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

JOSEPH A. LASZLO[1]

Cereal Chem. 68(1):21-24

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 2*N* HCl. Fe(II) and Fe(III) content of extracts was measured with bathophenanthrolinedisulfonic 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, Sczekan and Joshi 1987, Yoshida 1989), whereas the hull is composed largely of cell wall polymers (Aspinall and Whyte 1964; Aspinall et al 1966, 1967; Rasper 1979). Phytic acid (Cheryan 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 far 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 2*N* 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,

[1]Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 1815 N. University St., Peoria, IL 61604.

The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. American Association of Cereal Chemists, Inc., 1991.

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 m*M* 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

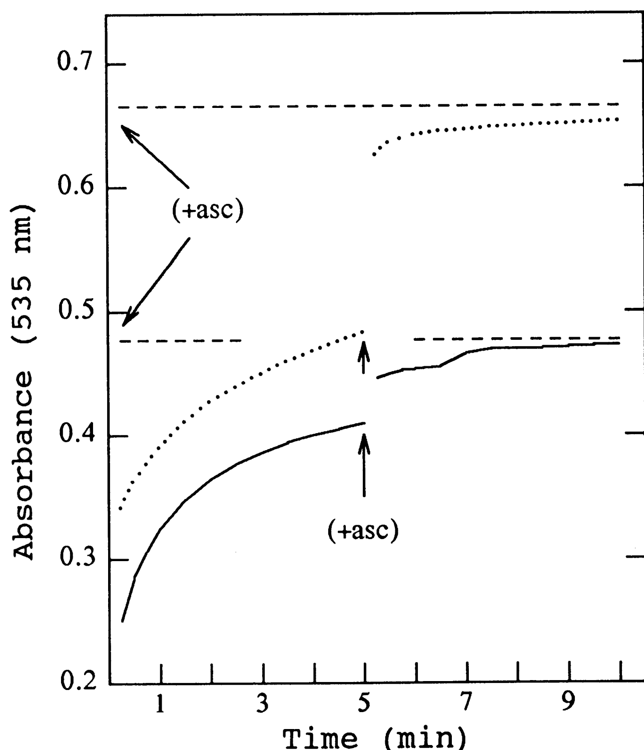

Fig. 1. Change over time in the absorbance of a mixture of bathophenanthrolinedisulfonic acid (BPS) assay and sample extract. Century (50 mg, solid line) and Williams 82 (54 mg, dotted line) hulls (stage R8) were extracted with 2*N* HCl (5 ml) for 2 hr at room temperature. Arrows indicate time of addition of ascorbate (20 μl, 100 m*M*) to cuvette. Dashed lines represent absorbance of assay/sample mixture with ascorbate added to sample extract just prior to mixing with BPS solution. Total iron content of these samples was 264 and 345 μg/g dry weight for Century and Williams 82, respectively.

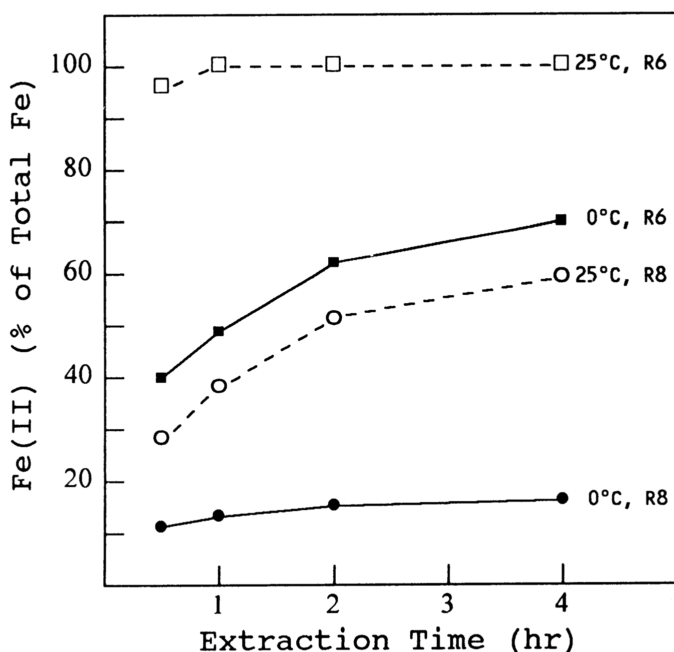

Fig. 2. Apparent Fe(II) content of Century hulls as a function of extraction time and temperature and of developmental stage (R6 or R8).

determined by adding 20 μl of 100 m*M* 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., 2*N* 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 2*N* 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 artifactual reduction of iron. However, quantitative extraction of hull iron could be achieved only at room temperature. Treatment of hulls (any stage) with 2*N* HCl at 0°C resulted in only partial extraction (~80%). Extraction with more dilute HCl (200 m*M*) 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 2*N* 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, 2*N* 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_{527}/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 possiblity cannot be excluded that hull iron is reduced by anthocyanins in situ.

TABLE I
Fe(III)-Reducing Capacity of Acid Extracts of Century Soybean Hulls

Developmental Stage	Apparent Fe(II) Content[a] No Added Fe(III)	Apparent Fe(II) Content[a] Added Fe(III)[b]	Reduction of Added Fe(III) (%)
R8	29 ± 4	42 ± 3*[c]	13
R7	102 ± 1	138 ± 12**	36
R6.5	91 ± 16	158 ± 2**	67

[a] Fe(II) (μg/g dry wt) determined by BPS-assay method. Extraction by 2*N* HCl at 0°C for 30 min.
[b] 1.0 μg/ml Fe(III).
[c] Asterisks indicate that the difference was significant at $P < 0.05$ (*) or $P < 0.01$ (**), $n = 3$.

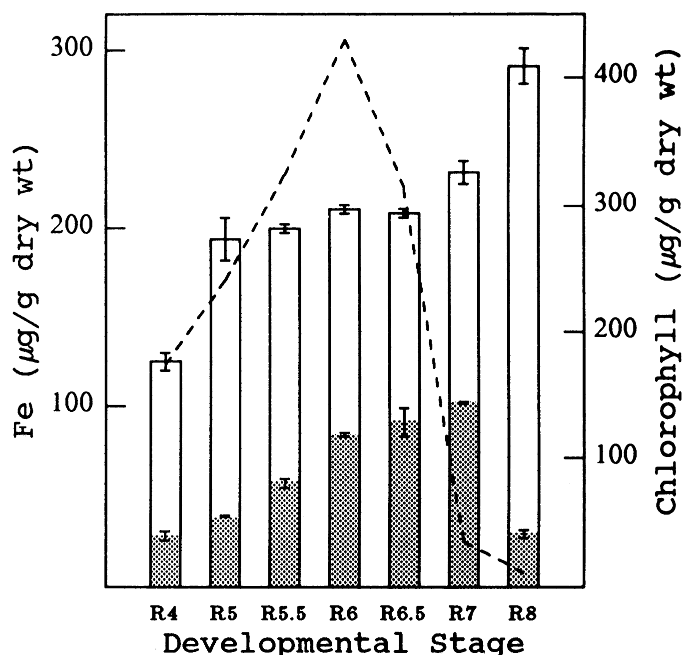

Fig. 3. Iron and chlorophyll content of Century hulls during development. Total iron (open bar) determined on 2*N* HCl extracts (2 hr, 25°C). Fe(II) content (shaded bar) measured on 0°C extracts (30 min, 2*N* HCl). Error bars represent standard deviation of means ($n = 3$). Chlorophyll content (dashed line) was determined following the method of Arnon (1949).

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, etc.). 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

AMBE, S., AMBE, F., and NOZAKI, T. 1987. Mössbauer study of iron in soybean seeds. J. Agric. Food Chem. 35:292-296.

ARNON, D. I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. 24:1-15.

ASPINALL, G. O., and WHYTE, J. N. C. 1964. Polysaccharides of soy-beans. I. Galactomannans from the hulls. J. Chem. Soc. 1964, 5058-5063.

ASPINALL, G. O., HUNT, K., and MORRISON, I. M. 1966. Polysaccharides of soy-beans. II. Fractionation of hull cell-wall polysaccharides and the structure of a xylan. J. Chem. Soc. 1966, 1945-1949.

ASPINALL, G. O., HUNT, K., and MORRISON, I. M. 1967. Polysaccharides of soy-beans. Part V. Acidic polysaccharides from the hulls. J. Chem. Soc. C 1967, 1080-1086.

BEARD, J., WEAVER, C. M., LYNCH, S., JOHNSON, C. D., DASSENKO, S., and COOK, J. D. 1988. The effect of soybean phos-

phate and phytate content on iron bioavailability. Nutr. Res. 8:345-352.
CHERYAN, M. 1980. Phytic acid interactions in food systems. CRC Crit. Rev. Food Sci. Nutr. 13:297-335.
FEHR, W. R., CAVINESS, C. E., BURMOOD, D. T., and PENNINGTON, J. S. 1971. Stage of development descriptions for soybeans, *Glycine max* (L.) Merrill. Crop Sci. 11:929-931.
FERNANDEZ, R., and PHILLIPS, S. F. 1982a. Components of fiber bind iron in vitro. Am. J. Clin. Nutr. 35:100-106.
FERNANDEZ, R., and PHILLIPS, S. F. 1982b. Components of fiber impair iron absorption in the dog. Am. J. Clin. Nutr. 35, 107-112.
FUNK, M. O., CARROLL, R. T., THOMPSON, J. F., and DUNHAM, W. R. 1986. The lipoxygenases in developing soybean seeds, their characterization and synthesis in vitro. Plant Physiol. 82:1139-1144.
HAYES, R. E., BOOKWALTER, G. N., and BAGLEY, E. B. 1977. Antioxidant activity of soybean flour and derivatives—A review. J. Food Sci. 42:1527-1532.
JACOB, R. A., SANDSTEAD, H. H., KLEVAY, L. M., and JOHNSON, L. K. 1980. Utility of serum ferritin as a measure of iron deficiency in normal males undergoing repetitive phlebotomy. Blood 56:786-791.
JOHNSON, C. D., BERRY, M. F., and WEAVER, C. M. 1985. Soybean hulls as an iron source for bread enrichment. J. Food Sci. 50:1275-1277, 1305.
JURD, L., and ASEN, S. 1966. The formation of metal and "co-pigment" complexes of cyanidin 3-glucoside. Phytochemistry 5:1263-1271.
LASZLO, J. A. 1988. Content and stability of ferrous iron in soybean hulls. Cereal Chem. 65:20-23.
LASZLO, J. A. 1989. Effect of gastrointestinal conditions on the mineral-binding properties of dietary fibers. Pages 133-145 in: Mineral Absorption in the Monogastric GI Tract. F. R. Dintzis and J. A. Laszlo, eds. Plenum Press: New York.
LASZLO, J. A. 1990. Mineral contents of soybean seed coats and embryos during development. J. Plant Nutr. 13:231-248.
LEE, K., and CLYDESDALE, F. M. 1979. Iron sources used in food fortification and their changes due to food processing. CRC Crit. Rev. Food Sci. Nutr. 11:117-153.
LEVINE, S. E., WEAVER, C. M., and KIRLEIS, A. W. 1982. Accumulation of selected trace elements in hydroponically grown soybeans and distribution of the elements in processed soybean fractions. J. Food Sci. 47:1283-1287.
LOLAS, G. M., PALAMIDIS, N., and MARKAKIS, P. 1976. The phytic acid-total phosphorus relationship in barley, oats, soybeans, and wheat. Cereal Chem. 53:867-871.
LYKKEN, G. I., MAHALKO, J. R., NIELSEN, E. J., DINTZIS, F. R. 1987. Availability of soybean hull iron to humans. J. Food Sci. 52:1545-1548.
LYNCH, S. R., BEARD, J. L., DASSENKO, S. A., and COOK, J. D. 1984. Iron absorption from legumes in humans. Am. J. Clin. Nutr. 40:42-49.
MORR, C. V., and SEO, A. 1986. Comparison of methods to remove endogenous iron from soy protein isolates. J. Food Sci. 51:994-996.
MORRIS, E. R., and ELLIS, R. 1982. Phytate, wheat bran, and the bioavailability of dietary iron. Pages 121-141 in: Nutritional Bioavailability of Iron. C. Kies, ed. American Chemical Society: Washington, DC.
PICCIANO, M. F., WEINGARTNER, K. E., and ERDMAN, J. W., JR. 1984. Relative bioavailability of dietary iron from three processed soy products. J. Food Sci. 49:1558-1561.
PRATT, D. E. 1985. Natural antioxidants of soybeans. Pages 145-153 in: Flavor Chemistry of Fats and Oils. D. B. Min and T. H. Smouse, eds. American Oil Chemists' Society: Champaign, IL.
RASPER, V. F. 1979. Chemical and physical characteristics of dietary fiber. Pages 93-115 in: Dietary Fibers: Chemistry and Nutrition. G. E. Inglett and S. I. Falkehag, eds. Academic Press: New York.
REILLY, C. 1979. Zinc, iron and copper binding by dietary fibre. Biochem. Soc. Trans. 7:202-204.
REINHOLD, J. G., GARCIA, L. J. S., and GARZON, P. 1981. Binding of iron by fiber of wheat and maize. Am. J. Clin. Nutr. 34:1384-1391.
REINHOLD, J. G., GARCIA ESTRADA, J., GARCIA, P. M., and GARZON, P. 1986. Retention of iron by rat intestine in vivo as affected by dietary fiber, ascorbate and citrate. J. Nutr. 116:1007-1017.
SCZEKAN, S. R., and JOSHI, J. G. 1987. Isolation and characterization of ferritin from soybeans (*Glycine max*). J. Biol. Chem. 202:13780-13788.
THORNE, J. H. 1981. Morphology and ultrastructure of maternal seed tissues of soybean in relation to the import of photosynthate. Plant Physiol. 67:1016-1025.
WEAVER, C. M., NELSON, N., and ELLIOTT, J. G. 1984. Bioavailability of iron to rats from processed soybean fractions determined by intrinsic and extrinsic labeling techniques. J. Nutr. 114:1042-1048.
WELCH, R. M., and VAN CAMPEN, D. R. 1975. Iron availability to rats from soybeans. J. Nutr. 105:253-256.
YOSHIDA, S. 1989. Distribution of the molecular weight and chemical form of intrinsic iron compunds in soybeans. Agric. Biol. Chem. 53:1071-1075.
YOSHIKURA, K., and HAMAGUCHI, Y. 1969. Anthocyanins of the black soybean. Eiyo to Shokuryo 22:367-370.
ZEMEL, M. B. 1984. In vitro evaluation of the effects of ortho-, tripoly- and hexametaphosphate on zinc, iron and calcium bioavailability. J. Food Sci. 49:1562-1565.

[Received December 21, 1989. Revision received June 19, 1990. Accepted June 21, 1990.]