Iron Content and Ferritin in Leaves of Iron Treated Xanthium pensylvanicum Plants

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Abstract. Iron administration to iron-starved cocklebur (Xanthium pensylvanicum) plants causes an increase in the iron content of ferritin fractions extracted from mature leaves. Xanthium plants grown under long days (vegetative stage) have more iron and ferritin than similarly iron-treated plants induced to flower under short day regimes. This first demonstration of ferritin in cocklebur (Compositae) leaves suggests that a substantial portion of iron that enters the iron-starved plant appears as this protein-iron macromolecule.

Plant ferritin (phytoferritin) is a protein-iron complex closely resembling ferritin from other sources; it is located within the plastids and chloroplasts in regions not occupied by the thylakoid system. Most of the phytoferritin studies and observations in higher plants (3, 12, 15, 19) have been electron microscopical. As yet the biosynthesis and biochemistry of the plant ferritin molecule have not been investigated. Likewise there have been no reports on the iron core of phytoferritin. A recent review by Price (14) does not give any information on ferritin.

Recovery of iron-chlorotic plants by application of iron has been known for over a century (6) but as yet the role of iron or its function in deficient plants remains uncertain and vague (14). Seckbach (16) has shown that iron supply to iron-chlorotic bean plants results in the deposition of ferritin in the plastids and chloroplasts of the mature green leaves. The uptake of iron compounds is associated with ferritin formation also in mammalian tissues (4, 8, 13).

This paper reports experiments dealing with ferric iron application and the examination of the ferritin-iron fractions extracted from a Compositae (Xanthium pensylvanicum) plant grown under different photoperiods. The object of this study was to add more information concerning plant ferritin formation under specially stimulated conditions.

Materials and Methods

Plant Material. Burrs of cocklebur (Xanthium pensylvanicum Wallr.) were soaked in water over-night and planted into flats containing sand-vermiculite mixture and irrigated with Hoagland No. 1 nutrient solution (9). Seedlings remained at 23° (day)/19° (night) under 16 hr photoperiod for about 2 weeks. When they had produced 2 to 4 foliage leaves all plants were transferred into liquid culture (Hoagland solution containing 5 or 10 μg iron/ml, supplied as Sequestrene NaFe, 13% Fe-monosodium iron3+ EDTA) for 1 week. The plants were divided into groups of 7. Each plant was placed into a 1 liter plastic container lined with a disposable plastic bag which contained fresh nutrient solution supplemented with increasing levels of iron (5, 10, 15 and 20 μg/ml) to be kept as controls. A set of additional plants was placed into an equivalent solution to which iron was not added. Fresh solutions were supplied to all plants every few days during the growth period. About 12 days after the iron-starved plants showed chlorotic symptoms; they were pale green or yellowish. The plants from the iron deficient set were provided with one of the complete nutrient solutions containing 5, 10, 15 or 20 μg iron/ml and placed together with the rest of the plants (control) in growth chambers. Within 3 days following this iron addition the chlorotic plants regreened (they are designated as iron treated plants because their iron content is higher than the control). Small and undamaged leaves (about 20 mm in length) or larger blades (about 70 mm midrib) were harvested from all the plants.

Plant Ferritin Purification. The extraction and isolation of Xanthium ferritin followed basically a method used for legume ferritin (10,16), and is outlined in Fig. 1. The brei obtained after grinding fresh leaves in distilled water and filtered through 3 layers of cheesecloth was kept frozen. Filtrate was thawed and centrifuged, the supernatant (SN1) was made to 70% saturated with solid ammonium sulfate and the precipitated fraction (PPT1) was collected by centrifugation. Gray pellets were redissolved in 0.1 M tris buffer, pH 7.6, and dialyzed

1 This work was supported by a United States Public Health Service Grant (GM 06965).
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Ground leaves were filtered and kept frozen overnight or longer
Centrifugation at 10,000 rpm, 20 min

SN
70% saturated with (NH₄)₂SO₄
Centrifugation at 10,000 rpm, 20 min

PPT₁

SN₂
Pellets were resuspended in Tris buffer, pH 7.6, and dialyzed at 4°C overnight then incubated with 10
μg/ml RNase at room temperature

PPT₂
Ultracentrifugation at 55,000 rpm, 2 hr

SN₃
Sedimented ferritin in PPT₃

Fig. 1. Flow diagram for the isolation of phytoferritin particles from legumes and cocklebur leaves.

against the same buffer overnight in the cold room.
Bovine pancreatic RNase was added and the brownish solution was dialyzed against tris buffer at room temperature for a few hr. Ferritin pellet (PPT₃) was obtained after ultracentrifugation and was dissolved in water or in buffer. The purity of ferritin fraction was determined by absorption spectra in the ultraviolet region (Fig. 2) and by the appearance of the PPT₃ solution in the electron microscope.

Iron Determination. The estimation of iron in ferritin extracts followed the Fischer-Price method (5) using tripyridyl-triazine as a chromogen. One ml of leaf solution was digested with an equal volume of 2 N HCl and 20% (w/v) trichloroacetic acid (1:1) in a conical graduated centrifuge tube. One ml of supernatant obtained by 20 min centrifugation (clinical centrifuge) was mixed with 0.4 ml of buffered chromogen, and the absorbance was read at 593 μm. For the standard calibration curve increasing concentrations of ferrous ammonium sulfate solutions were mixed with buffer chromogen and treated as the samples. The data are presented in absolute terms as μg Fe per gram fresh wt of leaf.

Results

Absorption Spectra of Ferritins. The nature of ferritin absorption from the PPT₃ fractions was compared to horse ferritin for purification establishment. Both ferritins have similar absorption spectra (Fig. 2) in the visible and ultraviolet wavelengths. The optical density increases slowly from ca. 600 μm with a shoulder in the curve around 275 μm. Similar observations from tuna fish and horse ferritin were reported elsewhere (11). The main absorption value in these wavelengths is due to the ferritin-iron and not because of the protein (4).

Recovery of Ferritin From Long Day Iron Treated Plants. The iron content of solutions obtained from ammonium sulfate RNase fraction (PPT₃-RNase) and from the pellets obtained by ultracentrifugation (PPT₄) are shown in Fig. 3 and 4 respectively. These fractions were extracted from leaves harvested from plants grown under long day regimes in liquid cultures which contained 0 to 20 μg Fe per ml nutrient solution. In general the more iron supplied in the media, the higher the leaf iron content (Fig. 3). The iron level of the iron-treated leaves is higher than the control among all fractions (Fig. 3 and 4). The amount of iron in the treated plants (Fig. 3) is about 12 to 15-fold higher at lower Fe levels (5 and 10 μg) in nutrient solution, than in the control plants, and only 9-fold higher at 20 μg Fe/ml level.

Fig. 4 represents the iron content of the ferritin fraction. Iron level within the nutrient solution and the ferritin iron detected from leaf extracts are less correlated than the previous fractions (Fig. 3). Iron treated plants show about 6-fold higher iron (ferritin) than the corresponding controls.

Photoperiod and Iron Treatments. After establishing that light-grown and iron-treated plants have a much higher content of iron (and ferritin) than similar plants which were transferred to the dark (unpublished data) the question was asked whether the photoperiods (short or long day conditions)
might have affected this accumulation. Xanthium being a short-day plant will flower when the dark period exceeds 8 hr with accompanying light periods of shorter than 15 hr (7). Table I gives the actual amount of iron from Xanthium plants grown under 16-hr (long days) and 10-hr (short days) photoperiods from 3 fractions. Both the total and the relative ratios (over the 5 μg/ml control) of iron content determined from ferritin fractions (PPT₃-RNase, PPT₄) were twice as concentrated in the plants grown under long days (16 hr photoperiods). The supernatant from the final ultracentrifugation (SN₃) showed about 3-fold more soluble iron in the long day group.

In order to determine additional clues for the high ferritin formation under long day condition another photoperiodic experiment was designed. These plants received similar total light energy but the illumination distribution varied. Iron chlorotic Xanthium plants were transferred to either short days of 10 hr photoperiod (10H) or given long days through 8 hr continuous illumination and 2 hr in the middle of the 14-hr dark period (10I). Light intensity was about 1300 ft-c at the plant level illuminated from fluorescent tubes supplemented by incandescent bulbs. Four days later 20 μg Fe/ml was supplied to the chlorotic plants and the leaves were harvested and lyophilized and ferritin was extracted from them. The iron content within the ferritin fractions (PPT₃) from these plants showed 2-fold more for the long day (10I) grown leaves. It seems that the Xanthium in the vegetative stage takes up iron to a higher level (and deposits more ferritin) than the plants exposed to floral induction under short days (10H).

**Discussion**

Iron application to iron-deficient Xanthium pensylvanicum plants results in the accumulation of iron in the leaves and the deposition of ferritin. Such iron uptake was reported to follow ferritin deposition in plants (16) and in mammalian tissues (4, 8, 13). The iron (ferritin) level is much higher in leaves from plants which received iron following an iron starvation period (iron treatment) than from control plants grown on constant amounts of ferric iron (Fig. 3 and 4). This finding agrees with reports by Brown et al. (2) and Tiffin and Brown (20) that chlorotic plants have the capacity to reduce and absorb ferric iron many times heavier and more rapidly than green plants and to accumulate iron in leaves. From the results of the present study it is confirmed that such iron accumulation is accompanied by a substantial increase in the presence of ferritin.

Since the iron treated leaves show quite a similar concentration of iron (ferritin) within the PPT₃ fraction (Fig. 4), it is not excluded that the formerly starved plants received saturating amounts of ferric iron or that the chloroplasts had recovered from their deficient stages by the time of harvest. In addition, one can realize that the treated leaves contain more iron in partially purified extracts (Fig. 3) than in the purified ferritin fraction (Fig. 4). This means that a substantial fraction of iron absorbed by the chlorotic plants might appear in a different form (e.g., as soluble iron shown in SN₃ fraction of table I).

As reviewed elsewhere, the main bulk of plant iron is located in chloroplast fraction (14); the plastid is found to be the exclusive organelle which accumulates ferritin (10, 15, 16, 19). In addition, thin sections of these iron-treated Xanthium leaves have heavy clusters of ferritin within their plastids (unpublished data).

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**Table I. Iron Levels in Leaf Extracts of Iron-Treated Xanthium Plants Subjected to Long Day and to Short Day Photoperiods**

<table>
<thead>
<tr>
<th>Fraction¹</th>
<th>Long day</th>
<th>Short day</th>
<th>Long day</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPT₃-RNase</td>
<td>μg Fe per g fresh wt</td>
<td>Ratio</td>
<td></td>
</tr>
<tr>
<td>16 hr</td>
<td>10 hr</td>
<td>Short day</td>
<td></td>
</tr>
<tr>
<td>10.80</td>
<td>(19)²</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>4.56</td>
<td>(8)</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>SN₃</td>
<td>3.56</td>
<td>1.20</td>
<td>3.0</td>
</tr>
<tr>
<td>(11)</td>
<td>(3.5)</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>PPT₄</td>
<td>2.55</td>
<td>1.39</td>
<td>1.8</td>
</tr>
<tr>
<td>(8)</td>
<td>(4.5)</td>
<td>1.8</td>
<td></td>
</tr>
</tbody>
</table>

¹ See plant ferritin purification under Materials and Methods.
² Iron content was normalized (from 20 μg/ml) to the concentration of controls (5 μg/ml) on a fresh wt basis.
Iron and Ferritin in Xanthium Leaves

**Xanthium pensylvanicum** being a short day plant, can be induced to initiate floral primordia after exposure to one long dark period (7). Biochemical changes of flowering induction are associated with iron demand (18). In this study Xanthium plants were starved for iron under non-flower-inductive conditions (long days) and were transferred to different photoperiods (short/long days) for an additional 4 days and were then resupplied with 20 μg/ml iron for 3 more days before leaves were harvested. The total iron-ferritin content in the vegetative stage (long day conditions) is double that of the flower-induced (short day) plants. Thus the non-flower induced plant seems to deposit more iron (ferritin) than the short day plant under these experimental conditions regardless of the distribution of total illumination period and intensities.

Although the fine structure of chloroplasts from green and iron-deficient Xanthium leaves was studied (1), this is the first report on ferritin found in the Compositae. Ferritin seems to be important in the iron chemistry of plants and might regulate reactions which involve iron. There is a negative correlation between the degree of chloroplast development and the presence of ferritin within this organelle. The greater the build-up of the photosynthetic apparatus the less ferritin occurs in the cell. Previous works pointed out that the primary function of ferritin is iron storage for the supplying photosynthetic machinery (3,10,12) such as ferredoxin, heme enzymes and the process for chlorophyll synthesis.

From the present study it seems that plant ferritin is an iron storehouse for the maturing chloroplasts whose development was arrested through the chlorotic period. This iron-protein complex protects the cell by providing a "protective protein" shell around the iron against excess doses of iron absorbed by the chlorotic plants.

**Acknowledgment**

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**Literature Cited**