Content and Stability of Ferrous Iron in Soybean Hulls

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ABSTRACT

Soybean hulls are a rich source of highly bioavailable dietary iron. The chemical properties of iron in soybean hulls were examined to identify characteristics that would explain the availability of iron from this source. Ferric and ferrous ions were extracted from the hull with 2*N* HCl and quantitated by ion chromatography. It was found that most of the iron was in the ferrous state; the proportion varied with soybean variety and crop year. The Fe(II) was stable to chemical oxidants, yet readily solubilized by acidic conditions comparable to those found in the human gastrointestinal tract. These studies suggest that the high bioavailability of soybean hull iron is the result of the presence of stable Fe(II) in this tissue.

Key words: Iron bioavailability, Ion binding

The poor bioavailability of iron from plant-derived food sources is generally attributed to the presence of iron-binding moieties such as phytic acid (Cheryan 1980, Morris and Ellis 1982, Gordon and Chao 1984, Zemel 1984) and dietary fiber (Reilly 1979, Fernandez and Phillips 1982, Reinhold et al 1981), as well as to the overall insolubility of ferric hydroxides (Forth and Rummel 1973, Lee and Clydesdale 1979). An apparent exception is provided by the iron present in soybean seed coats (hulls). The bioavailability of iron from soybean hulls is equivalent to that of ferrous sulfate (Weaver et al 1984, Johnson et al 1985, Lykken et al 1987). The reason for the high availability of iron from this source is unknown.

Soybean hull may be viewed as desiccated plant cell wall tissue that is devoid of phytic acid (Dintzis et al 1985). It has a high dietary fiber content, yet is more digestible than other fiber sources such as corn and wheat brans (Mitaru et al 1984, Dintzis et al 1985). The polysaccharide composition of soybean hulls has been extensively characterized (Aspinall and White 1964; Aspinall et al 1966, 1967), and the hull's mineral-binding attributes have been examined (Thompson and Weber 1979, Laszlo 1987). Iron in this tissue accounts for about one-third of the iron in the whole bean, whereas the hull represents only about 8% of the bean mass (Levine et al 1982). Soybean hulls may be incorporated into human foodstuffs without adversely affecting quality (Johnson et al 1985) and are presently utilized in animal feeds and some commercial products for human consumption (Scott and Aldrich 1983).

The present study examines the oxidation state, chemical stability, and acid solubility of soybean hull iron in order to identify those characteristics that lead to good bioavailability of iron from this source.

MATERIALS AND METHODS

Sample Preparation

Soybeans were stored at ambient room temperature and humidity. Unless otherwise noted, experiments were performed on variety Century soybeans from 1983 and 1984 crop years. Beans were cracked with mortar and pestle, then separated manually into hull and cotyledon fractions. The hulls were stored under vacuum without further processing. The cotyledon fraction was ground to a coarse powder, then extracted with acetone. The acetone-extracted powder was air-dried and stored under vacuum.

Iron Extraction

Typically, 60–80-mg hull or 200-mg cotyledon samples were extracted with degassed 2*N* HCl (4 ml) containing 1 m*M* Na_2SO_3. The atmosphere above the extraction vials was flushed with N_2 immediately after adding acid. The vials were sealed, then stirred for 2 hr at room temperature. After extraction, the aqueous phase was separated from insoluble sample residue by centrifugation of a 0.8-ml aliquot through a Centrifree micropartition unit (Amicon Corp.). Wet-ashing of hulls was performed as described by Garcia et al (1972).

Chromatographic Conditions

Ferric and ferrous iron in the 2*N* HCl extracts were quantitated by ion chromatography. Chromatography was performed on a Dionex 2010 advanced chromatography module equipped with HPIC-CG2 guard and HPIC-CS5 separating columns. Detection was achieved by post-column derivatization with 4-(2-pyridylazo) resorcinol and monitoring at 520 nm with a Hitachi 100-40 spectrophotometer equipped with an 80 μl flow cell (1-cm path length). Data collection and quantitation were performed with a Spectra-Physics 4270 reporting integrator. Columns were purged with 100 m*M* Na_2SO_3, prepared fresh daily, flowing at 1 ml/min for 2 hr. The column eluent was 100 m*M* Li-acetate, pH 4.8, containing 6.0 m*M* 2,6-pyridinedicarboxylic acid (Aldrich). Eluent was degassed before use. The eluent flow rate was 1.0 ml/min. Sample was introduced into the 50-μl loading loop with a Hamilton gas-tight syringe. Calibration standards were prepared by dilution of a 1,000 ppm ferric nitrate stock (Fisher Scientific) into 0.2*N* HCl containing 40 μM potassium dichromate or 1.0 m*M* ascorbic acid, for ferric or ferrous standards, respectively.

Statistical Evaluation

Reported iron values represent the mean of analyses performed on duplicate samples.

RESULTS

The protocols devised for this work had to meet several criteria. The procedures had to provide quantitative extraction of iron without altering the iron oxidation state and allow quantitation of the extracted ions. The total iron extracted from the soybean hulls by 2*N* HCl was equivalent to that obtained by wet-ashing samples. Analysis of hull samples (variety Century, 1983) by 2*N* HCl extraction and by wet-ashing gave 360 ± 30 and 370 ± 20 μg/g dry wt of iron, respectively. The 2*N* HCl also stabilized the extracted iron against oxidation by molecular oxygen. A solution of ferrous sulfate was completely stable in the extraction medium for at least 8 hr, which is consistent with the observation of Dintzis and Watson (1984). Furthermore, 2*N* HCl did not interfere with the analysis of Fe(III) and Fe(II) species by ion chromatography. Fe(III) and Fe(II) eluted at 4.7 and 9.5 min, respectively, under the conditions stated in Materials and Methods, and were separated from other transition metals (Zn and Cu) present in the tissue in significant quantities. Identities of the Fe(III) and Fe(II) peaks in the chromatograms of the 2*N* HCl extracts were confirmed by coelution of authentic ferric and ferrous nitrate. Further confirmation of the identity of the two ions in the extracts was

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produced by oxidation of the sample Fe(II) by dichromate, producing a single Fe(III) peak, and ascorbate reduction to produce a single Fe(II) species. Replicate analyses of a single sample extract indicated a 2.0% coefficient of variation in quantitation of Fe(III) and Fe(II). However, analysis of multiple 60–80 mg samples indicated that the soybean hull heterogeneity at this sampling size increased the coefficient of variation to approximately 10% in the reported values. Thus, 2*N* HCl extraction, followed by fractionation and detection by ion chromatography, provided a simple procedure by which to measure the Fe(III) and Fe(II) contents of soybean hulls.

To quantitatively extract Fe(III) and Fe(II) from soybean hulls, 2*N* HCl was necessary, as well as sufficient. Table I lists the extent of Fe(III) and Fe(II) solubilization at various HCl concentrations. At HCl concentrations less than 2*N*, extraction was incomplete. Furthermore, there was a substantial difference between the extent of Fe(III) and Fe(II) extraction. Because neither iron species forms insoluble hydroxides at the pH values studied, the lower degree of Fe(III) extractability under acidic conditions indicates that ferric ions are more tightly bound to the hull than ferrous ions.

Various chemical treatments were applied to the soybean hulls to test in situ oxidation state stability of Fe(III) and Fe(II) (Table II). Hulls were treated in the presence or absence of ascorbate or dichromate. Treated hulls were recovered and their retained-iron content was analyzed. Ascorbate treatment combined with 0.005*N* HCl diminished the retained Fe(III) and Fe(II) by about two thirds and one third, respectively, compared to hulls treated with only 0.005*N* HCl. This result suggests that the lost Fe(III) fraction was reduced to Fe(II) and solubilized, because ascorbate did not increase the amount of Fe(II) in the hull. That one third of the iron remained in the Fe(III) state implies that Fe(III) is bound to at least two different kinds of sites, one in which Fe(III) is ascorbate-reducible and one in which it is not reducible.

Treatment of soybean hulls with dichromate, a strong oxidant of soluble Fe(II), diminished the retained Fe(II) content only slightly (Table II). This indicated that the Fe(II) fraction was bound in the hulls as a complex that is highly stable to chemical oxidation.

TABLE I
Effect of HCl Concentration on Fe(III) and Fe(II) Extraction[a]

HCl Concentration (*N*)	Percentage of Total Fe Species Extracted[b]		
	Fe(III)	Fe(II)	Total Fe
0.005	18	19	19
0.05	25	67	51
0.2	29	96	70
2	100	100	100

[a] Century (harvested in 1984) soybean hulls. Composition given in Table III.
[b] Percentage of Fe(III) or Fe(II) solubilized is calculated based on total of each species in untreated hulls. The 2*N* HCl percentages are 100 by definition, based on the inferred extractive capabilities of 2*N* HCl, but are included for comparison.

TABLE II
Influence of Chemical Treatments on the Oxidation State of Iron in Soybean Hulls[a]

Sample Treatment[b]	Retained Iron (μg/g dry wt)		
	Fe(III)	Fe(II)	Total Fe
0.005*N* HCl	150	140	290
0.005*N* HCl + ascorbate	50	100	150
0.005*N* HCl + dichromate	150	120	270

[a] Variety Century (harvested in 1984).
[b] All samples were extracted with 0.005*N* HCl containing the indicated additions. Ascorbate and dichromate concentrations were 1 m*M* and 40 μM, respectively. Samples were treated for 2 hr, then collected on a fritted-glass filter and washed successively with water and acetone. The recovered hulls were dried under vacuum. Retained iron was measured by 2*N* HCl extraction as described in Materials and Methods. The iron content of the untreated hulls is presented in Table III.

The stability of the Fe(II) fraction was further tested by subjecting it to a moist heat treatment. Hulls were held in a water-vapor-saturated atmosphere at 50°C for up to a week. Under these conditions the moisture content of the hulls was 24%. The Fe(II) complement of the hulls did not change over this time, supporting the hypothesis that the endogenous Fe(II) fraction is stable in the presence of chemical oxidants, which in this case was molecular oxygen.

The Fe(III) and Fe(II) contents in hulls of a large number of soybean varieties from different harvest years were inspected (Table III). Two notable observations resulted from this survey. First, in hulls of soybeans up to a decade old, a large portion of the iron was in the ferrous oxidation state. Thus, the endogenous Fe(II) must be stable in the presence of molecular oxygen essentially indefinitely and probably inert to microbial and fungal activity as well. Second, whereas the average distribution between Fe(III) and Fe(II) oxidation states for all of the varieties surveyed was approximately 50:50, the iron in three varieties, Sooty, Peking, and Hardee, was nearly all Fe(II). The Sooty and Peking varieties were distinct in that the hulls were darkly colored. The Hardee beans were yellow, as were the rest of the varieties. Otherwise, there was no discernable trend in Fe(III) and Fe(II) ratios across several years within a single variety, or as a function of time (harvest year) across all of the varieties. This is reasonable because there was no control of the growing conditions for these samples.

Finally, the oxidation state of the iron in the cotyledon fractions was inspected. The Fe(III) to Fe(II) ratio in the cotyledons was found to be similar to that of the hulls, albeit at much lower concentrations. The Fe(III) and Fe(II) concentrations were 12 and 16 μg/g, respectively, in the Century (1983) cotyledon fraction. Thus, ferrous iron is not exclusively present in the soybean hull. The chemical stability and acid extractibility of iron in the cotyledon was not investigated.

DISCUSSION

A large fraction of iron present in soybean hull is in the ferrous oxidation state. The ferrous iron is insensitive to oxidation by molecular oxygen or by other chemical oxidants, yet it is readily extracted under acidic conditions comparable to those found in the human gastrointestinal tract. Although extractability of iron does not necessarily ensure its bioavailability, correlation of the two phenomena suggests that presence of easily solubilized ferrous iron in soybean hulls may account for the high iron availability from this source. The observed varietal differences in Fe(II) content

TABLE III
Fe(III) and Fe(II) Composition of Soybean Hulls

Variety	Harvest Year	Iron Content (μg/g dry wt)			% of Total Iron	
		Fe(III)	Fe(II)	Total	Fe(III)	Fe(II)
Century	1986	90	130	210	38	62
Century	1985	220	90	310	71	29
Century	1984	120	200	320	37	63
Sooty	1984	<5	180	180	0	100
Lee	1984	160	170	330	48	52
Century	1983	160	200	360	44	56
Raiden	1981	270	110	380	70	30
Matsuura	1981	190	170	360	48	52
Hawkeye	1981	210	180	390	54	46
Peking	1981	6	180	190	3	97
York	1980	140	230	370	38	62
Harosoy-63	1980	100	130	230	43	57
Beeson	1980	80	150	230	35	65
Hardee	1980	7	340	350	2	98
Williams	1979	280	110	390	72	28
SRF-200	1979	140	230	370	38	62
Clark-63	1979	110	210	320	34	66
Cutler	1979	110	170	290	39	61
Pomona	1979	160	150	310	52	48
Amsoy	1978	150	210	360	41	59
Wayne	1977	130	260	390	33	67

suggest that this attribute should be considered when hulls are to be utilized for iron-fortification of diets.

The presence of stable ferrous iron in soy hulls was unexpected, given that Fe(II) is susceptible to oxidation by molecular oxygen and organic compounds commonly occurring in plant tissues and that iron is transported to the developing seed as a ferric citrate complex (Tiffin 1970; White et al 1981a,b). To the author's knowledge, this is the first report of ferrous iron in metabolically quiescent plant cell walls.

Two unresolved questions are raised by this work. First, the nature of the chemical moieties responsible for binding Fe(III) and Fe(II) in the hull is unclear. The major ion-binding component of soybean hulls, the acidic polysaccharides or pectins, may account for only a small portion of the iron-binding sites, because most of the endogenous ferric and ferrous iron remained firmly bound at pH values well below the pK_a of the pectin in the hull (Laszlo 1987). Also, iron is purportedly bound by phytic acid in some seed tissues (Morris and Ellis 1982), but this is not a source of iron-binding sites in soybean hulls as they do not contain phytate. Because the endogenous iron fills less than 1% of the potential ion-exchange sites in the hull (Laszlo 1987), a number of minor cell wall constituents may afford iron-binding sites. Lignin has a well-documented affinity for iron (Fernandez and Phillips 1982, Platt and Clydesdale 1985), as do the functional groups in extensin and other cell wall-associated proteins (Cassab et al 1985). Phytoferritin (Van der Mark and van den Briel 1985) and phytochelatins (Grill et al 1985) are also potential iron-binding peptides, but it is not known whether they are present in soybean hull. Soybean lipoxygenase, which contains stable Fe(II) at its catalytic center, accumulates to significant levels (approximately 0.1% by weight) in the cotyledon fraction of mature seeds (Vernooy-Gerritsen et al 1984, Funk et al 1986). The lipoxygenase content of soy hull is very low (Omura et al 1986) and can explain only an insignificant amount of the iron. Furthermore, even the concentration of lipoxygenase in the cotyledon is insufficient to account for more than a small fraction of the iron present in the cotyledon.

The properties of the ferric and ferrous ions in soybean hull follow the general trends observed with the redox solution chemistry and stability of iron-coordination complexes (Basolo and Johnson 1964). Ligands generally form much more stable complexes with Fe(III) than with Fe(II); hulls bound Fe(III) more tightly than Fe(II). Strong coordination of Fe(III) by ligands in solution decreases its reduction potential; one third of the hull Fe(III) was not reducible by ascorbate. Conversely, weakly bound Fe(II) was not readily oxidized by oxygen or dichromate. However, the lack of reactivity of the hull iron to oxidizing or reducing chemicals may reflect the inability of the tested reagents to permeate to the bound iron rather than a change in the redox potential of the iron. Future spectroscopic and potentiometric studies of iron in soybean hulls should help elucidate the chemistry of the iron-binding sites.

The second conundrum pertains to the origins of the hull-resident ferrous iron. Again, there are several possibilities. Soybean root cells have an iron-reductase activity associated with their cell walls for iron-uptake from the rhizosphere (Tipton and Thowsen 1985). Iron-reductase activity involved in phloem unloading, or other iron-requiring enzymatic functions—such as lignin, cutin, and extensin biosynthesis during seed maturation—may be responsible for iron deposition in the hull. The hull fraction of several soybean varieties contains substantial quantities of peroxidase (Buttery and Buzzell 1968). An iron-requiring, suberin-specific peroxidase activity has been identified in *Phaseolus vulgaris* roots (Sijmons et al 1985). Such activity may be involved in synthesis of the waxy deposits on the surface of soybean hull (Wolf et al 1981), although preliminary investigation by X-ray microprobe analysis showed a nearly even distribution of iron throughout the hull rather than restriction to the region of the waxy cuticle (Laszlo, Dintzis, and Baker, *unpublished work*). A number of antioxidant compounds in soybean flour have been identified (Hayes et al 1977, Pratt 1985), which may serve as nonenzymatic reductants of soybean iron; however, the extent to which these compounds occur in the hull is unknown. Alternatively, iron may be imported to the bean in the ferrous state, contrary to its supposed state of transport in the plant. These suggested mechanisms by which ferrous iron may come to occur in soybean hulls are presently unsupported by evidence and must await further investigation, as does the identification of the iron-binding sites.

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