Changes in Endogenous and Exogenous Iron-Reducing Capability of Soybean Hull During Development

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ABSTRACT

Hulls (seed coats) of soybean (Glycine max) seeds contain relatively high levels of bioavailable iron. The presence of some iron in the Fe(II) oxidation state may be a contributing factor to its availability. Apparent Fe(II) and Fe(III) content of hulls of several soybean cultivars was followed throughout development. Iron was extracted from hulls with 2N HCl. Fe(II) and Fe(III) content of extracts was measured with bathophenanthroline disulfonic acid or by ion chromatography. Extracts of immature Century and Williams 82 hulls had higher levels of Fe(II) than fully mature hulls. Iron was all Fe(II) in extracts of Sooty, Wilson, and Peking hulls from late growth stages (late linear seed fill to harvest maturity). Conclusive assessment of the in situ hull iron oxidation state was not possible because extracts of all cultivars and stages contained reductants that were endogenous to the tissue or were generated during extraction. These reductants may influence bioavailability to a greater extent than hull iron valence.

Soybean seed coats (hulls) have been identified as a highly concentrated source of dietary iron (Levine et al. 1982, Weaver et al. 1984). Unlike the extremely poor bioavailability of iron in soy meal (Welch and Van Campen 1975, Lynch et al. 1984, Beard et al. 1988), hull iron is readily assimilated by humans (Jacob et al. 1980, Johnson et al. 1985, Lykken et al. 1987). The reason for this difference is unknown. The composition of these two tissues with respect to iron-chelating components is quite different. Soybean cotyledons contain phytic acid (Lolas et al. 1976) and a range of iron-binding proteins (Funk et al. 1986, Szczekan and Joshi 1987, Yoshida 1989), whereas the hull is composed largely of cell wall polymers (Aspinall and Whyte 1964; Aspinall et al. 1966, 1967; Raper 1979). Phytic acid (Chetyan 1980, Morris and Ellis 1982, Zemel 1984), soy protein (Picciano et al. 1984, Morr and Seo 1986), and plant cell walls (Reilly 1979; Reinhold et al. 1981; Fernandez and Phillips 1982a,b; Reinhold et al. 1986) have all been identified as potential inhibitors of iron absorption. The oxidation state of dietary iron also can have a profound influence. Fe(II) is believed to be more bioavailable than Fe(III) (Lee and Clydesdale 1979). Thus, the difference in iron availability between cotyledon and seed coat tissue could be attributed to many causes.

Previous work (Laszlo 1988) indicated that soybean hulls contain a substantial quantity of Fe(II). Hull Fe(II) is solubilized to a greater extent than hull Fe(III) under simulated gastrointestinal conditions (Laszlo 1989). These observations of hull Fe(II) and good bioavailability provide an appealing correlation. The Mössbauer study by Ambe and co-workers (1987) seemingly provides corroborative evidence for the presence of Fe(II) in soybean seeds. Their work, which did not distinguish between signals arising from seed coat or cotyledons, suggested that immature seeds contain a higher percentage of Fe(II) than mature seeds. Given the high concentration of iron in the hull relative to the rest of the seed, much of the Fe(II) signal may have originated from the seed coat. One objective of the present work was to examine hull Fe(II) and Fe(III) content during development.

Mature soybean cotyledons contain reductants or antioxidants (Hayes et al. 1977, Pratt 1985) that may influence the oxidation state of iron. Although the hull is not known to contain significant quantities of these chemicals, the possibility that endogenous reductants contribute to the formation of Fe(II) in hulls was explored.

MATERIALS AND METHODS

The soybean (Glycine max (L.) Merrill) cultivar Century was field grown locally under standard cultivation practices. Cultivars Williams 82, Wilson, Sooty, and Peking were greenhouse grown with supplemental lighting. Greenhouse conditions are detailed elsewhere (Laszlo 1990).

Developing soybean seeds were collected and segregated following the classification scheme of Fehr and co-workers (1971): R4, initial seed formation; R5, beginning linear seed fill; R5.5, intermediate linear seed fill; R6, late linear seed fill; R6.5, maximum seed fresh weight; R7, physiological maturity; and R8, harvest maturity. Hulls were removed from the seeds and placed immediately in a container on ice, then freeze-dried overnight. The dry hulls were ground to a coarse powder and stored at room temperature under vacuum.

Colorimetry was the principal method used to measure sample Fe(II) and total iron content. Hull samples (50 mg) were extracted with 2N HCl (5 ml) in 6-ml screw-capped vials. Vials were masked with black tape to avoid inadvertent photoreduction of Fe(III) during extraction. Sample extracts were passed through a 5-μm nitrocellulose filter (Alltech, Deerfield, IL). After filtration,
sample extract (1 ml) was mixed rapidly in a cuvette (1-cm optical path) with an equal volume of 2.2 M Tris (base form) containing 5.0 mM bathophenanthrolinedisulfonic acid (BPS). Tris and BPS were obtained from Sigma Chemical Co. (Saint Louis, MO). The absorbance of the BPS-Fe(II) complex was measured at 535 nm within 30 sec of mixing. The total Fe content of the extract was determined by adding 20 μl of 100 mM ascorbic acid to the cuvette to reduce Fe(III) to Fe(II). Calibration standards were prepared by dilution of 1,000 μg/ml of Fe solution (Fisher Scientific).

Anthocyanins present in dark-pigmented hulls (Wilson, Sooty and Peking) interfere with the BPS-iron assay. For these samples, Fe(II) and Fe(III) content of the acid extracts was quantitated by ion chromatography. Chromatographic conditions were as described previously (Laszlo 1988).

Chlorophyll content of hulls was determined following the method of Arnon (1949). Hulls (25 mg) were stirred with 1-ml of water for 2 hr; acetone (4 ml) was added and stirred for an additional 4 hr (25°C). Unsolubilized material was removed by brief centrifugation, and the absorbance of the chlorophyll extract was determined at 663 and 645 nm.

Anthocyanin content of hulls was measured following extraction in 1% HCl/methanol at 527 nm (Yoshikura and Hamaguchi 1969).

**RESULTS**

The mixing of Fe(II) with BPS results in rapid color formation that is complete within 30 sec and essentially invariant thereafter. The behavior of the reaction differed with iron derived from a Century or Williams 82 (stage R8) hull extract (Fig. 1). After an initial, rapid color formation, the absorbance of the solution continued to rise slowly. Addition of ascorbate to the extract prior to mixing with BPS resulted in fast and full color formation. This suggests that Fe(III) in the hull extract was being slowly reduced under the conditions of the BPS assay. Addition of ascorbate to the sample during the BPS assay produced rapid additional color formation, indicating that the BPS-Fe(II) complexation step was not rate limiting. Simultaneous analysis by ion chromatography and BPS indicated that the 30-sec absorbance value of the BPS assay represented the Fe(II) content of the hull extracts more accurately than longer time periods (data not shown). Inorganic salt sources of Fe(III) did not form Fe(II) under conditions of the hull extraction procedure (i.e., 2N HCl in capped, dark vials) during the BPS assay (without added reductant). Thus, iron reductants were generated during the BPS assay from solubilized hull components.

Iron-reducing agents also were generated during the 2N HCl extraction of iron from hulls, dependent on time and temperature of extraction and on hull developmental stage (Fig. 2). The apparent Fe(II)/Fe(III) ratio of hull iron increased with extraction time and temperature. This suggested that short extraction time (30 min) and low temperature (0°C) would minimize artificial reduction of iron. However, quantitative extraction of hull iron could be achieved only at room temperature. Treatment of hulls (any stage) with 2N HCl at 0°C resulted in only partial extraction (~80%). Extraction with more dilute HCl (200 mM) at 0°C solubilized even less iron—either Fe(II) or Fe(III)—yet still generated reducing equivalents (data not shown). Attempts to find preextraction treatments that removed reductants but not iron from the hull were not successful. Thus, conditions could not be found that gave quantitative, or even substantial, extraction of hull iron without also producing detectable quantities of reducing equivalents. Therefore, extraction for 30 min at 0°C with 2N HCl was settled upon for measuring Fe(II) content as a compromise condition, albeit imperfect, between being too mild to liberate hull iron and overly harsh (generating reductants). Extraction at room temperature (2 hr, 2N HCl) served for quantitation of hull total iron content.

The changes in apparent Fe(II) and total iron content of Century hulls during development are shown in Figure 3. The apparent Fe(II) content increased during development, then dropped sharply in the final stage of maturation (R8). The chlorophyll content of hulls roughly parallels the Fe(II) pattern, except the decrease in chlorophyll precedes somewhat the (apparent) loss of Fe(II). A similar pattern was observed for Williams 82 hulls (data not shown). This suggests that the apparent Fe(II) content of immature hulls is related to photosynthetic activity, or at least to metabolic activity.
Because ideal Fe(II)-extraction conditions could not be found, it was necessary to assess the extent of reductant generation. Addition of Fe(III) to the medium at the beginning of the extraction gave an estimation of the reducing equivalents produced. Table I demonstrates that more Fe(II) was generated when Fe(III) was added. The last column of Table I gives the percentage reduction of exogenously supplied Fe(III). Immature hulls reduced added Fe(III) to a greater extent than mature (R8) hulls. This implies that much, and perhaps all, of the apparent Fe(II) content of soybean hulls is the result of reduction of endogenous Fe(III) during extraction.

A similar situation arises with the Fe(II) content of hulls containing anthocyanins. Previous measurements of the black-hulled cultivars Peking, Sooty, and Wilson (all at stage R8) indicated that iron was completely in the ferrous oxidation state (Laszlo 1988). Anthocyanins appear in the hulls of these cultivars starting as early as stage R6. Anthocyanin content (expressed as A$_{650}$/g dry weight) of Wilson soybeans through development was as follows: R4-R5.5, 0.0; R6, 9.2; R6.5, 45.7; R7, 107; and R8, 134. Stages lacking anthocyanins, R4-R5.5, had apparent Fe(II) contents similar to Century and Williams 82 hulls of the same stages. Fe(III) added to anthocyanin-containing hull extracts was completely reduced (measured by ion chromatography). Addition of potassium dichromate to these hull extracts, in small excess of the endogenous iron, failed to produce Fe(III). Thus the high apparent Fe(II) content of the mature stages of these cultivars may also be due to reduction during extraction. However, the possibility cannot be excluded that hull iron is reduced by anthocyanins in situ.

### DISCUSSION

The presented results demonstrate the sensitivity of the ferrous-ferric couple to the oxidation-reduction potential of cellular extracts. Tissues such as soybean hull are replete with potential oxidants and reductants, particularly the photosynthetically active cells of immature stages. Mature stage (R8) hulls are less likely to have endogenous reductants present since they lack metabolic activity and are nearly devoid of intracellular constituents (Thorne 1981). However, hulls may retain indefinitely certain reductants such as anthocyanins. Furthermore, new reducing equivalents may be generated during extraction, perhaps through hydrolysis of cell wall polymers, that might alter the redox poise of the extract. The change in Fe(II)/Fe(III) ratio with extraction time and temperature (Fig. 2) likely represents such an effect. Thus the rise and fall of the Fe(II)/Fe(III) ratio during development (Fig. 3) reflects change in the balance of oxidants and reductants in the tissue but does not necessarily reflect the in situ oxidation state of the iron.

Based on analysis of tissue extracts alone, a case can be made that the valency of cellular iron is indeterminate, regardless of whether additional oxidants or reductants can be detected. Therefore, in situ spectroscopic evidence is required. Mössbauer examination of whole soybean seeds suggested that immature beans had a higher level of Fe(II) than harvest-mature seeds (Ambe et al 1987). Preliminary examination of mature hulls lacking anthocyanins by Mössbauer spectroscopy failed to find evidence for the presence of Fe(II) (S. Ambe, personal communication). Thus, the percentage of Fe(II) in fully mature hulls probably is far less than previously thought (Laszlo 1988), except, perhaps, for the anthocyanin-containing hulls (i.e., cultivars Sooty, Wilson, and Peking).

The extent to which anthocyanin reduces endogenous iron in situ during maturation is unknown. Fe(II) would also be expected to occur in immature hulls as a result of normal metabolic activities (i.e., photosynthesis, chlorophyll synthesis, et c.). Anthocyanin production in hulls during maturation (seemingly a wound response) may result in Fe(II) formation as well. Anthocyanins are known to complex iron and other metals (Jurd and Asen 1966). The evidence presented here indicates that reduction accompanies complexation.

Whether hull iron is Fe(II) or Fe(III) does not alter the conclusion that soybean hulls represent an excellent source of dietary iron and fiber. The reason why hull iron is more available for absorption than iron from other plant-derived sources remains a mystery. The iron reductants generated from soybean hulls by acid extraction may play a role in vivo, but this hypothesis needs testing under physiological conditions. Utilization of hulls in food products need not be abated due to uncertainty over the cause for enhanced iron bioavailability.

### LITERATURE CITED


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