Development of Hard-to-Cook Defect in Cowpeas: Role of Pectin Methylesterase

Keshun Liu,* R. Dixon Phillips, and Yen-Con Hung

Department of Food Science and Technology, Georgia Agricultural Experiment Station, University of Georgia, Griffin, Georgia 30223-1797

Development of the hard-to-cook (HTC) defect in cowpeas has been shown to consist of two stages: an increase in cation-uptake capacity followed by binding of divalent cations. Activation of pectin methylesterase (PME) leading to demethylation of cell wall pectin was believed to be responsible for the first-stage development. To confirm this hypothesis, a headspace gas chromatographic method was used to monitor changes in free methanol, PME activity, and methoxy content in cowpeas during HTC-inducing treatments (heat incubation and/or $CaCl_2$ soaking) and adverse storage (30 °C/64% relative humidity). Results showed that PME activity decreased progressively with incubation temperature and time. The difference in PME activity between the control and aged seeds was insignificant, while the difference in methanol released was significant. The degree of pectin methylation of the cell wall remained unchanged in both treatment- and storage-induced seeds. It was concluded that an increase in cation-uptake capacity during HTC development is not due to PME action or pectin demethylation, implying inadequacy of the cations–pectin egg-box model to explain the HTC defect.

INTRODUCTION

It is well-known that certain legume seeds stored under conditions of elevated temperature and humidity progressively increase in resistance to softening during cooking (Stanley and Aguilera, 1985). This so-called "hard-to-cook" (HTC) defect is one of the major constraints associated with legume consumption, imposing economical and nutritional implications.

An early hypothesis on HTC development is that degradation of phytin releases divalent cations which migrate into the middle lamella of the cell wall to bind pectin (Mattson, 1946). Pectin methylesterase (PME) is also believed to be involved in this process by creating new carboxyl groups (Jones and Boulter, 1983; Mafuleka et al., 1991). However, the complex mechanism remains unsolved.

To gain a better insight of HTC mechanism, Liu et al. (1992) found that incubation in water at temperatures between 60 and 85 °C dramatically enhanced the hardening effect of $CaCl_2$ soaking applied subsequently. However, for aged seeds with a certain degree of HTC induced by storage at 30 °C/64% relative humidity, the behavior was different from the soft control seeds; the Ca soaking alone greatly induced the HTC state, while heat incubation only slightly enhanced the hardening effect of Ca soaking. They proposed that HTC development, either treatment-induced or storage-induced, consists of two sequential stages: an increase in cation-uptake capacity and binding of divalent cations. Both moderate heat incubation and adverse storage increase cation-uptake capacity.

One possible explanation for increased cation-uptake capacity is via demethylation of cell wall pectin since PME may be activated during heat incubation or adverse storage. The objective of this study was to verify the role of PME on HTC development in cowpeas. A headspace gas chromatographic (GC) technique (Bartolome and Hoff, 1972a) was modified to monitor changes in free methanol content, PME activity, and methoxy content in cowpeas during treatments and after storage.

MATERIALS AND METHODS

Aged and Control Cowpeas. Cowpeas (*Vigna unguiculata*, cv. California Blackeye No. 5) were purchased from Kerman Warehouse (Kerman, CA). Aged seeds were obtained via storage in a tightly covered polyethylene container (Consolidated Plastics Co., Twinsburg, OH) at 30 °C/64% relative humidity. The humidity was maintained by placing a glass jar of saturated sodium nitrite solution inside the container. After 12 months of storage, seeds were kept at ambient temperature and humidity for a week and then stored at 4 °C until used. Seeds stored at −18 °C for 12 months served as a storage control. For each storage condition, duplicate samples were obtained by means of two separate containers.

Free Methanol Released during Incubation. Seeds (15 g, 11.2% moisture) were hydrated with 86.5 mL of water and 0.6 mL of 0.5% (v/v) 1-propanol internal standard solution in a stoppered Erlenmeyer flask for 60 min. The total volume of the content was 100 mL. The flask was then transferred to a water bath held at selected incubation temperatures of 45, 60, 70, 85, and 100 °C and mounted to a condenser to minimize loss of alcohols. Incubation at 25 °C served as a treatment control. After 90 min (for 100 °C, 20 min), the incubation was terminated by transferring the system to an ice bath. The cooled contents were then blended in a Oster blender (Osterizer Galaxie) at the highest speed for 8 min, interspersed by four cooling periods (each 1 min) in a cold room. One milliliter of slurry was taken into a reaction bottle for nitrite conversion.

PME Activity. Seeds (15 g) wrapped in cheesecloth were hydrated at room temperature for 60 min and then incubated at a selected temperature (25–100 °C) for 90 min (for 100 °C, 20 min). The incubated sample was cooled in an ice bath and washed with deionized water before being blended with 70 mL of cold 1 M phosphate buffer, pH 7.4, in a cold room. One milliliter of slurry was reacted with 9.0 mL of 0.5% pectin (from citrus fruits, Sigma Chemical Co.) solution at 30 °C for 5 min. The pectin solution was prepared in 0.2 M phosphate buffer, pH 7.5, containing a proper level of the internal standard. Zero time reaction was used as a blank. One milliliter of the mixture was taken for nitrite conversion.

Conversion of Alcohols to Nitrites. Alcohols in the test solutions were converted to nitrite esters in a closed reaction bottle (50 mL, serum bottle, Fisher Scientific), sealed with a rubber stopper (Fisher Scientific, size 13). Conversion took place in an ice bath by mixing 1 mL of test solution, 2.5 mL of 7% (v/v) phosphoric acid, and 2.5 mL of 2.5% (w/v) KNO_2 solution. All solutions had been held below 2 °C. The mixture was shaken

0021-8561/92/1440-0949$03.00/0

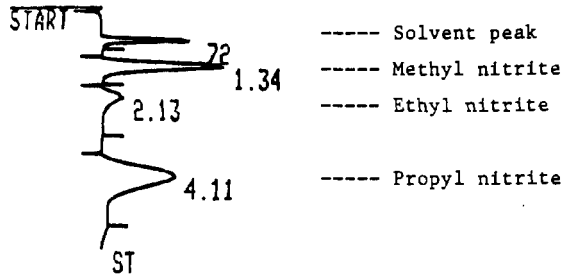

Figure 1. Chromatogram of a headspace chromatographic separation of acyl nitrites.

well and allowed to react for at least 4 min before 1 mL of headspace gas was withdrawn with a 2-mL syringe.

Gas Chromatography. A gas chromatograph (Hewlett-Packard 5890A) equipped with a single flame ionization detector was used. The column was 6 ft × 0.25 in. o.d. stainless steel, packed with 15% Ucon nonpolar LB1715 on Chromosorb WAW, 60/80 mesh (Alltech Applied Science Laboratories, Deerfield, IL). Oven temperature was 60 °C; the injector and detector were maintained at 110 and 120 °C, respectively. Nitrogen was used as a carrier gas at a flow rate of 55 mL/min. Signal responses were recorded in an integrator (Hewlett-Packard 3390A). The relative peak areas were used for concentration calculation. Duplicate measurements were made for each sample.

Standard Solutions. Standard solutions of methanol at concentrations ranging from 0.001 to 0.004% (v/v) and a solution of 1-propanol internal standard at a concentration of 0.003% (v/v) were used to construct a standard curve.

Degree of Pectin Methylation. Estimation of the degree of pectin methylation was carried out by measuring the methoxy content and total galacturonic acid in the cell wall. Before cell wall material (CWM) was isolated, control seeds were subjected to heat incubation, which was done by maintaining hydrated seeds in water in a water bath at 25, 60, or 70 °C for 90 min. Aged seeds were subjected only to 25 °C incubation.

Isolation of CWM. For starchy tissues, the CWM isolation procedure described by Bartolome and Hoff (1972b) involving washing with water and sonication was adopted with modification (Liu et al., 1992).

Methoxy Content of CWM. Total methanol released from the cell wall after alkaline digestion was analyzed by the aforementioned GC method. Dried CWM (100.0 mg) was digested overnight at 4 °C with 20 mL of water, the appropriate amount of the internal standard, and 20 mL of 0.5 N NaOH solution containing 0.25% EDTA. The final volume was brought to 50 mL, and a 1-mL aliquot was taken for nitrite conversion.

Uronic Acid in CWM. Colorimetric determination of total galacturonic acid content in CWM was done with 3,5-dimethylphenol (Eastman Kodak Co.) according to the procedure described by McFeeters and Armstrong (1984). Dry CWM was wetted with water rather than with 95% ethanol. Standard D-galacturonic acid was purchased from Sigma.

RESULTS

GC Chromatogram. A typical GC separation of nitrites is shown in Figure 1. The first peak was a solvent one. This was followed by methyl, ethyl, and propyl nitrites. We found that the fifth peak with a retention time of about 5.3 min would appear if (a) the reaction temperature during nitrite conversion was above 2 °C and/or (b) the concentration of KNO_2 for alcohol conversion was not reduced from 5% of the original method (Bartolome and Hoff, 1972a) to 2.5% of this study. Therefore, to have better separation, these two critical conditions must be well controlled.

Conversion of alcohols to nitrites was very rapid. The original paper recommended a 3-min reaction, but we found that the data became more reproducible when headspace gas was withdrawn after a 4-min reaction. The esters inside the reaction bottle were stable for up to 1 h if the temperature was kept below 2 °C. As reported in the original paper, the headspace GC method for determining PME activity and methoxy content was sensitive and convenient, when compared with the method of titration for free carboxyl group (Schultz, 1965) and the method of direct measurement of methanol (McFeeters and Armstrong, 1984).

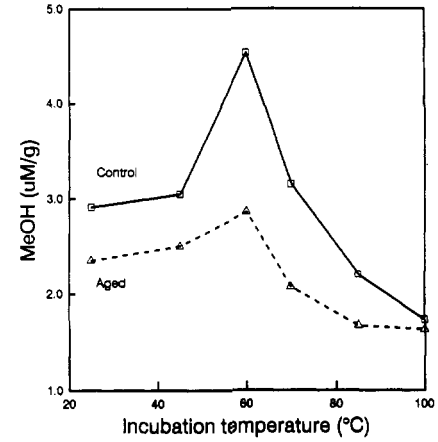

Figure 2. Effect of incubation temperature on free methanol (MeOH) released from control and aged cowpea seeds.

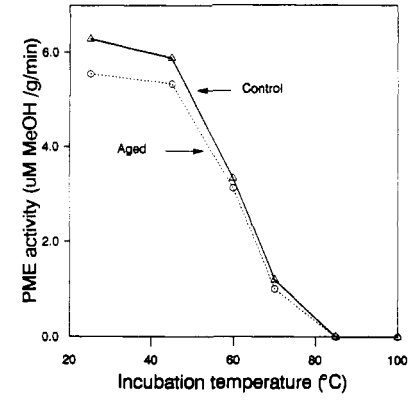

Figure 3. Effect of incubation temperature on pectin methylesterase activity in control and aged cowpea seeds.

Free Methanol Released during Incubation. Methanol released from both control and aged cowpeas during incubation was a function of incubation temperature (Figure 2). Between 25 and 45 °C, there was a slight increase in methanol, but after 45 °C, a sharp increase was noticed, reaching a peak at 60 °C. This was followed by a sharp decrease. The temperature-dependent changes in free methanol were similar for both control and aged samples, but the aged sample released significantly less methanol than the control.

PME Activity. As the incubation temperature increased from 25 to 45 °C, PME activity in both control and aged samples decreased slightly, but beyond 45 °C, a sharp decrease was observed, approaching 0 at 85 °C (Figure 3).

Since the PME activity shown in Figure 3 was assessed after 90 min of incubation, it might be argued that the PME underwent activation and deactivation during this prolonged period. Therefore, an experiment was conducted in which the methanol released and PME activity were both monitored against incubation time at 60 °C where a methanol peak was observed (Figure 2). Results indicate that as incubation time increased, PME decreased gradually and started to level off after 90 min while free methanol increased continually, reaching a plateau after 90 min (Figure 4).

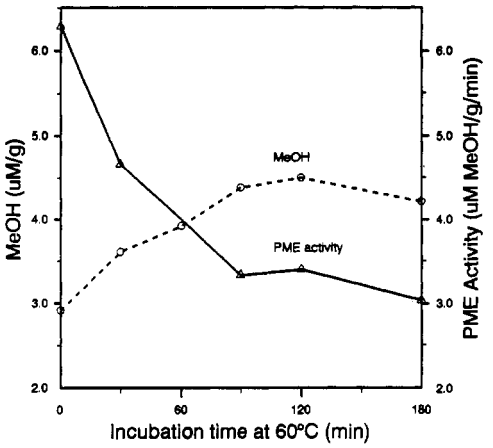

Figure 4. Effect of incubation time on pectin methylesterase activity in and free methanol released from control cowpea seeds.

Table I. Effect of Heat Incubation and Adverse Storage on Galacturonic Acid, Methoxy Content, and Degree of Pectin Methylation in Cell Wall Materials Isolated from Cowpea Cotyledon[a]

cowpea	incubation temp, °C	uronic acid, mmol/g	CV,[b] %	methoxy, mmol/g	CV,[b] %	degree of methylation, %	CV,[b] %
control	25	1.041	2.5	0.284	4.5	27.3	5.1
	60	0.980	4.6	0.275	3.9	28.1	6.0
	70	0.967	5.0	0.267	4.6	27.6	6.8
aged	25	0.830	7.2	0.240	2.9	28.9	7.8

[a] Quadruplicate samples were analyzed, dry weight basis. [b] Coefficient of variation.

Galacturonic Acid, Methoxy Content, and Degree of Pectin Methylation in CWM. Incubation of control seeds at 60 or 70 °C led to a slight decrease (0.061–0.074 mmol/g) in galacturonic acid content in CWM while adverse storage (30 °C/64% relative humidity, 12 months) resulted in a large reduction (0.211 mmol/g) (Table I). However, due to similar changes in the methoxy content of the cell wall, the degree of pectin methylation in CWM remained relatively constant regardless of incubation temperature and adverse storage.

DISCUSSION

Pectin methylesterase is widely distributed in higher plants and associated with demethylation of pectin (Kertesz, 1951). The enzyme has received considerable attention due to its influence on plant texture (Hsu et al., 1965; Collins, 1970; McFeeters et al., 1985).

In a textural study with other species, Bartolome and Hoff (1972b) observed that preheating potato tubers at temperatures between 60 and 70 °C reduced sloughing during cooking. They also noted increases in free methanol and CWM Ca^{2+} and Mg^{2+} contents and a decrease in methoxy content of CWM at the effective temperature and concluded that activation of PME leading to demethylation of cell wall pectin was responsible for the firming effect of the preheating. However, results of this study suggest that the hardening effect of moderate heat incubation with subsequent divalent cation soaking on cowpea seeds observed previously (Liu et al., 1992) was apparently not due to PME action or demethylation of pectin. First, methanol released during incubation was not directly related to HTC development, since the methanol–temperature curve (Figure 2) did not parallel the HTC state–temperature curve reported previously (Liu et al., 1992). The methanol curve had a peak at 60 °C, while the textural curve had a peak around 70 °C. Second, methanol released during incubation was not due to activation of PME. This conclusion resulted from two observations: (1) PME decreased progressively with either incubation temperature (Figure 3) or incubation time (Figure 4), and (2) the difference in PME activity between control and aged seeds was insignificant (Figure 3), while the difference in methanol released was significant (Figure 2). It appears that methanol released as a result of incubation mainly came from intracellular components rather than from cell wall pectin because the methoxy content of the cell wall was only slightly affected by incubation (Table I). Third, an increase in cation-uptake capacity during HTC development did not result from demethylation of cell wall pectin since the degree of pectin methylation in CWM remained rather constant regardless of heat incubation or storage condition (Table I). The observed decrease of galacturonic acid in aged seeds may result from pectin decomposition or from a change in pectin solubility during adverse storage.

It is commonly agreed that the effect of PME on plant tissue texture is manifested by demethylation of cell wall pectin, which then binds cations. The cation cross-linked pectate structure is known as the "egg-box" model, which postulates that divalent cations of an appropriate size could fit into ionic pockets formed by adjacent, negatively charged polysaccharide chains (Grant et al., 1973). In the previous study (Liu et al., 1992), the observation of a similar hardening effect on cowpeas among the divalent cations as well as the observation of a constant Ca^{2+} content in the cell wall during adverse storage suggested that the egg-box model may not be mainly responsible for development of the HTC defect. Failure to reveal a role of PME in increasing cation-uptake capacity in this study further suggests that the egg-box model is inadequate in explaining the role of cations in inducing the HTC defect in cowpeas.

Several recent studies likewise showed evidence that the egg-box model fails to explain the effect of Ca^{2+} on plant tissues adequately. This evidence includes a lack of correlation between the degree of pectin methylation and cucumber softening (McFeeters and Fleming, 1989) and lentil cookability (Bhatty, 1990). There is also a lack of correlation between the affinity of cations for pectin and the ability of these ions to inhibit cucumber softening (McFeeters and Fleming, 1989) and the ability of these ions to inhibit acid-induced cell wall loosening (Tepfer and Taylor, 1981). More important evidence is the observation made by McFeeters and Fleming (1991) that the pH effect on Ca^{2+} inhibition of cucumber softening exhibits a pattern opposite to that expected.

In summary, our previous study (Liu et al., 1992) showed that in terms of sequential events HTC development in cowpeas progresses through an increase in cation-uptake capacity followed by binding of cations to the sites. The present study further indicates that an increase in cation-uptake capacity is apparently not due to the action of PME or demethylation of cell wall pectin. Alternative causes for the increased cation-uptake capacity during HTC development in cowpeas, such as cell membrane damage leading to an improved access of cations to the existing binding sites, are under investigation.

ACKNOWLEDGMENT

This study was supported by a grant from the Bean/Cowpea Collaborative Research Support Program, U.S. Agency for International Development. We thank Ms. Kay McWatters for her useful comments in preparing the manuscript and Mr. Larry Hitchcock for his assistance in GC analysis.

LITERATURE CITED

Bartolome, L. G.; Hoff, J. E. Gas chromatographic methods for the assay of pectin methylesterase, free methanol, and methoxy groups in plant tissues. *J. Agric. Food Chem.* **1972a**, *20*, 262–266.

Bartolome, L. G.; Hoff, J. E. Firming of potatoes: biochemical effects of preheating. *J. Agric. Food Chem.* **1972b**, *20*, 266–270.

Bhatty, R. S. Cooking quality of lentils: the role of structure and composition of cell walls. *J. Agric. Food Chem.* **1990**, *38*, 376–383.

Collins, J. L. Pectin methyl esterase activity in southern peas (*Vigna sinensis*). *J. Food Sci.* **1970**, *35*, 1–4.

Grant, G. T.; Morris, E. R.; Rees, D. A.; Smith, P. T. C.; Thom, D. Biological interactions between polysaccharides and divalent cations: the egg-box model. *FEBS Lett.* **1973**, *32*, 195–198.

Hsu, C. P.; Deshpande, S. N.; Desrosier, N. W. Role of pectin methylesterase in firmness of canned tomatoes. *J. Food Sci.* **1965**, *30*, 583–588.

Jones, P. M. B.; Boulter, D. The cause of reduced cooking rate in *Phaseolus vulgaris* following adverse storage conditions. *J. Food Sci.* **1983**, *48*, 623–626, 649.

Kertesz, Z. I. Pectic enzymes. In *The Pectic Substances*; Kertesz, Z. I., Ed.; Interscience: New York, 1951; Chapter XIV, pp 353–374.

Liu, K.; Hung, Y.-C.; Phillips, R. D.; Shewfelt, R. L.; McWatters, K. H. Development of hard-to-cook defect in cowpeas: storage-induced and treatment-induced. *J. Food Sci.* **1992**, in press.

Mafuleka, M. M.; Ott, D. B.; Hosfield, G. L.; Uebersax, M. A. Dry bean (*Phaseolus vulgaris*) hardening and the consequence of pectin methylesterase activity in storage. *J. Food Process. Preserv.* **1991**, *15*, 1–18.

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

McFeeters, R. F.; Armstrong, S. A. Measurement of pectin methylation in plant cell walls. *Anal. Biochem.* **1984**, *139*, 212–217.

McFeeters, R. F.; Fleming, H. P. Inhibition of cucumber tissue softening in acid brines by multivalent cation: inadequacy of the pectin "egg box" model to explain textural effects. *J. Agric. Food Chem.* **1989**, *37*, 1053–1058.

McFeeters, R. F.; Fleming, H. P. pH effect on calcium inhibition of softening of cucumber mesocarp tissue. *J. Food Sci.* **1991**, *56*, 730–732.

McFeeters, R. F.; Fleming, H. P.; Thompson, R. L. Pectin esterase activity, pectin methylation, and texture changes during storage of blanched cucumber slices. *J. Food Sci.* **1985**, *50*, 201–205.

Schultz, R. W. In *Methods in Carbohydrate Chemistry*; Whistler, R. L., Ed.; Academic Press: New York, 1965; Vol. 5, pp 189–194.

Stanley, D. W.; Aguilera, J. M. A review of textural defects in cooked reconstituted legumes—the influence of structure and composition. *J. Food Biochem.* **1985**, *9*, 277–323.

Tepfer, M.; Taylor, I. E. The interaction of divalent cations with pectic substances and their influence on acid-induced cell wall loosening. *Can. J. Bot.* **1981**, *59*, 1522.

Received for review August 14, 1991. Revised manuscript received December 26, 1991. Accepted March 17, 1992.

Registry No. PME, 9025-98-3; MeOH, 67-56-1; pectin, 9000-69-5.