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Purification and Characterization of Phytoferritin

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Abstract: Ferritins from germinated pumpkin seeds were isolated by ammonium sulfate precipitation (0.55 saturation), ion-exchange chromatography on DEAE-cellulose, and gel filtration chromatographies on Sephacryl S-300 and Sephadex G-100. Pumpkin ferritin contains less iron than soybean ferritin. Pumpkin ferritin cross-reacted with anti-soybean ferritin antiserum made in rabbit, and showed two distinct antibody reactive bands, both of equal intensity. The pumpkin ferritins corresponding to the two bands were separable by centrifugation in a sucrose gradient (20~50%). The molecular weights of the native pumpkin ferritins based on the estimation of sucrose gradient centrifugation, gel filtration on Sephacryl S-300 and non-denaturing polyacrylamide gel electrophoresis appeared to be: 530~580 KD (the large molecular weight pumpkin ferritin) and 330~360 KD (the small molecular weight pumpkin ferritin). The large molecular weight pumpkin ferritin contains less iron. Both pumpkin ferritins cross-reacted with anti-soybean ferritin antibody with a spur formation suggesting partial antigenic recognition.

Key words: antigenic recognition ferritin, germination, pumpkin.

Ferritin is a ubiquitous, constitutive protein whose synthesis is further enhanced by iron (Shull *et al.*, 1982; Fleming and Joshi, 1987; Joshi, 1990). It has 24 subunits which form a protein shell. The shell sequesters large amounts of iron as Fe(III) hydroxyphosphate (Aisen and Listowsky, 1980). Ferritin also binds large amounts of nonferrous metal ions such as Al(III), Zn(II), Be(II), Cu(II) (Price and Joshi, 1983; Flemig and Joshi, 1987). From this, an expanded role for ferritin in general metal toxicity was suggested (for discussion see Fleming and Joshi, 1987).

Since plants are unable to avoid toxic situations and also must instead endure them, some efficient defense mechanisms are crucial for their survival. Compared to dry nongerminating seeds, the metabolism in germinating seeds is very high, as is the mobility of metal ions. Therefore, a need for a metal detoxicant is more acute in growing tissue.

Soybeans and pumpkin seeds are the two richest sources of iron in plants (Adams, 1975). Ferritin from soybean has a M_r of 600,000 and contains 24 subunits. Fingerprint maps of the tryptic digest of soybean ferritin and horse spleen ferritin show substantial homology. Yet, the antibody against one does not cross-react with the other (Sczekan and Joshi, 1987).

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Early attempts to isolate ferritin from pumpkin seeds failed for unclear reasons (Sczekan and Joshi, 1987). We here report that pumpkin seeds contain at least two forms of ferritin separable by gel filtration and sucrose density gradient. Both forms appear only in the germinated seeds.

Materials and Methods

Materials

Pumpkin seeds (*Cucurbita Pepo*) were obtained from a market place. Phenylmethylsulfonyl fluoride (PMSF) and iron atomic absorption standard solution, acrylamid were obtained from Sigma (St. Louis, USA). Bisacrylamid, streptomycin sulfate and ammonium persulfate were obtained from Bio-Rad (Hercules, USA).

Preparation of ferritin

Ferritin was isolated from pumpkin seeds by using a modification of the procedures described for soybean seeds (Sczekan and Joshi, 1987). All steps were done at 4℃ unless otherwise stated. Four kg of seeds were soaked in flat-bottom trays with double-deionized water at room temperature. Soaking was carried out for 120 h to get germinated seed with 2 cm root size. Homogenization was performed in Waring blender set on high for 45 sec (twice) in 2 volumes of double-deionized water made to 0.1 mg/ml in PMSF. The slurry

was filtered through 4 layers of cheesecloth and centrifuged at 5,500×*g* for 20 min. Streptomycin sulfate was added to the supernatant to a final concentration of 0.3% (w/v) saturation and stirred for 2 h. The sample was centrifuged for 40 min at 12,500×*g*. The centrifuged pellet containing DNA was removed. The streptomycin sulfate supernatant was brought to 55% saturation with solid ammonium sulfate and was stirred for 1 h. The sample was centrifuged for 45 min at 12,500 ×*g*. The pellet was resuspended in 500 ml of 10 mM NaH_2PO_4-KOH, pH 7.4 buffer made to 0.1 mg/ml in PMSF and then dialyzed overnight at 4℃ against 5 liters of the 10 mM NaH_2PO_4-KOH, pH 7.4 buffer with 4 changes. The dialyzed sample was centrifuged for 1 h at 22,200×*g* and the supernatant was applied to a 5×40 cm column of DEAE-cellulose equilibrated in 10 mM NaH_2PO_4-KOH, pH 7.4 buffer, the column was washed with 1000 ml of equilibration buffer. The column was eluted with a linear NaCl gradient (0~0.5 M) in equilibration buffer. The eluent was monitored for protein and color by measuring absorbance at 280 nm and 405 nm, respectively. The major protein and color peak containing iron eluting in the region of 0.2 M NaCl was pooled and concentrated by adding solid ammonium sulfate to 55% saturation. The concentrated sample was applied to a 2.5×85 cm column of Sephacryl S-300 equilibrated in 10 mM NaH_2PO_4-KOH, pH 7.4 buffer. The column was eluted with this buffer and fractions of 4 ml were collected. The major iron peak was pooled and concentrated by ultrafiltration in an Amicon stirred cell with a PM-10 membrane. The concentrated protein was applied to a 2.5×90 cm column of Sephadex G-100 equilibrated in 10 mM NaH_2PO_4-KOH buffer (pH 7.4). The major iron fractions were pooled, concentrated by ultrafiltration and stored at 4℃ in 0.01% NaN_3.

Immunodiffusion test

Immunodiffusion precipitation was done according to Crowle (1973). 1% (w/v) agarose in PBS was used to prepare plate (5×7.5 cm, 1 mm thick). Samples were applied to 10 ml wells cut in a plate and allowed to diffuse for 3~5 days in a cold room. Polyclonal soybean-ferritin antisera raised in New Zealand White rabbits by subcutaneous injection (a gift of Dr. J. G. Joshi, University of Tennessee, Knoxville, USA) was used for cross-reactivity against pumpkin ferritin preparation. The immunoprecipitation proteins were stained with Coomassie Blue R-250.

Sucrose gradient centrifugation

The partially purified pumpkin ferritin up to Sephadex G-100 chromatography showed two distinct antibody reactive bands with almost the same intensity. In order to separate these two forms of pumpkin ferritin, we employed sucrose gradient centrifugation. The sample (2 mg protein) was loaded onto 30 ml of 20%, 30%, 50% step sucrose gradients. The gradients were spun in a SW27 Beckman rotor at 20,000 rpm for 41 h and fractionated with a peristaltic pump from the top. Fractions of 0.6 ml were collected, and ferritin was monitored by absorbance measurement at 280 nm and by atomic absorption spectroscopy. The standard used to calculate the sedimentation coefficient was apohorse spleen ferritin (16S).

Molecular weight estimation

Native molecular weight was estimated by Sephacryl S-300 gel filtration and gradient gel electrophoresis (3~15%) according to Manwell (1977) and sucrose gradient centrifugation according to Martin and Ames (1961).

Results and Discussion

Soybean and pumpkin seeds are the two iron rich sources in plants (Adams, 1975). Therefore, these two sources are likely to contain ferritin, a protein known to store, transport and detoxify iron. Earlier, Sczekan and Joshi (1987) reported a germination dependent proteolytic shortening of the subunits of soybean ferritin but failed to isolate phytoferritin from pumpkin seeds. Pumpkin ferritin was initially purified by ammonium sulfate precipitation (0.55 saturation), ion-exchange chromatography on DEAE-cellulose, and gel permeation chromatography on Sephacryl S-300. The sample was then chromatographed on Sephadex G-100 as described in the Materials and Methods. Gel filtration chromatography on Sephadex G-100 resulted in two major peaks with iron and color (Fig. 1). The small molecular weight peak corresponding to fraction numbers 148~162 was considered as cytochrome-C because: (a) cytochrome-C from horse heart was eluted from the same column with similar elution profile as fraction number of 155 for maximal absorbance at 280 nm; (b) there was no cross-reaction between the fraction and anti-soybean ferritin antibody; (c) the pattern of absorption spectrum of the fraction was very similar with that of cytochrome-C (data not shown). Therefore, the large molecular weight peak with fraction numbers ranging from 38 to 56 was considered as pumpkin ferritin because both the pumpkin ferritin and the soybean ferritin reacted well with antiserum against soybean ferritin, but neither horse spleen ferritin nor concanavalin A gave any detectable reaction (Fig. 2). Pumpkin ferritin contains less iron than soybean ferritin (Table 1;

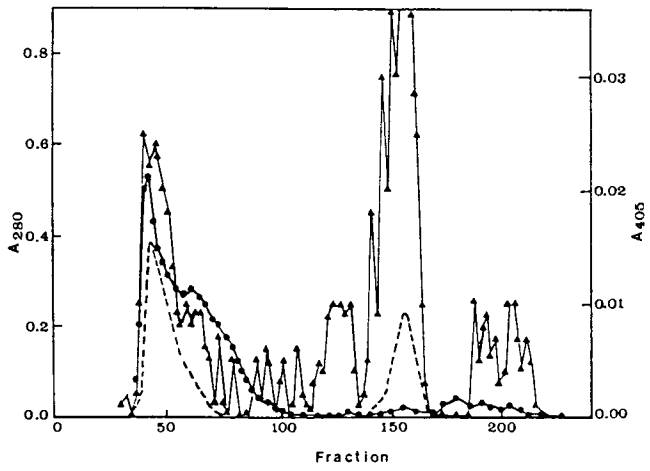

Fig. 1. Gel filtration chromatography on Sephadex G-100. The pooled factions from the Sephacryl S-300 column were concentrated by ultrafiltration in an Amicon stirred cell with a PM-10 membrane and applied to a column of Sephadex G-100 (2.5× 90 cm). Fractions of 2.0 ml were collected at a flow rate of 0.5 ml/min. The eluent was monitored for protein and color by measuring absorbance at 280 nm (closed circles) and 405 nm (closed triangles), respectively. The dashed line shows the monitored iron by atomic absorption spectroscopy with Fe hollow cathode lamp (wavelength, 248.3 nm).

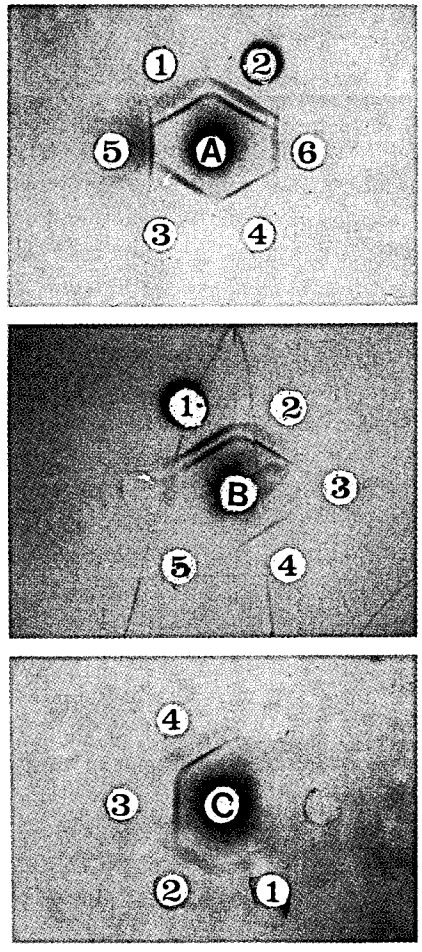

Fig. 2. Immunoprecipitation of pumpkin ferritin and soybean ferritin with anti-soybean ferritin antiserum. (A): rabbit anti-soybean antiserum, 4 μg; (1) and (2): pumpkin ferritin (after Sephadex G-100) (15 μg each); (3) and (4): pumpkin seed ferritin (18S fraction of sucrose gradient) (11 μg, 5.5 μg, respectively); (5) and (6): soybean ferritin, 8 μg each. (B): rabbit anti-soybean antiserum, 4 μg; (1) and (2): pumpkin ferritin (after Sephadex G-100) (15 μg each); (3): horse spleen ferritin, 5 μg; (4): pumpkin seed ferritin (18S fraction of sucrose gradient), 5 μg; (5): concanavalin A, 5 μg. (C): rabbit anti-soybean antiserum, 4 μg; (1) and (2): pumpkin ferritin (13S fraction of sucrose gradient), 11 μg each; (3): pumpkin ferritin (18S fraction of sucrose grsdient), 11.0 μg; (4): pumpkin ferritin (after Sephadex G-100), 15 μg.

Scezkan and Joshi, 1987). Thus, as isolated, the concentration of iron in horse spleen>soybean ferritin>pumpkin ferritin (Scezkan and Joshi, 1987). The pumpkin ferritin cross-reacted with anti-soybean ferritin antiserum made in rabbit, and showed two distinct antibody reactive bands, both of equal intensity (Fig. 2A, wells 1 and 2). The pumpin ferritins corresponding to the two bands were separable by centrifugation in a sucrose gradient (20~50%) (Fig. 3), and the separation was further identified by immunoprecipitation with anti-soybean ferritin antiserum (Fig. 2C, wells 2 and 3). Therefore, they have different molecular weights and it is possible that they have some different antigenic determinants. The molecular weights of the native pumpkin ferritins based on the estimation of sucrose gradient centrifugation (Fig. 3), gel filtration (Fig. 4) and non-denaturing polyacrylamide gel electrophoresis (data not shown) appeared to be: 530~580 kDa (the large molecular weight pumpkin ferritin) and 330~360 kDa (the small molecular weight pumpkin ferritin). The large molecular weight pumpkin ferritin contains less iron than the small molecular weight pumpkin ferritin does (Fig. 3 and Table 1). Both the pumpkin ferritins cross-reacted with anti-soybean ferritin antibody with a spur formation suggesting partial antigenic recognition (Fig. 2A).

A major question arises from the present study: is the germination step needed to induce the synthesis of phytoferritin? This question is interesting because seed germination is the early stage of the new plant growing. Plants are unable to avoid toxic situations and instead they are required to adapt to the changes in their environment. Therefore, some efficient mechanisms by which plants protect themselves from environmental attacks are crucial for their survival. The active seed accepts nutrients from soil and also accepts heavy metals. Thus, the seed is equipped with the ability to defend itself during its new life. Therefore, it has the machinary to synthesize proteins for defense purpose. Ferritin is known to sequester and thus detoxify iron. Ferritin also participates in nonferrous metal detoxification (Joshi and Clauberg, 1988). Therefore, it is possible that pumpkin seeds when exposed to osmotic

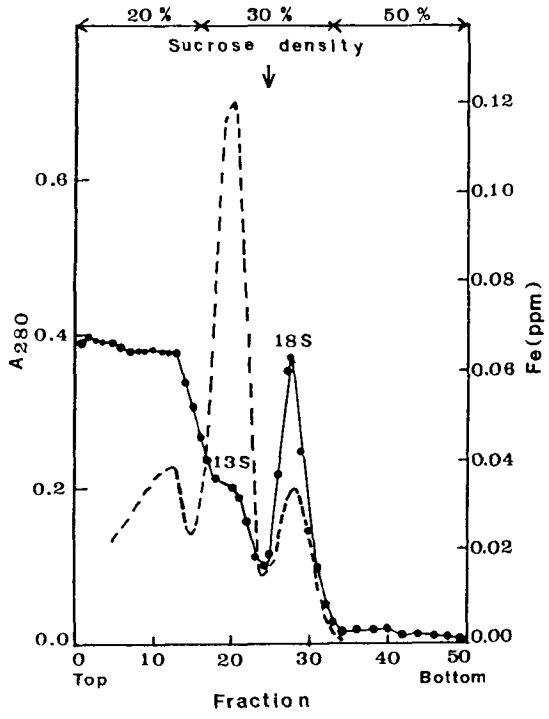

Fig. 3. Sucrose density gradient centrifugation. The pooled fraction from Sephadex G-100 were concentrated by ultrafiltration using a PM-10 membrane. The concentrated sample (~2 mg protein) was loaded into 30 ml of 20%, 30%, 50% step sucrose gradients. The gradients were spun and fractionated as described in the Materials and Methods. Fractions of 0.6 ml were collected and were examined for protein by measuring absorbance at 280 nm (closed circles) and for iron by atomic absorption spectroscopy as described in the legend in Fig. 1 (dashed line). The arrow indicates the position of standard apoferritin from horse spleen (16S).

Table 1. Comparison of iron concentration in pumpkin ferritin and soybean ferritin[a]

	g Fe/ μg protein[b]	mol Fe/ g protein	mol Fe/ mol protein[c]
small MW PFR	41.6×10^{-10}	0.745×10^{-4}	27
large MW PFR	2.0×10^{-10}	0.0358×10^{-4}	2
soybean FR	628.0×10^{-10}	11.245×10^{-4}	675

[a]Soybean ferritin was isolated from 72 h germinated soybean seeds by using the procedures used for pumkin ferritin isolation described in Materials and Methods.
[b]Iron was quantitated by atomic absorption spectrophotometer and iron atomic absorption standard solution (Sigma at St. Louis, USA). The concentration of protein was determined according to the method of Bradford (1976) by using the Bio-Rad reagent and gamma globulin as a standard protein.
[c]The ratio has been calculated using 360,000 for small MW PFR, 575,000 for large MW PFR, and 600,000 for soybean ferritin as molecular weight, respectively. PFR, pumpkin ferritin; FR, ferritin; MW, molecular weight.

shock or attack by heavy metal from soil actively synthesize ferritins or assemble preferritins (unassembled)

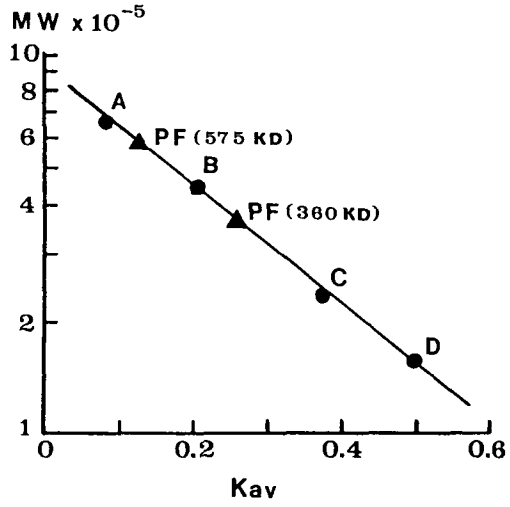

Fig. 4. Molecular weight determination of pumpkin ferritins by Sephacryl S-300 gel filtration. Purified pumpkin ferritins were applied to Sephacryl S-300 column (2.5×85 cm) which had been precalibrated with marker proteins. The marker proteins were as follows. A: thyroglobulin (669,000), B: horse spleen ferritin (445,000), C: catalase (232,000), D: aldolase (158,000). MW, molecular weight; PF, pumpkin ferritin. $K_{av}=(V_e-V_o)/(V_t-V_o)$.

for the defense purpose. In this regard it is of interest to note that phytoferritins from other sources such as soybean seeds (Sczekan and Joshi, 1987; Laulhere *et al.*, 1988), pea and maize seeds (Laulhere *et al.*, 1988) were isolated from swollen seeds for 48~72 h. However, a very low level of ferritin was obtained in the supernatant of a $MgCl_2$ precipitation from soybean seeds soaked for only 18 h (Sczekan and Joshi, 1987). In the present study we were able to obtain ferritin from 120 h soaked pumpkin seeds with approximately 2 cm root size. On the other hand, 72 h soaking was enough to get germinated soybean seeds with that size of roots. Therefore, the apparent discrepancies in soaking time between plant species seems due to the different characteristics of species.

When seeds are soaked in water for an appropriate time, seed embryos are formed and then germination starts. Sczekan and Joshi (1987) observed that there is a large efflux of iron from soybean seed into the surrounding water during the first 3~6 h imbibition. However, most of this iron-rich water is reabsorbed by the 72 h of soaking. Therefore, it is possible that an active mobilization and redistribution of iron occurs during the formation and germination of seed embryos. Thus, the soaking step to get further developed seeds such as germinated seeds seems like to be necessary to induce the synthesis of phytoferritins.

Of particular interest here is the observation that pumpkin ferritin from germinating seeds showed two distinct antibody reactive bands, both of equal intensity.

In addition, the pumpkin ferritins corresponding to the two bands were separable by sucrose gradient. The molecular weights of native pumpkin ferritins based on gel filtration (Fig. 4) have been estimated to be 575 kilodaltons and 360 kilodaltons, respectively. The molecular mass of ferritins from other plant sources were 540,000 for pea (Laulhere *et. al.*, 1988), 572,000 for soybean (Sczekan and Joshi, 1987), and 570,000 for soybean nodule (Ko *et al.*, 1987). In addition, Sczekan and Joshi (1987) have shown that germinated soybean seed has the light soybean ferritin which has a molecular mass ranging from 240 to 460 kilodaltons. It is possible that isoforms of phytoferritins could exist in relation to the stage of plant growth and development. Isoforms of ferritins have been detected in plants (Van Der Mark and Van Den Briel, 1985; Laulhere *et al.*, 1988) and in animal tissues (Theil, 1983). In animal systems, there are at least two different types of subunits (H and L subunits) encoded by different genes (Arioso *et al.*, 1978). Presently, little is known regarding the different genes for phytoferritin synthesis (Wicks and Entsh, 1993; Fobis-Loisy *et al.*, 1995). It is also possible that ferritin subunit(s) encoded by one gene assemble into two different types of ferritin due to post-translational modification or a specific proteolytic cleavage of the subunits. Sczekan and Joshi (1987) have shown that the 22 kilodalton subunit found mainly in the light soybean ferritin is easily generated from the 28 kilodalton form by treatment with subtilisin. Future studies with this model system may provide insight into the mechanism of regulation of ferritin during plant growth and development.

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