Hard-to-Cook Defect in Black Beans. Protein and Starch Considerations

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Black beans (*Phaseolus vulgaris*) were stored for 10 months under three environmental conditions [high temperature/humidity (HTHH: 30 °C, 85% RH); medium temperature/humidity (MTMH: 25 °C, 65% RH); low temperature/humidity (LTLH: 15 °C, 35% RH)] in order to determine changes in starch and protein and to assess their contribution in textural defects. Hard-to-cook defect developed in HTHH-stored seeds and partially in MTMH samples. An increase in the DSC peak starch gelatinization temperature during storage at all three conditions was not related to hardening. Five protein fractions were separated by gel filtration. A significant increase ($p < 0.05$) was found during storage for a low-MW fraction of HTHH and MTMH extracts. At the same time a high-MW fraction decreased significantly ($p < 0.05$), suggesting the breakdown of large proteins. After 10 months of storage, free aromatic amino acids increased significantly ($p < 0.05$) with increasing temperature and humidity of storage. The coincidence of the appearance of small polypeptides and aromatic amino acids with the development of the hard-to-cook defect suggested a relation between these phenomena.

Hardness of cooked seeds has been considered a fundamental textural quality of beans and other legumes. Hardness defects have been classified as hardshell, when the seeds do not absorb sufficient water during cooking and therefore do not soften when cooked, and as hard-to-cook (Stanley and Aguilera, 1985), when the seeds absorb enough water but fail to soften upon cooking. Many researchers have reported that extended storage under high temperature and high relative humidity conditions will promote hard-to-cook beans. It is thought that hardening of bean seeds is due to structural changes in the cotyledons, but the chemical reactions responsible are not completely understood.

The hard-to-cook defect has been shown to be the result of physical and chemical changes that occur at the intercellular level during storage, resulting in an increase in stability of the middle lamella upon cooking. The most widely accepted explanation relates to the insolubilization of the pectic substances due to the enzyme phytase. Other enzymatic reactions have been suggested to contribute to hardening. These include removal of methyl groups from pectins by pectinesterases, hydrolysis of storage proteins by proteases, oxidation of polyphenols assisted by peroxidases or polyphenolases, and, less likely, oxidation of lipids by lipoxygenases (Stanley and Aguilera, 1985). Little work has been reported, however, on the fate of bean intracellular starch and protein during storage and how these events relate to the development of textural defects. That is the purpose of this work.

MATERIALS AND METHODS

Materials. Common black beans (*Phaseolus vulgaris*) were obtained from the Faculty of Agronomy, University of Chile, Santiago, Chile. Beans were removed from the pods by hand and dried in single layers on screens by exposing them to sun for 8 h/day until a moisture content of 8.3% was obtained, after which they were transported to Guelph and placed in storage within 1 month of harvest.

Chemical reagents were obtained from the Sigma Chemical Co. (St. Louis, MO) and from Fisher Chemicals Ltd. (Don Mills, Ontario). Molecular weight standard proteins were purchased from Pharmacia Inc. (Dorval, Quebec).

Experimental Design. The experiment was carried out under three controlled environmental conditions: high temperature and high humidity (HTHH), 30 °C and 85% RH; medium temperature and medium humidity (MTMH), 25 °C and 65% RH; low temperature and low humidity (LTLH), 15 °C and 35% RH. These environments were selected because they resemble tropical, semitropical, and temperate climatic conditions. In addition, the HTHH and MTMH conditions have been reported to produce the hard-to-cook and the hardshell defects, respectively (Antunes and Sgarbieri, 1979; Stanley and Aguilera, 1985). Seeds were removed from the chambers after 1, 2, 3, 4, 6, 8, and 10 months of storage, and the following characteristics were determined; moisture, water uptake, texture of soaked and cooked beans, thermal properties of isolated starch and proteins, and gel filtration properties of salt-soluble proteins. A minimum of triplicate analyses was carried out on beans from the three storage conditions.

Separation Procedures. Starch was separated from dry beans by a wet procedure as described in Figure 1, adapted from Schoch and Maywald (1968). A protein extract was obtained by a NaCl extraction of homogenized cotyledons as depicted in Figure 2. The salt concentration and solvent conditions were chosen to maximize the protein recovery (Sathe and Salunkhe, 1981). Protein bodies were isolated by the nonaqueous method described by Prattley and Stanley (1982).

Proximate Analysis. Standard AACC methods were used for moisture (AACC 44-16), ash (AACC 08-03), and fat (AACC 30-20) determinations. Moisture was determined in an air-circulated oven at 103 °C. Protein was determined by a modified Lowry method (Hartree, 1972). Serum albumin was used as the standard.

Aromatic amino acids were determined from extracts (Figure 2) after protein was precipitated with 10% TCA overnight. The final solution was cleared by centrifugation (40000*g* for 30 min) and filtration (0.45 μm). Amino acids were quantified by UV absorption (280 nm) using tyrosine as the standard.

Physical Properties. *Hardness Measurements.* Hardness of soaked and cooked beans was determined on an Instron universal testing machine (Model TM-M). The wedge system described by Sefa-Dedeh et al. (1979) was employed for measuring soaked beans. The seeds were

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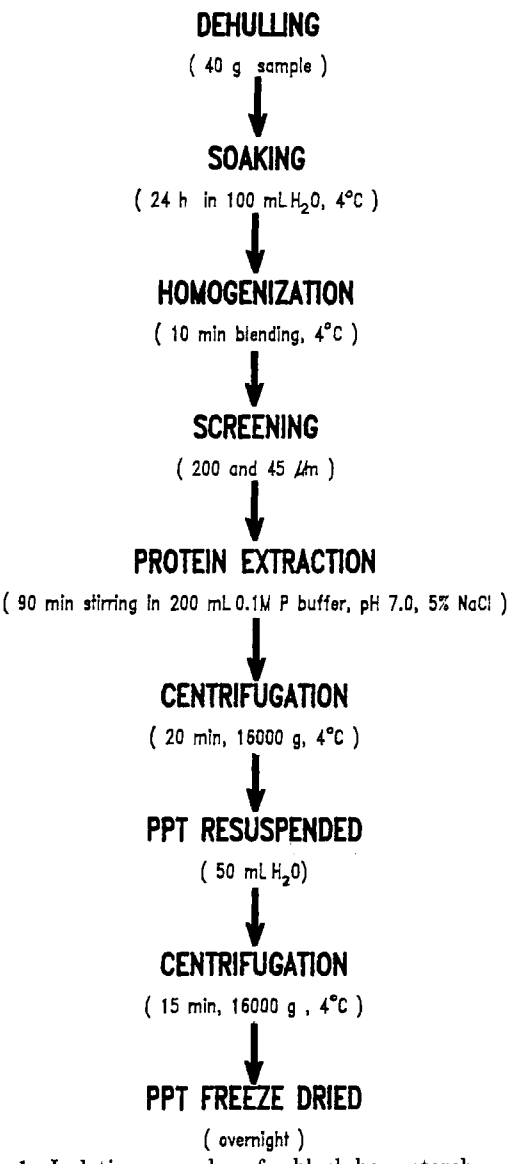

Figure 1. Isolation procedure for black bean starch.

soaked for 18 h in water at 25 °C before the measurement; 20 beans were tested for each treatment. Hardness of cooked 2-h beans (1:4 weight ratio in constant-volume boiling distilled water) was determined in an OTMS 10-cm^2 cell with an 8-bar extrusion grid. Maximum force was recorded for 30-g samples of cooked beans (Hincks and Stanley, 1987).

Water Absorption. Water uptake was followed on 10-g samples that had been soaked in 50 mL of distilled water for 1, 3, 6, 12, and 24 hr at 25 °C. After the beans were soaked for the appropriate time, water was drained from the samples and surface water removed prior to weighing.

Differential Scanning Calorimetry (DSC) of Starch. Aqueous suspensions of the isolated starch were prepared in concentrations between 20 and 60% (w/w). Samples were analyzed against a reference pan with sand, on a Du Pont Model 910 DSC at a heating rate of 10 °C/min. Gelatinization and melting transitions were analyzed with a Du Pont 1090 thermal analyzer using "Interactive Data Analysis" and "DSC Partial Area Integration" software. The DSC cell was calibrated against indium as reference. Suspensions weighing between 8 and 12 mg were run in triplicate for each sample.

Gel Filtration of Proteins. Molecular sieving of the bean protein extract was performed on an agarose prepacked (Superose 12 HR10/30) Pharmacia gel filtration column.

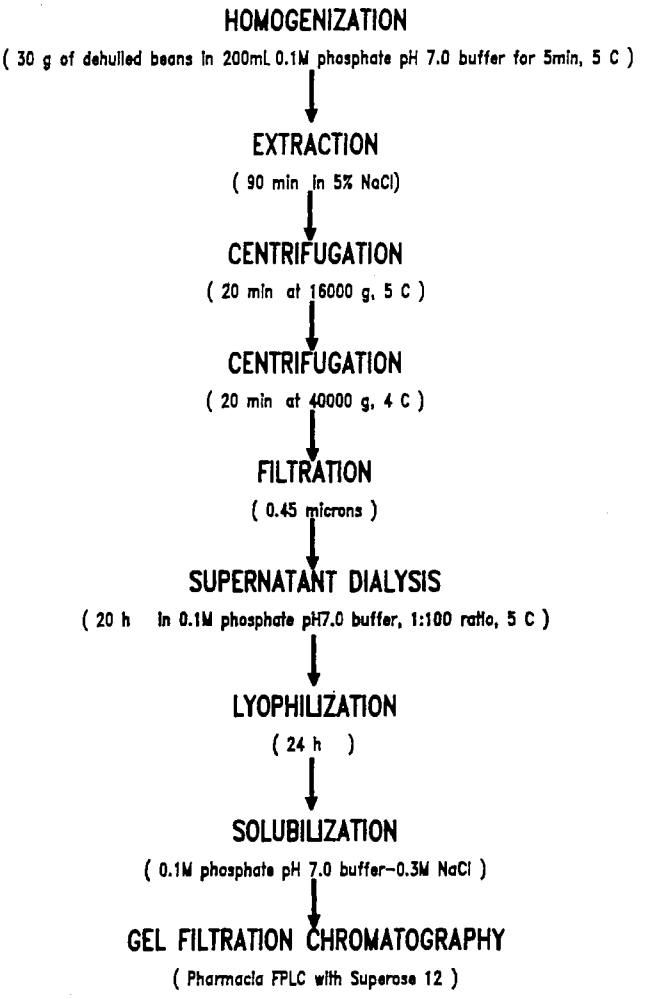

Figure 2. Separtion procedure for black bean protein extract.

The column was used with a Pharmacia fast protein–liquid chromatography (FPLC) system. Freeze-dried protein was redissolved (0.1% w/w) in phosphate buffer (pH 7.0, 0.3 M NaCl). The same buffer was used for eluting the sample through the column. A flow rate of 1.0 mL/min was selected, with a sample size of 100 μL and a monitor sensitivity of 0.1 AUFS. The column was calibrated with known proteins; acetone was used as a marker for the total permeation volume (V_t) and ferritin as a void-volume (V_0) marker. The optimal working range reported for the column was between 1 and 300 kDa. Determination of protein size was performed in terms of K_{av}, the adimensional parameter defined as (Siegel and Monty, 1966)

$$K_{av} = (V_e - V_0)/(V_t - V_0) \quad (1)$$

where V_e is the elution volume corresponding to the peak absorbance of the solute. The Lawrent–Killander relation was used for calibrating the molecular radius (a) of the proteins vs. the K_{av} factor. The resulting expression found using the standard 7 proteins was

$$-\log K_{av}^{0.5} = 0.32 + 0.00661a \quad (2)$$

$$R = 0.99,\ p < 0.01$$

A linear relation was found between log MW and K_{av} for MW calibration:

$$K_{av} = 1.669 - 0.259 \log \mathrm{MW} \quad (3)$$

$$R = 0.98,\ p < 0.01$$

Chemical Methods. *Amylose Determination.* The amylose content of the isolated starch was determined by the blue-value method (Gilbert and Spragg, 1964). Ab-

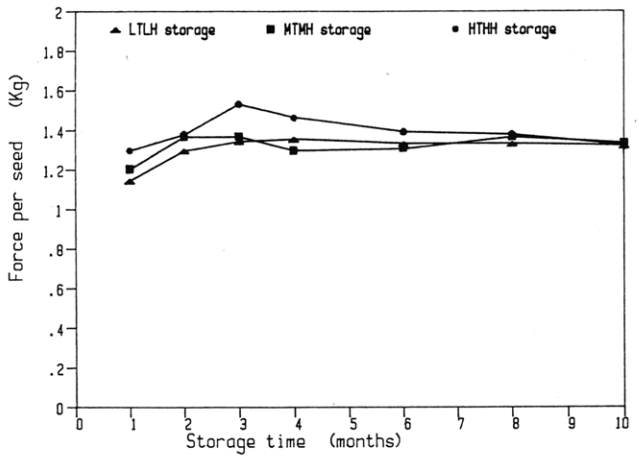

Figure 3. Hardness of soaked beans stored under LTLH, MTMH, and HTHH conditions.

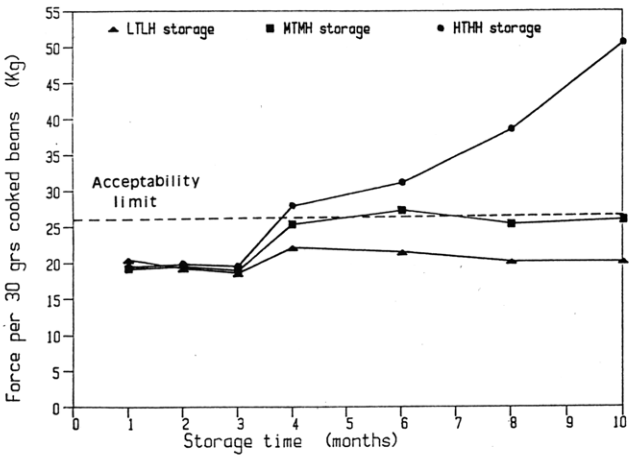

Figure 4. Hardness of cooked beans stored under LTLH, MTMH, and HTHH conditions.

sorbance was read at 700 nm.

Enzyme Assays. Peroxidase and polyphenolase activity were determined by colorimetric methods as described by Sharon-Raber and Kahn (1983). Proteases were assayed according to the colorimetric method described by Baumgartner and Chrispeels (1977). The substrates utilized were BOC-glutamine and CBZ-aspargine-ONp. The method was designed for measuring endopeptidases that hydrolyze vicilin from mung beans.

Microscopy. Light microscopy was used to examine the isolated starch as well as the cotyledon cells of uncooked beans. Sections were cut with a microtome and examined with a Zeiss photomicroscope (Model 62727). Samples for scanning electron microscopy (SEM) were critical-point dried, mounted on aluminum stubs, and coated with approximately 300 Å of gold/palladium. Specimens were viewed and photographed in an ETEC Autoscan electron microscope at acceleration voltages between 5 and 10 kV.

Statistical Methods. Statistical analysis was performed on an IBM-PC microcomputer with SAS and SYSTAT softwares. Factorial analysis of variance and regression were used to interpretate the data.

RESULTS AND DISCUSSION

Physical Properties. Proximate composition of black beans stored at the three different environmental conditions did not differ substantially after 4 months of storage. The moisture level of the seed was determined by the temperature and RH of storage and did not change significantly with storage time; average moisture contents for the beans stored in LTLH, MTMH, and HTHH environments were 9.6, 10.8, and 13.5%, respectively. Water absorption characteristics for the seeds kept under the three conditions did not change significantly ($p > 0.05$) with storage time. Seeds in all treatments absorbed between 100 and 115% of their original weight after 24-h soaking.

Hardness of soaked beans did not vary significantly during storage (Figure 3). The hardshell deffect was not detected in the beans, contradicting the report of Antunes and Sgarbieri (1979), under any of the storage conditions employed. This observation was based on the absence of seeds with high hardness after soaking and by visual inspection.

The hardness of cooked beans was significantly different ($p < 0.05$) for the three treatments after the third month of storage (Figure 4). It can be observed that HTHH and to some extent MTMH storage conditions promoted the hard-to-cook defect. After 4 months of storage the texture

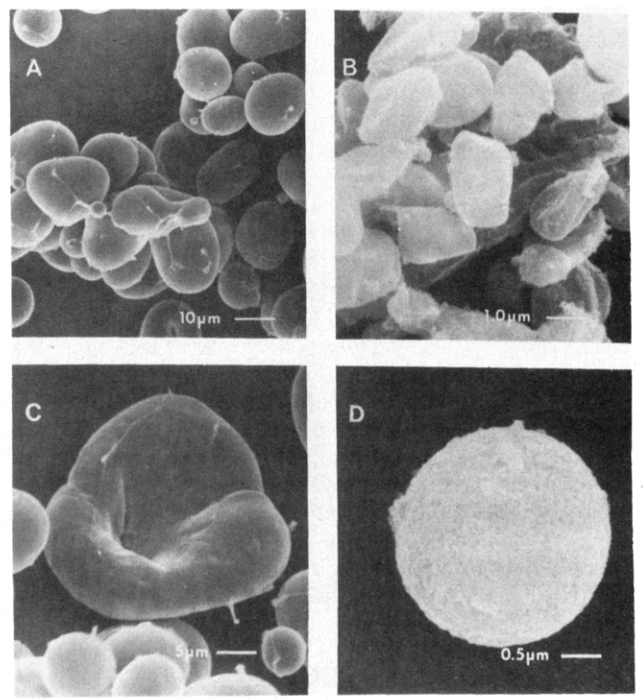

Figure 5. SEM micrographs from isolated bean organelles: A and C, intact starch granules; B and D, protein bodies.

of the HTHH beans exceeded the 26-kg force value acceptability limit determined through sensory analysis by Aguilera and Steinsapir (1985). The MTMH-stored beans remained at force values close to this limit after the fourth month. These hardness values were similar to those previously reported with the same type of beans under similar environmental conditions (Aguilera and Stanley, 1985; Hincks and Stanley, 1986).

Purification Results. Isolated starch had less than 0.5% (w/w) protein impurities and no measurable fat content. Light and electron microscopy were used to monitor the separation procedure. Plates A and C in Figure 5 show typical isolated starch granules. Amylose was found to account for 35–37% (weight basis) of the starch, and this did not vary with the storage conditions. Amylose concentrations were within the range reported for black beans (Lai and Varriano-Marston, 1979).

Micrographs B and D from Figure 5 depict protein bodies isolated from black beans. The presence of crystalloid inclusions in the aleurone is evident in plate D. The grains were eliptical or spherical in shape with sizes ranging between 1 and 5 μm.

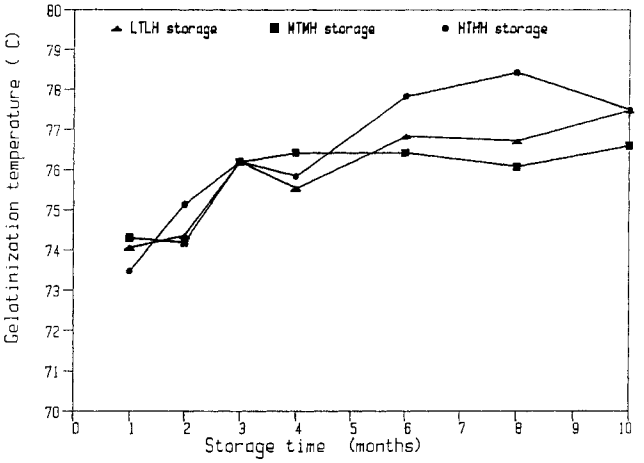

Figure 6. Effect of storage time on gelatinization temperature of black bean starch.

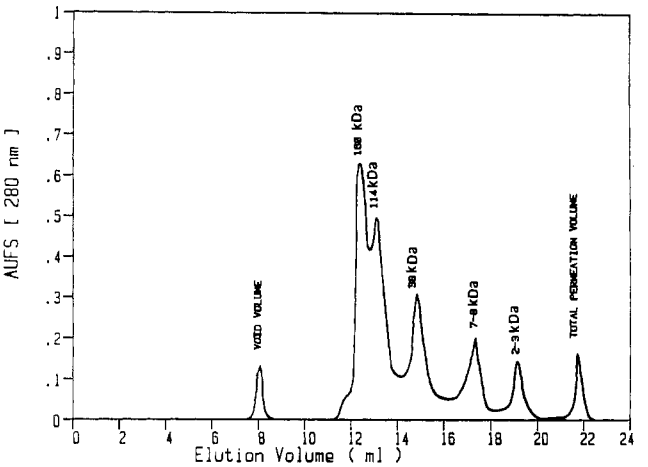

Figure 7. Typical gel filtration chromatogram for black bean proteins.

Calorimetry of Bean Starch. No significant differences were found for melt temperature, gelatinization energy, or melt energy of isolated starch as a result of either storage time or conditions. These parameters averaged 93.4 °C, 2.26 J/g, and 2.13 J/g, respectively. On the other hand, onset temperature and gelatinization temperature (Figure 6) were both significantly ($p \leq 0.05$) influenced by storage time but not conditions. This increase of gelatinization temperature, but not of melting temperature, could possibly be explained by changes in the crystallinity of starch granules during storage.

The foregoing results suggest that a chemical or structural change occurs in bean starch with storage independent of the environment. Textural problems in beans cannot be explained by this; however, other nutritional and quality deteriorations reported for beans may be a consequence of the granular alteration including the reported observations of intact starch granules in cooked beans (Aguilera and Steinsapir, 1985) and the decrease in starch digestibility during storage.

Separation of Proteins. Gel exclusion chromatography of the bean protein extracts produced five distinctive peaks. The resulting pattern for a typical chromatograph is shown in Figure 7. For a flow rate of 1 mL/min, the void volume was approximately 8 mL and the total permeation volume was 21.4 mL. The time required for each separation was less than 30 min, and acceptable reproducibility was obtained between replicates. The first peak detected had a retention time of 12.3 mL. Calculated values for MW and a for this protein fraction were 160 ± 9 kDa and 56 Å, respectively. This fraction accounted for 46.5% of the total protein extracted on the basis of the area under the peak. The protein was identified as phaseolin, the principal storage protein present in common beans, on the basis of its MW. Reported values for the MW of this protein are between 140 and 180 kDa; however, the real value is believed to be closer to 150 kDa because sugar moieties present in the molecule cause an overestimation of its MW with gel filtration (Chang and Satterlee, 1981).

Legumin, a large protein prominent in other legumes, was not identified in the chromatograms obtained. The second fraction eluted (12.8 mL) was not well resolved from the first peak. Its MW was determined to be 115 ± 12 kDa and its molecular radius 53 Å. The fraction accounted for 27% of the protein extracted and had a size similar to the previously described 6.8 S phytohemagglutinin in beans, but the proportion in which it was present was higher than the 10% reported by Baumgartner and Chrispeels (1977). Overestimation might have been caused by the overlap between peak 1 and peak 2.

The third protein fraction eluted from the column at 14.5 mL, although the base line did not return to normal between the second and this third peak. Computed values for the third fraction were 38 ± 5 kDa for MW and 37 ± 2 Å for a. This fraction is probably made of a group of proteins such as 4.9 S globulin, amylase inhibitors, and subunits from the lectin and phaseolin proteins. It represented approximately 10% of the protein extract; however, this percentage varied over storage. The presence of a fourth protein was detected close to 17-mL elution volume. This fraction accounted for approximately 11% of the total protein and had a MW of 7.4 ± 1.6 kDa and a of 15 ± 3 Å. Proteins possibly present in this fraction are albumins such as enzymes and trypsin inhibitors. Again, a complete base-line recovery was not obtained between this and the preceding peak. An explanation for this tailing effect might be the interaction of carbohydrate moieties present in the proteins with the agarose particles of the gel.

The last protein peak measured occurred after 18.5 mL and was found to be an approximately 2.7-kDa polypeptide(s). The area under this peak varied considerably between storage conditions and during storage. As can be seen from Figure 8, there was variation in the area of some of the peaks through storage. For beans stored under LTLH conditions no significant difference ($p > 0.05$) was detected in peak size during storage. Fraction 5 from the MTMH and HTHH stored beans increased significantly ($p < 0.05$) during storage. The area beneath peak 5 in the MTMH sample was highly correlated ($r = 0.93$; $p < 0.01$) with storage time, as was the area for the same peak in the HTHH sample ($r = 0.93$; $p < 0.01$). The appearance of these small polypeptides (2–3 kDa) in the samples that were stored under severe conditions may have resulted from the breakdown of larger proteins. A similar pattern was seen after ultracentrifugal analysis (Stanley and Aguilera, 1985). Fraction 1 (phaseolin) decreased significantly ($p < 0.05$) over storage in both HTHH and MTMH samples.

Relation between Protein Changes during Storage and Cooking Quality of Beans. It is most probable that phaseolin subunits were hydrolyzed either enzymatically or nonenzymatically after prolonged storage in an unfavorable environment. Breakdown of these proteins might be the result of an increase in membrane permeability and the relatively high water activities in the cotyledons (approximately 0.65 and 0.55) for the seeds stored under HTHH and MTMH, respectively (Aguilera and Stanley,

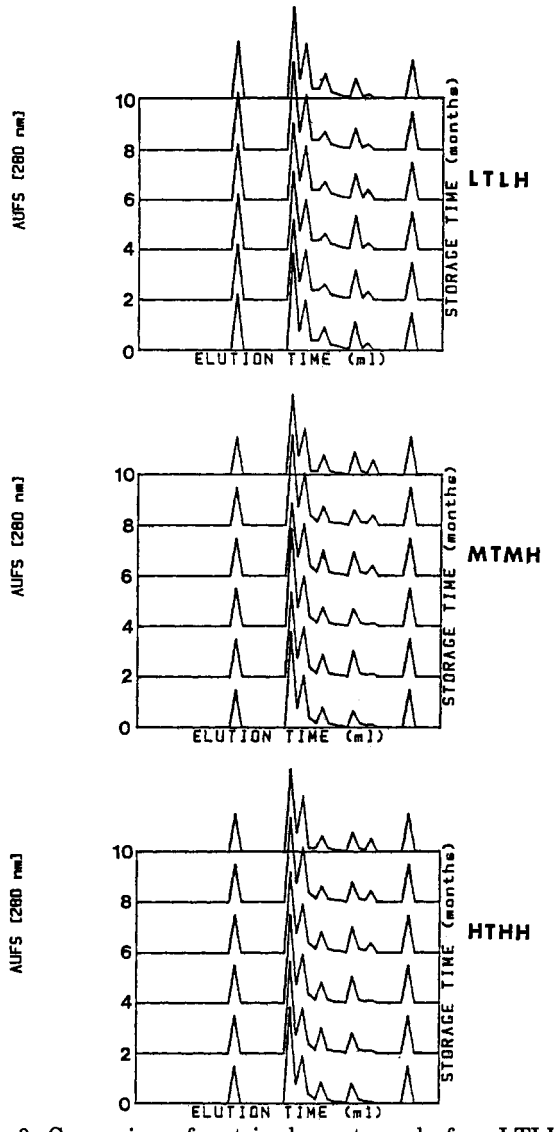

Figure 8. Comparison of protein chromatographs from LTLH-, MTMH-, and HTHH-stored beans.

Table I. Protease Activity in Bean Extracts, Average of Triplicates

protein[a]			activity,
storage	month	substrate[b]	AUFS/min
LTLH	3	BOC-GLN	0.016[c]
LTLH	6	BOC-GLN	0.020[c]
LTLH	10	BOC-GLN	0.016[c]
MTMH	6	BOC-GLN	0.016[c]
MTMH	10	BOC-GLN	0.021[c]
HTHH	6	BOC-GLN	0.016[c]
HTHH	10	BOC-GLN	0.015[c]
LTLH	3	CBZ-ASN	0.256[c]
LTLH	6	CBZ-ASN	0.256[c]
LTLH	10	CBZ-ASN	0.267[d]
MTMH	6	CBZ-ASN	0.304[c]
MTMH	10	CBZ-ASN	0.240[d]
HTHH	6	CBZ-ASN	0.256[d]
HTHH	10	CBZ-ASN	0.257[d]

[a] Activity measured in an aqueous solution of the protein extract (0.303 mg/mL). [b] Substrate concentration 0.121 mg/mL. [c,d] No significant difference between storage treatments ($p > 0.05$).

1985). These conditions may enhance the mobility of proteases within the cells. Hydrolysis of storage proteins has been reported to occur enzymatically in other pulses as water activity increases in the cotyledons upon germination (Gilford et al., 1983).

Table II. Peroxidase Activity in Bean Extracts, Average of Triplicates

protein[a]		activity,[b]
storage	month	AUFS/min
LTLH	3	0.376
LTLH	4	0.309
LTLH	6	0.272
LTLH	8	0.297
MTMH	4	0.346
MTMH	6	0.365
MTMH	8	0.305
HTHH	4	0.448
HTHH	6	0.342
HTHH	8	0.293

[a] Activity measured in aqueous solution of the protein extract (2 mg/mL). [b] No significant difference between storage treatments ($p > 0.05$).

Table III. Free Aromatic Amino Acids in Bean Extract after 10 Months of Storage, Average of Triplicates

storage	% aromatic AA[a] (w/w wet basis)
LTLH	0.776[b]
MTMH	0.828[c]
HTHH	0.987[d]

[a] Tyrosine equivalents. [b-d] Significant difference ($p < 0.05$).

Parallel experiments demonstrated the existence of protease activity in the bean cotyledons extracts. Table I presents the results obtained for protease activity of some of the protein extracts. It can be noted from the activities that the endopeptidases present in bean readily hydrolyzed BOC-glutamine and CBZ-aspargine substrates. No significant difference was found in enzyme activity ($p > 0.05$) during storage or between environments. The protein extract was also assayed for lignin-related enzymes, namely peroxidases and polyphenolases. Peroxidase activity was found in all samples tested; however, no significant difference ($p < 0.05$) was noted among samples (Table II). No polyphenoloxidase (catecholase) activity was detected in any of the protein extracts, contradicting previously reported results (Elias, 1982; Sierwright and Shipe, 1986).

Free aromatic amino acids were measured for LTLH-MTMH-, and MTHH-stored samples. Table III gives the percentages of free amino acids determined by UV absorption for protein-precipitated extractions from the beans after 10 months of storage. A significant increase ($p < 0.05$) in the percentage of free aromatic amino acids was determined as a function of storage conditions.

Rupture of the proteins during storage together with the presence of aromatic free amino acids and enzymatic activity within the bean cotyledons represent common initial steps for polymerization and/or lignification reactions in plants. Varriano-Marston and Jackson (1981) suggested that lignification may be an important cause for the loss of cookability of beans during storage. Hincks and Stanley (1987) provided evidence for lignification in black beans during storage.

These results support a possible alternative mechanism for the development of hard-to-cook beans. Small polypeptides and free aromatic amino acids are hydrolyzed from large proteins and may lead to polyphenol synthesis. These compounds presumably migrate to the middle lamella after a period of time at relatively high temperatures and humidities of storage. There they are lignified, perhaps by the action of mobilized enzymes. These reactions are known to be catalyzed by peroxidases, which were detected in extracts from the seeds. The production of lignin or other insoluble polymers in the middle lamella

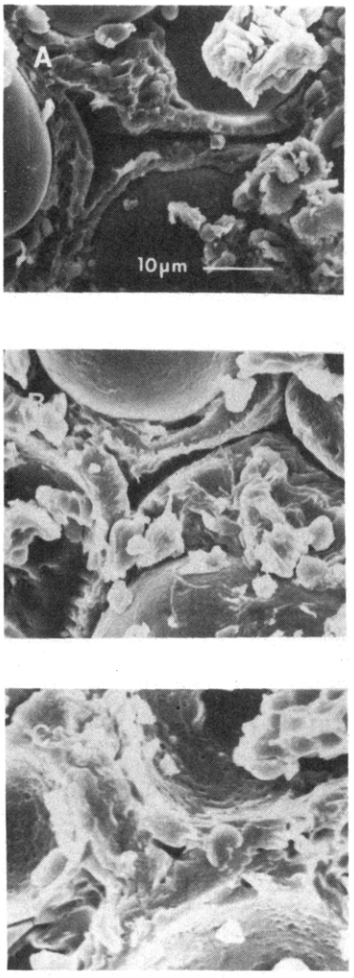

Figure 9. SEM micrographs of black beans after 8 months of storage: top, LTLH-stored beans; middle, MTMH-stored beans; bottom, HTHH-stored beans.

could certainly lead to a failure of the bean cells to separate upon cooking. Micrographs in Figure 9 depict the difference in intercellular material of raw beans after 8 months of storage. The presence of these deposits (lignin?) increased substantially from the LTLH to the HTHH storage. This observation supports the hypothesis that polymers are built up within the cell walls and in the middle lamella as the bean seeds age. Also, it is possible that water movement may be restricted within the cotyledons because of the deposited material.

ACKNOWLEDGMENT

We thank Tina Smajda for assistance with the enzyme and amino acid analyses and Ken Baker for help with SEM techniques.

Registry No. Protease, 9001-92-7; polyphenolase, 9002-10-2; peroxidase, 9003-99-0; amylose, 9005-82-7; starch, 9005-25-8.

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Received for review September 2, 1986. Accepted February 18, 1987. This work was supported in part by the International Development Research Centre and the Natural Sciences and Engineering Research Council of Canada.

Hard-to-Cook Defect in Black Beans. Soaking and Cooking Processes

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Water absorption, solids loss, and electrolyte leakage were determined for soft and hard black beans during the soaking and cooking processes. All three parameters increased in hard beans during soaking. It is hypothesized that membrane damage or deterioration is responsible for these differences. During cooking, hard beans lost fewer solids and minerals and did not continue to hydrate to the same degree as the controls, probably as a result of restricted cell separation. Microscopic evidence indicated reduced starch gelatinization in hard beans. This is attributed to reduced water absorption.

The hard-to-cook defect that develops in dry beans stored under conditions of high temperature and high humidity has been investigated extensively. One often-repeated approach has been to establish a relationship among the imbibition characteristics of the beans and their rates of softening. Though initial work indicated no differences in the absorption patterns of hard and soft beans (Burr, 1968; Molina et al., 1976), no corrections were made for the loss of solids during soaking. More recently, Jackson and Varriano-Marston (1981) emphasized that the differences in cooking times for soft and hard beans could not be accounted for by absorption differences during

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0021-8561/87/1435-0576$01.50/0