released iron. If this excess iron is deposited in ferritin and then results in the precipitation of the protein into the crystalline arrays, it would explain why no such arrays are extant in the first hours of water uptake. If this is in fact the case, the crystalline arrays would represent a plant counterpart of the mammalian hemosiderin. It would also explain why challenging the seeds with additional iron results in a decrease in the amount of ferritin which can be isolated, since much of the ferritin containing the excess iron would be deposited in the insoluble crystalline arrays.

Indications that the smaller ferritin (22 kdal.) may be an intermediate in a hemosiderin form are seen in the non-denaturing gradient electrophoresis. This technique separates proteins on the basis of native molecular weight (Manwell, 1977) by generating a series of decreasing pore sizes in the acrylamide gel, resulting in a progressive decrease in protein mobility until the protein comes to an effective stop. Even though the ferritin isolated from the $MgCl_2$ supernatant is composed of subunits of lower mass, the native protein migrates slower than the 28 kdal. counterpart. This may be explained by either assuming a tendency of the smaller weight protein to polymerize (covalently or non-covalently), into a molecule with greater than 24 subunits (a phenomenon observed for rat muscle ferritin; Linder et al., 1981), or else the protein has a markedly lower stability than the 28 kdal. form, and as such, progressively denatures as it is tightly sieved through the gel pores. If the 22 kdal. form was incompletely assembled (that is, comprised of an

assemblage of fewer than 24 subunits), it would be reasonable to assume a decreased stability for the protein.

The results of the sucrose gradient centrifugation would imply that the small form of ferritin is in fact incompletely assembled into a native molecule. If the protein had a complete shell of 24 subunits, it should migrate as a single peak ahead of the mammalian protein, which is composed principally of 20 kdal. subunits. Instead, the protein peak moves mainly behind the horse protein, indicating a smaller S value. In addition, the peak is abnormally broad, as compared to the 28 kdal. form. This would indicate that a variety of S values are present, which may correspond to diverse assemblages of the protein (perhaps complexes of 15-22 subunits).

Thus, there is good evidence that the 22 kdal. soya protein is in fact more labile than its 28 kdal. counterpart, presumably due to missing subunits in the protein shell. Additional evidence which implies that the 22 kdal. form may be incompletely assembled may be seen from the rate of iron uptake of the various ferritin species. While the 28 kdal. form alone, and the mixture of forms as isolated, give approximately equivalent rates of ferrous oxidation, the 22 kdal. molecule is able to catalyze the oxidation at a greatly increased rate, almost 200% over that seen with the other preparations. Although this form of the protein also represents that with the most iron present initially (approx. 1750), as compared to the other samples (approx. 400 and 1040), this difference is insufficient to account for such a large increase in activity. Harrison et al. (1974) have found that the rate of iron uptake by various species of ferritin

changes with the amount of iron initially present in the protein, with ferritin which contains approximately 1500 Fe per protein giving the fastest rate. However, ferritin with only 1000 Fe is able to uptake iron at about 75% that of the maximal value. Thus, the unusually high rate of oxidation associated with the 22 kdal. form cannot be explained by the extra iron initially present. A logical alternative would be that the iron core is more exposed in this form of the ferritin, presumably due to loss of protein subunits. This would provide increased access to the core by incoming ferrous ions, which would then undergo oxidation on the mineral surface. This situation is very similar to that seen for hemosiderin in mammals, where abnormally high Fe/protein ratios indicate the loss of protein subunits, resulting in the precipitation of the iron complex. Such incomplete ferritin shells may be seen in electron micrographs of hemosiderin preparations (Weir et al., 1984).

Generation of the small form of the protein may be a signal to remove the protein from the active iron pool. As proteolytic processing may play a role in hemosiderin formation (Richter, 1984; Weir, 1984), we reasoned that a similar mechanism may occur in plants. Surprisingly, we found that formation of the smaller form of the protein was easily achieved by incubation of the 28 kdal. form with subtilisin, a well-known serine protease. As ferritin is generally quite resistant to proteolysis in its native form, such a result was unexpected. Furthermore, the cleavage pattern appears quite specific, generating the 22-23 kdal. doublet seen on SDS PAGE when the native protein from the $MgCl_2$ supernatant is analyzed. Further degradation,

at least by this protease, does not occur. This raises the possibility of a specific, protease-susceptible sequence in the protein, allowing the ferritin to be easily marked for degradation. Such a phenomenon has been postulated as an intermediate in mammalian hemosiderin formation (Richter, 1984).

Our data indicate that ferritin isolated from soyabeans has many features in common with the mammalian proteins, including certain peptide homologies and subunits structures. In addition, there are indications that the plant protein may follow similar degradation pathways.

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PART II

METAL-BINDING PROPERTIES OF PHYTOFERRITIN AND SYNTHETIC IRON CORES

INTRODUCTION

Ferritin is a multisubunit protein which is capable of storing large amounts of iron. The iron is sequestered in the interior cavity of the protein in the form of an insoluble ferric oxyhydroxyl polymer. As such, one function of the protein coat is to render the iron core soluble, maintaining the iron in a bioavailable form (Theil, 1983).

While iron is the predominant metal stored, other divalent and trivalent cations may also be bound. Initial studies (Price and Joshi, 1983) indicated that ferritin could bind large amounts of beryllium. Indeed, the affinity of ferritin for this metal is such that it is able to remove Be^{++} from phosphoglucomutase, affording partial protection to the enzyme against inactivation (Joshi et al., 1984). As our interest in ferritin extends to the plant system, one of our main purposes was to investigate similar mechanisms in soyaferritin.

While apoferritin possesses certain specific binding sites for heavy metals (Clegg et al., 1980), the holoprotein is able to bind much higher numbers of the various metals. As the logical reason for this increased binding would implicate the iron core, we elected to focus on the inorganic structure of the core itself.

Various investigators have used artificial iron polymers as models for several naturally occuring iron systems. Gorun and Lippard (1986) found that a synthetic polynuclear iron complex could be constructed with phenyl rings mimicking the protein shell of ferritin and rendering the structure soluble in aprotic solvents. Yang et al. (1986) showed that various iron polymers prepared using either

carboxylate, hydroxyl or sulfate anions exhibited specific Mossbauer spectra, indicating differences in ordering patterns of the ferric-oxy species.

Preparation of artificial iron polymers using citrate results in a highly defined complex which in many respects mimics the core of ferritin (Spiro et al., 1967). These polymers are characterized by a narrow size distribution (72 $\pm$ 9 Å) containing approximately 1200 iron atoms linked by hydroxy and oxy bridges. The polymers have been used as models of the ferritin core in iron release studies (Jones et al., 1978).

In the present study, these citrate-iron polymers were used as a model to investigate metal binding to holoferritin. As the presence of inorganic phosphate in the ferritin core may be implicated in this interaction (Joshi et al., 1984), the polymers were loaded with P_i to more closely resemble the protein core. These results are then compared to the metal binding of soyaferritin.

MATERIALS AND METHODS

All metals were obtained from Baker Chemicals as their chloride, sulfate or nitrate salts and used without further purification. Metal concentration was in all cases determined on an Instumentation Laboratory Model 551 atomic absorption spectrophotometer, equipped with a Model 655 graphite furnace. Metal standards were obtained from Spex Industries, Inc. Synthetic iron cores were prepared as described by Spiro and Saltman (1967). The cores were prepared in batches of 500 mls. Titration of the ferric nitrate/citrate mix was with 0.01 N

NaOH, in 10 ml increments. The pH was constantly monitored with a Fisher Accumet pH meter, and titration was continued until the pH stabilized at 9.2 - 9.4. The polymer fraction was enriched by ultrafiltration through a PM-30 Amicon filter to a final volume of 10 mls. At this time, the quality of the preparation was monitored by electron microscopy (Figure II-1). The polymer was then dialyzed exhaustively against 0.1 M Na MES, pH 6.0, for 7-10 days to remove excess citrate. Dialysis against 0.05 M Na PO_4, pH 7.0 was then carried out for 12-14 hrs, at which time extensive precipitation of the polymer was evident. The sample was again dialyzed exhaustively against MES buffer for 3-4 days to remove excess phosphate, at which time the accreted polymer was used for metal binding analysis. Bound phosphate was measured by the method of Ames (1966), and iron by flame atomic absorption after dissolution in 50% HNO_3 (ULTREX), followed by dilution. The binding analyses of the metals to synthetic cores were performed with polymer which had been preloaded with phosphate, in order to more closely approximate the _in vivo_ situation with ferritin. To quantitate the actual binding of phosphate to the core, inorganic phosphate spiked with $^{32}P_i$ was incubated with the cores as described, and the amount of free phosphate in the supernatant was determined by scintillation counting. From these values, a Scatchard plot of the binding of the phosphate anion to the synthetic cores was obtained.

Metal Binding

Metal stocks were prepared in 1 mM HCl, then diluted into 0.1 M MES, pH 6.0. Metal concentration was determined by AAS. Phytoferritin was isolated from soyabean as described in Section I.

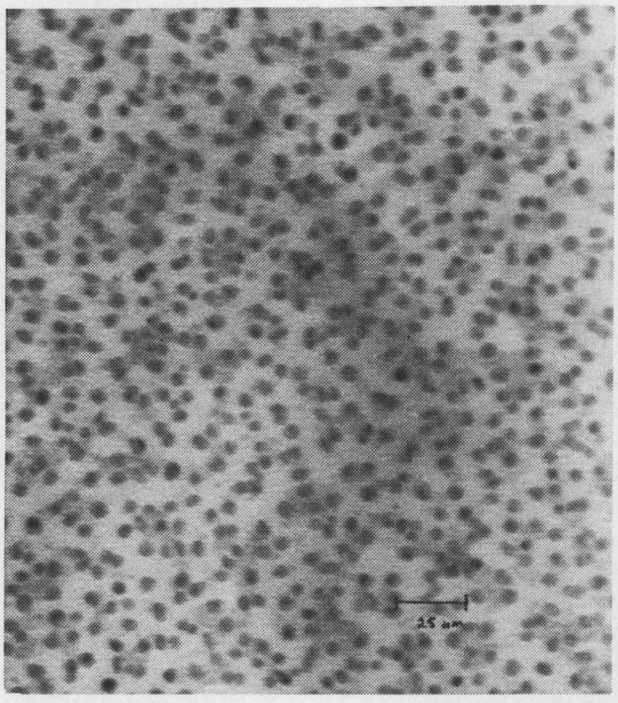


Figure II-1. Electron microscopy of synthetic cores (250,000x).

For phytoferritin stoichiometry, the Centrifree system by Amicon was employed. The cartridges were first centrifuged with 1 ml of MES buffer to remove glycerin, azide and residuals from the filter. Omission of the rinse step resulted in significant metal retention by the filter, leading to false high readings of metal bound. In a separate test tube (borosilicate), was added MES buffer, metal ion in MES buffer, and finally an aliquot of protein in MES. The total volume was 1 ml. After incubation at 23°C for 5 min., the sample was transferred to the prewashed Centrifree cartridge, and centrifuged at 12,000 rpm in a clinical fixed angle rotor for 5 min., or until approximately 0.7 ml had collected in the filtrate cup. The filtrate was immediately analyzed for the particular metal via AAS. Controls were treated as described, minus the protein.

For metal binding to synthetic cores, 1.5 ml Eppendorf centrifuge tubes were used. As the synthetic cores were insoluble in the buffer used (MES), simple centrifugation was sufficient for separation of bound vs. free metal. To the premeasured volume of MES buffer was added an aliquot of the metal prepared as above, followed by addition of the core suspension. Incubation at room temperature was done for 5 min., at which time the samples were centrifuged for 2 min. in a Brinkman Eppendorf centrifuge at 12,000 rpm. The supernatant was immediately analyzed as above. Controls were treated as described, minus the cores.

RESULTS

The Scatchard plots of binding of metals to soyaferritin can be

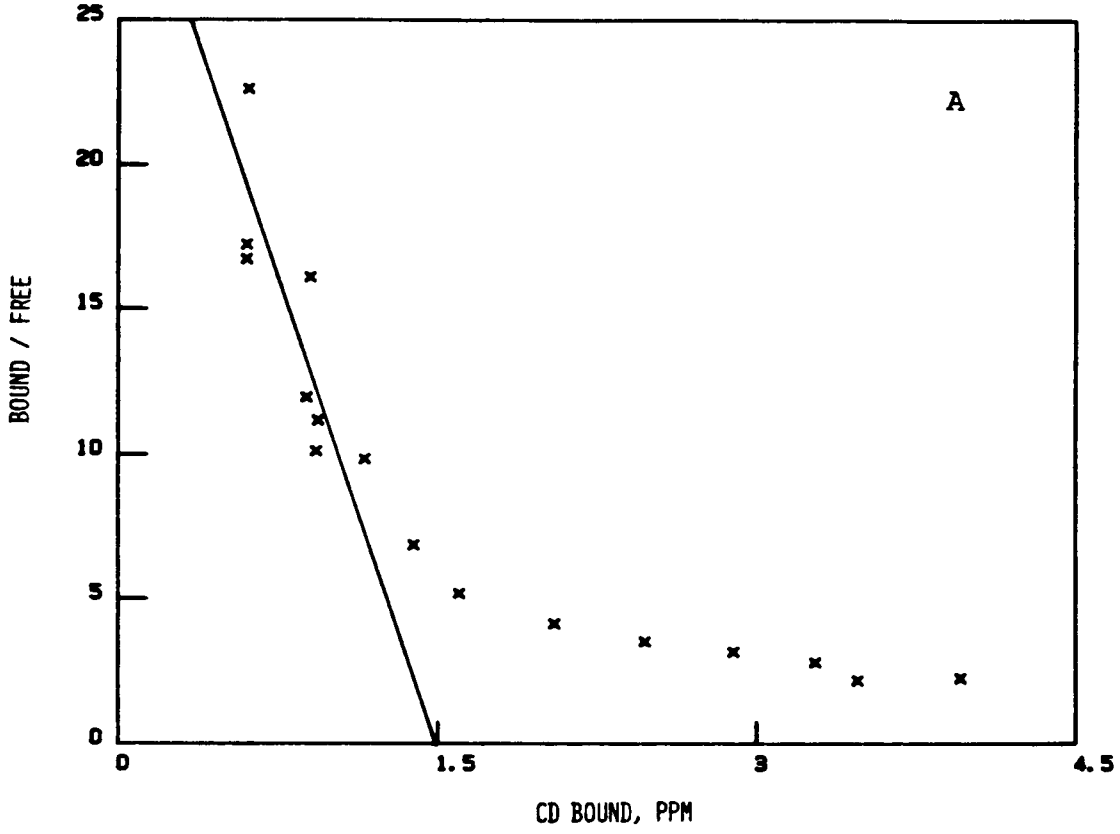
seen in Figure II-2. All of the metals tested (Cd, Zn, Be and Al) were found to bind in a significant manner to the plant protein. According to the Scatchard anlysis, there is evidence of at least one class of high-affinity ($Kd \simeq 10^{-6}M$) binding sites for all four metals. In addition, there was some indication of binding of the metals to a second class of rather low affinity sites. This is indicated by the "tailing" of the graphs in the region of high numbers of metals bound, rather than the line approaching directly to the x axis. This phenomenon occurred with all the metals tested.

The results of the metal binding by the synthetic cores can be seen in Figure II-3. Again, each of the metals tested were found to be sequestered by the polymer, with affinities ranging from 10^{-5} to $10^{-7}M$. Additionally, the same phenomenon of "tailing" in the high bound region of the graph was in evidence.

The dissociation constants and stoichiometries of the metal binding are displayed in Table II-1. The Kd's were calculated from the slopes of the Scatchard analyses, and the numbers' bound from the x intercepts. The lines displayed on the graphs were generated by least-squares linear regression analyses.

The binding of inorganic phosphate to the synthetic core is shown in Figure II-4. The results of the experiment indicate that the binding is very strong, with a Kd of $2.5 \times 10^{-9}M$. As the total time of exposure of the phosphate to the core preparation was 5 minutes, these values are most likely a measure of the binding to the surface only.

Figure II-2. Scatchard plots of metal binding by soya ferritin. (A) Cd; (B) Zn; (C) Be; and (D) Al.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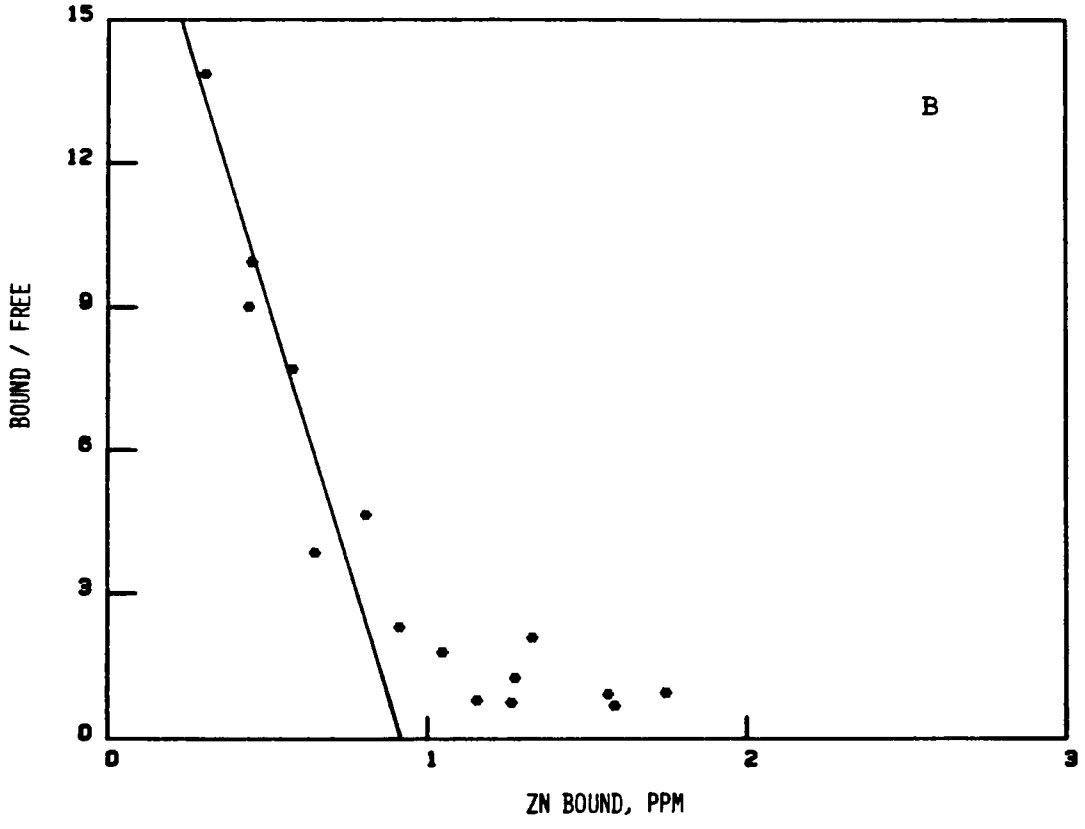
CD BOUND, PPM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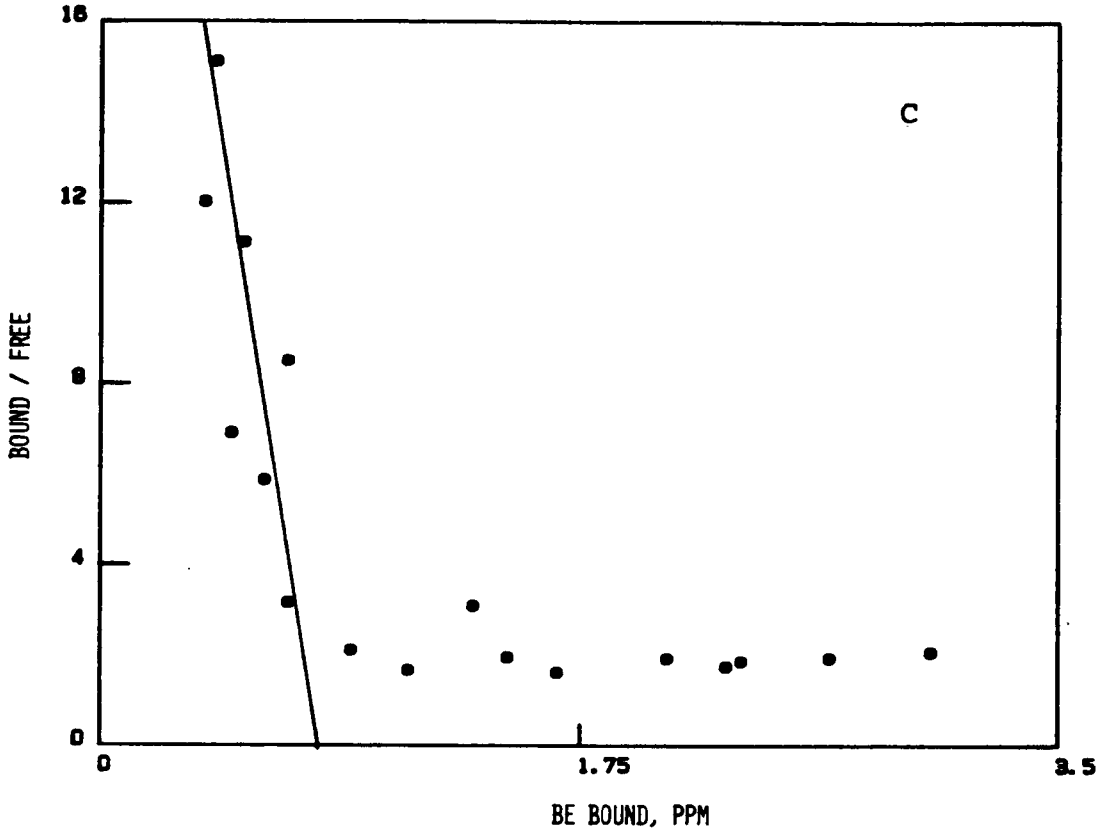
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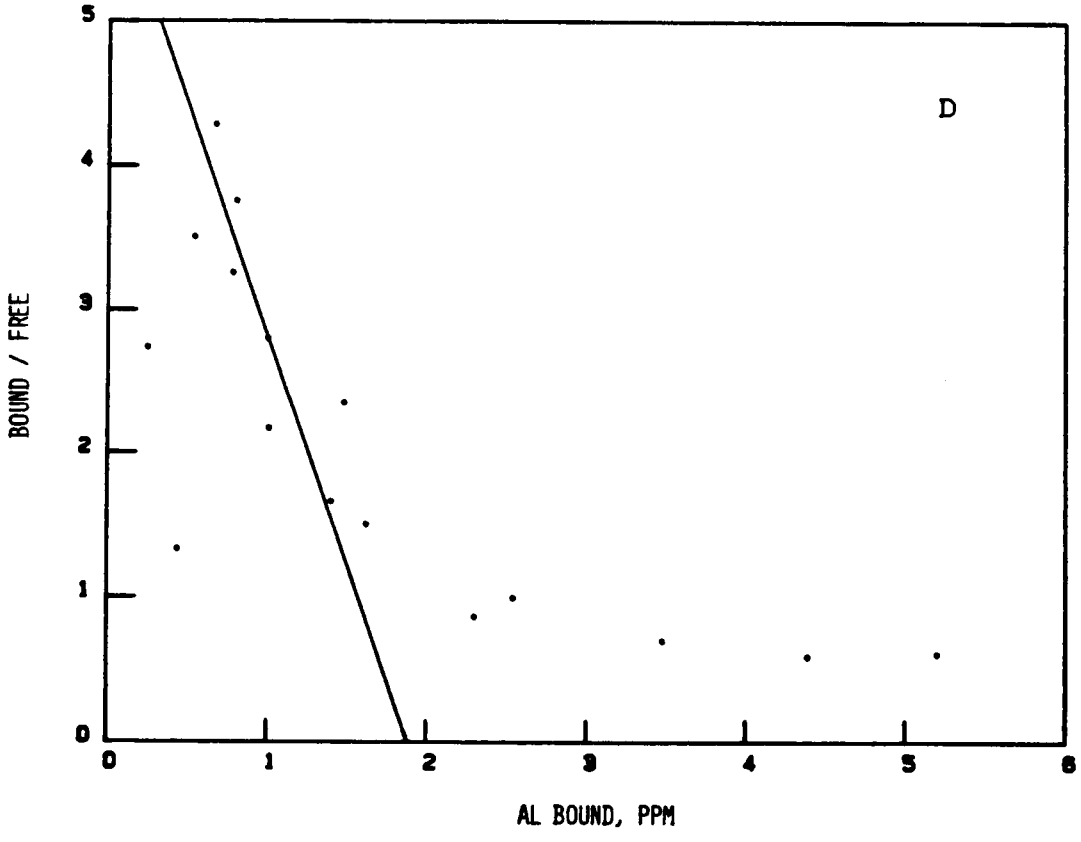
BE BOUND, PPM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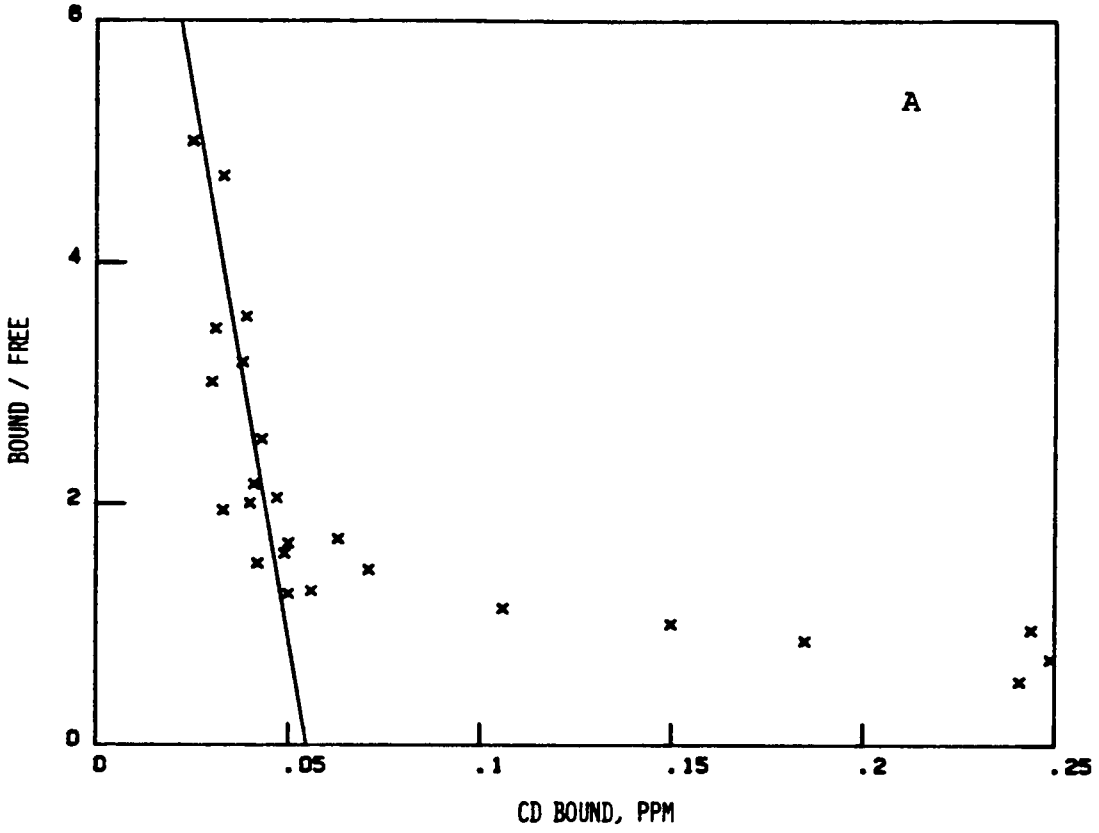
AL BOUND, PPM

Figure II-3. Scatchard plots of metal binding by synthetic cores. (A) Cd; (B) Zn; (C) Be; and (D) Al.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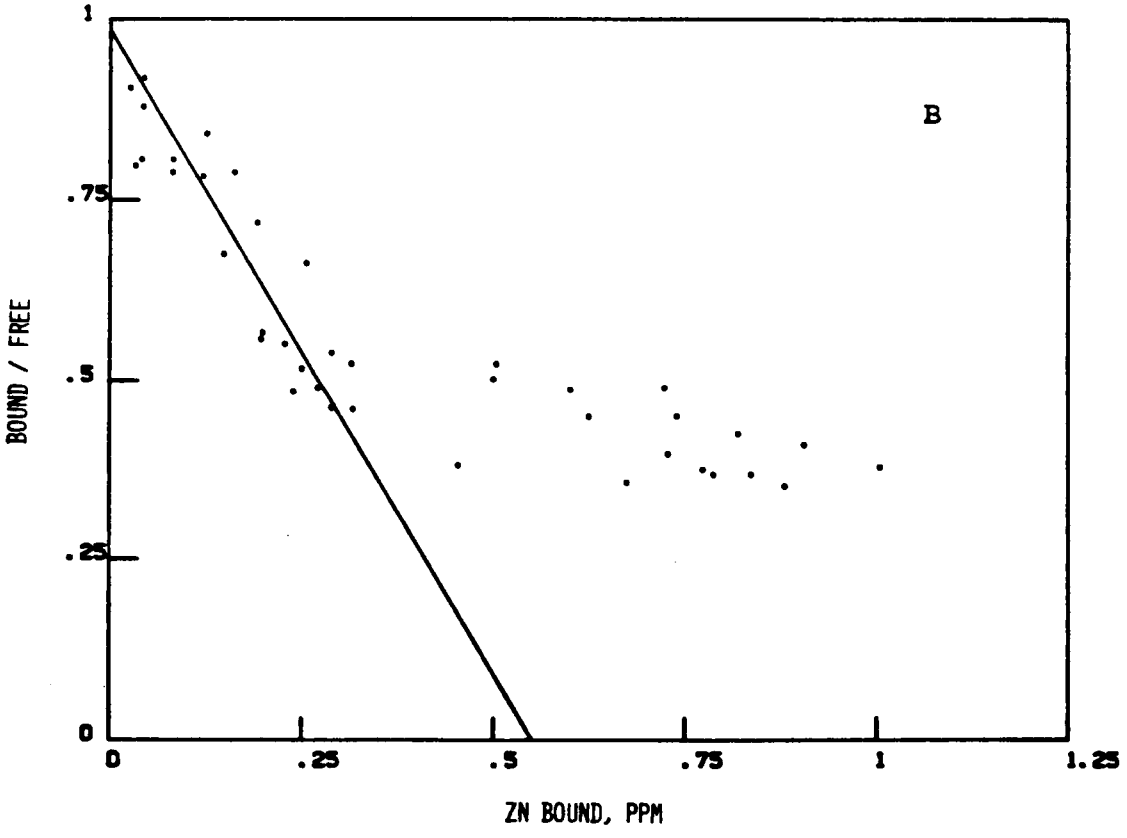
CD BOUND, PPM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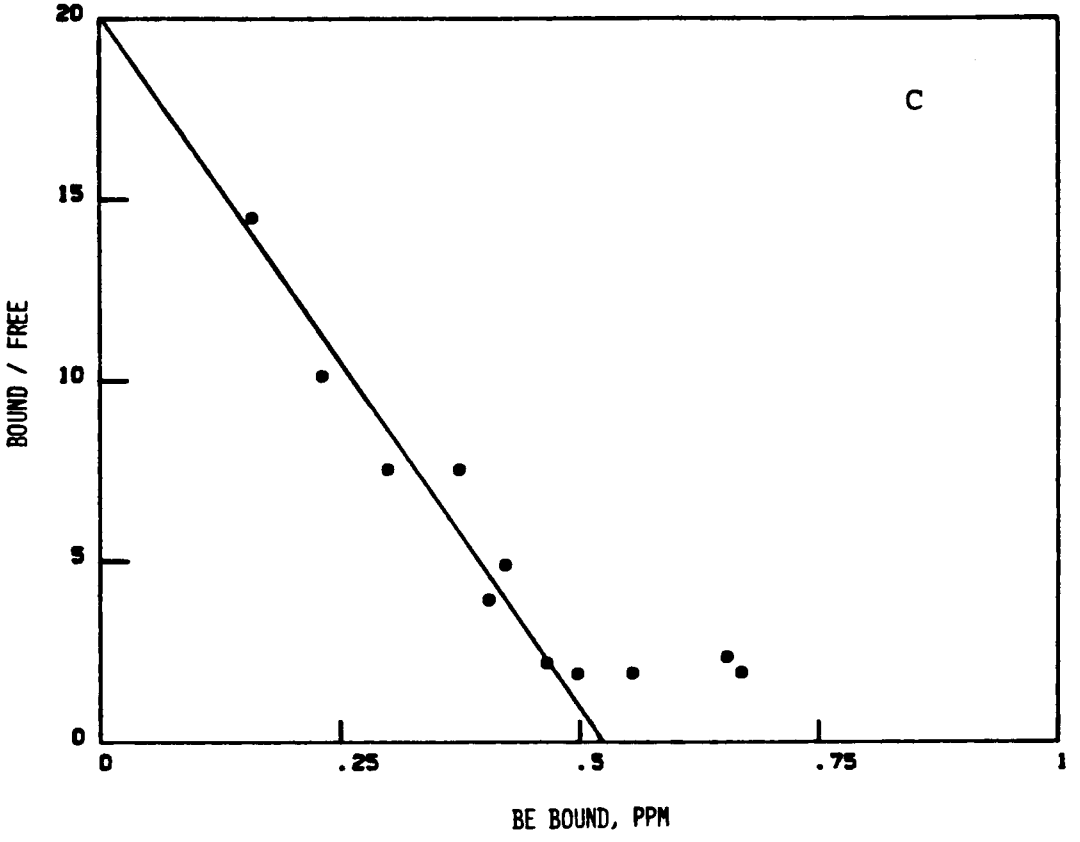
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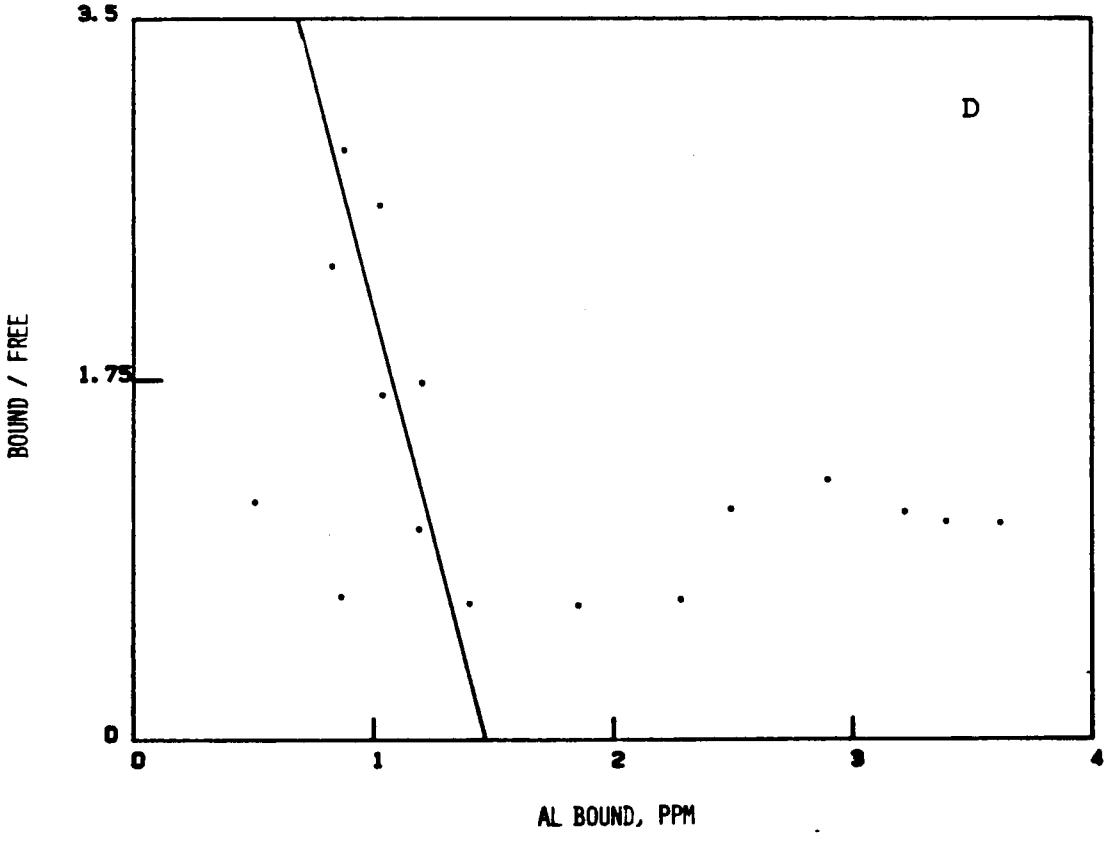
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TABLE II-1

METAL-BINDING CONSTANTS OF FERRITINS AND SYNTHETIC CORES

	Fe Content	PO_4 Content	Zn		Cd		Be		Al	
			K_d	N	K_d	N	K_d	N	K_d	N
Core	1200*	186	9.8×10^{-6}	8	1.27×10^{-7}	2.2	1.83×10^{-6}	74	1.05×10^{-5}	211
Soya	1400	288	8.01×10^{-7}	75	5.62×10^{-7}	73	5.55×10^{-6}	882	3.06×10^{-5}	652
Horse**	1500	322	5.25×10^{-6}	135	8.01×10^{-7}	76	6.8×10^{-6}	800	4.52×10^{-5}	164

Note: Values expressed in the table are the dissassociation constants (K_d) and extrapolated number of binding sites (N) for the various metals bound to either the synthetic cores or the specific protein. Number of binding sites was obtained from the Scatchard analysis X-intercept, and the relative K_d from the slope of the Scatchard plot.

*The synthetic cores contain approximately 1200 Fe at the time of preparation. However, during dialysis, as much as 30 - 40% of the iron is removed prior to accretion. All values in the table for cores were derived as ratios of Fe to metal, or Fe to phosphate, and subsequently normalized to the original value.

** Data taken from Price, D.J. and Joshi, J.G. (1983) J. Biol. Chem. 258, 10873-10880.

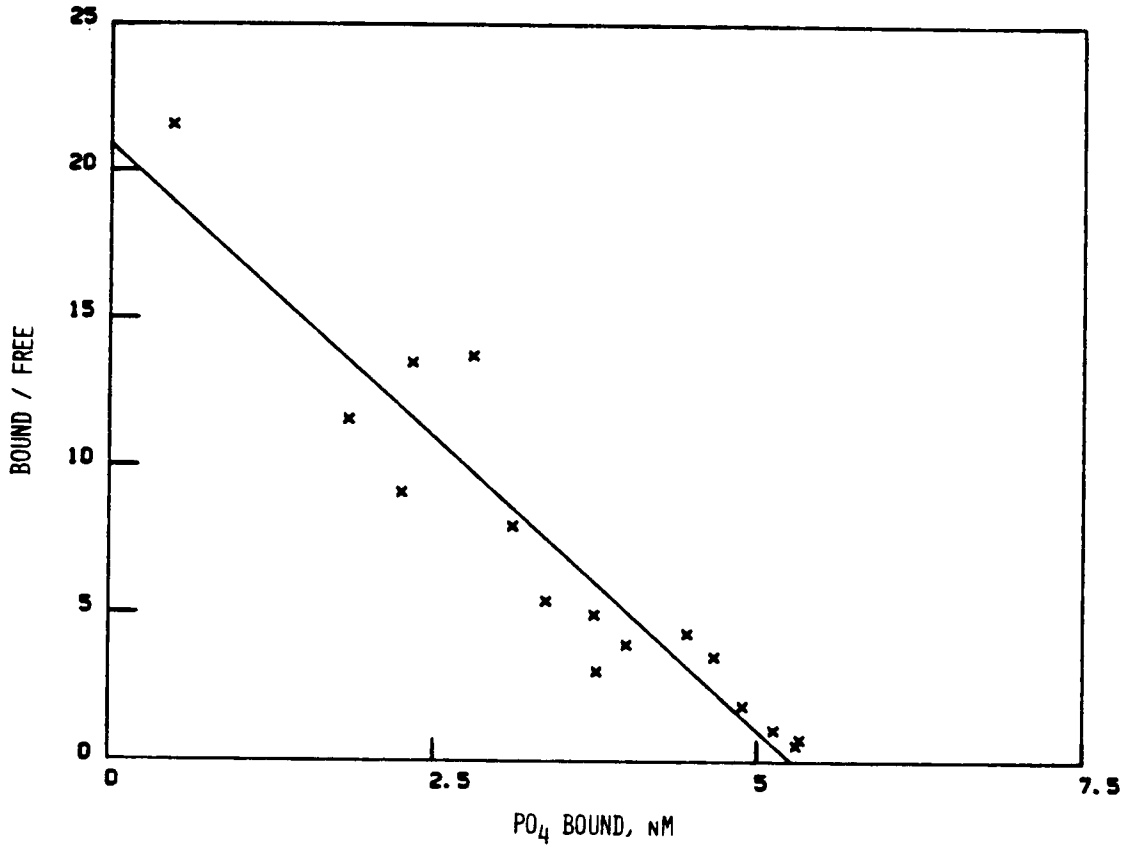

Figure II-4. Scatchard plot of inorganic phosphate binding by synthetic cores.

Quantitation of the binding of beryllium to cores from which the phosphate had been omitted is shown in Figure II-5. As can be seen, the effect of omission of phosphate on metal binding is principally a reduction in the number of binding sites, without significant alteration of the disassociation constant. It is conceivable that the binding we detected was due to trace amounts of phosphate in the glassware used, which was subsequently sequestered by the core sample. In our hands, the phosphate assay used (Ames, 1966), was sensitive enough to detect 0.1 nM of phosphate, or approximately 8-10 phosphate per 1200 iron. At this level, no phosphate could be detected in the preparation.

DISCUSSION

Various mechanisms exist for the detoxification of heavy metal poisoning in living systems. The induction of the protein metallothionein results from the exposure of mammalian systems to lead, cadmium and zinc, with a similar, cysteine-rich protein induced by cadmium in higher plants (Jackson et. al., 1985).

We have demonstrated earlier the binding of various heavy metals by the iron-storage protein, ferritin (Price and Joshi, 1983). This binding appears to have an in vivo significance, as elevation of ferritin levels reduced the toxicity of beryllium dosage (Lindenschmidt et. al., 1986). As such, it seems reasonable to ascribe to ferritin an expanded role in metal toxicity.

The unique situation of fixed habitat makes the defense mechanisms of plants at least as important as those of animals. As a

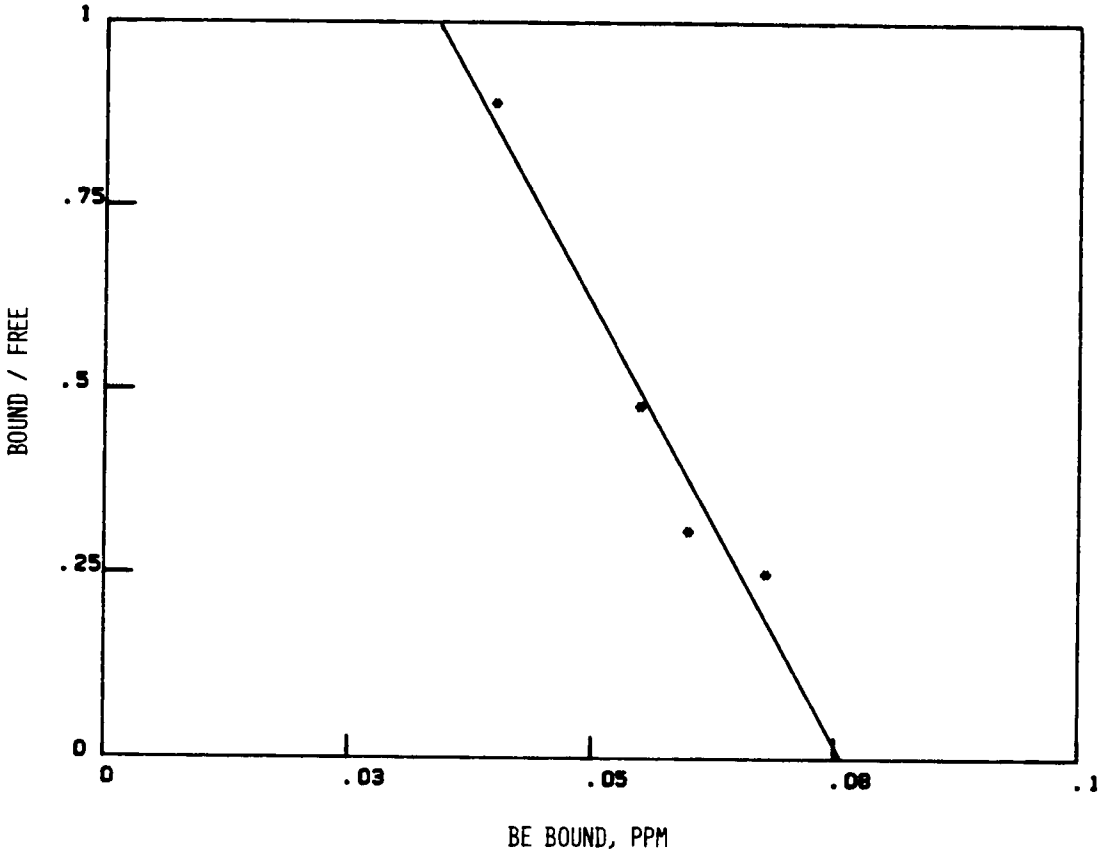

Figure II-5. Scatchard plot of beryllium binding by dephospho-cores.

toxic fluctuation in the environment must be endured, rather than avoided, efficient mechanisms must be available to enable the plant to survive. In the case of acid rain, this has been amply illustrated by the recent work of Tam and Williams (1986), showing the rapid increase in metal release from geologic deposits, particularly aluminum, as a function of lowered pH. This example may have unusual applicability to living systems, as aluminum has been shown to bind specifically to transferrin and as such may be implicated in perturbated iron metabolism (Trapp, 1983). With the increase in available metal toxins, the need for adequate sequestration and detoxification becomes acute. As such, it seems reasonable to postulate an analogous protective system in plants.

The data presented demonstrate that ferritin from plant sources is well-adapted to absorb significant amounts of heavy metals, in a manner analogous to the mammalian form. As the non-ferrous metal-binding capabilities of ferritin seem principally restricted to the holoprotein (at least for high stoichiometries), it is reasonable to predict that the iron core of the protein plays a leading role in non-ferrous metal binding.

The data presented clearly establish the iron core of ferritin as being responsible for high levels of non-ferrous metal binding. Not only do the synthetic cores bind the test metals with affinities nearly identical to these of ferritin, the preference of metals in terms of stoichiometries also follows the order found in the protein. The smaller metals, Al and Be, are bound in much higher numbers than are Cd or Zn. Only the absolute numbers of metals bound are altered,

with the synthetic preparation binding fewer metals in every case examined. As we were constrained to the use of accreted cores in this study, the reduction in numbers of binding sites may be adequately explained by the concommitant reduction of available surface area which results when the cores are precipitated.

Of particular interest is the affinity of the iron polymer for the phosphate anion. Iron-phosphate complexes are found in a variety of environments, including oceans and soils (Berner, 1973; Juo and Fox, 1971). Such interactions may play an important role in nucleotide metabolism, where ATP-Fe(III) complexes have been characterized (Theil, 1985). Several authors have investigated the existence of phosphate in the core of ferritin (Van Kreel et al., 1972; Treffry and Harrison, 1978). Mann et al. (1986) reported that the cores of ferritin isolated from human spleen, limpet and bacteria all bind specific stoichiometries of phosphate, with the bacterial cores appearing unusually rich (Fe/Pi = 1.71), while St. Pierre et al. (1986) demonstrated distinct magnetic ordering parameters for these same sources, attributable to differences in crystallinity. Funk et al. (1985) cites "unambiguous evidence for surface complexes of Fe(III) with PO_4". Sigg and Stumm (1981) investigated the complexation of weak acids with the surface of hydrous goethite (α-FEOOH), and found that the mineral is able to bind various anions, notably sulfate and phosphate. As these cores prepared from Fe citrate hydrolysis carry a substantial cationic charge (Spiro and Saltman, 1967), and in view of the very low solubility of the ferric phosphate specie (Merck Manual), it is perhaps not surprising that the

anion should be so tightly sequestered. The dissassociation constant we obtained, 2.5×10^{-9}M, indicates an extremely tight complex. All the same, this does not demonstrate that phosphate should be a permanent resident in the iron polymer. However, in the present experiment, the phenomenon measured was the binding of the phosphate to the surface of the polymer. There is some evidence that the phosphate group is distributed throughout the volume of the core in vivo, probably associated with stacking faults (Treffry and Harrison, 1978). As such, the binding of the anion by the core would be even more stable. In preparing the phospho-cores for these experiments, the preparation was dialyzed exhaustively to remove unbound phosphate. By such techniques, we were not able to reduce the Pi/Fe ratio to below 0.15.

It seems probable that the binding of phosphate groups on the surface of the cationic core could result in the neutralization of the large surface charge, giving the surface a marked anionic character. This in turn would result in the creation of a large number of binding sites for non-ferrous metal cations, giving the binding phenomenon we have observed. This data is in agreement with that of Imai and coworkers (1980), who found that phosphate adhered to the iron core resulted in increased potassium binding. In support of this model, we observed a marked reduction in metal binding performance of the synthetic core when the preparation was not loaded with phosphate prior to non-ferrous metal loading (Figure II-5). While the K_d for the beryllium binding was unaffected, the number of binding sites

determined was drastically reduced. The extant sites detected may be due to trace amounts of phosphate in the system.

PART II REFERENCES

PART II REFERENCES

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Mansour, A.N., Thompson, C., Theil, E.C., Chasteen, N.D. and Sayers, D.E. (1985) J. Biol. Chem. 260, 7975-7979.

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Tam, S.C. and Williams, R.J.P. (1986) J. Inorg. Biochem. 26, 35-44.

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PART III

ISOLATION OF A CHEMOTACTIC FACTOR FROM SEEDS OF THE SOYABEAN (*GLYCINE MAX*)

INTRODUCTION

Chemotaxis, the movement of an organism in response to a chemical gradient, is a widespread phenomenon throughout the biological world. In mammalian systems, many of the ceilular immune responses originate through a chemotactic mechanism (Myrvik and Weiser, 1984), while reproduction in higher plants is also affected by such processess (Glenk et al., 1971). In the microbial field, chemotaxis mediates such diverse functions as reproduction, nutrition, pathogenesis and ecologic distributions (Stainer et al., 1976).

Perhaps the most intensely studied area of chemotaxis concerns the response of bacteria to a nutritional gradient, that is, a positive response to a sugar or amino acid which may be used as a carbon or nitrogen source. Many motile bacteria display such a response, and by doing so frequently achieve a higher rate of growth than their non-motile counterparts.

The distribution of motile microorganisms in the soil can markedly affect the growth of various plant species. Many plants are actively colonized by certain fungi, which appears to result in increased growth rates for the plant (Rai and Strobel, 1966; Chet and Mitchell, 1976). Others, notably the legumes, have a nutritional advantage over other plants by virtue of the Rhizobium bacteria which nodulate their roots, thus giving the plant access to extra nitrates via atmospheric nitrogen fixation (Stanier et al., 1976). Surprisingly, it has also been found that certain gram-negative bacteria, notably Pseudomonas, are able to actively colonize the root surface and render the plant disease-suppresive (Schroth and Hancock,

1982). As such, these plants (e.g., potato and sugar beet) are able to flourish in soils which would normally be considered unsuitable. This ability of the bacteria to protect the plant against potential pathogens appears to be a function of its iron sequestration capacity. These procaryotes manufacture and secrete compounds, termed siderophores, which have an extremely high affinity for the ferric ion. As the association constant for these compounds and iron are unusually high ($K_a=10^{30}-10^{50}M$) (Neilands, 1982), bacteria which utilize these structures for iron recruitment are believed to outcompete other, potentially detrimental, microbes.

The mechanisms by which these bacteria are able to infect the plant are the subject of numerous studies. It is well known that the area immediately surrounding the root surface (the rhizosphere) contains a high amount of organic material, including exudates from the roots themselves. This concentration of organic material results in an influx of bacteria to the area, particularly gram-negative species. The levels of these microbes may be as high as 20 times that found in non-vegetative areas (for a review, see Stacey and Brill, 1982). The idea that the excess organic debris in the area can serve as a chemoattractant, resulting in an influx of these bacteria to the area for nodulation, seems at least possible, although this concept has been discounted (Schmidt, 1979). Specifically, evidence suggests that chemotaxis most probably plays little or no role in the actual host/symbiont nodulation, which most likely is mediated by a lectin/carbohydrate complex (Dazzo and Hubell, 1975; Stacey and Brill, 1982). However, the means of initial recruitment of the bacteria to

the rhizosphere is still unknown. Recent reports indicate that there is a positive chemotactic response of Rhizobium to pea root exudates, principally the low molecular weight fraction (Gaworzewska and Carlile, 1982). These investigators also report unpublished data showing that a mutant which exhibits slow migratory ability is also less efficient at nodulation. An additional report (Currier and Strobel, 1977, 1981) indicates that the Rhizobium bacteria which nodulates the birdsfoot trefoil, Lotus corniculatus, is positively attracted to a large glycoprotein (65 kdal.) secreted by the plants' root system. The previous investigations lend credence to the belief that the host plant recruits bacteria to form an active rhizosphere, either for specific host/symbiont relationships (Rhizobia-legume), or non-specific interactions (Pseudomonas and disease suppression).

Our interest in plant chemoattractants originated when we discovered that soaking soyabeans (Glycine max) in water for protein preparations led to the efflux of a great deal of iron from the seeds. As iron is generally a highly conserved metal (necessary for a host of metabolic functions), we endeavored to determine if the liberated iron might constitute a signal for the recruitment of beneficial bacteria to the germinating plant. Accordingly, we analyzed the seed effluent for factors which were able to act as chemoattractants for Rhizobia bacteria.

METHODS AND MATERIALS

All chemicals used were reagent grade quality obtained from Sigma Chemical Co. or Fisher Scientific Co. and used without further

purification. Absorption spectra were measured with a Beckman DU-7 spectrophotometer. Thin-layer chromatography was performed on Whatman silica gel 200 um plates, in butanol/acetic acid/water/pyridine (15/3/12/10).

Soyabeans (Glycine max) were obtained from Mayo Garden Center (Knoxville, Tenn.). The dried seeds were soaked for 8-10 hours in two volumes of double-deionized water. The resulting brown-colored liquid was decanted, filtered through four layers of cheese cloth and centrifuged at 15,000 x g for 20 minutes. The clarified solution was brought to 95% ammonium sulfate saturation (713 g/l) and left at $4^{o}C$ overnight. The suspension was again centrifuged as above, and the pellet redissolved in 0.01 M $NaPO_4$, pH 7.4. This sample (usually about 5-10 mls.) was heated in a boiling water bath for 10 min. Following centrifugation to remove denatured protein, the clear supernatant was mixed with one volume of acetone at $-70^{o}C$, allowed to stand for 10 min., and centrifuged at 20,000 x g for 15 min. The supernatant was decanted and mixed with 8 volumes of acetone as described, the pellet redissolved in 0.05 M NH_4HCO_3, and chromatographed on a BioGel P-4 column (1.5 x 85 cm) in the same buffer. Monitoring of the column was at 280 nm., and the fractions of interest were pooled and lyophilized.

Rhizobium leguminosarum stocks were prepared by growing a succession of three motility plates to isolate a motile strain. 0.3% TYC plates were stabbed in the center, incubated until a well-established growth was evident, and subcultured from the outermost ring onto a new plate. Inoculum from the third plate was placed in 50

mls. of TYC broth, grown for 24 hrs., then aliquoted and frozen at -70°C (0.5 ml. broth culture + 0.5 ml. 50% glycerol).

For chemotaxis assays, a stock solution of bacteria was grown for 24-48 hrs. in minimal media (6.9 g sodium succinate, 1.1 g sodium glutamate, 0.11 g K_2HPO_4, 0.11 g $CaCl_2 \cdot 2\ H_2O$, 0.1 g $MgSO_4 \cdot 7\ H_2O$, 3.3 mg $FeCl_3 \cdot 6\ H_2O$, 0.372 mg Na_2EDTA, 0.75 mg thiamine HCl, 0.75 mg Ca pantothenate, 0.75 mg biotin, distilled water to 1 liter, filter sterilized (0.45 um Nalge filter)). Cultures were grown to an A_{590} O.D. of 0.1-0.2 and used directly for assay. Chemoattractants were dissolved in 0.01 M potassium phosphate buffer, pH = 7.2.

CHEMOTAXIS ASSAY

For an assessment of the chemoattraction to various components, we used a modification of an established chemotaxis assay (Adler, 1966; Moulton and Montie, 1979). The standard assay consists of placing a microcapillary tube (1 ul volume) filled with attractant in a culture of bacteria grown in minimal media, permitting the bacteria to migrate into the capillary against the attractant gradient, and then quantitating the bacteria in the capillary by plating out various dilutions. In this way, high levels of positive chemotaxis may be measured as an increase in colony-forming units (CFU's) per capillary.

Since Rhizobia bacteria are rather slow-growing, this assay for chemotaxis may take up to 3-4 days before growth is sufficient to allow for counting. Furthermore, this technique requires a considerable amount of practice to perfect, is very laborious, and is subject to several external variables, such as dilution errors and

contamination of the seeded plates. Accordingly, we developed a faster method of quantitation. By observing the placement of the filled capillary into the droplet of bacterial suspension with a compound phase-contrast microscope (1000 x), we found that the bacteria could easily be seen aggregating near the entrance to the capillary and migrating to the interior. By measuring the time between insertion of the capillary and entrance of the bacteria into the capillary, using glutamate as a standard attractant, we found that there was an inverse correlation between the time required for the bacterial entrance and concentration of chemoattractant. This is what would be expected if indeed a stronger chemoattractant (or a higher concentration) resulted in the bacteria responding in a shorter time.

We also checked this new method of chemotaxis quantitation against the conventional methodology (Moulton and Montie, 1979) in order to determine if both techniques would yield similar estimates of activity. By generating plate counts of Rhizobium tested against a known attractant (glutamic acid), we found that the new method gave values which were in good agreement with the standard method (data not shown). Accordingly, we adopted this method for chemotaxis analysis for all future Rhizobia work.

The chemotactic response of the bacteria to a particular component was calculated as the difference in time (as measured with a stopwatch) required for the bacteria to enter a control (no attractant) capillary and the time required for entry into a test (attractant) capillary. For example, if random movement resulted in bacterial entrance to a control capillary (buffer only) in 30 seconds,

and a particular attractant resulted in bacterial entrance in only 5 seconds, the difference would be recorded as 25 seconds, indicative of a strong response. Each attractant concentration was tested 3 times and the mean response determined. This activity is termed the relative chemotactic response.

RESULTS

By testing the crude extract of the seed wash, we found that there was a significant amount of chemotaxis activity which occurred with Rhizobia. Our initial idea was that the attractant was most likely an iron chelate released by the seed, and we attempted to purify this component. As we hypothesized that the putative factor may be a small peptide, we attempted to concentrate the factor with NH_4SO_4 precipitation. This step was quite successful. As seen in Table III-1, the chemotactic activity of the ammonium sulfate precipitate is 37.5 times that of the crude wash. Further purification was achieved by heating the concentrate; this step resulted in an increase of activity of approximately 75000-fold that of the crude extract.

Based on these preliminary experiments, we developed the purification scheme outlined in Figure III-1. In addition to the steps described above, we included an acetone precipitation series followed by gel filtration chromatography of the final acetone precipitate. When this preparation was chromatographed, a relatively simple peak pattern was obtained. When analyzed, this elution profile revealed that maximum chemotactic activity did not correspond to

TABLE III-1

PARTIAL PURIFICATION OF THE CHEMOATTRACTANT FROM SOYA BEANS

Step	Volume	A_{260}	A_{260} mls	Total Units Specific Activity: Relative Chemotactic Response*	Total Units Specific Activity: R.C.R. X Volume	Total Units Specific Activity: Un/ml / A260
Wash	188 mls	12.3	2312.2	1	188	0.081
$(NH_4)_2SO_4$	12 mls	33.9	406.8	37.5	450	1.1
Heat	11.5 mls	36.2	416.3	75,000	862,500	2073.3

Note: 200 g of soya beans were soaked for 18 hours in an equal volume of water and the wash was decanted and used for the purification.

*The relative chemotactic response was calculated as the reciprocal of the dilution required to give a response equal to that of the wash.

PREPARATION OF CHEMOTACTIC FACTOR

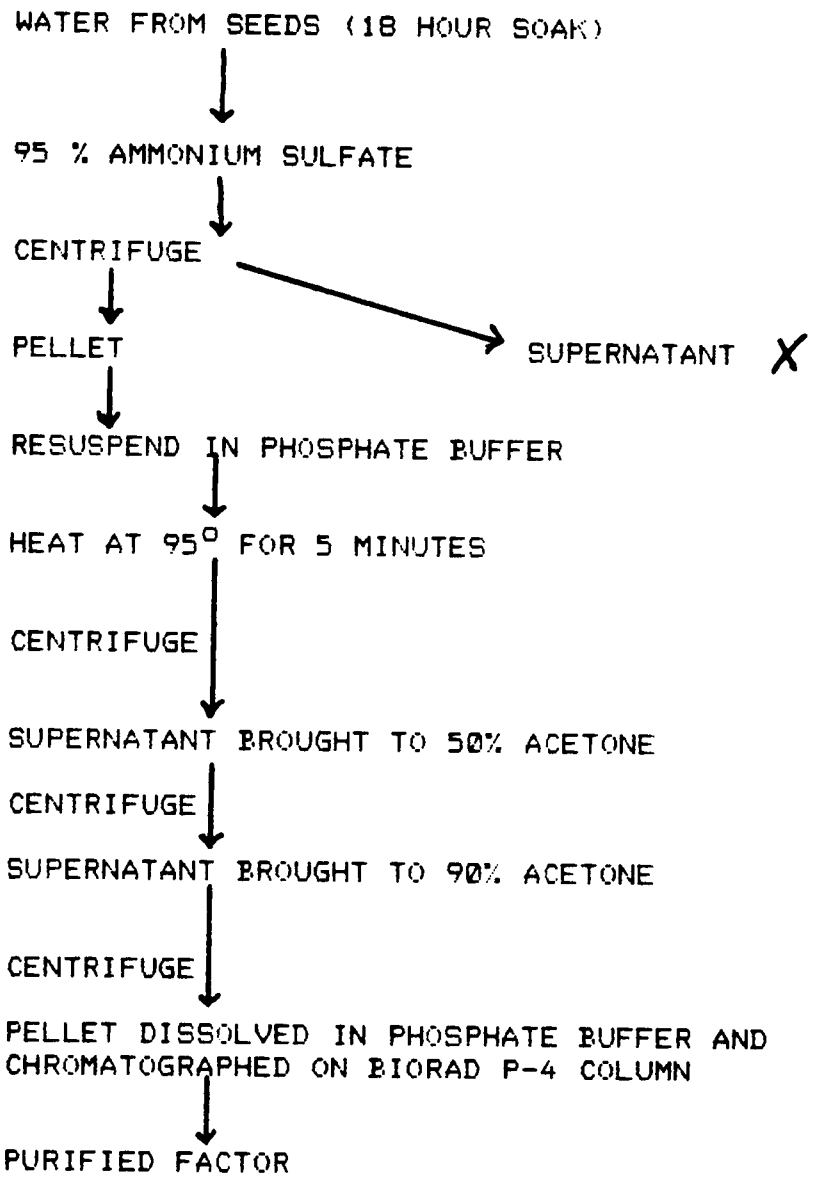

Figure III-1. Purification scheme for soya bean seed wash chemotactic factor.

regions of maximal iron and phosphate content (Figure III-2). This A_{280} peak containing the maximal activity was concentrated by lyophilization and used for further characterization.

An assessment of the purity of this peak was attained by thin layer chromatography. Since a peptide was a likely candidate for the chemotactic factor, we sprayed the plate with ninhydrin; this revealed two components (Figure III-3). The upper spot was determined to be ammonia (as measured by control). The lower spot, while detected as a faint yellow area, did not yield the characteristic Ruhemann's purple, indicative of a free amino group. No phosphate or tryptophan was detected in this region (phosphate was detected at a lower Rf). The spot was recovered from a parallel, unstained plate and tested for chemotactic activity. As seen in Figure III-4, this lower spot exhibited a high level of activity in comparison to the either the pre-chromatographed fraction or the spot containing ammonia. The activity of the lower spot was comparable to that of 0.01 M glutamate.

The ammonium sulfate precipitate was used to calculate a dose-response curve for chemotactic activity (Figure III-5). It was seen that the chemoattractant abililty of the precipitate decreased proportionally with increasing dilution, thus indicating that the response followed a typical concentration-dependent pattern.

Several other assays were performed in an attempt to identify the composition of this purified factor. The UV-vis spectral analysis of the factor is seen in Figure III-6 and is reminiscent of phenylalanine. However, acid hydrolysis and subsequent amino acid analysis revealed no detectable amino acids. Analyses for phosphate,

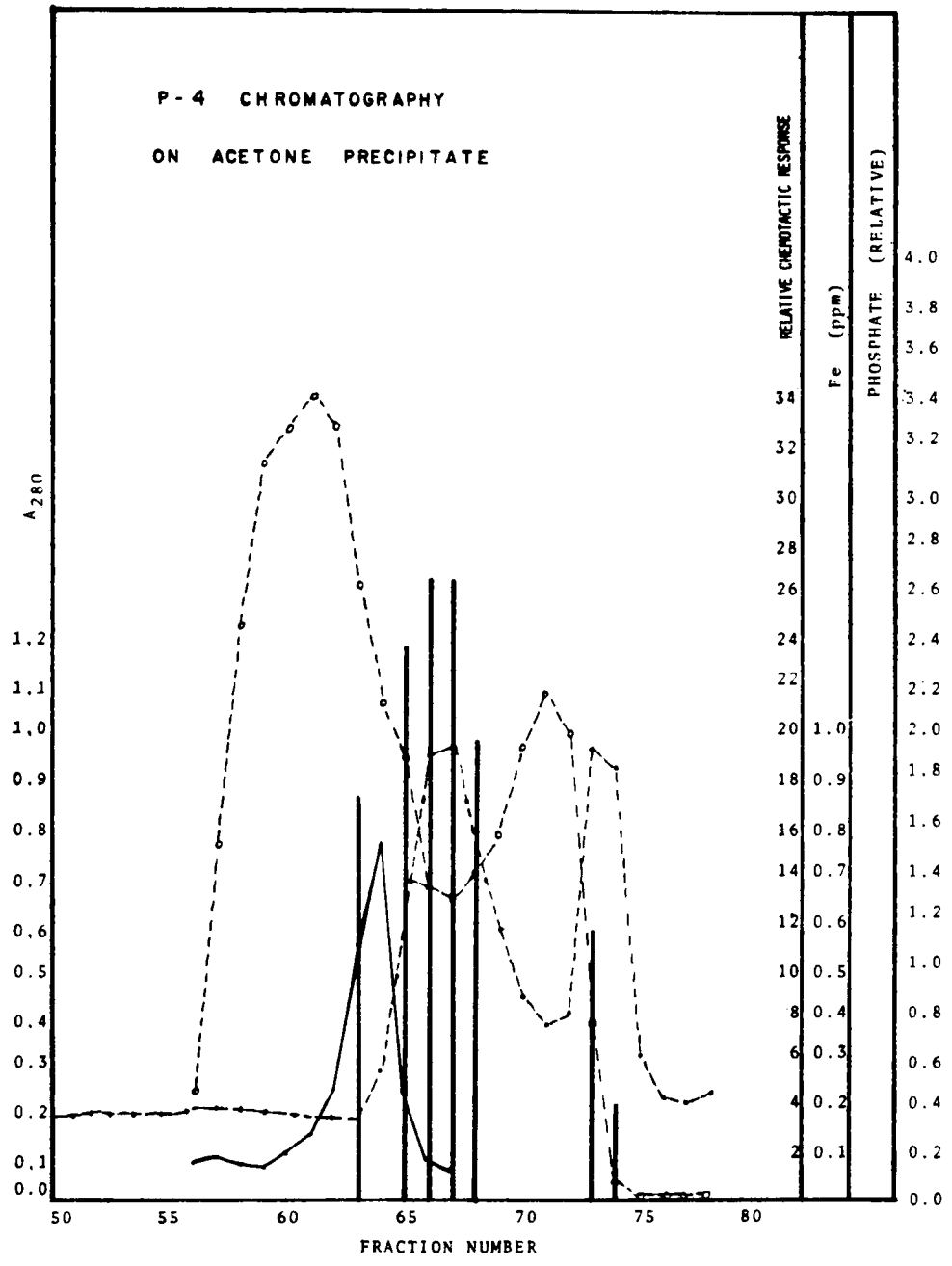

Figure III-2. Chemoattractant elution profile from P-4 column. (o) PO_4; (●) A_{280}; (-) Fe; and (▌) chemotactic activity. 1 ml of the 90% acetone precipitate, redissolved in dilute buffer, was applied to the P-4 column and eluted with 0.05 M NH_4HCO_3. 2 ml fractions were collected and analyzed for phosphate (Ames, 1966), iron (atomic absorption), and chemotactic activity.

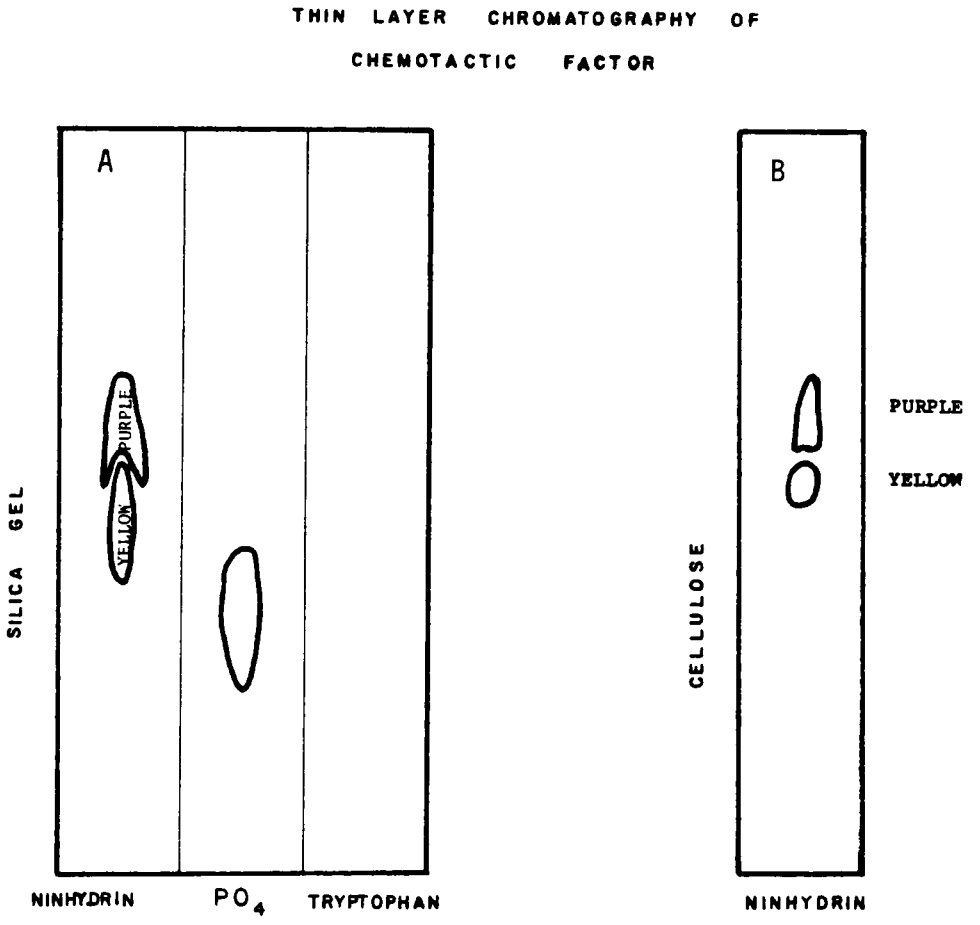

Figure III-3. Thin-layer chromatography of chemotactic factor. (A) on silica gel, and (B) on cellulose, both sprayed with ninhydrin. 1 mg of the sample lyophilyzed from the P-4 column was dissolved in 10 ul of dH_2O and chromatographed on 0.25 mm silica gel H (Supelco) and 0.25 mm microcrystalline cellulose (Kodak) thin layer chromatography plates in n-butanol/acetic acid/water/pyridine (15:3:12:10, by vol.). The plates were dried and sprayed with ninhydrin (0.2% in ethanol/ acetic acid, 20:1, v/v).

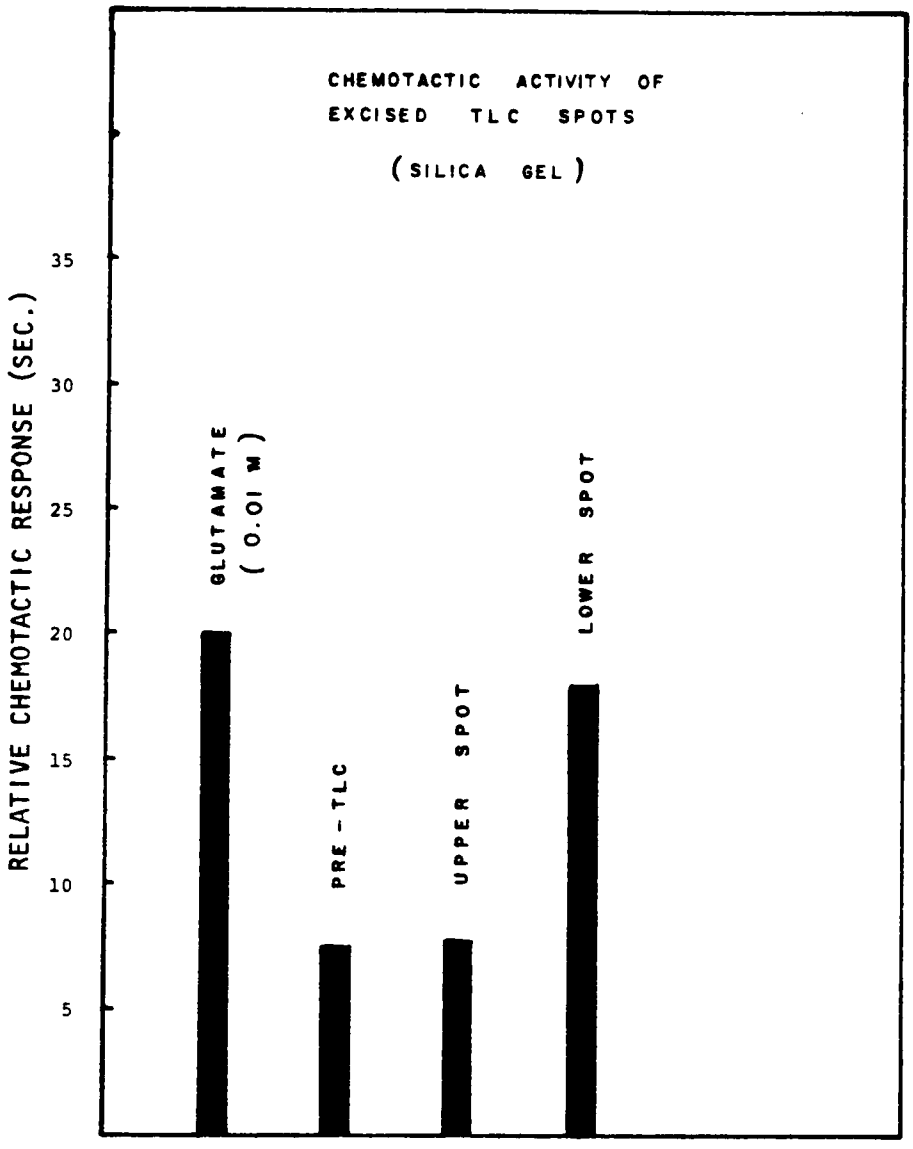

Figure III-4. Relative chemotactic response of excised TLC spots. Glutamate (0.01 M) was used as the standard. A parallel plate was run according to the conditions in Figure III-3, the unsprayed spots were excised from the plate, eluted in dH_2O for 20 min., centrifuged to remove the silica gel and assayed for activity as described.

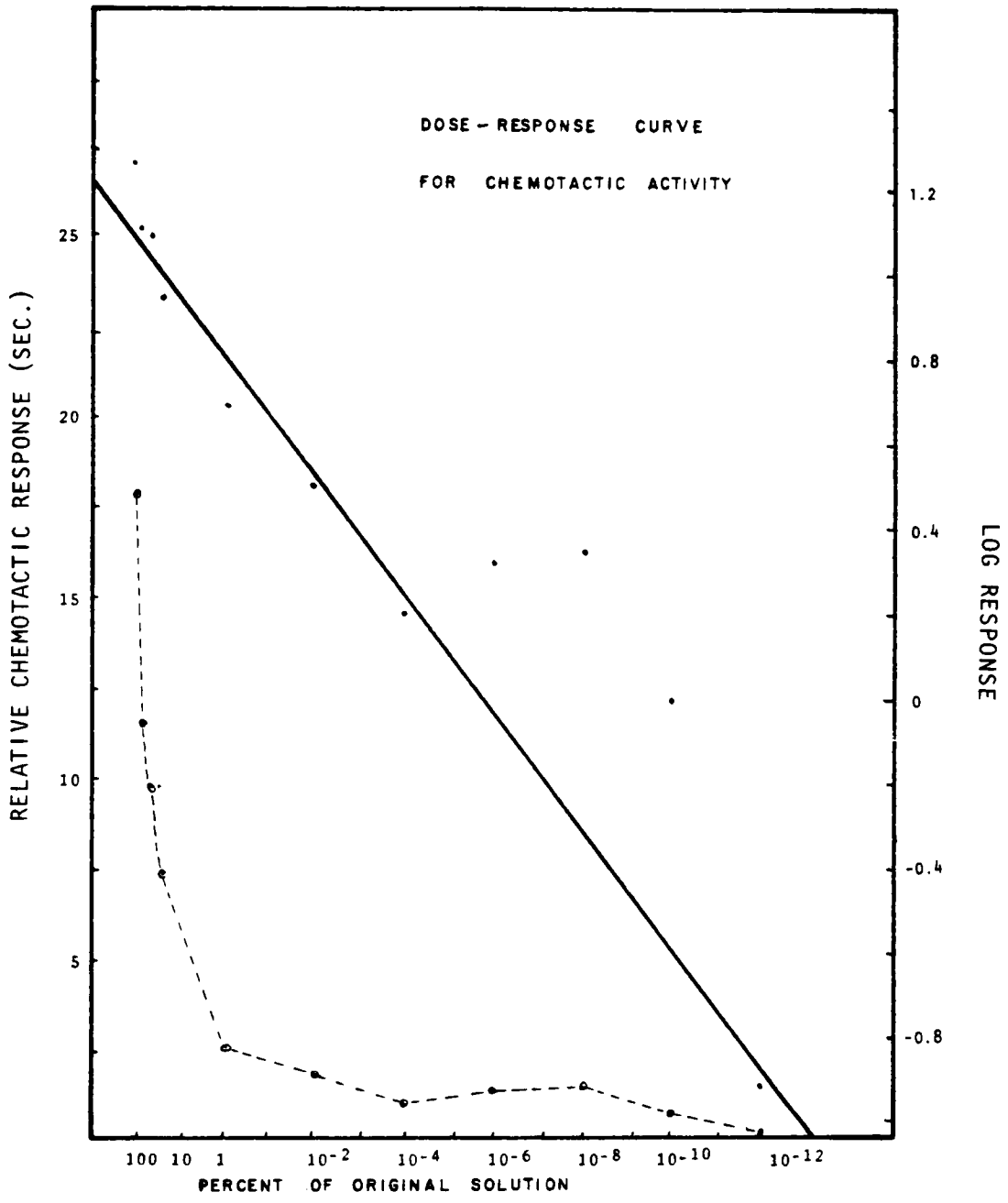

Figure III-5. Dose-response curve for relative chemotactic response. (o) The response to the ammonium sulfate precipitate, and (●) the response plotted in log fashion.

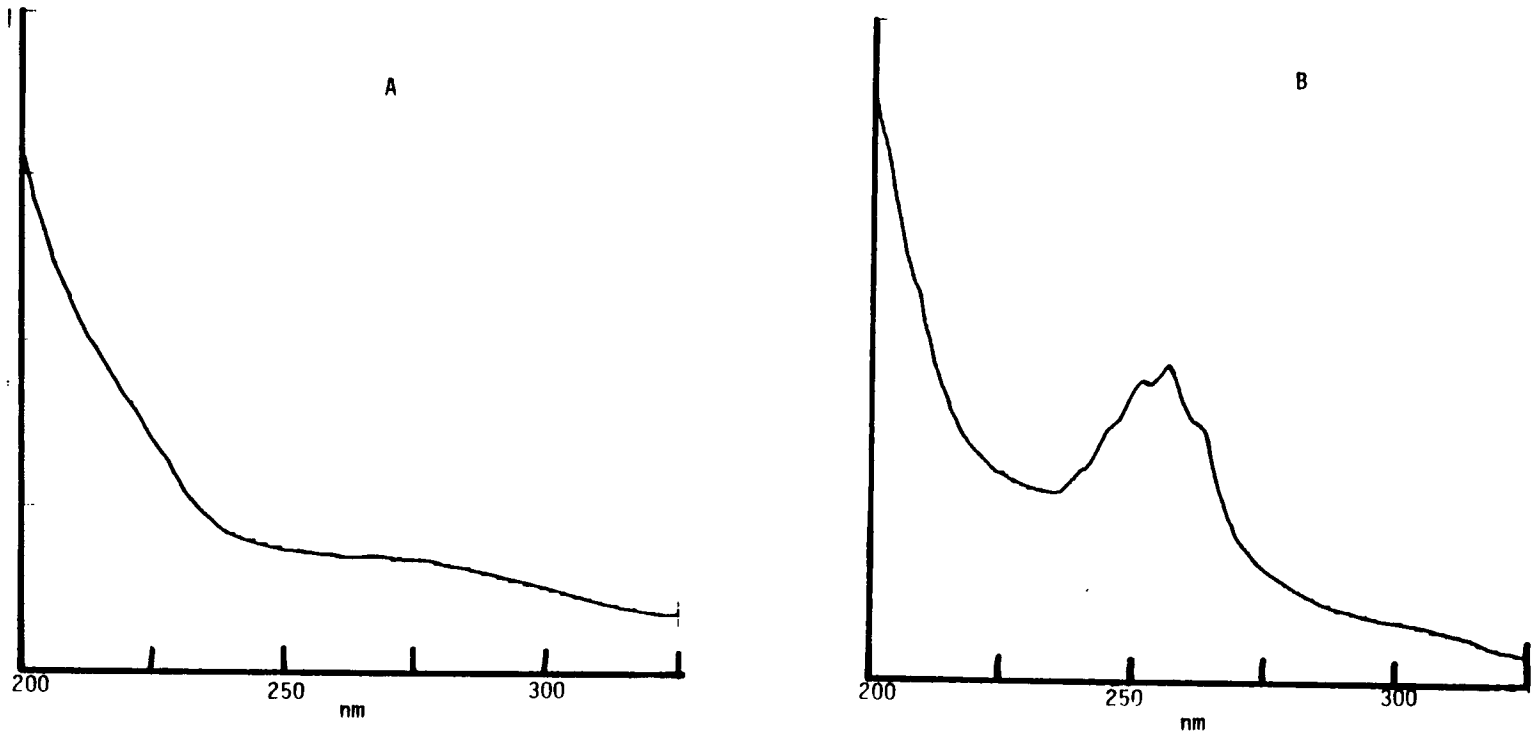

Figure III-6. The UV-vis spectral analysis of soya bean chemotactic factor. (A) before TLC, and (B) the lower spot from the TLC seen in Figure III-3.

deoxyribose, free amines (dansyl chloride and ninhydrin), and iron (atomic absorption) all yielded negative results; tests for hexoses (phenol-sulfuric acid and indole) were weakly positive.

DISCUSSION

The data presented demonstrate that substances released from soyabean seeds during imbibition and germination can function as chemoattractants for Rhizobia bacteria. While we have not conclusively identified the purified factor, evidence indicates a low-molecular weight component with an unusual UV absorption spectra, possibly an amino acid/sugar conjugate. Such a component would be well-suited to function in bacterial recruitment to embryonic legumes for future nodulation.

By achieving a high density of bacteria in the immediate vicinity of the seed, the plant can optimize the rhizosphere for future growth. Whether or not the effluent released from both seeds and maturing roots is specifically designed to attract bacteria, or if th normal leakage of compounds which occurs during imbibition simply happens to be nutritive to the microflora is not known.

When dried seeds are placed in an environment of high water potential, rapid uptake of water into the seed (imbibition) occurs. If the rate of water uptake is too high, severe damage and even loss of seed viability may occur. However, even under mild conditions, many seed embryos release large quantities of electrolytes (eg., K^+, Na^+ and Cl^-) as well as simple sugars, organic acids, ions, amino acids and proteins (Bewely and Black, 1978). The source of this

leakage is principally the outermost layers of the seed embryo.

There is a certain amount of evidence which indicates that the plasma membrane of dried seeds is not present in the well-known bilayer configuration, but due to lack of sufficient water to maintain hydration, is instead configured in a hexagonal-phase arrangment (Bewely and Black, 1985). Since such a configuration would presumably require a lag time in which to reorient to a bilayer structure upon rehydration, several layers of cells would be subject to excessive leakage during the initial stages of imbibition. Thus, material leached from the seeds during the early phase of hydration could arise from at least two areas: material associated loosely with the outer coat or testa of the seed, or intracellular components of the first few cell layers.

The development of the rhizosphere of a plant may begin as early as the point of seed germination. While numerous factors undoubtedly play a role in this process, the predominant factors appear to be nature of the root exudates (Rovira, 1969), various soil properties (Dommergues, 1978), and the specific conditions which affect the ability of a particular microorganism to compete effectively. As the concentration of microbes in this area is unusually high, it follows that ecological interactions, such as competition and predation, will play expanded roles in these cases. Such relationships have been found to affect control of plant pathogens (Baker and Cook, 1974), and curiously, even nodulation failure in legumunous species (Bowen and Rovira, 1976). In terms of microbial selection, several investigators report that the establishment of certain species of the rhizosphere

may be delayed by inhibitory diffusates from the seed coat (Vincent, 1965; Hala and Mathers, 1977). These studies serve to outline the importance of plant exudates in influencing the microbial population near the root environment. The component which we have isolated may constitute an additional method for recruitment of beneficial bacteria to the plants' rhizosphere.

PART III REFERENCES

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CONCLUSIONS

CONCLUSIONS

This dissertation has dealt with the characteristics of the iron-storage protein, ferritin, as isolated from a plant source, the soya bean. As an extension of our earlier work regarding the binding of non-ferrous metals, we elected to investigate the plant protein in an effort to determine if similar mechanisms occur in a plant system. Previous results indicated that ferritin was able to absorb large amounts of the toxic metal, beryllium, and therefore able to at least partially protect the animal from the poisonous effects. In a plant, it is reasonable to assume similar mechanisms should exist, as a plant is even less able to regulate its environment than an animal, and therefore requires efficient defense systems to enable it to survive. For these reasons, the metabolism of phytoferritin is of prime importance.

Our results here indicate that ferritin from soya beans is in many respects similar to its mammalian counterpart. The protein is able to store large amounts of iron, can release the iron to the environment upon reduction to the ferrous state, can absorb additional iron added exogenously, and even shares a high degree of sequence homology with the animal protein, as determined via thin-layer peptide mapping. However, the exterior of the plant protein apparently differs considerably from that of the animal, as immunological analysis revealed no shared determinants between the two intact proteins.

Phytoferritin is composed of subunits of approximately 28 kdal., generating a parent molecule of 670 kdal. However, we found that

during germination of the seeds, the ferritin is apparently processed to a smaller size, containing 22 kdal. subunits. Peptide mapping indicates that this smaller subunit has extensive homology with the parent protein, and as such is most likely derived from it, rather than from a separate gene. As ferritin is normally quite resistant to proteolysis, this was an unexpected finding. Surprisingly, we were able to accurately reproduce this cleavage pattern in vitro by using the protease subtilisin. The cleavage pattern was quite specific, generating the 22 kdal. form from the larger, with no further degradation in evidence. Thus, it appears that the smaller form of the protein, present only after germination of the seed, may represent a plant form of the mammalian hemosiderin, the degradation product of animal ferritin. This hypothesis is supported by the electron microscopy of the fresh and germinated seeds, where no crystalline arrays of ferritin can be detected in new seeds, but are in abundance in the germinated seeds. Furthermore, sucrose density centrifugation of the metal-free proteins indicates that the 22 kdal. form actually has a size inconsistent with an assemblage of 24 subunits, but rather is considerably smaller and exhibits a large range of sedimentation values, suggesting that the protein is probably in the process of losing subunits and as such is less stable. Evidence of this can also be seen in native gradient electrophoresis, where the 22 kdal. protein does not migrate as a discrete band (the 28 kdal. protein generates a single, well-defined band), but produces a large, smeared area behind the 28 kdal. form, suggesting the protein is considerably less stable and in the process of denaturation. All of these results are

consistent with the idea that the 22 kdal. protein is a stage of ferritin turnover in the plant, most likely similar to the mammalian hemosiderin.

We also analyzed the ability of the protein to sequester additional, non-ferrous metals, and as such to function in metal detoxification. Our results are entirely consistent with the previous data obtained for the mammalian protein, horse spleen ferritin. The soya ferritin is able to bind large numbers of beryllium and aluminum, as well as some quantities of cadmium and zinc. Our interest in aluminum as a toxicant comes from data implicating this metal in various diseases, notably Alzheimers' dementia and dialysis dementia. Additionally, aluminum is responsible for several metabolic disorders in plants. In support of the possible role of ferritin as a metal detoxificant, we found a significant amount (approx. 25-30 g. atoms) of the metal already bound to the plant protein after isolation. This would substantiate the protective role the protein may play.

We also attempted to determine the precise mechanism of non-ferrous metal binding to the plant protein. Since the holoprotein is able to bind much higher numbers of these metals than the apoprotein, we focused on the iron core as a likely site for the binding. By manufacturing synthetic iron polymers without any protein present, we developed a model to ascertain the role of the proteins' iron center in this binding. Our results indicate that these artificial iron cores are indeed able to bind the non-ferrous metals in a fashion entirely analogous to the holoferritin protein. In particular, the

binding affinities of synthetic cores for these metals were very similar to those obtained for the plant protein.

The iron core of ferritin is known to contain an appreciable amount of inorganic phosphate. This is perhaps not surprising, as iron-phosphate interactions are widespread in nature. Indeed, the ability of phosphate to catalyze the oxidation of the ferrous ion to ferric is well known. Unpublished results from this lab indicate that other phospho compounds, including phospholipids, are excellent catalysts for this reaction. In order to determine the role of the phosphate ion in metal binding, we prepared the synthetic cores both with and without phosphate, and analyzed the metal binding to these preparations. The results indicate that the majority of this metal binding is in fact due to the presence of phosphate, as the binding was dramatically reduced when phosphate was absent.

An additional area of interest which concerned us was the high amount of iron found to be released from the seeds during germination. Since iron is frequently a limiting nutrient, we hypothesized that the metal may be serving as a signal for the recruitment of beneficial bacteria to the site of seed germination, possibly for future nodulation (Rhizobium, eg.). By analyzing the seed wash for chemotactic activity, we were able to detect and partially purify a compound which generated a strong chemotactic response in Rhizobium bacteria. This compound, while devoid of iron, appears to be some sort of amino acid-sugar conjugate, and as such may be admirably suited for bacterial recruitment to the growing rhizosphere of the

root. This may also have significance for generating a disease-suppressive environment in which the plant may become established.

VITA

Steven R. Sczekan was born in Denver, Colorado on July 30, 1956. He graduated from Walla Walla College in College Place, Washington in 1978 with a B.S. in Biology. After working two years with Cutter Laboratories, Inc. in Chattanooga, Steve entered the graduate program in Biochemistry at The University of Tennessee, Knoxville. He completed work for the Doctor of Philosophy degree in this field in March, 1987 and then began work with Dr. E. C. Theil at North Carolina State as a post-doctoral associate.