JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Characterization of the Temperature Activation of Pectin Methylesterase in Green Beans and Tomatoes

GORDON E. ANTHON* AND DIANE M. BARRETT

Department of Food Science and Technology, University of California, Davis, California 95616

Low-temperature blanching of vegetables activates the enzyme pectin methylesterase (PME), which demethylates cell wall pectins and improves tissue firmness. This temperature activation of PME has been investigated by measuring the formation of methanol in intact tissue of green beans and tomatoes. Rates of methanol formation at temperatures of 35−65 °C were obtained by measuring the release of methanol from thin slices of tomato pericarp or green bean pod material. Activation energies of 112 and 97 kJ mol^{-1} were calculated for PME activity in green beans and tomatoes, respectively. These activation energies indicate that the rate of pectin demethylation at 65 °C will be nearly 100 times that at 25 °C. PME activity was also determined titrimetrically using a solubilized form of the enzyme and purified pectin at temperatures from 30 to 60 °C. Under these conditions, much lower activation energies of 37 and 35 kJ mol^{-1} were obtained for green beans and tomatoes, respectively. Methanol accumulation during heating of whole intact green beans was also determined and yielded an activation energy similar to that obtained with sliced beans. Whole green beans held at room temperature did not accumulate any methanol, but sliced or homogenized beans did. If whole beans were first heated to 45 °C and then cooled, methanol accumulation was observed at room temperature. These results indicate that two factors contribute to the observed high rate of pectin de-esterification during low-temperature blanching: (1) An irreversible change, causing PME to become active, occurs by heating to ≥45 °C. (2) The high activation energy for pectin de-esterification means that the rate of de-esterification increases substantially with increasing temperature.

KEYWORDS: Pectin methylesterase; temperature activation; methanol; low-temperature blanching; tomato; green bean

INTRODUCTION

Maintaining a firm product texture is an important quality consideration in the thermal processing of vegetables. In fresh vegetables texture is determined by both the mechanical strength of the cell wall and cellular turgor pressure. Because membrane integrity and thus turgor pressure are rapidly lost during thermal processing, the most important determinant of texture in processed vegetables is the structure of the cell wall, particularly the degree of polymerization and the level of methylesterification of the galacturonic acid residues within the pectins (*1*−*3*). The level of methylesterification, which is controlled by the activity of the enzyme pectin methylesterase (PME), can affect texture in two ways. First, the free carboxylic acid groups in the unesterified regions of the polygalacturonic acid chains can bind calcium. Calcium bound to these sites can cross-link pectin chains of the middle lamella, creating greater cell to cell adhesion and a firmer texture (*4*). This is the basis for the longstanding practice of adding calcium to products such as diced tomatoes to improve firmness. Second, prolonged high-temperature treatments, such as those needed to sterilize low-acid vegetables such as green beans, can result in the β-eliminative cleavage and solubilization of pectins. This pectin breakdown leads to a further loss of firmness. Because β-elimination occurs only at methylesterified galacturonic acid residues (*5*, *6*), a reduced level of methylesterification reduces the tendency of the pectins to undergo this type of cleavage during high-temperature processing.

It was first demonstrated more than 40 years ago that the texture of thermally processed vegetables could be improved by subjecting the material to a low-temperature (generally 50−70 °C) blanch prior to high-temperature processing (*7*, *8*). This firming effect of low-temperature blanching prior to high-temperature processing has subsequently been studied in a number of vegetables (*9*−*15*). A substantial body of evidence indicates that the firming effect is due to the temperature activation of PME. The resulting reduction in the degree of methylesterification of the pectins in the middle lamella allows for more calcium cross-linking between pectin molecules and a reduced amount of pectin chain cleavage by β-elimination during high-temperature processing. Although both factors may contribute to the observed increase in firmness, previous studies have indicated that increased calcium binding may be the more

* Corresponding author [telephone (530) 752-2585; fax (530) 754-7677; e-mail geanthon@ucdavis.edu].

10.1021/jf051877q CCC: $33.50
Published on Web 12/06/2005

important factor for texture improvement in tomatoes (*16*), whereas reduced β-elimination may be the main factor in green beans (*9*).

It is generally accepted that PME activity increases in a low-temperature blanch, but the mechanism for temperature activation is less well understood. A simple explanation for the increased activity would be that, as with most other enzymes, PME activity simply increases with temperature. Studies on the temperature dependence of PME activity, employing an extracted and, in some cases, purified form of PME and assayed under optimal conditions using purified pectin, have been reported by a number of workers. In most cases only a 2−3-fold increase in activity was observed when the temperature was raised from 25 to 60 °C (*17*−*20*). This relatively small increase in PME activity cannot explain the large increase in pectin de-esterification and texture improvement that occurs during blanching at 50−70 °C. If the temperature response of PME was this small, the benefits of a low-temperature blanch would be small compared to the effect of simply allowing material to sit at room temperature. It is likely then, that the temperature response of the enzyme, once extracted and assayed under artificial conditions, is different from the temperature response of the enzyme acting on the endogenous pectins in intact tissue.

It is possible that the temperature activation of PME involves more than simply the increase in activity with temperature. At elevated temperatures some change in the PME enzyme or its environment may occur such that the enzyme is converted to a different, more active form. One proposal along these lines, originally put forward by Bartolome and Hoff (*10*), is that the activation of PME occurs through an increase in the calcium concentration around the enzyme. This model is based on the observation that in in vitro assays, PME activity can be increased by the presence of calcium or other cations. In this model, elevated temperatures cause a loss of membrane integrity, which allows calcium to leak out of the cells and into the cell wall, activating PME. Although this model has been widely cited, there is actually very little evidence to support it. One serious flaw is that it assumes that the calcium concentration in the cell wall is initially low. Recent measurements made with tomatoes have shown, however, that the calcium concentration in the apoplast is actually much higher than that of the bulk cell sap (*21*). Rupturing the cells would lower rather than raise the calcium concentration surrounding the PME enzyme. In addition, many plants have been shown to contain multiple forms of PME, some of which are only weakly affected by cations such as calcium. For example, in both green beans and tomatoes the most abundant form of PME shows considerable activity even in the absence of added salts (*22*, *23*). One would expect these enzymes to be active even in the absence of any ions leaking out of cellular compartments.

One of the products of pectin methylesterase activity is methanol. Following the production of methanol during a low-temperature blanch is thus one way to measure the activity of PME in intact tissue (*10*, *11*). We have previously shown that when thin pieces of tomato or other vegetable tissue are immersed in water and heated to activate PME, methanol rapidly accumulates in the bathing solution (*24*). Sampling the solution surrounding the plant material provides for a simple nondestructive way to follow the time course of PME activity during low-temperature blanching. Here we have examined the kinetics and temperature response of this process to gain a better understanding of the mechanism for PME activation during low-temperature blanching

MATERIALS AND METHODS

Materials. Alcohol oxidase (from *Pichia pastoris*), Purpald, and apple pectin (degree of methylesterification ~75%) were obtained from Sigma, St. Louis, MO. Roma type tomatoes and green beans (variety not known) were obtained from a local market. Green beans were stored at 4 °C and tomatoes at room temperature for up to 1 week prior to use.

Determination of Methanol. Methanol was quantified using alcohol oxidase and Purpald, as described previously (*24*). Our standard reaction contained 90 μL of 200 mM phosphate buffer (pH 7.2), 10 μL of alcohol oxidase at 0.01 unit/μL in 200 mM phosphate buffer (pH 7.2), 25−50 μL of the sample solution, and H_2O to give a final volume of 200 μL. In experiments when the sample solution contained added calcium, 100 mM citrate buffer (pH 6.5) was used in place of phosphate buffer to avoid the formation of insoluble $Ca_3(PO_4)_2$. For samples that were homogenized in trichloroacetic acid (TCA), an aliquot of 0.5 N NaOH sufficient to neutralize the acid was added and the amount of H_2O in the assay reduced to maintain a final volume of 200 μL. Assays were started by the addition of the alcohol oxidase solution and then incubated in a water bath at 30 °C. After 10 min, 200 μL of a freshly prepared 5 mg/mL Purpald solution in 0.5 N NaOH was added, and the samples were vigorously vortexed to ensure oxygenation. After an additional 30 min at 30 °C, the samples were removed from the water bath and 0.6 mL of H_2O was added for a final volume of 1.0 mL. Absorbance at 550 nm was then determined. Methanol concentrations were determined from a standard curve between 0 and 50 nmol of methanol.

Methanol Production by Tissue Slices. Thin slices (~1−2 mm) of green bean pods and tomato pericarp were prepared using a sharp knife. For incubations a 20 mL serum bottle containing 18 mL of water was preheated to temperatures up to 65 °C in a circulating water bath. The incubation was started by the addition of 2.0 g of the sliced vegetable material. The bottle was closed with a rubber serum stopper and incubated at the desired temperature, with occasional shaking. At various time points 0.1 mL aliquots of the incubation solution were removed with a Hamilton syringe, transferred to small sealed vials, and held on ice. To determine methanol content, 50 μL of the incubation solution was then assayed with alcohol oxidase and Purpald as described above.

In experiments comparing the methanol content of the incubation solution with that of the tissue slices, the procedure was modified as follows. The initial amount of water in the serum bottle was reduced to 10 mL. This water was preheated to 60 °C, and then to start the incubation, 2.0 g of sliced material was added. At the times indicated in the figures the incubation was terminated by adding 5.0 mL of ice-cold 50% TCA, then removing the sample from the water bath and placing it on ice. A 200 μL sample of the incubation solution was saved, and then the remaining 14.8 mL of solution plus 2.0 g of plant material was transferred to a mortar and pestle and homogenized. After clarification of an aliquot of the homogenate by centrifugation at 16300*g* for 3 min, a 30 μL sample of the supernatant and the incubation solution was analyzed for methanol content.

Methanol Production by Whole Beans and Homogenates. Whole green beans were placed in plastic zip-lock bags and sealed, excluding as much air as possible. The bag was then placed in a circulating water bath at the desired temperature. At the times indicated in the figures three beans were removed from the bag and placed on ice. After cooling for at least 2 min, a 1.0 g sample of pod material was cut from each bean and homogenized in a small mortar and pestle in 2.0 mL of 50% TCA. The addition of acid was necessary to ensure inactivation of PME during homogenization. After homogenization, an additional 1.0 mL of H_2O was added, and then an aliquot of the homogenate was transferred to a 1.5 mL centrifuge tube and held on ice or frozen for later assay. Prior to analysis for methanol content, samples were centrifuged for 3 min at 16100*g*. A 25 μL aliquot of the supernatant was then assayed for methanol content using alcohol oxidase and Purpald as described above.

To determine methanol production by homogenates, 2.0 g of plant material was homogenized with 6 mL of H_2O in a mortar and pestle. At the desired times, 100 μL aliquots of the homogenate were removed and mixed with 100 μL of 50% TCA. These samples were then

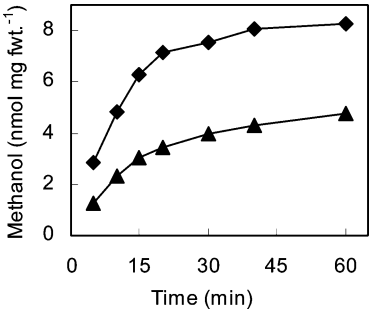

Figure 1. Methanol generation by green bean (▲) and tomato (◆) slices at 60 °C.

processed and assayed for methanol following the same procedure as used for the homogenates prepared from whole beans.

Extraction and Titrimetric Assay of PME. To solubilize and extract PME, green bean pod or tomato pericarp material was homogenized in a mortar and pestle in an equal weight of a solution containing 2.0 M NaCl and 10 mM phosphate buffer (pH 7.5). The homogenate was clarified by centrifugation at 16100*g* for 2 min. An aliquot (equivalent to 0.5−25 μL of the supernatant) was then assayed for PME activity. The high activity level in the tomato supernatant meant that in some cases it was necessary to dilute the tomato supernatant 10-fold with water prior to taking the aliquot for assay. To assay for PME activity, 2.5 mL of a solution containing 0.5% apple pectin, 0.2 M NaCl, and 0.1 mM phosphate buffer (pH 7.5) was put in a stirred, water-jacketed cell and preheated to the desired temperature (30−60 °C). After starting the assay by the addition of the plant supernatant, the pH of the incubation was adjusted to 7.5 by the addition of small aliquots of 0.1 M NaOH. Once the activity of PME had caused the pH to drop to 7.00, the time was noted and a small aliquot of 0.1 N NaOH (1−5 μL) was added. This raised the pH to ∼7.3, and then the elapsed time for the pH to return to 7.00 was recorded. PME activity was then calculated from the amount of added NaOH consumed in the elapsed time.

Data Analysis. Linear regression was used to determine the best straight lines for estimation of rates of methanol production as well as for determining the activation energies in Arrhenius plots. When error estimates are given for activation energies, they are derived from these linear regressions. Error bars, when present in the figures, indicate one standard deviation.

RESULTS

Methanol Production by Tissue Slices. Heating thin slices of both tomatoes and green beans to 60 °C caused the rapid accumulation of methanol in the surrounding solution (**Figure 1**), consistent with what we have observed previously with other vegetables (*24*). In both beans and tomatoes, methanol production slowed considerably after ∼15 min at 60 °C. This reduction in the rate of methanol production could be due to either the depletion of the available pectin methylester substrate by the PME enzyme or a loss of PME activity as the enzyme is slowly inactivated at this temperature. Previous work with tomato PME suggests that inactivation is not likely to be a factor at 60 °C. Although we have no direct evidence that this methanol is the product of PME catalyzed de-esterification of pectin, we know of no other reaction in plant material that could account for its production. Other workers have shown that pectin de-esterification is the predominant source of methanol from plant material (*25*−*27*). The initial rate of methanol production is thus a measure of the PME activity in the plant material at that temperature.

The methanol measurements in **Figure 1** were made by sampling the solution surrounding thin slices of plant material. To establish that the initial rate of methanol accumulation in the surrounding solution accurately reflects the rate of methanol

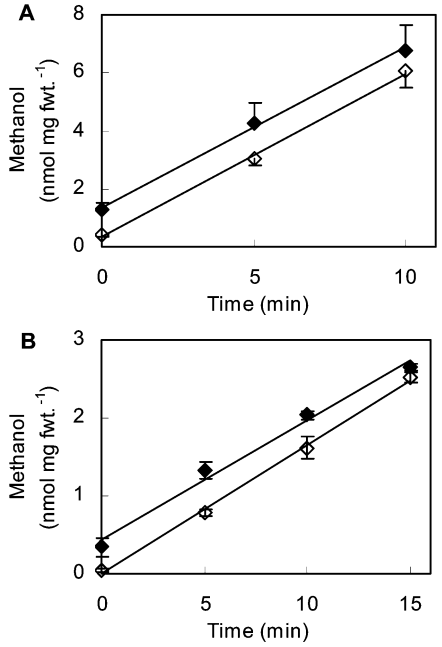

Figure 2. Time course for methanol formation by tomatoes (**A**) or green beans (**B**), as determined by analyzing the whole sample (plant material plus surrounding water; ◆) or the surrounding water alone (◇). Data points are the means of three replicates.

production in the plant material, the two quantities were compared. To do this, a sample of plant material in water was heated to 60 °C as in **Figure 1**, and then after incubation for up to 15 min, an aliquot of the surrounding water was taken and saved. Immediately following this, cold TCA was injected into the sample to stop any further PME activity; then the entire sample, plant material plus surrounding solution, was homogenized. The methanol content of this homogenate was then compared with that of the reserved aliquot of the surrounding water. In both tomatoes and green beans the initial rate of methanol accumulation in the surrounding water was nearly the same as the rate of accumulation in the total homogenized sample (**Figure 2**). Although the slopes are nearly the same, in both cases the apparent amount of methanol in the complete homogenate is slightly higher than that in the surrounding water. Because our method for determining methanol, enzymatic conversion to formaldehyde followed by colorimetric detection by Purpald, is not completely specific for methanol, it is possible that this additional amount of apparent methanol in the homogenates actually arises from other substances in the plant material that interfere with our assay. For example, it has been shown that most plant materials, including tomatoes, can contain up to 1 nmol/mg of fresh weight of bound formaldehyde (*28*).

To determine the temperature dependence of PME activity, initial rates of methanol production were determined for both green beans and tomatoes at a series of temperatures. For this measurement, the accumulation of methanol in the surrounding solution was used to estimate the production of methanol by the plant material. At each temperature investigated a linear rate of methanol production was obtained (**Figure 3**). From the slopes of these lines, rates of methanol production were calculated and plotted versus temperature in an Arrhenius plot. For both green bean and tomato slices, continuous linear plots were obtained (**Figure 4**). From the slopes of these plots activation energies of 97 and 112 kJ mol^{-1} were calculated for tomatoes and green beans, respectively. Similar determinations were done with thin slices of pea pods and carrots over this

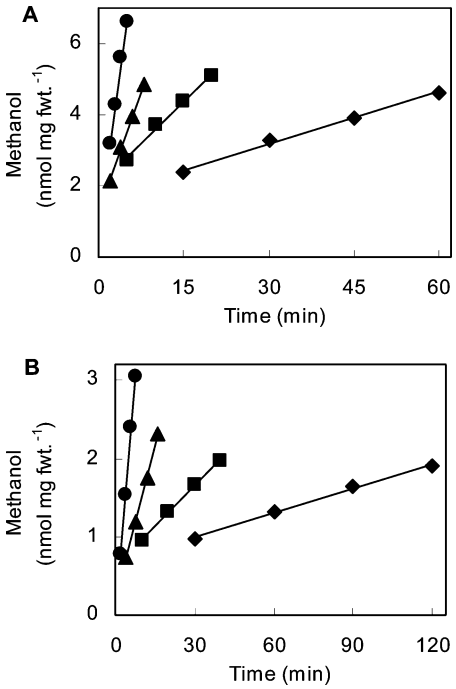

Figure 3. Time course for methanol generation by thin slices of tomato pericarp (**A**) or green bean pod (**B**). Plant material was incubated at 35 °C (◆), 45 °C (■), 55 °C (▲), and 65 °C (●).

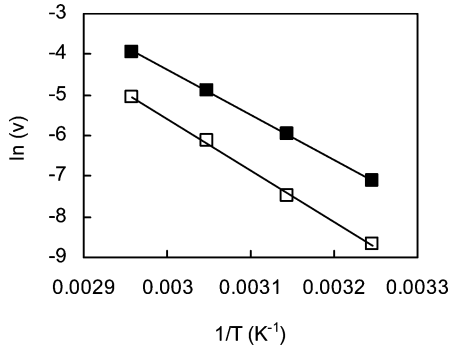

Figure 4. Arrhenius plot for the temperature dependence of the rate of methanol formation by thin slices of tomato pericarp (■) or green bean pods (□). Rates (*v*) were derived from the slopes of the lines in **Figure 3**.

same temperature range, and linear Arrhenius plots were again obtained (data not shown). For both pea pods and carrots activation energies of 130 kJ mol^{-1} were obtained.

The activation energy for pectin de-esterification by PME under commonly used laboratory assay conditions was also determined. In this case PME was extracted from tomatoes and green beans by homogenizing the plant material in high salt to solubilize the enzyme. Activity was then measured titrimetrically at pH 7, using a saturating concentration of purified apple pectin as a substrate. From the activity measured at temperatures between 30 and 60 °C, Arrhenius plots were constructed for both tomatoes and beans (**Figure 5**). In both cases linear plots with similar slopes were obtained. From these slopes activation energies of 35 and 37 kJ mol^{-1} were calculated for tomatoes and green beans, respectively. This value for tomato is in good agreement with the value of 31 kJ mol^{-1} recently reported for PME purified from tomatoes and assayed under similar conditions but between 0 and 25 °C (*29*). These values are also in good agreement with results obtained from titrimetric assays of PME extracted or purified from apples (*17*), potatoes (*18*),

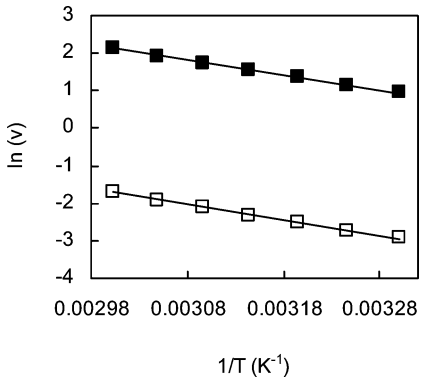

Figure 5. Arrhenius plot for the temperature dependence of PME activity (*v*), assayed titrimetrically with a salt extract enzyme from tomatoes (■) or green beans (□) and purified apple pectin.

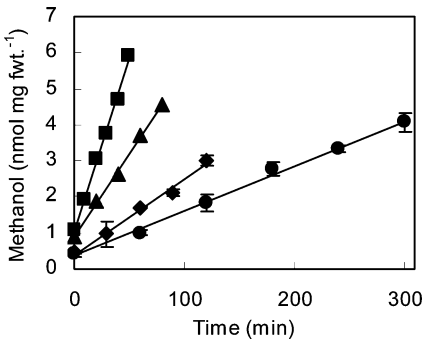

Figure 6. Time course for methanol generation by whole green beans at 45 °C (●), 50 °C (◆), 55 °C (▲), and 60 °C (■). Data points for 45 and 50 °C are the means of three replicates; data points for 55 and 60 °C are single determinations.

onions (*19*), and green peppers (*20*), which gave activation energies of 31, 26, 33, and 23 kJ mol^{-1} respectively.

Experiments with Whole Green Beans. Using thin slices of plant material to measure the kinetics of methanol generation by intact tissue during heating offers several advantages. Heat penetration times will be short and not a significant factor. The methanol produced is rapidly released to the medium, allowing for nondestructive sampling and the collection of many time points. Nevertheless, it could be argued that results obtained with sliced material do not necessarily reflect what occurs in larger intact pieces of tissue. We thus looked at the effects of heating on methanol accumulation in whole green beans. Whole tomatoes were not examined due to their large size and the associated long heat penetration times. For this measurement beans were sealed in a plastic bag, immersed in a water bath for various times, and then cooled and homogenized, and the methanol was content determined. Consistent with the results obtained with thin slices of material, linear increases in methanol content were observed when green beans were incubated at temperatures between 45 and 65 °C. The temperature range examined with whole green beans was narrower than that used with green bean slices because, unlike the sliced material, with which a linear rate of methanol accumulation could be measured at 35 °C (**Figure 3**), methanol accumulation in whole green beans at temperatures below 45 °C was very low and linear time courses were not obtained. Typical time courses of methanol accumulation at several temperatures are given in **Figure 6**. Rates calculated from the slopes of such time courses were plotted versus temperature in an Arrhenius plot (**Figure 7**). From this plot an activation energy of 117 kJ mol^{-1} was obtained, which is similar to the 112 kJ mol^{-1} obtained using

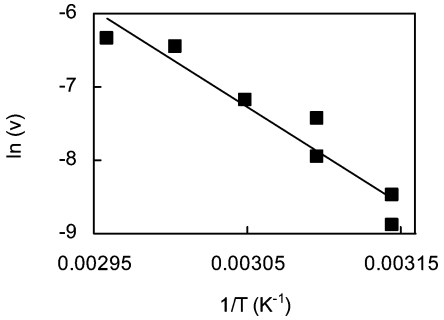

Figure 7. Arrhenius plot for the temperature dependence of the rate of methanol formation (v) by intact whole green beans.

Table 1. Activation Energies for Methanol Production by Whole or Sliced Material and for PME Activity of the Salt Extracted Enzyme, Assayed Titrimetrically

	methanol production (kJ mol^{-1})		
	slices	whole	PME activity
green bean pods	112 ± 3.0	117 ± 15	37 ± 0.8
tomato pericarp	97 ± 0.9		35 ± 0.5

thin slices of beans. A summary of the activation energies is given in **Table 1**.

PME Activity at Room Temperature. Extrapolation of the lines in **Figures 4** and **7** to lower temperatures indicates that a low but measurable rate of methanol accumulation should occur in both sliced and whole green beans held at room temperature. When sliced beans were incubated at room temperature (23 °C) for times up to 30 h, this expected production of methanol was indeed observed (**Figure 8**). From the slope of the line in **Figure 8**, a rate of methanol production of 0.075 nmol mg of fwt^{-1} h^{-1} was calculated. This is in reasonable agreement with a value of 0.11 nmol mg of fwt^{-1} h^{-1} that can be obtained by extrapolating the line in **Figure 4** to 23 °C. By contrast, with intact whole beans no methanol accumulation occurred over 48 h at room temperature (**Figure 8**). Extending this time course up to 7 days also failed to show any methanol accumulation. The lack of methanol accumulation indicates either that no PME activity is occurring at room temperature or that any methanol produced is further metabolized. This latter possibility cannot be excluded because plants are able to metabolize methanol (*30*) and plant surfaces are known to harbor methylotrophic bacteria (*31*). It is possible that these pathways are sufficient to consume the small amount of methanol produced at room temperature, but at higher temperatures they are either thermally inactivated or simply overwhelmed by the increased methanol production, allowing for the observed accumulation.

We also investigated the activity of PME in a green bean homogenate. When green beans were homogenized and incubated at room temperature (23 °C), methanol was produced (**Figure 8**). The rate of methanol production by the homogenate was >5 times that of the sliced material incubated at the same temperature. Similarly, tomatoes homogenized at room temperature rapidly generated methanol (data not shown). The rate of methanol production by tomatoes was many times greater than that of green beans, consistent with a much higher total level of PME activity in tomatoes versus green beans (*22*). Rapid pectin demethylation upon homogenization agrees with the well-known observation from commercial tomato processing that once the tomatoes are comminuted, PME and polygalacturonase will rapidly degrade pectins if not immediately inactivated by heat in the so-called "hot break" process. The high rate of

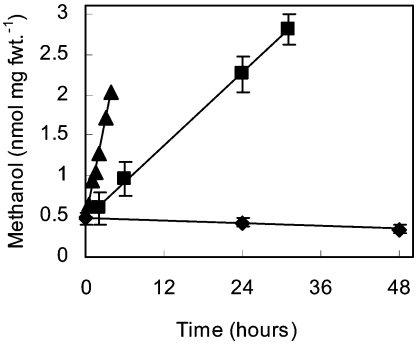

Figure 8. Time course for methanol generation at room temperature (23 °C) by whole green beans (◆), green bean slices (■), or a green bean homogenate (▲). Each point is the mean of three replicates.

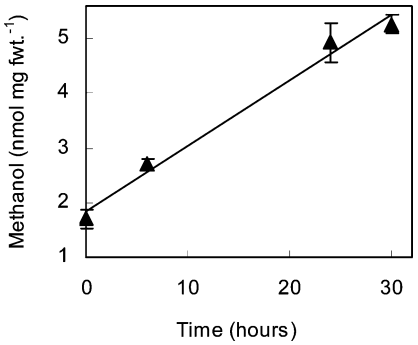

Figure 9. Methanol production at room temperature by preheated green beans.

methanol production upon homogenization indicates that a loss of compartmentation within the plant material leads to a very large increase in PME activity.

Although whole green beans did not accumulate methanol at room temperature, if they were preincubated at 50 °C for 1 h, then cooled and allowed to stand at room temperature, methanol production was observed (**Figure 9**). The rate of methanol production at room temperature by these preheated beans was comparable to that obtained with the sliced material. The higher initial level of methanol in these heated beans reflects the activity of PME occurring during the 1 h preheating step. It is apparent then that heating intact beans to 50 °C causes PME to become active and that the enzyme remains active even after the material is cooled to room temperature. The temperature dependence of this PME activation was investigated by incubating whole beans for 1 h at a series of temperatures, measuring the methanol content, then allowing the beans to incubate for a further 23 h at room temperature, and again measuring methanol (**Figure 10**). One hour incubations at temperatures of ≤40 °C did not lead to any accumulation of methanol either initially or after the extended room temperature incubation. Above 40 °C, methanol was produced both during the 1 h at elevated temperature and during the subsequent room temperature incubation.

Effect of Calcium and EDTA. Because calcium is known to activate PME activity, it has been proposed that an increase in the cell wall calcium concentration is an essential element in the temperature activation of PME. Consequently, the effects of calcium and the calcium chelator EDTA on the production of methanol from green bean slices were examined. Green bean slices were vacuum infiltrated with either water, 50 mM $CaCl_2$, or 10 mM EDTA. They were then immersed in these same solutions and preheated to 60 °C, and the production of methanol over time was measured. Samples treated with calcium produced

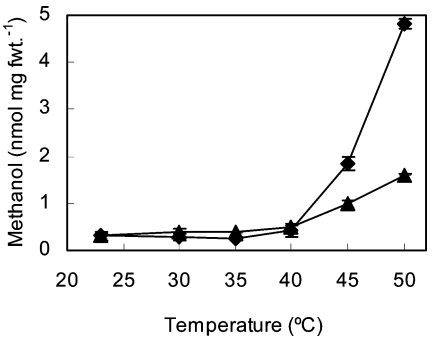

Figure 10. Temperature dependence for methanol formation by whole green beans heated for 1 h at the indicated temperatures (▲) and after a subsequent 23 h incubation at room temperature (◆).

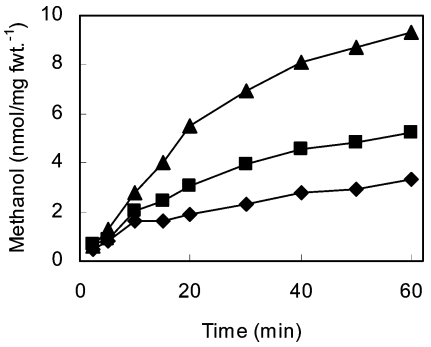

Figure 11. Effect of added calcium or EDTA on the time course of methanol generation by green bean slices. Green bean slices were incubated at 60 °C in water (■), 50 mM $CaCl_2$ (▲), or 10 mM EDTA (◆).

~2 times the amount of methanol as those in water (**Figure 11**). This enhanced production of methanol by added calcium agrees with our previous results for tomato slices, which showed a similar doubling of methanol production by added calcium (*16*), and is consistent with the known activating effect of calcium on PME. Treatment with EDTA reduced the amount of methanol produced but did not entirely prevent it. This indicates that a portion of the PME activity in green bean pods does not require calcium for activity, which is consistent with what has been reported (*22*).

DISCUSSION

The rate of methanol production determined by measuring the methanol content of the surrounding solution closely parallels the rate of methanol production in the plant material. This indicates that the methanol produced by the action of PME rapidly diffuses out of the plant material into the surrounding solution. This is not surprising given that thin (1−2 mm thick) slices of material were used and that the methanol produced from pectin de-esterification is formed outside the plant cells in the cell wall where no barrier to diffusion would be present. The advantage of measuring the methanol content of the surrounding solution rather than the plant material directly is that the sampling is nondestructive and multiple time points can be taken on a single sample. Using thin slices also greatly reduces the come-up time during heating and permits accurate kinetic measurements over relatively short time courses. For many commodities, such as carrots or tomatoes, the large size and slow heat penetration times would confound any interpretation of methanol accumulation during heating if whole vegetables were used. In the case of green beans reasonable kinetics could be obtained with whole intact material and the calculated activation energy for PME activity agrees with that obtained from slices (**Table 1**).

The most important factor in the large increase in PME activity during low-temperature blanching is the high activation energy for PME to act on the endogenous pectins in intact tissue. This high activation energy results in a very large increase in activity with temperature. For example, the activation energy of 112 kJ mol^{-1}, obtained for methanol generation by tissue slices of green beans (**Table 1**), translates into an 88-fold increase in activity at 60 versus 25 °C. Tomatoes, carrots, and pea pods had similarly high activation energies and thus would have similarly large increases in PME activity with temperature. The Arrhenius plots in **Figure 4** are linear and continuous over the temperature range of 35−65 °C, indicating that when thinly sliced material immersed in water is assayed, the increase in activity with temperature does not involve any specific activation of the PME enzyme or change in the reaction mechanism over this temperature range. Using this sliced material, pectin demethylation can be measured even at room temperature (**Figure 8**), although the rate is very low.

The activation energy for PME activity, determined using a salt-extracted PME preparation and assayed with the standard titrimetric assay, was 37 kJ mol^{-1}. This value is in line with what is found for others for soluble enzyme acting on soluble substrates where activation energies are typically in the range of 30−60 kJ mol^{-1} (*32*). At the same time it is only one-third that obtained by measuring PME activity by methanol production from intact tissue (**Table 1**). There are many possible origins for this 3-fold difference in activation energy between the two assays. The solubility and effective concentration of the pectin substrate, as well as the pH and ion concentration surrounding the enzyme, are likely very different in the two assays. Any one of these factors could alter the observed activation energy of the enzymatic reaction. It is also possible that some step in the overall process of converting the substrate in intact tissue, other than the actual demethylation reaction, is strongly temperature dependent and determines the observed temperature dependence. For example, elevated temperatures may induce changes in the structure of the pectin matrix of the cell wall and middle lamella and allow for the PME enzyme to come in contact with its substrate. The observed activation energy could reflect the temperature dependence of this process rather than that of the demethylation reaction.

The activation energy of 37 kJ mol^{-1}, found with the titrimetric assay of the extracted green bean enzyme, implies only a 4-fold increase in activity at 60 versus 25 °C. This is in contrast to the 88-fold increase in methanol production by tissue slices over the same temperature range. Measuring the effect of temperature on PME activity using a solubilized or purified PME preparation and the standard titrimetric assay thus does not give information relevant to the temperature activation of PME in intact tissue. In addition, salt-extracted PME is likely to have a thermal stability different from that of the enzyme in the intact tissue. Together these factors would explain why a poor correlation was previously noted between the temperature dependence of firming by low-temperature blanching and the temperature dependence of PME activity measured titrimetrically with an extracted enzyme (*15*).

A high activation energy and the resulting steep temperature dependence of PME activity in intact tissue has consequences for the design of low-temperature blanching procedures. For practical application of such blanching processes, heating times need to be as short as possible. It is apparent from the kinetic data that temperature differences of only a few degrees will

lead to substantial differences in the rate of pectin demethylation. With green beans, for example, blanching at 60 °C would require twice as long as blanching at 65 °C to achieve the same level of pectin de-esterification. It is thus apparent that the highest possible temperature, one just short of that leading to rapid inactivation of the PME enzyme, should be used. In green beans and tomatoes the principle form of PME is relatively thermostable and is not rapidly inactivated at temperatures below 70 °C (*22*, *33*). We have recently shown that a blanching treatment as short as 5 min at 70 °C caused a significant improvement in the firmness of diced tomatoes (*16*). Most of the available information on PME inactivation is derived from solubilized or purified preparations. Obtaining inactivation kinetics for PME in intact tissue would be very useful for designing low-temperature blanching processes. The high activation energy for PME activity in intact tissue is not the only factor involved in the activation of PME during low-temperature blanching. Whole green beans showed no evolution of methanol when maintained at room temperature. Only when heated above 40°C was any methanol production observed (**Figure 10**). More significantly, once activated by heating to ≥45 °C, the PME enzyme remained active even when the material was brought back to room temperature. This would indicate that some type of irreversible change took place in the PME enzyme or its environment during the heating. A loss of membrane integrity and leakage between cellular compartments at temperatures >40 °C would be a simple explanation for this irreversible change. Consistent with this, we found that other treatments that rupture cellular membranes, such as freezing and thawing the tissue, also caused green beans to produce methanol at room temperature (data not shown). This would not, however, explain why the sliced material gave continuous Arrhenius plots down to 35 °C () and green beans were active even at room temperature. It is possible that in the sliced material most of the observed activity is occurring at the cut surfaces where tissue disruption has occurred.

An inhibitor protein that binds specifically to PME and inhibits its activity has recently been identified. This protein (designated PMEI) has thus far been isolated from only a few plant species, but it has been speculated that these inhibitor proteins are ubiquitous in plants and act to control PME activity in vivo (*34*). The irreversible activation of PME we observed by heating intact green beans to temperatures >40 °C could involve changes in the interaction of between PME and PMEI. This would be consistent with the recent observation that the PME−PMEI complexes are labile and dissociate at temperatures >40 °C (*35*).

The appearance of PME activity upon tissue homogenization (**Figure 8**) and the stimulation of activity by added calcium (**Figure 11**) could be taken as support for the ion leakage hypothesis (*10*). However, there are other aspects of our data that do not support this model. Activation of PME at room temperature occurred by slicing and immersing green beans in water (**Figure 8**). It is hard to imagine a mechanism by which this treatment would increase the cation content of the cell wall. Even if one assumes that the calcium concentration within the cells is high and that cutting the material releases cations from the cut cells, immersing the material in a large excess of water would dilute out these ions. Adding calcium to the immersion solution enhanced the production of methanol at elevated temperatures (**Figure 11**), indicating that at least a portion of the PME activity is stimulated by calcium. However, methanol production occurred even when the slices were infiltrated with EDTA, indicating that at least some of the PME activity does not require calcium. This is consistent with the results of PME purification from green beans, where the isozyme designated PE-1 showed little salt stimulation (*22*).

A simpler explanation for the increase in PME activity upon tissue disruption would be that in intact tissue, some or all of the PME enzyme is fully active but physically separated from its substrate. The increase in pectin de-esterification after tissue disruption is simply the result of the enzyme and substrate coming together. There are many other well-known enzymes in plants, such as alliinase, myrosinase, or polyphenol oxidase, where the enzyme and its substrates are normally found in separate compartments, leading to a sudden burst of enzyme activity upon tissue disruption. Immunolocalization data from tomatoes, however, indicates that most of the PME protein is associated with the cell wall and not some other compartment (*36*). It is still possible that the bulk of the pectin with high methoxy content and the PME enzyme are located in different parts of the cell wall or even different regions of the tissue. The activity of PME in tomato pericarp during ripening has been shown to be highly localized (*37*).

The exact temperature dependence and mechanism for the irreversible activation of PME in intact tissue are of little interest in low-temperature blanching applications because these processes will involve temperatures well above 40 °C. Understanding this activation may be of more interest to postharvest handling of materials. For example, air temperature during the harvesting of tomatoes can reach 40 °C, and harvested material often sits in the sun for hours before processing. The internal temperature of this material may well reach a level high enough to activate PME. A more detailed study of the time and temperature dependence of PME activation in tomatoes would thus be of interest.

LITERATURE CITED

(1) Greve, L. C.; Shackel, K. A.; Ahmadi, H.; McArdle, R. N.; Gohlke, J. R.; Labavitch, J. M. Impact of heating on carrot firmness. Contribution of cellular turgor. *J. Agric. Food Chem.* **1994**, *42*, 2896−2899.

(2) Greve, L. C.; McArdle, R. N.; Gohlke, J. R.; Labavitch, J. M. Impact of heating on carrot firmness. Changes in cell wall components. *J. Agric. Food Chem.* **1994**, *42*, 2900−2906.

(3) Stolle-Smits, T.; Beekhuizen, J. G.; Recourt, K.; Voragen, A. G. J.; Van Dijk, C. Changes in pectic and hemicellulosic polymers of green beans (*Phaseolus vulgaris* L.) during industrial processing. *J. Agric. Food Chem.* **1997**, *45*, 4790−4799.

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

(5) Albersheim, P.; Neukom, H.; Deuel, H. Splitting of pectin chain molecules in neutral solutions. *Arch. Biochem. Biophys.* **1960**, *90*, 46−51.

(6) Sajjaanantakul, T.; Van Buren, J. P.; Downing, D. L. Effects of methylester content on heat degradation of chelator soluble carrot pectin. *J. Food Sci.* **1989**, *54*, 1273−1277.

(7) Van Buren, J. P.; Moyer, J. C.; Wilson, D.; Robinson, W. B.; Hand, D. B. Influence of blanching conditions on sloughing, splitting, and firmness of canned snap beans. *Food Technol.* **1960**, *14*, 233−236.

(8) Hoogzand, C.; Doesburg, J. J. Effect of blanching on texture and pectin of canned cauliflower. *Food Technol.* **1961**, *15*, 160−163.

(9) Stolle-Smits, T.; Beekhuizen, J. G.; Recourt, K.; Voragen, A. G. J.; Van Dijk, C. Preheating effects on the textural strength of canned green beans. 1. Cell wall chemistry. *J. Agric. Food Chem.* **2000**, *48*, 5269−5277.

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

(11) Lee, C. Y.; Bourne, M. C.; Van Buren, J. P. Effect of blanching treatments of the firmness of carrots. *J. Food Sci.* **1979**, *44*, 615−616.
(12) Stanley, D. W.; Bourne, M. C.; Stone, A. P.; Wismer, W. V. Low temperature blanching effects on chemistry, firmness and structure of canned green beans and carrots. *J. Food Sci.* **1995**, *60*, 327−333.
(13) Wu, A.; Chang, W. H. Influence of precooking on the firmness and pectin substances of three stem vegetables. *Int. J. Food Sci. Technol.* **1990**, *25*, 558−565.
(14) Ng, A.; Waldron, K. W. Effect of cooking and pre-cooking on cell wall chemistry in relation to firmness of carrot tissues. *J. Sci. Food Agric.* **1997**, *73*, 503−512.
(15) Ni, L.; Lin, D.; Barrett, D. M. Pectin methylesterase catalyzed firming effects on low-temperature blanched vegetables. *J. Food Eng.* **2005**, *70*, 246−254.
(16) Anthon, G. E.; Blot, L.; Barrett, D. M. Improved firmness in calcified diced tomatoes by temperature activation of pectin methylesterase. *J. Food Sci.* **2005**, *70*, C342−347.
(17) Denes, J.-M.; Baron, A.; Drilleau, J.-F. Purification, properties and heat inactivation of pectin methylesterase from apple (cv golden delicious). *J. Sci. Food Agric.* **2000**, *80*, 1503−1509.
(18) Puri, A.; Solomons, T.; Kramer, A. Partial purification and characterization of potato pectinesterase. *Food Chem.* **1982**, *8*, 203−213.
(19) Garcia, E.; Alviar-agnew, M.; Barrett, D. M. Residual pectinesterase activity in dehydrated onion and garlic products. *J. Food Process. Preserv.* **2002**, *26*, 11−26.
(20) Castro, S. M.; Van Loey, A.; Saraiva, J. A.; Smout, C.; Hendrickx, M. Activity and process stability of purified green pepper (*Capsicum annuum*) pectin methylesterase. *J. Agric. Food Chem.* **2004**, *52*, 5724−5729.
(21) Almeida, D. P. F.; Huber, D. J. Apoplastic pH and inorganic ion levels in tomato fruit: a potential means for regulation of cell wall metabolism during ripening. *Physiol. Plant.* **1999**, *105*, 506−512.
(22) Laats, M. M.; Grosdenis, F.; Recourt, K.; Voragen, A. G. J.; Wichers, H. J. Partial purification and characterization of pectin methylesterase from green beans (*Phaseolus vulgaris* L.). *J. Agric. Food Chem.* **1997**, *45*, 572−577.
(23) Warrilow, A. G. S.; Jones, M. G. Different forms of tomato pectinesterase have different kinetic properties. *Phytochemistry* **1995**, *39*, 277−282.
(24) Anthon, G. E.; Barrett, D. M. Comparison of three colorimetric reagents for the determination of methanol with alcohol oxidase. Application to the assay of pectin methylesterase. *J. Agric. Food Chem.* **2004**, *52*, 3749−3753.
(25) Nemecek-Marshall, M.; MacDonald, R. C.; Franzen, J. J.; Wojciechowski, C. L.; Fall, R. Methanol emission from leaves. *Plant Physiol.* **1995**, *108*, 1359−1368.
(26) Frenkel, C.; Peters, J. S.; Tieman, D. M.; Tiznado, M. E.; Handa, A. K. Pectin methylesterase regulates methanol and ethanol accumulation in ripening tomato (*Lycopersicon esculentum*) fruit. *J. Biol. Chem.* **1998**, *273*, 4293−4295.
(27) Obendorf, R. L.; Koch, J. L.; Gorecki, R. J.; Amable, R. A.; Aveni, M. T. Methanol accumulation in maturing seeds. *J. Exp. Bot.* **1990**, *41*, 489−495.
(28) Trezl, L.; Csiba, A.; Juhasz, S.; Szentgyorgyi, M.; Lombai, G.; Hullan, L. Endogenous formaldehyde level in foods and its biological significance. *Z. Lebensm. Unters. Forsch. A* **1997**, *205*, 300−304.
(29) Terefe, N. S.; Delele, M. A.; Van Loey, A.; Hendrickx, M. Effects of cryostabilizers, low temperature, and freezing on the kinetics of pectin methylesterase-catalyzed de-esterification of pectin. *J. Agric. Food Chem.* **2005**, *53*, 2282−2288.
(30) Cossins, E. A. The utilization of carbon-1 compounds by plants. I. The metabolism of methanol-C14 and its role in amino acid biosynthesis. *Can. J. Biochem.* **1964**, *42*, 1793−1802.
(31) Corpe, W. A.; Rheem, S. Ecology of the methylotrophic bacteria in living leaf surfaces. *FEMS Microbiol. Ecol.* **1989**, *62*, 243−250.
(32) Laidler, K. J.; Peterman, B. F. Temperature effects in enzyme kinetics. *Methods Enzymol.* **1979**, *63*, 234−257.
(33) Anthon, G. E.; Sekine, Y.; Watanabe, N.; Barrett, D. M. Thermal inactivation of pectin methylesterase, polygalacturonase, and peroxidase in tomato juice. *J. Agric. Food Chem.* **2002**, *50*, 6153−6159.
(34) Giovane, A.; Servillo, L.; Balestrieri, C.; Raiola, A.; D'Avino, R.; Tamburrini, M.; Ciardiello, M. A.; Camardella, L. Pectin methylesterase inhibitor. *Biochim. Biophys. Acta* **2004**, *1696*, 245−252.
(35) Ly Nyugen, B.; Van Loey, A.; Smout, C.; Verlent, I.; Duvetter, T.; Hendrickx, M. E. Effect of intrinsic and extrinsic factors on the interaction of plant pectin methylesterase and its proteinaceous inhibitor from kiwi fruit. *J. Agric. Food Chem.* **2004**, *52*, 8144−8150.
(36) Pogson, B. J.; Seymour, G. B.; Brady, C. J.; Jones, M.; Goodchild, D. Immunolocalization of pectinases in tomato fruit. *N. Z. J. Crop Hortic. Sci.* **1992**, *20*, 137−146.
(37) Steele, N. M.; McCann, M. C.; Roberts, K. Pectin modification in cell walls of ripening tomatoes occurs in distinct domains. *Plant Physiol.* **1997**, *114*, 373−381.

Received for review August 1, 2005. Revised manuscript received October 19, 2005. Accepted November 4, 2005.

JF051877Q