Effects of Minerals and Apparent Phytase Activity in the Development of the Hard-to-Cook State of Beans[1]

O. L. KILMER,[2] P. A. SEIB,[3] and R. C. HOSENEY[3]

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ABSTRACT

Orthophosphate, potassium, magnesium, and calcium ions were leached from pinto, red kidney, and Great Northern white beans during 16 hr of soaking in water at 25°C. The extent of leaching of the ions increased concurrently with the hard-to-cook (HTC) condition of the beans as they were aged at warm temperature and high humidity (WT/HH). Beans also developed the HTC defect during soaking at 41°C in acetate buffer of pH 4.8, but not in 0.05*M* fluoride ion. The inhibition by fluoride was reversed when fluoride-impregnated beans were soaked in an excess of tris-buffer at pH 6.8 and 25°C. Those results are consistent with fluoride ion inhibiting the hardening of beans through competitive inhibition of phytase, as opposed to its interference in the formation of magnesium and calcium pectinate through precipitation of magnesium and calcium ions as insoluble fluoride salts. Metaphosphate ion also inhibited the HTC condition in pinto beans, apparently by chelating the calcium and magnesium ions released during soaking at pH 4.8 and 41°C. A colorimetric method was developed to measure apparent phytase activity in ground cotyledons. Fifteen samples of beans from red, black, and white cultivars were found to have phytase activities that varied by a factor of three, whereas the half-lives for development of the HTC state at 46°C and 100% rh varied by a factor of 10.

Legumes and cereals are important sources of food energy, protein, fiber, and other nutrients. Legumes, in contrast to cereals, are not easy to preserve. Storage of legumes at warm temperatures (30–40°C) and high humidity (>75%) (WT/HH) results in the condition classified as "hard-to-cook" (HTC) (Stanley and Aguilera 1985, Hentges et al 1991). But even at low temperature (4°C) and high relative humidity (80%), beans (unknown moisture content) stored in metal containers over a period of five to seven years required a two- to-fivefold increase in cooking time (Hernández-Unzón and Ortega-Delgado 1989). Storage of legumes at high temperature and low humidity sometimes generates the "hardshell" condition (Bourne 1967).

HTC legumes have a defect between the cells in their cotyledons. The level of water that HTC seeds imbibe is approximately the same as that of normal seeds (Hincks et al 1987), but HTC beans do not soften during cooking because the cotyledon cells do not separate (Stanley and Aguilera 1985, Narasimha et al 1989, Shomer et al 1990). Hardshell beans, on the other hand, have a defective seed coat. They fail to soften during cooking because they do not imbibe sufficient water. HTC and hardshell beans have depressed economic value because of the increased energy requirements for cooking, the poor palatability, and the reduced quality of the protein (Stanley and Aguilera 1985).

Hincks et al (1987) proposed that the HTC defect is caused by a multistaged process. The first stage is attributed to insolubilization of pectic substances in the middle lamella. The second stage is attributed to cross-linking of products from protein hydrolysis or polyphenolics. Hentges et al (1990) questioned the lignification mechanism in the HTC state of two cultivars of cowpeas and three cultivars of dry beans. Those authors accelerated the development of the HTC state by storing the legumes for six months at 29°C and 65% rh, but they found that the HTC state was reversed by six to eighteen months of additional storage at 6.5°C and 71% rh.

Mattson (1946) was the first to propose a mechanism to explain the insolubilization of pectin in HTC peas. Phytate located inside cotyledon cells (Reddy et al 1989b) undergoes attack by phytase as temperature and humidity increase. A portion of the phosphate ester groups on phytate are hydrolyzed (Hernández-Unzóon and Ortega-Delgado 1989, Hentges et al 1991). The lower phosphate esters of *myo*-inositol no longer chelate magnesium and calcium ions. Those cations then diffuse to the middle lamella, where they cause insolubilization of pectinic acid. A decrease in the water solubility of pectin has been found in HTC beans (Jones and Boulter 1983, Shomer et al 1990). Fluoride ion, which is a potent inhibitor of phytase (Bitar and Reinhold 1972, Lolas and Markakis 1977, Reddy et al 1989a), has been shown to inhibit hardening of beans (Vindiola et al 1986).

Recent evidence cited to support the cross-linking mechanism in HTC legumes includes the detection of fluorescence by aromatic substances in cell walls of HTC beans (Stanley and Plhak 1989); the depolymerization of protein fractions to oligomers and aromatic amino acids (Hohlberg and Stanley 1987, Hussain et al 1989); and differential scanning calorimetry (DSC) data on protein denaturation and starch gelatinization (Garcia-Vela and Stanley 1989). The purpose of this study was to probe further the phytase-phytate hypothesis of bean hardening.

Vindiola et al (1986) devised two methods to accelerate the development of the HTC condition. In one method, a unilayer

[1]Contribution 93-187-J from the Kansas Agricultural Experiment Station, Manhattan.
[2]Instructor, Department of Chemistry, Southeast Missouri State University, Springfield.
[3]Professors, Department of Grain Science and Industry, Kansas State University, Manhattan.

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of beans was exposed to a large excess of warm (46°C) humid air in desiccators. The beans became HTC over a period of several days. To prevent the growth of microorganisms, the beans were rinsed with a solution of chloramphenicol, and the desiccators were sanitized with a disinfectant. In the second method, beans were subjected to soaking in pH 4.8 buffer at 41°C. They became HTC in a period of hours. Both acceleration methods were used in this investigation.

MATERIALS AND METHODS

Materials

All chemicals were reagent-grade unless otherwise noted. Water was purified by double distillation, followed by passage through a mixed-bed ion-exchange resin (Barnstead/Thermolyne, Dubuque, IA). Dodecasodium phytate (97% pure), chloramphenicol, tris-(hydroxymethyl)aminomethane, maleic acid, and acid phosphatase (Type V) were obtained from Sigma (St. Louis, MO). Standard solutions of potassium, sodium, magnesium, and calcium were certified atomic absorption standards from Fisher Scientific (Pittsburgh, PA). Phosphorus was a standard solution of potassium monobasic phosphate (20 μg of P per milliliter in 0.05M HCl) from Sigma. Rocal II, a disinfectant, was obtained from Lehn and Fink Industrial Products (division of Sterling Drug, Inc., Montvale, NJ).

Beans (*Phaseolus vulgaris*) were obtained from several sources: pinto, red, kidney, black, and Great Northern white (all unknown cultivars) from Vermont Seed Co. (Fair Haven, VT); white (cv. Sanilac), white-grey (cv. Nep-2), red (cv. 15-R-148), and black (cv. San Fernando) from USDA/ARS Food Legume Program (Lansing, MI); and black (cvs. Talamanca, Turrialba, and Huasteco) and red (cvs. Mexicano and Criollo) from the National Council of Production (San Jose, Costa Rica).

Sound beans of medium size were sorted by hand from the samples and stored in polyethylene bags at 5°C until used. AACC (1983) approved methods were used for moisture and protein determinations. The moisture content of whole beans (10–15 g) was determined in an air-oven for 72 hr at 103°C (method 44-15A). Protein (%N × 6.25) was determined by the micro-Kjeldahl method (46-13). Beans (0.25–0.5 g) were wet-ashed in either sulfuric acid/hydrogen peroxide for potassium and phosphorus assays (Lowther 1980) or in nitric acid/perchloric acid for magnesium and calcium assays (Blanchar et al 1965). Orthophosphate was measured colorimetrically by the method of Fiske-Subbarow (1925). Sodium and potassium ion concentrations were determined by flame-emission spectroscopy. Calcium and magnesium ions were determined by atomic absorption on a Perkin Elmer instrument (model 2380, Perkin Elmer, Norwalk, CT). Standard solutions were: sodium ion (1–5 ppm), sodium chloride in ammonium acetate; potassium ion (10–20 ppm), potassium chloride in water; and calcium (5-10 ppm) plus magnesium ions (0.5–1.0 ppm), a mixture of their chloride salts in 0.5% lanthanum chloride heptahydrate.

The cookability of beans was determined by the tactile method after boiling 60 min in an aqueous medium (Vindiola et al 1986). Gloves made from polyvinyl chloride (Fisher Scientific) were worn during testing of cookability when the beans had been soaked in buffers containing chloramphenicol or fluoride ions. The cookability of the beans was reported as the mean percentage of cooked beans from two or three replicates. The percentages between replicates agreed within five percentage points.

In some experiments, beans were soaked in aqueous media with different reagents and were not soaked again before cooking. The beans were removed from the soaking medium; water (150 ml) was added; and the medium was brought to a gentle boil. The beans were added, and cookability was determined.

Inhibiting Hardening of Pinto Beans by Soaking Beans at pH 4.8 with Fluoride Ion and then Reversing the Inhibition

Eight subsamples, each containing 12 pinto beans, were soaked 9 hr at 25°C in 0.1M sodium acetate buffer (pH 4.8, 150 ml) containing 0.002% chloramphenicol, with or without 0.05M sodium fluoride. Then, the temperature of two subsamples, with or without fluoride, was increased to 41°C, and the beans were soaked for another 16 hr. Cookability of beans was determined after each soaking period.

At the end of the first soaking period, subsamples of beans, with or without fluoride, were removed and placed in 0.05M tris-maleate buffer (pH 6.6, 150 ml) (Gomori 1955) for 3 hr at 25°C. Two subsamples of beans were removed from the tris-buffer and placed in 0.2M acetate buffer (pH 4.8, 150 ml) for 12 hr at 41°C. The cookability of those samples was determined after each soaking period.

Inhibiting Hardening of Pinto Beans at WT/HH by Impregnating Beans with Fluoride Ion

Pinto beans (30 g) were soaked at 25°C in 0.1M sodium acetate buffer (pH 4.7, 150 ml) containing 0.05M sodium fluoride for 14 hr. The beans were removed from the soaking solution and placed one layer deep in perforated plastic petri dishes. The loaded dishes were covered with lids and submerged in 0.002% aqueous chloramphenicol then removed after 1 min. Excess antibiotic solution was shaken from each dish. A blank sample of beans was treated in the same manner, but without sodium fluoride.

Dishes containing fluoride-impregnated beans and dishes containing blank beans were placed in desiccators prerinsed with a 10% aqueous solution of Rocal II to prevent mold growth. Water was placed in the well of the desiccators. The lids were carefully sealed using high-vacuum silicon grease. The loaded desiccators were placed in a constant temperature chamber at 46°C. One desiccator was removed at a given storage time up to 13 days. Immediately upon removal from storage, the cookability of the beans was determined. Subsamples of 12 beans were soaked in water (150 ml) for 12 hr at 25°C and then removed. Additional water (150 ml) was added to the soaked beans, and the water was brought to a gentle boil. Cookability was determined. The experiment was replicated. The data are the means of the two tests.

Inhibiting Hardening of Pinto Beans by Soaking with a Chelating Agent and then Reversing the Inhibition

Subsamples of 12 pinto beans were soaked at 25°C in 0.1M sodium acetate buffer (pH 4.4, 150 ml) for 12 hr. Others were soaked at 25°C in 0.1M sodium metaphosphate buffer (PO_3, pH 4.4, 150 ml) for 12 hr. Metaphosphate buffer (pH 4.4, 0.1M) was prepared by mixing 0.2M sodium trimetaphosphate solution with 0.2M metaphosphoric acid. Cookability was determined after each soaking. The temperature of the subsamples was increased from 25 to 41°C for 16 hr. Cookability was again determined.

After the first soaking at 25°C, subsamples were placed in 0.1M tris-maleate buffer (pH 6.8, 150 ml) and allowed to soak for 3 hr at 25°C. Subsamples were removed from the tris-buffer, placed in 0.1M acetate buffer (pH 4.4), and soaked 16 hr at 41°C. Cookability was determined after each soaking. The experiment was duplicated. The data are the means from the two tests.

Apparent Phytase Activity in Beans

Using a razor blade, the hulls were removed from beans by hand. The naked cotelydons (5 g) were ground together with dry ice using a high-speed micromill (S-61691-02, Sargeant-Welch Scientific Co., Skokie, IL). Ground cotelydons (0.5 g, as is basis) were added to test tubes containing 5.0 ml of 0.05M sodium acetate buffer (pH 5.3) with 3 mM sodium phytate and 2 mM magnesium chloride. The tubes were held at 37°C, and the contents were agitated occasionally over a period of 4 hr. The enzyme reaction was terminated by adding 24% aqueous trichloroacetic acid (5.0 ml) with mixing. The acidified mixture was allowed to stand 2 min and then filtered through a glass-fiber filter pad (934-AH, Whatman). Sodium sulfate (1.0 g) was added to the filtrate (5.0 ml). The mixture was allowed to stand 10 min at 25°C. A white flocculant precipitate (probably pectin) was removed by filtration through a glass-fiber filter pad. An aliquot (1.1 ml, which was equivalent to 1.0 ml in the absence of sodium sulfate) of the clear filtrate was made to volume (10 ml or 25 ml)

with water, and the concentration of orthophosphate was determined (Nahapetian and Bassiri 1975). Phytase activity was defined as the increase of micromoles of orthophosphate per hour per gram of cotelydon at 37°C and pH 5.3. All phytase activity plots were adjusted assuming the orthophosphate (Pi) determined at zero digestion time was Pi endogenous to the beans. Phytase activities reported are the means of at least two replicates. Activities were reproduced with a standard error of the mean of 0.5–2.0.

Accelerated Hardening of Beans at HT/HH to Different Cookabilities and Leaching the Minerals

Samples of pinto, red kidney, and Great Northern white beans were rinsed with purified water and placed in desiccators at 46°C and 100% rh as previously described (Vindiola et al 1986). A desiccator was removed at different times up to 7 days, and the beans were dried overnight under ambient conditions and kept at 5°C for 1–6 hr. Duplicate samples of 12 beans (moisture 9–12%, wb) were soaked in deionized and distilled water (15.0 ml) at 25°C for 16 hr in glassware that had been cleaned with acid and rinsed with purified water. The liquid from one duplicate sample was decanted, diluted with pure water, and the concentrations of phosphorus, potassium, magnesium, and calcium were determined. The duplicate soaked sample was used to determine cookability.

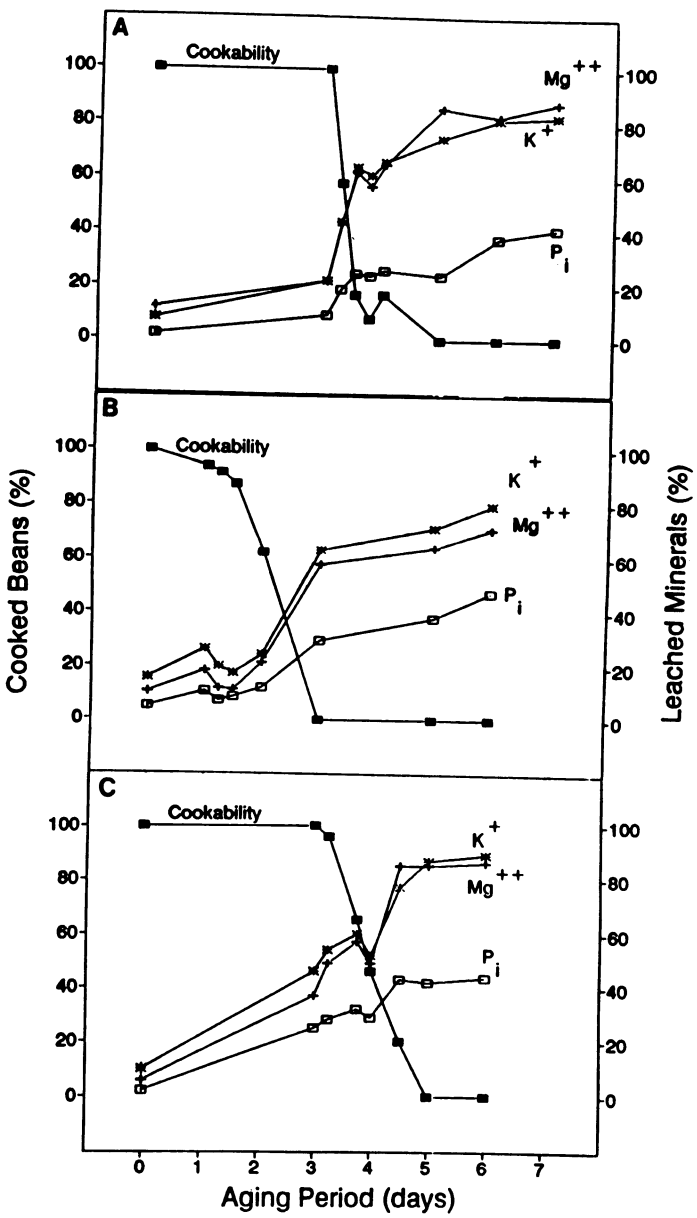

Fig. 1. Minerals leached (%) were calculated using mean levels of magnesium, potassium, and phosphorus in the pinto, Great Northern white, and kidney beans (A–C, respectively).

Leaching of Calcium and Magnesium Ions from Beans in Metaphosphate Buffer, and of Orthophosphate in Acetate Buffer with Fluoride

Two subsamples of 12 pinto beans each were soaked 12 hr at 25°C in 150 ml of 0.1*M* acetate buffer (pH 4.8), with and without 0.05*M* sodium fluoride. The temperature was increased to 41°C, and the beans were allowed to soak an additional 16 hr. The cookability of the beans and the orthophosphate in the soaking water were determined.

Pinto beans were soaked 12 hr at 25°C in 150 ml of either 0.1*M* acetate buffer (pH 4.4) or 0.1*M* metaphosphate (pH 4.4). The pH of the solution was determined. The temperature was increased to 41°C for 16 hr, and the cookability was determined. The pH values of the clear soaking liquors were recorded, and the concentrations of calcium and magnesium ions in the soaking liquors were determined.

RESULTS AND DISCUSSION

Releasing Minerals in Concert with the Development of HTC State

When pinto beans, white beans, and kidney beans were subjected to accelerated hardening in desiccators at WT/HH conditions (Vindiola et al 1986), their cookabilities differed with aging (Fig. 1). When the accelerated-aged beans were soaked in water 16 hr at 25°C, minerals were leached into the soaking water. From the average levels of endogenous phosphorus, potassium, magnesium, and calcium in the beans (Table I), the extent of leaching of each of the minerals was calculated. WT/HH aging of pinto, white, and kidney beans for 3.2, 2.2, and 4.0 days, respectively, reduced cookability by 50%. The rapid decline in cookability of the beans coincided with the rapid release of orthophosphate, potassium, and magnesium ions from the soaked beans (Fig. 1). Calcium ions behaved similarly (Vindiola 1992). For clarity, those data were not included in Figure 1.

A cause and effect relationship is apparent between the degree of cookability of beans and the release of potassium, magnesium, calcium, and orthophosphate ions in the soaking water. Several authors (Burr et al 1968, Parrish and Leopold 1977, Jackson and Varriano-Marston 1981, Moscoso et al 1984, Hincks et al 1987) have reported high leaching of minerals from HTC beans, but the correlation of the extent of hardening with increasing leachability was not demonstrated.

The concurrence of high leachability of minerals and the HTC condition in beans supports the dual-enzyme hypothesis (Fig. 2) (Jones and Boulter 1983). Phytate occurs in legumes as a protein-mineral complex inside cotelydon cells (Prattley and Stanley 1982, Lott and Ockenden 1986, Reddy et al 1989b). Upon hydration in a warm environment, the beans gain moisture and phytase hydrolyzes the phytate inside the cells. Pectin methylesterase deesterifies pectin in the cell walls. Subsequently, the Pi released from phytate, accompanied by its counter-ions of magnesium, calcium, and potassium, migrate towards the middle lamella, where cross-linking of pectinic acid occurs as the divalent ions form salt bridges.

TABLE I
Endigenous Levels of Minerals in Three Bean Cultivars

Bean	Concentration (mol/g of bean × 10^{-6})			
	P_t[a]	K^+	Mg^{++}	Ca^{++}
Pinto	109.7	393.9	65.8	27.4
Great Northern	96.8	383.6	61.7	39.9
Red kidney	114.5	386.2	57.6	15.0
Mean	107.0	387.9	61.7	27.4
Standard deviation	9.2	5.3	4.1	12.5

[a]Total phosphorus in beans.

An alternate explanation is based on the loss of integrity of the plasmalemma during bean hardening (Varriano-Marston and Jackson 1981). One proposal (I. Shomer, *personal communication*) is that pectin methylesterase is released from the cotelydon cell upon breakdown of the plasmalemma. It forms pectinic acid in the middle lamella. The pectinic acid then is intractable in the presence of even low concentrations of calcium and magnesium ions because of the polysaccharide's high molecular weight.

Phytate phosphorus in beans accounts for ~75% of the total phosphorus level in seeds (Lolas and Markakis 1975, Reddy and Pierson 1987, Reddy et al 1989b). Only about one third to one half of the phytate phosphate needs to be hydrolyzed to release divalent ions from inside the cells (Fig. 2), because *myo*-inositol hexa-, penta- and tetraphosphates are much stronger chelators than are tri- and diphosphate esters (Kaufman and Kleinberg 1971, Lasztity and Lasztity 1988, Xu et al 1992). Figure 1 shows that 25–40% of bean phosphorus was leached in the form of Pi when the three types of beans become HTC. The amounts of magnesium and calcium ions released into the soaking waters accounted for 60–85% of those ions in the beans. Because low levels of divalent cations are sufficient to cross-link the large pectinic acid molecules in the middle lamella, the remainder of the minerals would be leached into the soaking water.

Impregnating Pinto Beans with Fluoride Ion and Hardening at HT/HH

Vindiola et al (1986) showed that fluoride ion (0.05*M*) inhibited the hardening of pinto beans when they were soaked at pH 4.7 in acetate buffer for 12–16 hr at 41°C. In the present work, pinto beans that had been impregnated with fluoride ion at pH 4.7, and never dried after impregnation, failed to harden when stored 13 days at WT/HH, whereas the control beans without fluoride became HTC (Table II). For unknown reasons, if the fluoride-impregnated beans were dried overnight under ambient conditions using forced convection, and then subjected to WT/HH, they hardened at or before three days (Table II). Unfortunately, cooking data were not recorded at or before three days on the fluoride-impregnated beans that were air-dried. Possibly, upon drying, the fluoride ion became immobile and could not reach the active site of the phytase before arrival of the mobile hydrogen ions (initial pH 4.7). Soaking water of pH 3.0–4.8 accelerates the hardening of beans (Vindiola et al 1986).

The inhibition of bean hardening by fluoride might be explained by precipitation of local high concentrations of calcium and magnesium ions as their insoluble fluoride salts (0.2–0.9% solubility in water at 25°C), which would prevent the cross-linking of pectinic acid as shown in Figure 2. But the inhibition of the HTC condition by fluoride ion was reversible in pinto beans. Pinto beans that had been soaked at 25°C, with and without fluoride, were still 100% cookable after 9 hr (first soak, Table III). On the other hand, accelerating the hardening by increasing the soaking temperature to 41°C (final soak) caused the control beans (Sample 1, Table III) to be come HTC after 16 hr. Beans in 0.05*M* fluoride at the same pH (Sample 2) were 88% cookable. These findings are in agreement with previous reports (Vindiola et al 1986).

Another bean sample (Sample 4, Table III), which was also impregnated with fluoride, might have contained precipitated magnesium and calcium fluorides. But when Sample 4 was soaked 3 hr at 25°C in tris-buffer at pH 6.7, probably the fluoride ion was leached from the beans, because subsequent soaking (final soak, Table III) at pH 4.8 and 41°C caused the beans to become HTC. Those data, plus the observation that fluoride ions decreased the release of phosphorus into the soaking medium by ~40% (Table IV), support the concept that fluoride ion inhibits the development of the HTC state by inhibiting phytase activity. Lolas

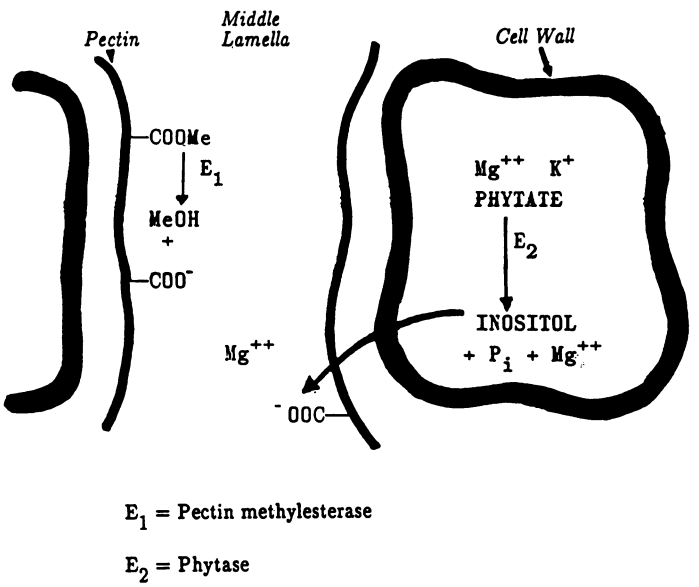

Fig. 2. Proposed phytase/methyl esterase mechanism of the hard-to-cook phenomenon in legumes. Arrow depicts diffusion of mineral ions from inside a cotelydon cell into the middle lamella.

TABLE II
Hardening of Beans at Warm Temperature/High Humidity After Presoaking at 25°C in 0.1*M* Acetate Buffer (pH 4.7) With and Without 0.05*M* Fluoride

Fluoride Ion	Days Stored at 48°C, 100% rh	Cookability, % Not Dried After Soaking	Cookability, % Dried After Soaking[a]
+	0	100	NT[b]
−	0	100	NT
+	1	100	NT
−	1	100	NT
+	2	100	NT
−	2	88	NT
+	3	100	NT
−	3	75	NT
+	4	100	0
−	4	52	0
+	5	100	0
−	5	58	0
+	13	100	0
−	13	0	0

[a]Impregnated beans dried overnight with forced-convection ambient air.
[b]Not tested.

TABLE III
Reversing the Inhibiting Effect of Fluoride Ion on the Development of the Hard-to-Cook State in Pinto Beans

Bean Sample	1st Soak[a] 25°C 0.05*M* Fluoride	pH	Time (hr)	Cookability (%)	2nd Soak[b] 25°C Tris Buffer pH	Time (hr)	Cookability (%)	Final Soak[a] 41°C 0.05*M* Fluoride	pH	Time (hr)	Cookability (%)
1	−	4.8	9	100	...	...	...	−	4.8	16	0
2	+	4.8	9	100	...	...	...	+	4.8	16	88
3	−	4.8	9	100	6.7	3	100	−	4.8	12	0
4	+	4.8	9	100	6.7	3	100	−	4.8	12	0

[a]Soaked in 0.1*M* acetate buffer containing 0.002% chloramphenicol with or without fluoride.
[b]Soaked in 0.1*M* tris-(hydroxymethyl) aminomethane sodium maleate buffer.

and Markakis (1977) and Bitar and Reinhold (1972) previously showed 0.01–0.02*M* fluoride inhibited purified phytase.

Inhibition of HTC State in Pinto Beans by Metaphosphate

Pinto beans that had been soaked at pH 4.4 and 41°C for 16 hr in acetate buffer became uncookable. Pinto beans soaked in metaphosphate buffer were 100% cookable (Table V). Removing the metaphosphate ion from the beans by soaking in tris-buffer caused the beans to harden after subsequent soaking in acetate buffer at 41°C (Sample 4, Table V).

The inhibiting of hardening by metaphosphate was previously attributed to its metal chelating effect (Vindiola et al 1986). However, sodium metaphosphate buffer contains some linear phosphate chains because of ring-opening hydrolysis of the metaphosphate ring. Those linear phosphates could competitively inhibit phytase (Tangkongchitr et al 1982).

The level of calcium plus magnesium ions leached from beans after soaking in metaphosphate buffer at 41°C was 13% less than that leached after soaking in acetate buffer (Table VI). Some inhibition of phytase might have occurred in the metaphosphate buffer, as evidenced by the somewhat reduced release of divalent cations in metaphosphate vs. acetate buffers. However, chelation of those ions from the pectinic acid in the middle lamella apparently is the major mechanism by which metaphosphate blocks bean hardening.

Apparent Phytase Activity in Beans Related to Hardening

We devised a procedure to measure apparent phytase activity in ground decorticated beans (Fig. 3). It was based on the colorimetric determination of orthophosphate released from added sodium phytate (Irving 1980, Nayini and Markakis 1986). The success of the procedure depended upon sample preparation prior to orthophosphate determination. Adding sodium sulfate to a digest after removal of TCA-precipitated protein presumably caused precipitation of pectic substances. If those substances were not removed during sample preparation, they precipitated when color was developed in the orthophosphate assay.

The pH of 5.3 and substrate concentration (3 m*M*) in our phytase assay was derived from the work of Lolas and Markakis (1977), who purified phytase from a white bean and found it had optimum activity at pH 5.3 at a subtrate concentration of 1–2m*M*. Their enzyme was almost totally inhibited at 10m*M* phytate. The beans contained an average of 107 μmol of phosphorus per gram, most of which occurs in the cotelydons (Cosgrove 1980). If ~75% of the phosphorus in beans occurs as phytate (Lolas and Markakis 1975, Reddy and Pierson 1987), then the endogenous phosphorus in beans contributed 1.3m*M* of phytate to a digest, in addition to 3.0m*M* sodium phytate. The endogenous phytate would occur probably as insoluble Mg^{++}/K^{+} salt (Lott and Ockenden 1986), as suggested by the ionic excess in beans of magnesium ions compared to that of monophosphate ester groups (Mg^{++} and P, 61.7 and 107 μmol/g, respectively). In our assay, magnesium ion was added to stabilize phytase (Bitar and Reinhold 1972).

The rate of release of orthophosphate in the assay was slow, which indicated low levels of phytase (Fig. 4). Plots of the orthophosphate released in digests were linear with time up to ~15–40% release of the total phosphorus (~145 μmol = 53.5 μmol from the bean + 90 μmol from added sodium phytate).

Linear regression analysis was used to calculate apparent phytase activities (ΔPi/hr/g of dry cotelydon) from plots such as those in Figure 4. We expressed phytase activities based on

TABLE IV
Cookability and Percentage of Phosphorus Leached from Pinto Beans upon Soaking at pH 4.9 in 0.05*M* Fluoride Ion

Bean Sample	Final Soak[a] 41°C: 0.05*M* Fluoride	Final Soak[a] 41°C: Time (hr)	Final Soak[a] 41°C: Final pH	After Soaking: pH	After Soaking: Cookability (%)	After Soaking: Phosphorus Concentration in Soaking Medium (mol/g bean × 10^{-6})
1	−	16	4.8	4.9	0	56.7 ± 3.5 (52%)[b]
2	+	16	4.8	4.9	70	34.9 ± 1.5 (32%)[b]

[a] Beans were soaked 12 hr at 25°C in the same medium before increasing the temperature of the mixtures to 41°C.
[b] Pinto beans contained an equivalent of (110 ± 0.7) × 10^{-6} mol of P_i/bean. The percent phosphorus leached from the bean is given in parenthesis.

TABLE V
Cookability of Pinto Beans Soaked in Acetate or Metaphosphate Buffers at pH 4.4

Bean Sample	1st Soak 25°C: Buffer[a]	1st Soak 25°C: pH	1st Soak 25°C: Time (hr)	2nd Soak 25°C: Buffer[a]	2nd Soak 25°C: pH	2nd Soak 25°C: Time (hr)	2nd Soak 25°C: Cookability (%)	Final Soak 41°C: Buffer	Final Soak 41°C: pH	Final Soak 41°C: Time (hr)	Final Soak 41°C: Cookability (%)
1	OAc	4.4	12	...	...	...	100	OAc	4.4	16	0
2	PO_3	4.4	12	...	...	...	100	PO_3	4.4	16	100
3	OAc	4.4	12	Tris	6.8	3	100	OAc	4.4	16	0
4	PO_3	4.4	12	Tris	6.8	3	100	OAc	4.4	16	0

[a] Buffers were OAc = 0.1*M* sodium acetate; PO_3 = 0.1*M* sodium metaphosphate; and Tris = 0.1*M* tris-(hydroxymethyl)aminomethane sodium maleate.

TABLE VI
Calcium and Magnesium Ions Leached from Pinto Beans upon Soaking at pH 4.5–4.7 in Acetate or Metaphosphate Buffer, and Their Cookabilities

Bean Sample	Final Soak[a] 41°C: Buffer	Final Soak[a] 41°C: pH	Final Soak[a] 41°C: Time (hr)	Cookability (%)	Concentration of Leached Mineral Ions[b] (mol/g bean × 10^6): Ca^{++}	Concentration of Leached Mineral Ions[b] (mol/g bean × 10^6): Mg^{++}	Total Divalent Ions Leached (%)
1	OAc	4.5[c]	16	0	8.61 ± 0.17	55.3 ± 2.0	69
2	PO_3	4.7[d]	16	100	19.0 ± 0.36	33.6 ± 0.6	56

[a] Beans soaked 12 hr at 25°C in the buffers before increasing soaking temperature to 41°C.
[b] Mean levels of calcium and magnesium ions in pinto beans was (27.4 ± 0.4) × 10^{-6} and (65.8 ± 2.3) × 10^{-6} mol/g of beans, respectively, for a total of 93.2 × 10^{-6} mol/g of bean, which was used to calculate percentages leached.
[c] After soaking, pH 4.5.
[d] After soaking, pH 4.9.

Decorticated beans

Dry ice (excess)

↓

Ground cotyledons (0.50 g),

pH 5.3 acetate buffer (0.05M, 5.0mL) with

sodium phytate (3 mM)

and magnesium chloride (2 mM)

↓

Incubate 0-4 h, 37°C,

Add

24% TCA (5.0 mL),

mix and filter

↓

Mix filtrate (5.0 mL) with

sodium sulfate (1.0 g) and

let stand 5-10 min, filter

↓

Determine P_i in filtrate,

Phytase activity = Δ μmole P_i/h/g dry cotyledon

Fig. 3. Procedure for determining apparent phytase activity in decorticated beans.

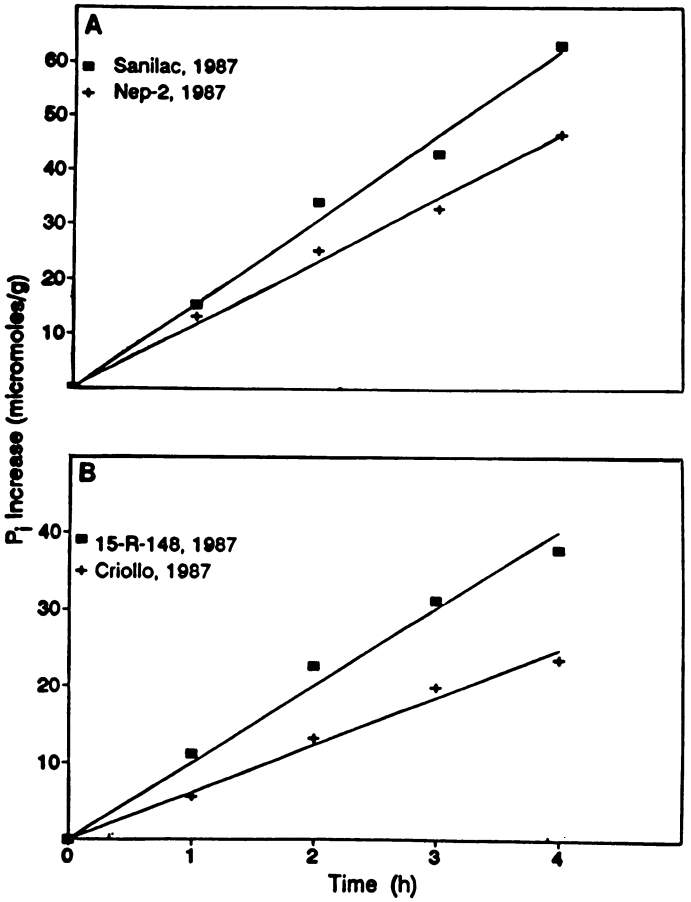

Fig. 4. Phytase activity in ground cotelydons from two white (A) and from two red (B) beans.

TABLE VII
Accelerated Hardening of Beans at High Temperature/High Humidity and the Apparent Phytase Activities in Ground Cotelydons

Bean	Cookability (%) After Accelerated Hardening							Half-life (days)	Phytase activity (Δμmol Pi/hr/g of bean)
	0	1	2	3	4	5	6		
Black Beans									
San Fernando 87	100	100	100	50	63	4	0	3.5	9.2
Talamanca	100	...	83	54	13	0	...	2.8	8.5
Turrialba	100	...	75	29	8	0	...	2.5	7.9
Huasteco	92	...	13	0	...	...	...	1.2	5.4
Black 84	100	95	95	60	35	20	10	3.7	10.4
White Beans									
Sanilac 87	100	100	54	42	33	0	0	2.7	15.5
Nep-2 87	100	100	54	46	0	0	...	2.4	11.7
Great Northern	100	94	63	0	...	0	0	1.9	11.4
Red Beans									
15-R-148 87	100	87	29	13	0	0	...	1.8	10.1
Criollo 88	88	...	17	0	0	...	...	1.2	6.2
15-R-148 82	100	...	...	80	...	60	...	4.9	10.5
Pinto Beans									
Pinto 84	100	95	90	95	50	10	5	3.8	8.5
Pinto 86	100	...	100	100	17	0	0	3.4	7.4

the weights of cotelydons because the protein levels of the beans were approximately equal (22.3–24.8%).

The apparent phytase activities of 15 samples of beans were determined along with their half-lives of hardening upon exposure to WT/HH (Table VII). The expected negative correlation between those two parameters was not observed. Surprisingly, the subset of black beans showed a high positive correlation (R^2 = 0.987) between those two factors.

The lack of correlation between apparent phytase activity in the ground cotelydons and the rate of bean hardening indicates that the rate of migration of ions through the cell walls in the cotelydons (Fig. 2), or the action of pectin methylesterase, may control the rate of development of the HTC state.

CONCLUSIONS

The concurrence of mineral leachability with the development of the HTC condition in beans, and the inhibition of the HTC condition by fluoride ion, indicates that phytate hydrolysis occurs in the main pathway by which beans become HTC when stored at WT/HH conditions. Phytase activity in cotyledon does not appear to control the rate-limiting step in the development of the HTC state.

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