Protein Insolubilization and Thermal Destabilization during Storage As Related to Hard-To-Cook Defect in Cowpeas†

Keshun Liu,* Kay H. McWatters, and R. Dixon Phillips

Food Safety and Quality Enhancement Laboratory, Department of Food Science and Technology, The University of Georgia, Georgia Agricultural Experiment Station, Griffin, Georgia 30223

As storage duration at 30 °C/64% RH increased from 0 to 18 months, hard-to-cook (HTC) state of cowpeas (*Vigna unguiculata*) increased from 15.8 to 91.2 N/g, protein water extractability decreased from 76.5 to 11.2%, protein thermal transition temperature (T_m) decreased from above 100 to 56 °C, and starch gelatinization temperature remained constant at 64–73 °C. A decrease in tissue pH from 6.64 to 5.57 was also found with storage duration and correlated well with HTC state. Seeds stored at −18 °C and ambient humidity showed no changes in all of these parameters. The pH change is likely to be a cause of decreased protein solubility and thermal stability since extractability or T_m vs tissue pH fit well into pH-dependent curves of extraction and heating. Extraction was minimal when water extract pH was 4 and increased at more acid and alkaline pH values. T_m was above 100 °C at pH 6.5 and decreased as pH dropped. It is hypothesized that the hard-to-cook defect is due in part to protein insolubilization and thermal destabilization (reversible denaturation) during storage. Those changes could lead to formation of a protein network, limiting starch gelatinization during cooking.

INTRODUCTION

A major constraint associated with consumption of certain legume seeds is the storage-induced hard-to-cook (HTC) defect. The term indicates resistance of seeds to softening during cooking. Two explanations for the HTC defect have been proposed: (1) pectin insolubilization via binding with divalent cations as a result of phytate breakdown (Mattson, 1946; Jones and Boulter, 1983) and (2) cell lignification via cross-linking of phenolics with cell wall proteins (Hincks and Stanley, 1987). However, the complex mechanism of HTC defect has not been clearly elucidated. Our previous studies showed inadequacy of the cell wall pectin–cation model to explain HTC development in cowpeas (Liu et al., 1992a,b) and implied possible involvement of intracellular protein and starch (Liu et al., 1992c).

Cowpea cotyledon cells contain starch granules embedded in a protein matrix consisting of protein bodies. Interactions among proteins and between protein and starch or other components could occur during storage. These interactions may lead to changes in functional properties of seed protein and starch, which in turn affect seed cookability and utilization. Evidence supporting this expectation is that cooked HTC seeds exhibit limited starch gelatinization under microscopic examination (Varriano-Marston and Jackson, 1981; Carabez-Trejo et al., 1989). Among the functional properties of proteins, solubility is the most critical one because it affects many others, such as foaming and emulsifying capacity and gelling ability (Kinsella, 1976). Changes in total protein extractability/solubility with storage have been reported in soybeans (Nash et al., 1971; Saio et al., 1980) and cottonseeds (Conkerton et al., 1991). Changes in solubility of different protein fractions with storage were also reported with cowpeas (Hentges et al., 1991). Thermal stability is another important functional property since it determines coagulation/gelation which occurs normally during heating. There is limited information regarding storage effects on thermal stability of seed proteins (Garcia-Vela and Stanley, 1989).

Objectives of this study were (1) to investigate the effect of adverse storage on (a) protein solubility and thermal stability in cowpeas and (b) gelatinization temperature of isolated starch and (2) to relate these changes to the hard-to-cook defect. The cause of protein denaturation as a result of storage was also investigated.

MATERIALS AND METHODS

Cowpea Storage. Cowpeas (*Vigna unguiculata* cv. California Blackeye pea No. 5, 11.4% moisture) were obtained from Kerman Warehouse (Kerman, CA) and stored in a covered polyethylene container at 30 °C/64% relative humidity (RH) for 6, 12, and 18 months. The humidity was maintained by placing a glass jar of saturated $NaNO_2$ solution inside the container. At the end of each storage time, samples were kept at ambient temperature (ca 23 °C) and humidity (40–50%) for a week to bring moisture to the ambient and then stored at −18 °C and ambient humidity until analyzed. Cowpeas stored at −18 °C and ambient humidity for the entire period of storage served as a control. For each treatment, duplicate samples were prepared with separate containers. At the time of laboratory measurement, newly harvested cowpeas were obtained from Kerman Warehouse as a gift.

Hard-To-Cook State Measurement. Dry seeds (30 g) were wrapped in cheesecloth, soaked in water at room temperature for 4 h, and cooked in water preheated to 100 °C in a beaker covered with a watch glass on a hot plate for 90 min. Cooking was terminated by transferring the samples into an ice bath. For textural measurement, an Instron universal testing machine (Model 1122, Instron, Inc., Canton, MA) was used. Details are described in a previous study (Liu et al., 1992a).

Protein Extraction. Dry seeds were ground into flour which passed through a screen of No. 50 mesh size. Five grams of the flour was mixed with 100 mL of water in a beaker and stirred for 45 min at ambient temperature. The pH of the mixture was recorded before centrifugation at 9800*g* for 15 min. The supernatant was saved for protein assay and thermal stability determination. Protein extractability was expressed as percentage of Bio-Rad protein (Bio-Rad protein assay procedure, Bio-

* Author to whom correspondence should be addressed at Jacob Hartz Seed Co., 901 N. Park Ave., Stuttgart, AR 72160.

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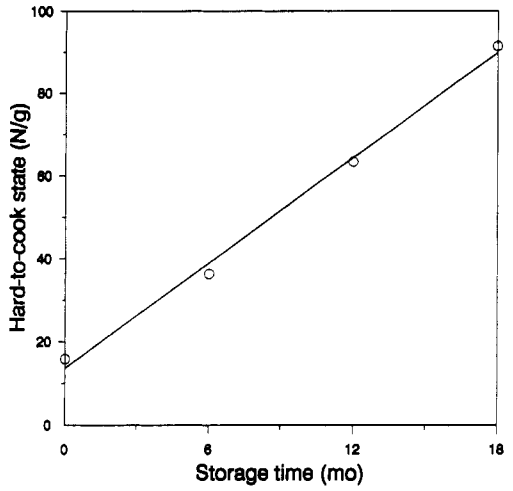

Figure 1. Hard-to-cook state of cowpea seeds as a function of storage time at 30 °C/64% RH.

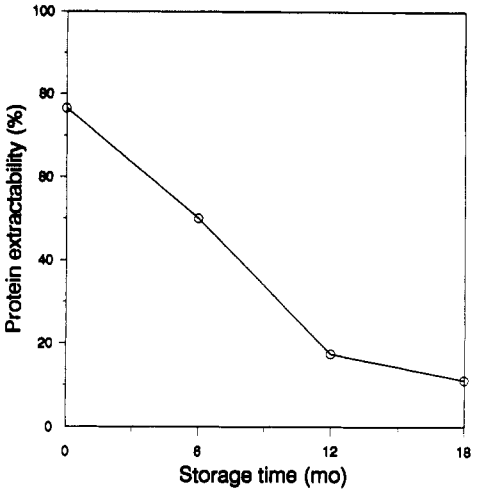

Figure 2. Protein water extractability from cowpea seeds as a function of storage time at 30 °C/64% RH.

Rad Chemical Division, Richmond, CA) in the extract vs Kjeldahl protein (AOAC, 1984) of dry seed. Duplicate measurements were performed for an extract.

The effect of extract pH on protein extraction was studied by slurrying 5 g of the flour with 90 mL of water. The pH of the mixture was adjusted to values from 2.0 to 12.0 with either 0.1 or 1.0 N NaOH or HCl. Since some pH drift occurred, the pH was checked every 15 min and readjusted when necessary. After 45 min of stirring, the volume was brought to 100 mL.

Protein Thermal Stability. The spectrophotometric technique of Deng et al. (1976) was used to study protein–protein interaction during thermal denaturation. The method is based on the principle that association or aggregation of protein molecules during denaturation leads to increasing turbidity of the solution. The protein concentration of a solution was adjusted to 1.0 mg of protein/mL before heating. Change in turbidity with heating temperature was monitored by measuring absorbance at 320 nm.

Isolation of Starch. The procedure described by Bartolome and Hoff (1972) involving water washing was adopted with modification. Samples (30 g) were hydrated and hand-decorticated before being blended in an Oster blender (Osterizer Galaxie). The slurry was washed with water through two screens (No. 60 and 200). Starch granules were collected from the washing liquors. Repeated washing was done by allowing the fraction to settle in a beaker and decanting the supernatant. Finally, the isolated starch was dried overnight in a forced-air oven at 40 °C.

Starch Gelatinization Temperature. Starch slurry (5 g in 50 mL) in a set of tubes was heated in a beaker with water. Between 60 and 80 °C, a tube was withdrawn every 4 °C and cooled in an ice bath. A drop of the slurry was observed under a polarizing microscope. Using this sort of double layer was expected to eliminate the hot-wall effect associated with the starchy system.

RESULTS AND DISCUSSION

Seed HTC State. Cowpeas stored at 30 °C/64% RH exhibited an apparently linear increase in HTC state with time (Figure 1). As the time increased from 0 to 18 months, seed hardness increased from 15.8 to 91.2 N/g. Aguilera and Rivera (1992) found a lag period for the first few weeks, a period for which we obtained no data.

Protein Extractability. A gradual decrease in total water-soluble protein was observed with time of storage at 30 °C/64% RH (Figure 2). As the storage time increased from 0 to 18 months, protein extractability decreased from 76.5 to 11.2%. This was in sharp contrast to the study by Hentges et al. (1991), who reported slight changes in solubility of different protein fractions from cowpeas with storage. However, a dramatic reduction in the salt-soluble or water-soluble protein as a result of adverse storage was reported in other seeds (Conkerton et al., 1991; Saio et al., 1980).

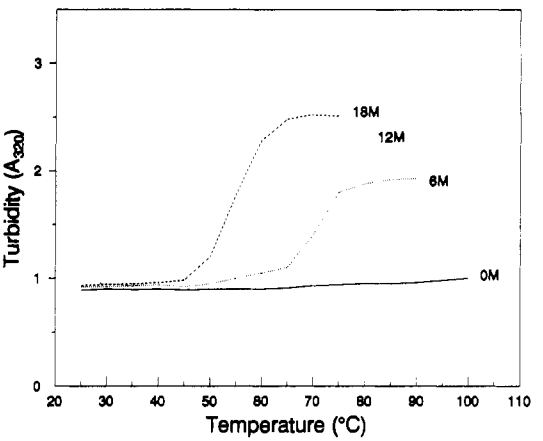

Figure 3. Turbidity change in solutions of water-extractable proteins from cowpeas as a function of heating temperature. 0M, 6M, 12M, and 18M represent 0, 6, 12, and 18 months of storage, respectively, at 30 °C/64% RH.

Protein Thermal Stability. Figure 3 records turbidity change in solutions of water-extractable proteins with heating temperature. Curves corresponding to each storage time (0, 6, 12, and 18 months) at 30 °C/64% RH exhibited a temperature range of very rapid rise to a maximum level. The slope of the steepest line, which could be drawn from the rising point to the point where the rate of change started to taper off, was used to determine the rate and maximum extent of protein–protein interaction. The corresponding temperature of the maximum interaction was referred to as the protein thermal transition temperature (T_m).

A decrease in thermal stability of water-extractable protein was observed with time of adverse storage (Table I). Protein from control seeds exhibited a T_m higher than 100 °C, and no coagulant was observed. Seeds stored for 18 months exhibited a T_m of 56 °C. In contrast to protein denaturation, the gelatinization temperature of isolated starch remained constant during storage. Protein denaturation and starch gelatinization in hard-to-cook black beans have been studied previously by Garcia-Vela and Stanley (1989), using differential scanning calorimetry. However, in contrast to our study, they observed no significant difference in protein denaturation temperature between hard and soft beans.

Seed Tissue pH. Further study was needed to determine the cause of decreased protein solubility and thermal stability during storage. First, the pH profile of seed tissue

Table I. Effect of Storage at 30 °C/64% RH on Thermal Stability of Water-Extractable Protein from Cowpeas and Starch Gelatinization[a]

	storage time			
	0 months	6 months	12 months	18 months
thermal transition temp, °C	>100	72	64	56
temp at which coagulants appeared, °C		76	67	58
starch gelatinization temp, °C	64–73			64–73

[a] Duplicate measurements; concentration of protein solutions for heat stability study was 1 mg/mL.

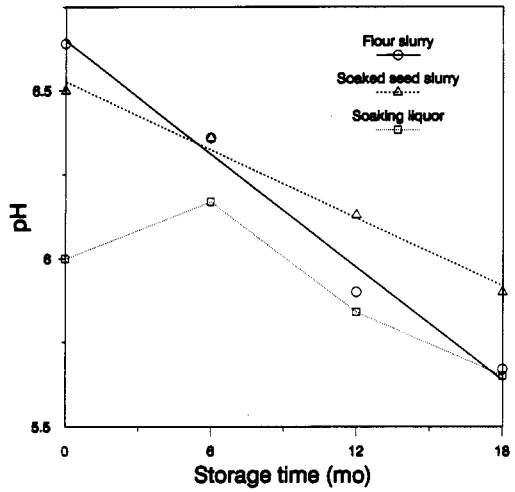

Figure 4. pH change of cowpea preparations with storage time at 30 °C/64% RH.

was recorded with storage time at 30 °C/64% RH. The pH measurement was performed on three preparations: flour slurry, 6-h soaking liquor, and slurry of soaked seeds with fresh water. Results show that as storage time increased, pH of the soaking liquor increased up to 6 months of storage and then decreased (Figure 4). The pH of the flour slurry and soaked seed slurry decreased linearly with storage time, but the slope of the flour slurry curve was steeper than that of the soaked seed slurry. The difference was apparently due to acid leakage during soaking. Therefore, flour slurry pH best represents tissue pH. An increase in acidity during storage was also reported in soybeans (Saio et al., 1980; Thomas et al., 1989).

Ching and Schoolcraft (1968) observed increases in amino acids and inorganic phosphates in leaching solutions of aged crimson clover and ryegrass seeds. Hydrolysis of lipids into fatty acids, oxidation of these acids into organic acids, and other active biological processes might also be causes of pH decrease during adverse storage (Saio et al., 1980). Hydrolyses of phytates (Mattson, 1946) and of storage proteins (Hohlberg and Stanley, 1987) have been also reported.

Effect of Control Storage. Newly harvested cowpea seeds were also tested for HTC state, protein water extractability and thermal stability, and tissue pH. None of these parameters were significantly different from those of seeds stored for 18 months at −18 °C and ambient humidity. This implies that the control storage had no effect on the parameters studied.

HTC State and Tissue pH. When HTC state of four groups of seeds stored at 30 °C/64% RH was plotted against the corresponding tissue pH, as measured via flour slurry, a linear relationship was observed with a coefficient of 0.924 (Figure 5). This observation was significant in two

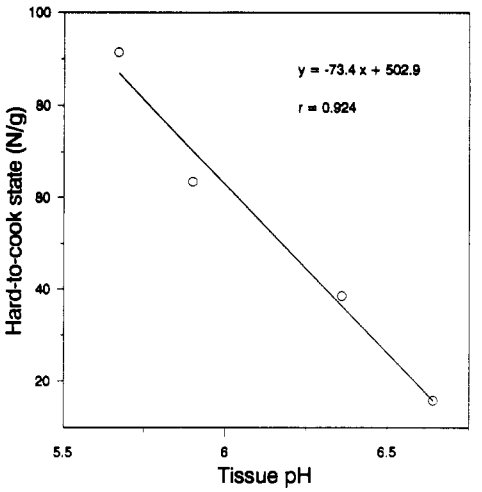

Figure 5. Relationship between hard-to-cook state of cowpea seeds stored at 30 °C/64% RH for four time periods (0, 6, 12, and 18 months) and tissue pH of the corresponding samples, as measured via flour slurry.

aspects. First, it implies that acidification during storage leads to textural defect. Second, it suggests that tissue pH can be a convenient and reliable indicator of seed HTC defect induced by adverse storage.

There have been reports of plant tissue texture being controlled via acidification prior to heat processing (Doesburg, 1961; Sistrunk, 1971; Walter et al., 1992). Inducement of the hard-to-cook defect in legume seeds by acidification was also reported (Vindiola et al., 1986; Carabez-Trejo et al., 1989). Pectin insolubilization is often considered to be the mechanism of hardening via acidification (Vindiola et al., 1986; Walter et al., 1992). Walter et al. (1992) also speculated partial inactivation of endogenous amylolytic enzyme to be another possible route. However, since the opposite effect was observed, they preferred a hypothesis that chemical processes were responsible. On the other hand, pH change with storage has been found with plant seeds (Ching and Schoolcraft, 1968; Saio et al., 1980; Thomas et al., 1989), but the present study is the first to report a relationship between tissue pH and storage-induced textural defect.

Water Extract pH. Protein extraction as affected by water extract pH was investigated with control seeds and seeds stored at 30 °C/64% RH for 12 months (aged seeds). Results show that minimum extraction occurred around pH 4 and that extraction increased at more acid and alkaline pH values (Figure 6). This characteristic pH dependence is apparently determined by isoelectrical properties of seed proteins. Although both control and aged seeds had a similar pH-dependent pattern, aged seeds had slightly lower extraction. For example, at pH 6.5, about 63% protein was extracted from control seeds and 48% from aged seeds. Protein extractability as a function of pH has also been studied in other leguminous seeds (Pant and Tulsiani, 1969).

A decrease in protein solubility during storage of soybeans has been attributed to a decrease in tissue pH (Saio et al., 1980), although divalent cation binding has also been hypothesized (Thomas et al., 1989). Our present study with cowpeas supports the role of tissue pH in causing decreased protein solubility. When protein extractability from four groups of seeds stored at 30 °C/64% RH (0, 6, 12, and 18 months) was plotted against the pH of the corresponding flour slurry, all four points fit well into pH-dependent extraction curves (solid dots, Figure 6).

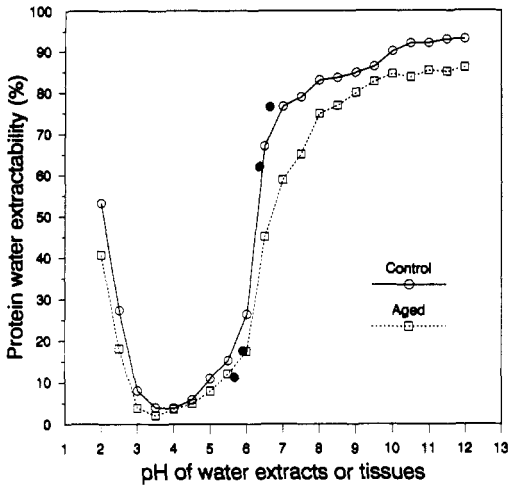

Figure 6. Effect of water extract pH on protein extractability from cowpeas. Control: 12-month storage at −18 °C and ambient humidity. Aged: 12-month storage at 30 °C/64% RH. Solid dots represent protein water extractability from four groups of stored seeds (0, 6, 12, and 18 months at 30 °C/64% RH) vs the corresponding tissue pH.

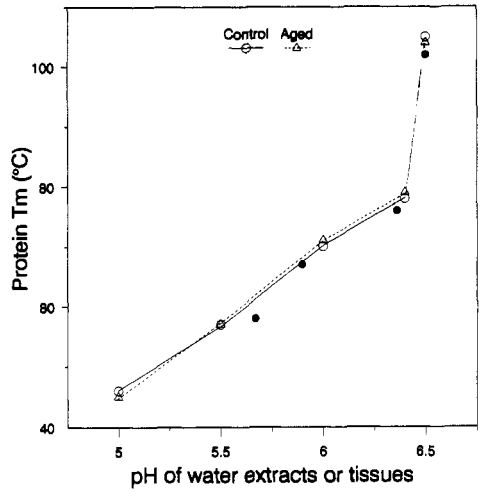

Figure 7. Effect of water extract pH on thermal stability of water-soluble proteins extracted from cowpeas. Control: 12-month storage at −18 °C and ambient humidity. Aged: 12-month storage at 30 °C/64% RH. Solid dots represent thermal transition temperature of protein from four groups of stored seeds (0, 6, 12, and 18 months at 30 °C/64% RH) vs the corresponding tissue pH.

The effect of pH on protein thermal stability was also investigated. In a solution at pH 6.5, protein T_m was beyond 100 °C and there was no visual appearance of coagulants (Figure 7). As pH reduced to 6.4, T_m sharply decreased to 78 °C. As pH continued to be reduced to 5.0, a linear decrease was observed. Unlike protein extraction, both control and aged seeds gave the same T_m at a given pH value, suggesting that pH was the only factor affecting protein thermal stability. Indeed, when T_m for four groups of adversely stored samples was plotted against pH of the corresponding flour slurry, all points fell well within the pH-dependent line (solid dots, Figure 7). Thus, a decrease in protein thermal stability with storage time also resulted from increased acidity of seed tissues. The pH dependence of thermal denaturation was also reported with salt-soluble proteins from chicken muscles (Xiong and Brekke, 1990).

Finally, observations of increased extractability and thermal stability of aged seed proteins as a result of pH change (Figures 6 and 7) indicate that storage-induced protein denaturation is reversible. This finding is very significant in terms of reversibility of bean HTC state, which will be discussed shortly.

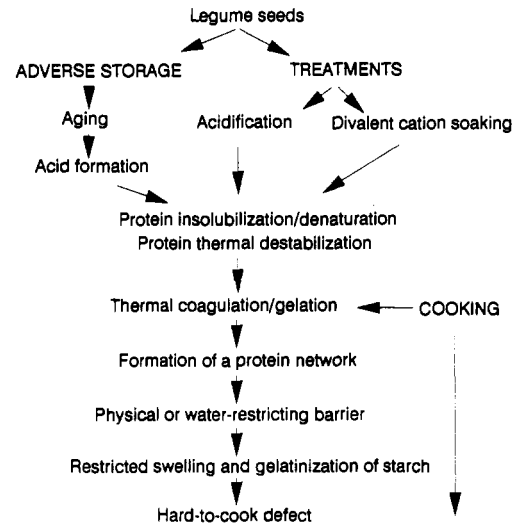

Figure 8. Proposed mechanism and sequential events leading to hard-to-cook defect (storage-induced or treatment-induced) in legume seeds.

Proposed HTC Mechanism. Cowpea cotyledon cells contain starch granules embedded in a protein matrix consisting of protein bodies. During cooking, protein–protein interaction could occur. The consequence of the heat-induced interaction would be unfolding, aggregation, coagulation, or gelation. All of these changes depend on solubility and thermal stability of seed protein (Kinsella, 1976). We propose that decreased protein solubility and thermal stability during storage are directly responsible for the HTC defect in cowpeas. In control seeds, water-extractable proteins constitute about 76% of the total protein and their thermal coagulation during cooking is restricted because of high T_m. In aged seeds, most protein is water-insoluble and those soluble could coagulate readily upon cooking. In addition, protein coagulation/gelation is expected to occur prior to starch gelatinization since protein T_m had decreased below starch gelatinization temperature. Thus, there could be a protein network formed around starch granules which functions as a physical and/or water-restricting barrier. This barrier could lead to restricted starch gelatinization during cooking, which contributes to the hardness of cooked seeds.

Accordingly, any treatments or conditions leading to insolubilization or denaturation of seed protein can induce HTC defect in legume seeds. These include adverse storage (storage-induced), acidification (Vindiola et al., 1986; Carabez-Trejo et al., 1989), and divalent cation soaking (Liu et al., 1992a). The sequence of events leading to the HTC defect is described in Figure 8.

It appears that the proposed mechanism of protein insolubility and heat destabilization during storage and its effect on starch gelatinization during cooking is consistent with the theory of pasta cooking quality proposed and verified by Resmini and Pagani (1983). According to this theory, pasta cooking quality is determined by a physical competition for water between protein coagulation into a continuous network (I) and starch swelling with spherulite scattering (II) during cooking. If the former (I) prevails, starch particles are trapped in the network alveoli, promoting firmness in cooked pasta. If the latter (II) prevails, the protein coagulates in discrete masses lacking a continuous framework. This allows starch to swell fully, and pasta will show softness and usually stickiness.

The proposed mechanism can also account for observations of acid firming in plant tissues containing starch reported previously (Vindiola et al., 1986; Carabez-Trejo et al., 1989; Walter et al., 1992). In addition, since storage-induced protein denaturation is reversible, the practical significance of this mechanism would be that it points out a possibility of reversing bean HTC state via alkalifying. Indeed, softening seeds by soaking in alkaline salt solutions has been a household tradition in some regions of the world for many years (Ankrah and Dovlo, 1978), although the scientific basis behind this practice has not been understood.

Conclusions. Storing cowpeas at 30 °C/64% RH led to decreased tissue pH. This decrease caused reversible protein denaturation, which was reflected in a gradual decrease in water extractability and thermal stability with storage time. Insolubilized and thermal coagulated proteins in aged seeds would restrict starch from full swelling during cooking, leading to HTC defect. Thus, seed protein and starch could play a partial role in directly causing the HTC defect. Further study is underway to verify the proposed mechanism via microstructural examinations of seeds heated at different temperatures.

LITERATURE CITED

Aguilera, J. M.; Rivera, R. Hard-to-cook defect in black beans: hardening rates, water imbibition and multiple mechanism hypothesis. *Food Res. Int.* **1992**, 101–108.

Ankrah, E. K.; Dovlo, F. E. The properties of Trona and its effect on the cooking time of cowpeas. *J. Sci. Food Agric.* **1978**, *29*, 950–952.

AOAC. *Official Methods of Analysis*; Association of Official Analytical Chemists: Washington, DC, 1984.

Bartolome, L. G.; Hoff, J. E. Firming of potatoes: biochemical effects of preheating. *J. Agric. Food Chem.* **1972**, *22*, 266.

Carabez-Trejo, A.; Paredes-Lopez, O.; Reyes-Moreno, C. Microstructure of cotyledon cells from hard-to-cook common beans. *Starch/Staerke* **1989**, *41* (9), 335–339.

Ching, T. M.; Schoolcraft, I. Physiological and chemical differences in aged seeds. *Crop Sci.* **1968**, *8*, 407–409.

Conkerton, E. J.; Schneider, G. R.; Bland, J. M.; Marshall, H. F., Jr.; Goynes, W. R., Jr. Microwave heating to prevent deterioration of cottonseed during storage. *J. Am. Oil Chem. Soc.* **1991**, *68*, 834–839.

Deng, J.; Toledo, R. T.; Lillard, D. A. Effect of temperature and pH on protein-protein interaction in actomysin solutions. *J. Food Sci.* **1976**, *41*, 273–277.

Doesburg, J. J. Relation between the behavior of pectic substances and changes in firmness of horticultural products during heating. *Qual. Plant. Mater. Veg.* **1961**, *8*, 115.

Garcia-Vela, L. A.; Stanley, D. W. Protein denaturation and starch gelatinization in hard-to-cook beans. *J. Food Sci.* **1989**, *54*, 1284–1286, 1292.

Hentges, D. L.; Weaver, W. M.; Nielsen, S. S. Changes of selected physical and chemical components in the development of the hard-to-cook bean defect. *J. Food Sci.* **1991**, *56*, 436–442.

Hincks, M. J.; Stanley, D. W. Lignification: evidence for a role in hard-to-cook beans. *J. Food Biochem.* **1987**, *11*, 41.

Hohlberg, A. I.; Stanley, D. W. Hard-to-cook defect in black beans. Protein and starch consideration. *J. Agric. Food Chem.* **1987**, *35*, 571–576.

Jones, P. M. B.; Boulter, D. The cause of reduced cooking rate in *Phaseolus vulgaris* following adverse storage conditions. *J. Food Sci.* **1983**, *48*, 623.

Kinsella, J. E. Functional properties of proteins in foods: a survey. *Crit. Rev. Food Sci. Nutr.* **1976**, *15*, 219–280.

Liu, K.; Phillips, R. D.; Hung, Y.-C.; Shewfelt, R. L.; McWatters, K. H. Hard-to-cook defect in cowpeas: storage-induced and treatment-induced development. *J. Food Sci.* **1992a**, *57*, 1155–1160.

Liu, K.; Phillips, R. D.; Hung, Y.-C. Development of hard-to-cook defect in cowpeas: role of pectin methylesterase. *J. Agric. Food Chem.* **1992b**, *40*, 949–952.

Liu, K.; Phillips, R. D.; McWatters, K. H. Induced hard-to-cook state in cowpeas by freeze-thawing and calcium chloride soaking. *Cereal Chem.* **1992c**, in press.

Mattson, S. The cookability of yellow peas: a colloid-chemical and biochemical study. *Acta Agric. Suec.* **1946**, *2*, 185–231.

Nash, A. M.; Kwolek, W. F.; Wolf, W. J. Denaturation of soybean proteins by isoelectric preparation. *Cereal Chem.* **1971**, *48*, 360–368.

Pant, R.; Tulsiani, D. R. P. Solubility, amino acid composition, and biological evaluation of proteins isolated from leguminous seeds. *J. Agric. Food Chem.* **1969**, *17*, 361–365.

Resmini, P.; Pagani, M. A. Ultrastructure studies of pasta. A review. *Food Microstruct.* **1983**, *2*, 1–12, 98.

Saio, K.; Nikkuni, I.; Ando, Y.; Otsuru, M.; Terauchi, Y.; Kito, M. Soybean quality changes during model storage studies. *Cereal Chem.* **1980**, *57*, 77–82.

Sistrunk, W. A. Carbohydrate transformations, color and firmness of canned sweet potatoes as influenced by variety, storage, pH, and treatment. *J. Food Sci.* **1971**, *36*, 39–42.

Thomas, R.; deMan, J. M.; deMan, L. Soymilk and tofu properties as influenced by soybean storage conditions. *J. Am. Oil Chem. Soc.* **1989**, *66*, 777–782.

Varriano-Marston, E.; Jackson, G. M. Hard-to-cook phenomenon in beans: structural changes during storage and imbibition. *J. Food Sci.* **1981**, *46*, 1379–1385.

Vindiola, O. L.; Seib, P. A.; Hoseney, R. C. Accelerated development of the hard-to-cook state in beans. *Cereal Foods World* **1986**, *31*, 538–552.

Walter, W. M., Jr.; Fleming, H. P.; McFeeters, R. F. Firmness control of sweetpotato French fry-type product by tissue acidification. *J. Food Sci.* **1992**, *57*, 138–142.

Xiong, Y. L.; Brekke, C. J. Thermal transitions of salt-soluble proteins from pre- and postrigor chicken muscles. *J. Food Sci.* **1990**, *55*, 1540–1570.

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