Gas Chromatographic Methods for the Assay of Pectin Methylesterase, Free Methanol, and Methoxy Groups in Plant Tissues

Linda G. Bartolome[1] and Johan E. Hoff*

Assay methods for pectin methylesterase (E.C. 3.1.1.11), free methanol, and methoxy groups in crude tissue homogenates were developed. The methods are based on the measurement of methanol by gas–liquid chromatography after conversion to methyl nitrite. Conversion is brought about by allowing the sample to react with nitrous acid in a closed tube. 1-Propanol, used as internal standard, is similarly converted to the nitrite derivative. An aliquot of the headspace gas is injected into the gas chromatograph. The enzyme assay is conducted at pH 7.5 and 30°C for 3 min with a buffered 1% pectin solution. Methoxy groups are determined after prior solubilization and hydrolysis of the pectic substances with an EDTA–NaOH solution for 30 min at room temperature. The determination of free methanol requires no pretreatment. Semi-micro modifications, which permit the analysis of as little as 50 mg of fresh tissue, are also presented. The probable error of the assays is 2% and the sensitivity is estimated as 3 ppm of methanol in the sample solution prior to nitrite conversion.

Methods for the assay of pectin methylesterase (PME, E.C. 3.1.1.11) and for the determination of the methoxy content of pectin are based on the measurement of the released methanol (Kertesz, 1951), the increase in free carboxyl groups (Kertesz, 1951; Primo *et al.*, 1966; Gee *et al.*, 1959), or changes in the physical properties of pectin (Koch and Bretthauer, 1952; Rukhlyadeva and Kretinina, 1968). Relatively large and pure samples are required in most of these methods for adequate precision and to prevent interference from foreign substances. None of these methods is sufficiently sensitive to permit studies of enzyme localization or sufficiently simple to allow the convenient handling of many samples.

The methods described in this paper overcome the problems by converting the methanol in crude and highly dilute solutions to methyl nitrite (Hoff and Feit, 1964) and determining the amount of methyl nitrite in the headspace by gas–liquid chromatography (glc) using a flame ionization detector. Crude tissue slurries can be used for the analyses and no purification of the enzyme or isolation of pectin is required.

MATERIALS

Reagent Solutions. PME was assayed in a 1% solution of pectin in 0.2 *M* K_2HPO_4, pH 7.5 containing a proper level of 1-propanol internal standard. Free methanol was converted to methyl nitrite in a 1:1 mixture of 5% (w/w) potassium nitrite and 7% (v/v) phosphoric acid. Both of these solutions should be cold (0 to 5°C) before mixing. Crude plant tissues were macerated in 1 *M* K_2HPO_4, pH 7.5. Pectic substances were extracted and demethoxylated in a 1:1 mixture of 1 *N* NaOH and 0.5% (w/v) EDTA. Standard curves were prepared with standard solutions of 1-propanol and methanol in the concentration range of 0.001 to 0.05% (v/v).

Gas–Liquid Chromatography. Any gas chromatograph with either a single or dual flame ionization detector and any column packing suitable for separation of methyl nitrite from propyl nitrite can be used. In this study, a gas chromatograph equipped with a single flame ionization detector (Model 600, Research Specialties Co.) was used. The column was 6-ft × 0.25-in. o.d. stainless steel, packed with 15% Ucon Non-Polar LB1715 on Chromosorb W, 60 to 70 mesh. It was operated at 50°C with a nitrogen carrier gas flow rate of approximately 60 ml per minute. Typical retention times were, under these conditions, approximately 1.2 min for methyl nitrite and 2 min for propyl nitrite.

Reaction Vessels. Conversion of methanol to methyl nitrite in the macro modification was performed in modified test tubes (Figure 1a), while, in the semi-micro modification, both maceration and conversion of methanol to nitrite were accomplished in smooth-walled tissue grinders (No. JT4830, Scientific Glass Apparatus Co., Inc., Figure 1b).

PROCEDURES

Macro Method. PREPARATION OF THE CRUDE TISSUE SLURRY. Grind a weighed amount of cold plant tissue in a pre-cooled blender with an equal amount of cold (0 to 5°C) 1 *M* K_2HPO_4 buffer until well homogenized. If a change in volume, due to incorporation of air, occurs during homogenization, weigh a known volume of the slurry to obtain a weight basis of the tissue.

PME ASSAY. Transfer 9.0 ml of pectin solution to test tubes and stopper tightly. Place the tubes in a 30°C water bath. Allow sufficient time for the pectin solution to reach 30°C. At convenient intervals, between samples, add 1.0 ml of the crude tissue slurry to the pectin. Mix well and allow to react for exactly 3 min. For blank determinations omit the 3 min reaction time. Transfer 1.0 ml of the mixture to nitriting tubes in an ice bath (see section on the conversion of alcohols to nitrites). Addition of the slurry for the enzyme determination and corresponding blank determination should be done at approximately the same time.

Determination of Free Methanol. Add 0.5 ml of the tissue slurry to a stoppered, pre-chilled test tube containing 5.0 ml of 0.001–0.02% 1-propanol internal standard solution. Mix well and immediately transfer 1.0-ml aliquots to nitriting tubes. This analysis should be done as soon as the tissue has been slurried since PME is active even at 0°C.

Determination of Total Methoxy Groups. Transfer 10 ml of the tissue slurry to 60-ml test tubes containing 10 ml of 0.01–0.05% 1-propanol internal standard. Add 20 ml of 0.5 *M* NaOH containing 0.25% EDTA. Stopper securely and shake for at least 30 min at room temperature. Transfer 1.0-ml aliquots to nitriting tubes.

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Semi-Micro Method. PME ASSAY. Grind the cold plant tissue with cold buffer in a smooth-walled tissue grinder until well macerated. The total weight of the crude tissue slurry should not exceed 100 mg. Warm the tissue grinder for 15 sec in the water bath and add 0.9 ml of pectin solution, previously pre-conditioned to 30°C. Mix well and react for exactly 3 min at 30°C. To stop the reaction, add 2.5 ml of cold H_3PO_4 solution. Mix well and chill the tissue grinder in an ice bath. Add 2.5 ml of cold KNO_2 solution and mix well.

For the blank determination, use pre-chilled tissue grinders and omit the 3-min reaction time. After mixing the pectin and tissue slurry, immediately add H_3PO_4 to stop the enzyme action.

Determination of Free Methanol and Total Methoxy Groups. Weigh sections of cold plant tissue containing at least 0.7 mg of pectin in a total quantity of not more than 300 mg of tissue.

For the free methanol determination, place the tissue in chilled tissue grinders. Add enough cold 1-propanol internal standard to make the final volume of slurry 1.0 ml. Keep the grinders cold, macerate the tissue well, and add immediately first H_3PO_4 and then KNO_2.

For the total methanol analysis, add to the tissue in tissue grinders 0.6 ml of the NaOH–EDTA reagent, internal standard solution, and distilled water, if necessary, to make a final volume of 1.0 ml. Grind the tissue until well homogenized. Allow to stand at room temperature for at least 30 min with frequent stirring.

Conversion of Alcohols to Nitrites. The alcohols in the test solution (such as standard solutions, enzyme–pectin, or pectin–alkali mixtures) are converted to the nitrite esters by reacting 1.0 ml of the test solution with 5.0 ml of nitrous acid (HNO_2) in a closed reaction vessel. HNO_2 is formed by mixing equal volumes of cold 5% KNO_2 and 7% H_3PO_4. It is preferred that this reaction occur at a low temperature because HNO_2 is unstable at room temperature (Latimer and Hildebrand, 1951).

In macro determinations, the aliquot is added to HNO_2 in chilled nitriting tubes. The tubes are capped with serum caps, immersed to the "shoulder" in the ice bath, and the contents mixed well by shaking at intervals. The number of times and manner of shaking should be standardized.

In the semi-micro method, after the reaction in the tissue grinder is completed, add 2.5 ml of cold H_3PO_4 to stop the reaction. The grinder is chilled at once in an ice bath and 2.5 ml of cold KNO_2 is added. Mixing is achieved by moving the plunger up and down several times.

For both methods, the reaction tubes are held in an ice bath, with occasional shaking, for at least 3 and up to 60 min. The tubes are shaken again before sampling and approximately 1.0 ml of the headspace gas is removed with a syringe and injected into the gas chromatograph.

Calculations. Prepare a series of standard solutions of methanol in the concentration range of 0.001 to 0.05% (v/v) containing similar amounts of 1-propanol. Obtain the average relative weight response (RWR) of methanol with respect to 1-propanol using peak height measurements:

$$RWR = \left[\frac{P_{M_s}}{P_{P_s}}\right] \cdot \left[\frac{\%P_s}{\%M_s}\right]$$

where P_{M_s} = peak height of methyl nitrite of the standard; P_{P_s} = peak height of *n*-propyl nitrite of the standard; $\%P_s$ = % 1-propanol in the standard; and $\%M_s$ = % methanol in the standard. Then, for free methanol and total methoxy (as methanol):

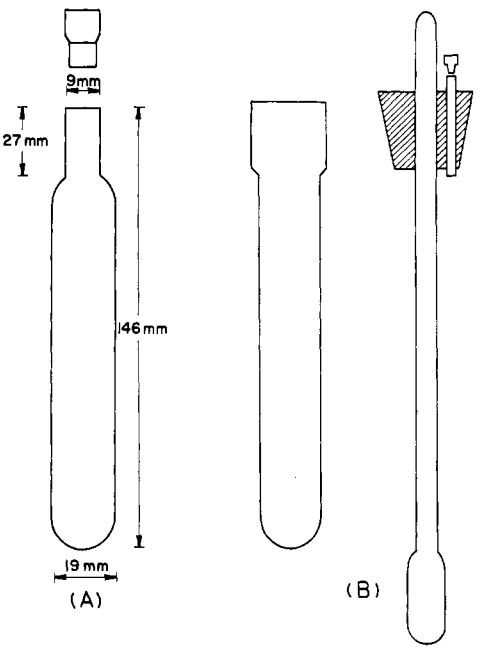

Figure 1. a. Reaction vessel for conversion of methanol to methyl nitrite in the macro methods. b. Tissue grinder for use in the semi-micro modifications

$$MeOH = \frac{(P_M)\,(\%P)\,(D_M)}{(P_P)\,(RWR)\,(D)} \cdot 10^4\ \mu g\ methanol/g\ tissue$$

where P_M = peak height of methyl nitrite of tissue sample; P_P = peak height of *n*-propyl nitrite of tissue sample; %P = % 1-propanol in sample solution; D = dilution factor of tissue in sample solution (g tissue/ml); and D_M = density of methanol. For PME activity determinations obtain:

$$\Delta MeOH = MeOH_t - MeOH_z$$

where t refers to the sample after incubation for t (usually 3) min and z to the zero time control. Then:

$$PME\ activity = \frac{\Delta MeOH}{t \cdot 32}\ \mu mole/min/g\ tissue$$

Comparison of Methods. The various glc methods were compared with corresponding methods based on titration of freed carboxyl groups.

The PME titration procedure by Kertesz (1951) was modified. To 27.0 ml of a 1% unbuffered pectin solution was added 3.0 ml of 0.01% PME solution (purified tomato PME, lot 107B, Sigma Chemical Co., in 4% NaCl) and the reaction allowed to proceed for exactly 10 min at 30°C. Changes in pH were followed potentiometrically and the initial pH of 7.5 was maintained by titration with 0.1 *N* NaOH. Nitrogen gas was bubbled through the mixture to prevent incorporation of carbon dioxide. The bubbling was sufficient for mixing and no additional stirring was required. Heated enzyme solution was used for blank determinations. The glc assay was, in this instance, also carried out for 10 min at 30°C.

For analysis of methoxy groups, 25 ml of unbuffered 1% pectin, initially at pH 7.5, were mixed with 25 ml of 1 *N* NaOH and allowed to stand at room temperature for 30 min with occasional shaking (Kertesz, 1951). The excess alkali was then titrated with 0.1 *N* HCl. Blank experiments were run with distilled water instead of pectin. For the corresponding

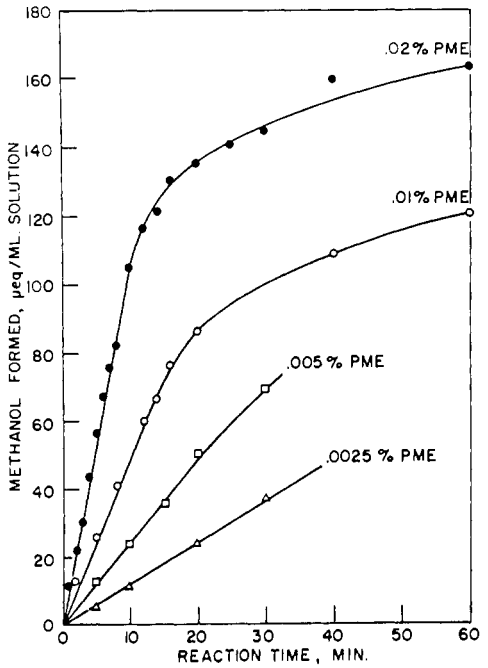

Figure 2. Pectin methylesterase curves. 1% pectin at different enzyme concentrations

glc determinations, 5.0 ml of 1% pectin (unbuffered, with 1-propanol internal standard) were reacted with 5.0 ml of 1 *N* NaOH for 30 min at room temperature. One milliliter of the mixture was then transferred to nitriting tubes. Blank values were similarly obtained with distilled water.

RESULTS AND DISCUSSION

The optimum temperature of PME from higher plants ranges from 25 to 40°C (Doesburg, 1965) and the optimum pH from 7 to 8 (Somogyi and Romani, 1964). The temperature and pH were, in our assay procedure, set at 30°C and 7.5, respectively. pH control during the enzyme reaction required the presence of a buffer. The concentration chosen (0.2 *M* phosphate buffer) is not inhibitory and provides pH stability as long as the recommended activity limit is not grossly exceeded.

An internal standard (1-propanol) was incorporated in the pectin solution as an aid in quantitation. 1-Propanol is normally not present in plant tissues and, similar to methanol, is easily converted to its corresponding nitrite.

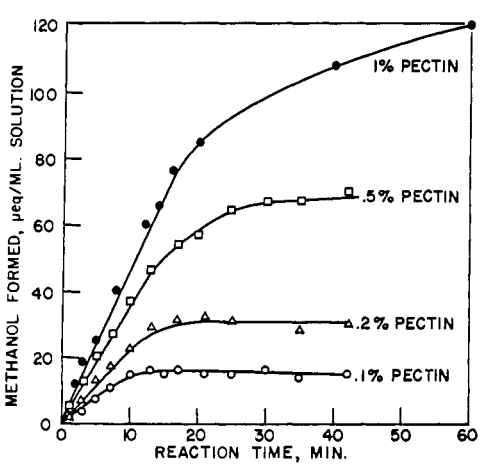

Figure 3. Pectin methylesterase curves. 0.01% PME at different pectin concentrations

Table I. Effect of K_2HPO_4 and NaCl on Crude Potato PME Activity

Extractant[a]		pH of extract	PME activity[b] μequiv methanol/min/g potato
H_2O			1.87 ± 0.09
K_2HPO_4	0.5 *M*	7.5	2.40 ± 0.17
	1.0 *M*	7.5	2.20 ± 0.14
	2.0 *M*	7.5	1.60 ± 0.07
NaCl	0.5 *M*	5.5	1.08 ± 0.18
	1.0 *M*	5.5	1.22 ± 0.13
	2.0 *M*	6.0	1.42 ± 0.16
NaCl + K_2HPO_4 (1:1)	0.5 *M*	7.3	2.20 ± 0.15
	1.0 *M*	7.2	2.18 ± 0.29
	2.0 *M*	6.9	1.98 ± 0.03
			WSD[c] = 0.31

[a] Equal amounts of potato tissue and extractant. [b] Mean of four determinations followed by standard deviation. [c] WSD = wholly significant difference between means at 95% confidence (Volk, 1958).

Table II. Effect of Potato Tissue on Purified PME Activity

PME source	PME activity[a] μmole methanol/min
Purified PME	2.14 ± 0.06
Potato tissue	0.95 ± 0.03
Sum	3.09 ± 0.09
Mixture of equal volume of PME and potato slurry	3.16 ± 0.08
	WSD 0.08

[a] Mean of six determinations.

Other parameters of the various methods were established on the basis of the following series of experiments. Enzyme reaction curves (Figures 2 and 3) gave information on suitable concentrations of substrate and on incubation time. Purified tomato PME (Lot 107B, Sigma Chemical Co.) was dissolved in cold 4% NaCl solution and filtered. One milliliter of PME solution was reacted with 9.0 ml of pectin solution under conditions defined earlier. At intervals, 1.0 ml of the mixture was removed and mixed with 5.0 ml of the nitrite–phosphoric acid mixture. Various concentrations of enzyme and of pectin were used. A standard incubation time of 3 min and a pectin concentration of 1% were selected on the basis of these curves as suitable for samples yielding between 3 and 30 μmol of methanol per milliliter in that period of time.

A relatively high concentration of buffer (1.0 *M*) was used on a 1:1 basis in maceration of the sample. This is sufficient to establish a pH of 7.5 in the resulting slurry from samples of even high acidity, such as tomatoes, and to desorb the enzyme from the cell wall (Kertesz, 1951; Jansen *et al.*, 1960) (Table I).

When tomato PME was added to potato tissue slurry, recovery was good (Table II), indicating that enzyme inhibitors were not present

Potato homogenate was mixed with an equal amount of NaOH–EDTA solution in a closed test tube and shaken at room temperature. Samples were withdrawn at intervals and analyzed for methanol after conversion to methyl nitrite (Figure 4). It is apparent that alkaline hydrolysis of pectin for determination of total methoxy should be carried out for at

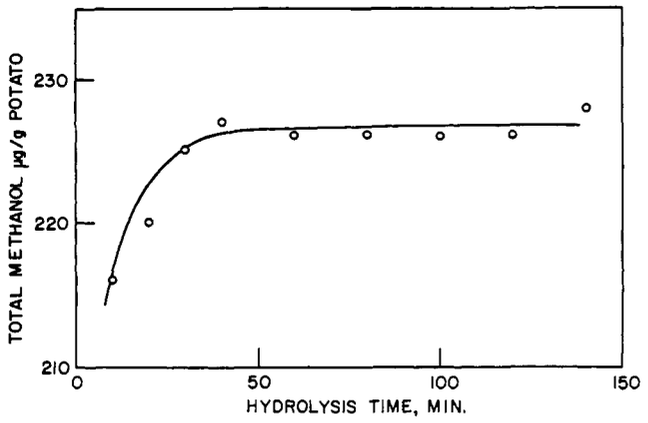

Figure 4. Effect of hydrolysis time on the measurement of total methanol in crude tissues

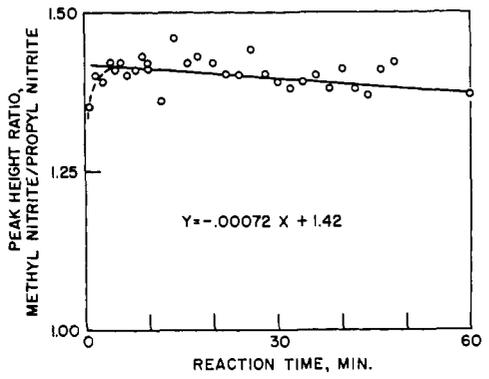

Figure 5. Stability and rate of formation of the methyl nitrite

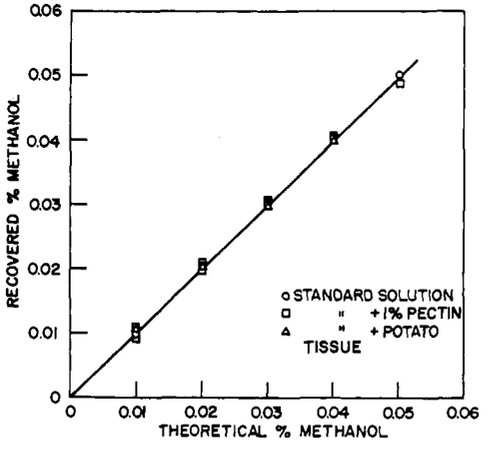

Figure 6. Effect of pectin or potato tissue on nitrite formation

least 30 min to ensure complete hydrolysis. A standard hydrolysis time of 30 min was adopted in the glc procedure.

The nitrite reaction occurs rapidly and is completed within 3 min (Figure 5). In this experiment one volume of methanol standard solution was mixed with five volumes of cold HNO_2 solution and the headspace gas sampled at intervals up to 60 min after mixing. It is evident that the ester is stable for at least 1 hr under the conditions used and that headspace sampling may take place at any convenient time within that period.

In other experiments it was demonstrated that pectin and crude tissue do not interfere with the reaction (Figure 6). Equal volumes of pectin solution (or potato tissue) without

Table III. Effect of Nitrous Acid on Pectin

Incubation time in HNO_2 min	Degree of esterification, %[a]
10	42.8 ± 1.2
60	43.4 ± 0.9
115	43.6 ± 1.9

[a] Mean of three determinations.

Table IV. Comparison of Methods

Glc and titration assays of PME

Enzyme preparation	PME activity glc	µmol methanol[a] titration	Difference	WSD
1	118 ± 3	111 ± 6	7	2
2	126 ± 5	121 ± 3	5	10
3	115 ± 5	116 ± 9	1	17

Glc and titration assays of methoxy groups

Run no.	% Methoxy[b] glc	% Methoxy[b] Titration	Difference	WSD
1	5.83 ± 0.17	5.76 ± 0.14	0.07	0.32
2	5.74 ± 0.06	5.76 ± 0.14	0.02	0.09
3	5.15 ± 0.17	5.28 ± 0.23	0.13	0.41

Macro and semi-micro assays of PME

Enzyme preparation	PME activity, µmol methanol/min[c] Macro	Semi-micro	Difference	WSD
1	21.3 ± 0.2	23.1 ± 0.6	1.8	1.4
2	23.2 ± 0.6	23.9 ± 0.4	0.7	1.1
3	36.0 ± 1.7	38.9 ± 2.9	2.9	2.5

Macro and semi-micro assays of crude PME

Enzyme source	PME activity,[d] µmol methanol/min/g tissue Macro	Semi-micro	Difference	WSD
Potato	2.0 ± 0.1	1.9 ± 0.8	0.1	0.9
Tomato	7.4 ± 0.6	7.9 ± 0.4	0.5	2.5

Macro and semi-micro assays of methoxy groups

Run no.	% Methoxy[e] Macro	Semi-micro	Difference	WSD
1	5.81 ± 0.31	5.52 ± 0.16	0.29	0.61
2	5.52 ± 0.09	5.44 ± 0.03	0.08	0.13
3	5.77 ± 0.19	5.73 ± 0.14	0.01	0.14

[a–e] Mean of 5, 4, 5, 5, and 6 determinations, respectively.

internal standard and standard solutions of different concentrations were mixed. Corrections for methanol in the pectin solution or the potato slurry were made. Recovery of methanol was, in each case, equal to that from the methanol solutions alone. Pectin was not affected during the nitriting reactions (Table III) as expected, due to its high stability under acidic conditions at 0°C (Deuel and Stutz, 1958).

Good agreements were obtained between results of the various glc macro methods presented here and those by the corresponding methods based on titration of freed carboxyl groups. Similarly, the glc semi-micro methods gave results in good agreement with those of the corresponding glc macro methods (Table IV).

During the development of the semi-micro PME method, it was observed that ground glass surfaces adversely affected the enzyme. When tissue grinders with ground glass surfaces were used, the assayed PME activity was as much as 50% lower than that determined by the macro method. Coating

the surfaces with wax reduced this loss, but full recovery was only obtained when tissue grinders with smooth surfaces were used.

The probable error of the glc methods is approximately 2% for macro and semi-micro purified PME and methoxy analysis, 6% for the macro and semi-micro crude PME analysis. Sensitivity is estimated as 3 ppm of methanol in the sample solution prior to nitrite conversion.

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Firming of Potatoes: Biochemical Effects of Preheating

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When potato tuber tissue is preheated at temperatures between 60 and 70°C the fully processed tissue (boiled) is firmer and sloughs less than the untreated control. The effect is time dependent and approaches a maximum in about 2 hr. The native pectin methylesterase (PME) of the tissue is inactive at temperatures below 50°C. It is activated above this temperature and reacts with the pectins of the cell wall, as evidenced by the production of free methanol and a decrease in the methoxy content of the cell wall pectin. Above 70°C the enzyme is rapidly destroyed and exerts no effect on the cell wall material. The calcium and magnesium contents of the cell wall increase at the effective temperatures. These observations are consistent with the following interpretation concerning the mechanism of tissue firming. Heating at temperatures above 50°C leads to a loss of integrity of the cellular membrane (plasmalemma) allowing intracellular electrolytes (predominantly K) to contact the cell wall materials, thereby activating PME. The enzyme increases the amount of free carboxyl groups of the cell wall pectin, and Ca and Mg from the cell interior increase the number of metal bridges. This leads to an increased resistance of the tissue to further thermal degradation.

When potato tissue is held for some time at moderate temperatures (50–80°C) and subsequently boiled, it attains a firmer texture than samples that are boiled without pretreatment. This firming effect has been ascribed to the behavior of the starch. Thus, Reeve (1967) and Potter *et al.* (1959) believed that the effect is due to starch retrogradation which results in decreased swelling power of the starch granules. It is held that starch generally affects potato texture by causing a distension of the cell wall when the granules swell during gelatinization (Bettelheim and Sterling, 1955). According to this view a "swelling pressure" leads to a rounding off of the cells, thereby causing rupture of the middle lamella and separation of the cells. Essential to this concept is the existence of an internal pressure caused by the swelling of the starch, although no experimental evidence in support of its existence has yet been reported in the literature.

An alternative view is offered by Linehan and Hughes (1969), who postulated migration of amylose from the starch granules to the middle lamella. Infiltration by amylose into the cell wall fabric was believed to result in reinforcement of the mechanical strength of the cell wall and the middle lamella.

Firming effects have been observed in cauliflower, snap beans, and tomatoes (Sterling, 1955; Hoogzand and Doesburg, 1961; Van Buren, 1968; Hsu *et al.*, 1965). Since these tissues contain only traces of starch, it appears unlikely that the effect is due to starch retrogradation and, in these cases, activation of pectin methylesterase has been postulated as the more likely cause of firming. According to this view, de-esterification of the pectic substances in the cell wall promotes firming, either by reaction of the free carboxyl groups with divalent ions, or more directly by formation of gel-like structures of the pectinic acid produced by the enzyme.

The present investigation was undertaken for the purpose of establishing to what degree activation of pectin methylesterase occurs in potato tubers during pretreatments involving heat and to what extent this reaction is responsible for the firming effect.

EXPERIMENTAL

Field-grown tubers of the cultivar "Superior" stored at 4°C were used throughout this study. The physical effects of firming were observed by determination of firmness by penetrometry and of sloughing by a modified sloughing test (Le Tourneau *et al.*, 1962). The effect of preheating on firmness was studied by heating potato dice (0.5 in.) in water for

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