Role of Cations in the Catalysis of Thermostable Pectinmethylesterase Extracted from Marsh Grapefruit Pulp

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The de-esterification of high methoxyl pectin by thermostable pectinmethylesterase (TS-PME) from Marsh grapefruit was studied at pH 7.5, in a temperature range between 25° and 50 °C and in the presence of various cations. Arrhenius plots were constructed for $CaCl_2$ (5 to 20 mM), $SrCl_2$ (5 to 20 mM), and spermidine (2.5 to 10 mM) added reactions. Enthalpy ($\Delta H^{\ddagger}$) and entropy ($\Delta S^{\ddagger}$) of activation changed with cation type and concentration. The presence of cations modified the free energy of the resulting enzyme/substrate complex. The entropy of activation was positive at all concentrations of $CaCl_2$ studied, and negative in the same concentration range with $SrCl_2$. Reactions with spermidine showed negative entropy of activation at concentrations <5 mM and positive values of entropy at higher concentrations.

Keywords: *Catalysis; pectinmethylesterase; kinetic parameters*

INTRODUCTION

Citrus juice appearance and consumer acceptability is closely dependent on cloud stability (Baker and Cameron, 1999). Clarification is a consequence of a series of events, which seem to originate from the action of pectinmethylesterase (PME). PME causes pectin demethoxylation and the formation of a pectate gel, which precipitates, resulting in loss of the stable, opaque appearance of the juice (Baker and Cameron, 1999). PME has been purified previously from several citrus fruits, and various isoenzymes have been described in lemon, grapefruit, and orange (Versteeg et al., 1978; Macdonald et al., 1993; Cameron and Grohmann, 1995). Multiple forms of PME differ not only in their kinetic properties, but also in stability to heating. The thermostable form of pectinmethylesterase (TS-PME) seems to be the main cause of loss of cloud in pasteurized orange juice (Versteeg et al., 1980).

Cations influence pectinesterases by releasing the enzyme from an inactive complex formed with soluble pectin (Nari et al., 1991; Charnay et al., 1992). The competition of cations with PME for carboxylic sites on pectins has been described by Nari et al. (1991). During ultrafiltration of PME extracts an increase in the permeability of PME in the presence of Ca^{2+} or Na^{+} was observed (Snir et al., 1995). Leiting and Wicker (1997) have reported that at the same ionic strength, monovalent and divalent cations activate PME extracts differently. Maximal PME activity was derived with lead acetate (6.8 mM), ferric chloride (0.2 mM), and $CaCl_2$ (20 mM), and higher cation concentrations inhibit PME activity (Leiting and Wicker, 1997). Spermidine, naturally occurring in plant cells (Smith, 1985; Bardocz et al., 1993), may have either an activating or inactivating effect on PME depending on concentration (Charnay et al., 1992; Leiting and Wicker, 1997). The optimal concentrations of polyamines for PME activity in crude extracts from Marsh grapefruit were spermidine (1.2 mM), putrescine (7.6 mM), and spermine (20 mM), (Leiting and Wicker, 1997). Charnay et al. (1992) suggested that polyamines might act similarly to small divalent cations by interacting with the negative charges of pectin, thus affecting the binding of PME to the substrate. The changes induced by mono- and divalent counterions on PME activity might be related to the higher selectivity of pectins for divalent than for monovalent cations (Goldberg et al., 1992).

In recent years, kinetic compensation relations have been published for physical, chemical, and biological reactions (Elizondo and Labuza, 1974; Özilgen and Özilgen, 1996; Rhim et al., 1990; Sun and Wicker, 1999) and have been used to describe differences in mechanisms for families of related reactions. In particular, by analyzing the kinetic parameters of TS-PME, Sun and Wicker (1999) showed that Ca^{2+} and Na^{+} influence pectin demethoxylation by PME differently. The level of inorganic cations varies widely in citrus juices, depending on the source and on the agricultural practices (Ting and Rouseff, 1986). For example, in orange juice strontium may vary between 0.095 and 0.98 ppm and calcium between 65 and 120 ppm (Ting and Rouseff, 1986).

The variation in naturally occurring inorganic and organic cations may influence PME activity, stability, and propensity to induce pectin aggregation. This paper presents the results of a kinetic study of TS-PME in the presence of three different cations: two divalent cations, $SrCl_2$ and $CaCl_2$, and a polyamine, spermidine [$H_2N(CH_2)_4NH(CH_2)_3NH_2$]. The objective of this work was to compare kinetics of activation of TS-PME by two divalent cations (Sr^{2+} and Ca^{2+}) of similar atomic radii and a naturally occurring polyamine.

EXPERIMENTAL PROCEDURES

Purification of TS-PME. Crude extract was prepared by diluting Marsh grapefruit pulp (donated by Citrus World, Lake Wales, FL) with 4 volumes of 1 M NaCl (Wicker, 1992). The extract was homogenized with a Proscientific homogenizer (Pro 300A, Proscientific Inc., Monroe, CT) for 25 s at 4 °C. After

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stirring for 1 h at 4 °C, the pulp extract was centrifuged at 5000*g* for 20 min (Sorvall RC-5B centrifuge, Dupont Instruments, Doraville, GA) and filtered through two layers of cheesecloth. Thimerosal was added as a preservative to a final concentration of 0.05 g/L. The extract was stored at 4 °C overnight to allow for the selective extraction of TS-PME (Corredig et al., 2000). After a concentration step carried out by addition of ammonium sulfate at 75% saturation, the extract was centrifuged at 5000*g* for 20 min. The pellet was resuspended in 50 mM acetate buffer, pH 5.5 and dialyzed with 10 volumes and two exchanges, of the same buffer. The dialysis tubing (Spectrapor, MWCO 6000, Fisher Scientific, Atlanta, GA) had been pretreated by boiling in 10% acetic acid for 10 min, and rinsing in deionized water, to prevent loss of enzymatic activity. After dialysis, the extract was filtered through a No. 1 Whatman filter (Fisher Scientific, Atlanta, GA). TS-PME was loaded onto a 5 mL cation exchange column (Hi-Trap SP, Pharmacia Biotech, Piscataway, NJ) at 5 mL/min. After loading the extract, the column was washed with start buffer (50 mM acetate pH 5.5) and eluted with a 0 to 1 M NaCl gradient in 15 column volumes. All chromatography buffers were filtered through a 0.45 μm filter (Gelman Scientific, Ann Arbor, MI). Chromatography separations were carried out at 4 °C using an FPLC system (P-500 pumps and GP-250 gradient programmer) (Pharmacia Biotech, Piscataway NJ). The pooled fractions from Hi-Trap SP were dialyzed in 50 mM acetate buffer, pH 5.5 and then loaded onto a 5 mL Heparin column (Hi-Trap Heparin Sepharose, Pharmacia Biotech). Fractions were eluted at 5 mL/min in 10 column volumes with a 0 to 1 M NaCl gradient in 50 mM sodium acetate buffer, pH 5.5. The fractions containing PME activity eluted from Heparin were dialyzed with 20 mM Tris buffer, 0.5M NaCl, 1 mM $CaCl_2$, pH 7.4. Dialyzed TS-PME was then loaded on a 1 mL Concanavalin A affinity column (Pharmacia Biotech). After the column was washed with binding buffer (20 mM Tris, 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4), a 3 mL fraction was collected by eluting with the same buffer, containing 0.5 M methyl-α-D-glucopyranoside.

Analytical Determinations. PME activity was determined by using a pH stat titrator (Brinkmann, Westbury, NY) at pH 7.5, 30 °C in 1% high methoxyl pectin, 0.1 M NaCl (Citrus Colloids Ltd., Hereford, U.K.). PME units were expressed as microequivalents of ester hydrolyzed per minute. The heat stability of the fractions (TS-PME) was determined as follows: aliquots (0.25 mL) of fractions were added to 2 mL of preheated buffer (50 mM potassium phosphate, 0.1 M NaCl pH 7.0) at 70 °C and then heated at 70 °C for 5 min and rapidly cooled in an ice bath. Residual activity of duplicate samples was determined by comparison with an unheated control diluted in the same buffer. Activity measurements were performed within 2 h of heating.

Protein determination was carried out according to the Bradford (1976) method (Protein assay kit, Biorad, Hercules, CA), using a microtiter plate assay, according to manufacturer's instructions. Calibration curve for the assay was determined with IgG as the standard.

Uronic acids were determined by the *m*-hydroxyphenyl phenol colorimetric method of Blumenkrantz and Asboe-Hansen (1973), reading absorbance in a microtiter plate reader at 520 nm (Wicker and Leiting, 1995).

Theoretical Background. The Arrhenius equation describes the relationship between the rate and the temperature of a reaction:

$$k = A \exp(-E_a/RT) \tag{1}$$

where k is the rate constant, the preexponential factor A is the frequency factor [s^{-1}], E_a is the energy of activation (J/mol), R is the gas constant [J/(mol·K)], and T is the absolute temperature [K]. Similarly, rate constants may be predicted with the absolute rate (Eyring) theory (Daniels and Alberty, 1975):

$$k = K_b T/h \exp\{\Delta S^{\ddagger}/R\} \exp\{-\Delta H^{\ddagger}/RT\} \tag{2}$$

where K_b and h are the Boltzmann and Plank constants, respectively; $\Delta S^{\ddagger}$ is the entropy of activation, and $\Delta H^{\ddagger}$ is the enthalpy of activation.

The relationship between $\Delta H^{\ddagger}$ and E_a, and $\Delta S^{\ddagger}$ and the frequency factor (A) may be obtained by calculating the slope of a plot of ln k versus $1/T$ from eqs 1 and 2 (Daniels and Alberty, 1975):

$$E_a = \Delta H^{\ddagger} + RT \tag{3}$$

$$A = 2.72RT/N_a h \exp\{\Delta S^{\ddagger}/R\} \tag{4}$$

where N_a is the Avogadro number, R is the gas constant, and h is the Planck constant.

Many authors have observed a thermodynamic compensation effect in a family of related reactions (Elizondo and Labuza, 1974; Özilgen and Özilgen, 1996; Rhim et al., 1990, Sun and Wicker, 1999). There are parallel enthalpy and entropy changes that compensate each other to produce minor changes in the free energy of the process under investigation. The compensation effect can be expressed by a simple linear relationship between enthalpy and entropy of activation:

$$\Delta S^{\ddagger} = \Delta H^{\ddagger}/T_c + b \tag{5}$$

where T_c is the isokinetic temperature, a theoretical temperature, characteristic for the entire sequence of reactions, and related to the E_a and ln A as well as the catalytic rate constant k (Rhim et al., 1990). Experimentally, kinetic compensation parameters (E_a and ln A) have been used instead of activation parameters ($\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$). The linear relationship which describes the systematic variation of the frequency factor (A) with the energy of activation (E_a) is as follows:

$$\ln A = \alpha E_a + \beta \tag{6}$$

where α and β are compensation parameter constants.

Experimental Measurements. Activity was measured by titrimetric method in 1% high methoxyl pectin, at pH 7.5, as described above. Analyses were carried out in a temperature range between 25 and 50 °C, and the temperature of the reaction vessel was controlled by a Haake F3C waterbath (Fisher Scientific, Atlanta, GA). Pectin was pretreated by dissolving a 2% solution in 10 mM EDTA and dialyzing with 10 volumes of deionized water, with four water changes. After dialysis the pectin was freeze-dried and stored at −20 °C until needed. Substrate solutions were prepared by dissolving 1% pectin and $CaCl_2$, $SrCl_2$, or spermidine at 70 °C to prevent pectin gel formation by addition of cation. $CaCl_2$ (Sigma Chemicals, St. Louis, MO) and $SrCl_2$ (Aldrich Chemicals, Milwaukee, WI) were prepared at 5, 10, 15, and 20 mM; spermidine (Fluka Biochemicals, Buchs, Switzerland) was prepared at 2.5, 5, 7.5, and 10 mM. Concentrations higher than those reported resulted in macroscopic appearance of gels in the pectin solutions. Controls without enzyme were run at each temperature, and those values were subtracted from those obtained in the presence of TS-PME. Activity determinations were carried out at least twice. The catalytic reaction rate k (s^{-1}) was calculated as $V_{max}/[E_t]$, where V_{max} was the maximum velocity of the reaction and $[E_t]$ was the total concentration of the enzyme in the reaction vessel. The initial concentration $[E_t]$ was determined under standard conditions at 30 °C, 1% pectin and at pH 7.5, using a molecular weight of 53500 for TS-PME from Marsh grapefruit (Seymour et al., 1991), as described by Sun and Wicker (1999). The catalytic rate constants k were plotted against $1/T$. A least-squares linear regression was performed on the Arrhenius plots, and E_a, and ln A were derived from the slope ($-E_a/R$) and the intercept (ln A). Numerical values for $\Delta S^{\ddagger}$ and $\Delta H^{\ddagger}$ were calculated at different temperatures from eqs 3 and 4 by substituting appropriate values for R, N_a, and h. Compensation reaction

Table 1. Purification of Thermostable Pectinesterase from Marsh Grapefruit Pulp

purification steps	vol (mL)	activity (U/mL)	protein (mg/mL)	spec act. (U/mg)	purification (fold)
crude extract	1390	3.9	0.15	26.0	1
ammonium sulfate precipitation (75%)	130	8.0	0.71	11.26	0.4
fractions from Hi-Trap SP[a]	17	20.6	0.80	25.75	0.99
fractions from heparin[a]	10	37.5	0.74	50.7	1.9
TS-PME from concanavalin A	3	71.4	0.08	892.0	34.3

[a] Values are reported for the pooled fractions. Hi-Trap SP (fractions 9, 10, 11) and heparin (fractions 6, 7).

parameters were determined by plotting ln A versus E_a and $\Delta S^{\ddagger}$ versus $\Delta H^{\ddagger}$.

RESULTS AND DISCUSSION

TS-PME Purification. A summary of the various steps in the purification of TS-PME is given in Table 1. Extraction of Marsh grapefruit pulp at low pH (pH 3.0) with 1 M NaCl selectively recovered TS-PME as described in previous work (Wicker, 1992; Corredig et al., 2000). The addition of ammonium sulfate to 75% saturation resulted in nonselective protein concentration, with the inactivated, thermolabile form of PME precipitating with the TS-PME, thereby causing a reduction in TS-PME specific activity. Generally, extraction of PME from citrus is carried out at higher pH values (between 7 and 8) (Seymour et al., 1991; Cameron and Grohmann, 1995), than that used in the present study. The chromatographic steps that followed the ammonium sulfate concentration resulted in higher fold purification and specific activity (Table 1). After dialysis of the ammonium sulfate precipitate, TS-PME was eluted on a Hi-Trap SP column as illustrated in Figure 1. Three peaks of PME activity were separated by a NaCl gradient, primarily in fractions 5, 7, and 10. The third peak, eluted at about 0.6−0.7 M NaCl, contained the highest specific activity, 40 U/mg. The fractions eluted were tested for PME (Figure 1A), protein, and uronic acid content (Figure 1B). Most of the protein bound to the column was eluted between fractions 3 and 8, with two peaks at fractions 5 and 7. Uronic acid analysis of the eluted fractions showed that pectin was present in all PME-active fractions, coeluting with the enzyme. The highest amount of uronic acid was found in fraction 5. The presence of pectin/PME complexes has been previously reported. MacDonald et al. (1993) showed that purification of PME from lemon juice is hindered by the presence of pectin, for which PME has great affinity. The presence of PME/pectin complexes may be responsible for some of the differences in behavior of some forms of PME described in the literature (MacDonald et al., 1993).

Fractions 9, 10, and 11 eluted from Hi Trap SP (those with the highest specific activity) were pooled, dialyzed with start buffer, and loaded on Heparin-Sepharose. TS-PME eluted in one peak in fractions 6 and 7, at a NaCl gradient of 0.6 M (Figure 2). The chromatography on Hi-Trap Heparin did not greatly increase the specific activity of the enzyme (U/mg protein) but the amount of uronic acids determined in fractions 6 and 7 (Figure 2B) was lower (about 5 times) than that of the PME fractions of the Hi-Trap SP column (Figure 1B). In this chromatographic separation, even though lower amounts of pectin were determined, pectin coeluted with PME. The specific activity of TS-PME increased after further purification of the fraction isolated from Heparin-Sepharose by affinity chromatography on Concanavalin A. This purification step resulted in a TS-PME fraction

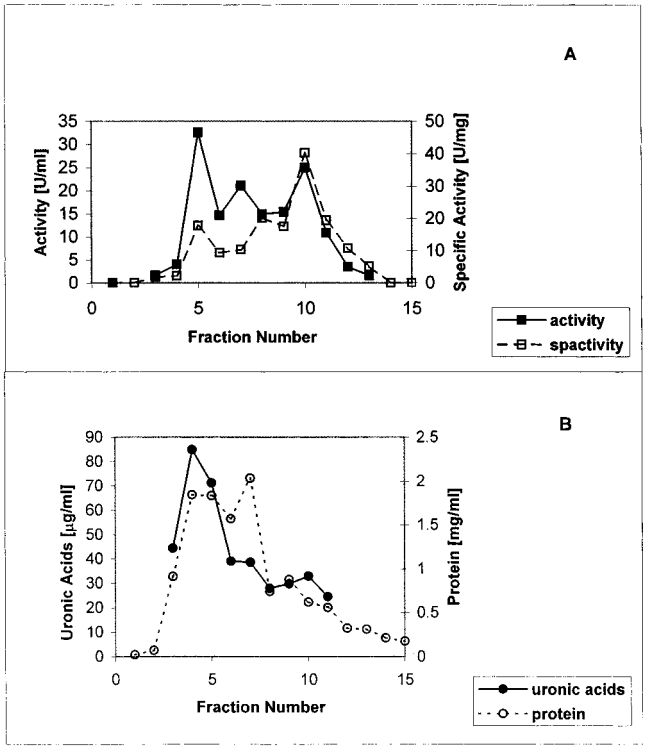

Figure 1. Fractionation of crude extract from Marsh grapefruit pulp after ammonium sulfate concentration on Hi-Trap SP (Pharmacia Biotech). A. PME activity [U/mL] [left-hand axis] and specific activity [U/mg] [right-hand axis]. B. Uronic acid content [μg/mL] [left-hand axis] and protein content [mg/mL] [right-hand axis] of the eluted fractions. Values are the average of two determinations.

of 890 U/mg, a 34-fold increase in specific activity from that of the initial crude extract (Table 1). Heating of the fraction at 70 °C for 5 min confirmed the presence of 100% TS-PME activity. No uronic acid in the eluted fraction was detected. The fraction prepared by this purification procedure had a higher specific activity than that reported by Seymour et al. (1991) of 217 U/mg, and Sun and Wicker (1999) of 313 U/mg. Comparable specific activity values (987 U/mg) for TS-PME from grapefruit pulp were obtained by Sun and Wicker (1996), by a combination of ion-exchange and affinity chromatography separations.

Kinetic Studies. In all $CaCl_2$-, $SrCl_2$-, and spermidine-containing reactions, an increase of PME activity was observed with increasing temperature to a maximal value (Figure 3). The activity profile obtained was unique for each cation type, with little variation in activity with salt concentration at low temperatures and an increase in activity at higher assay temperatures. For 5−15 mM calcium-added reactions (Figure 3A), the enzymatic activity increased with temperature up to 40 °C and decreased at higher temperatures. A shift in the maximum activity at 35 °C was shown for TS-PME in the presence of 20 mM $CaCl_2$. At all concentrations of

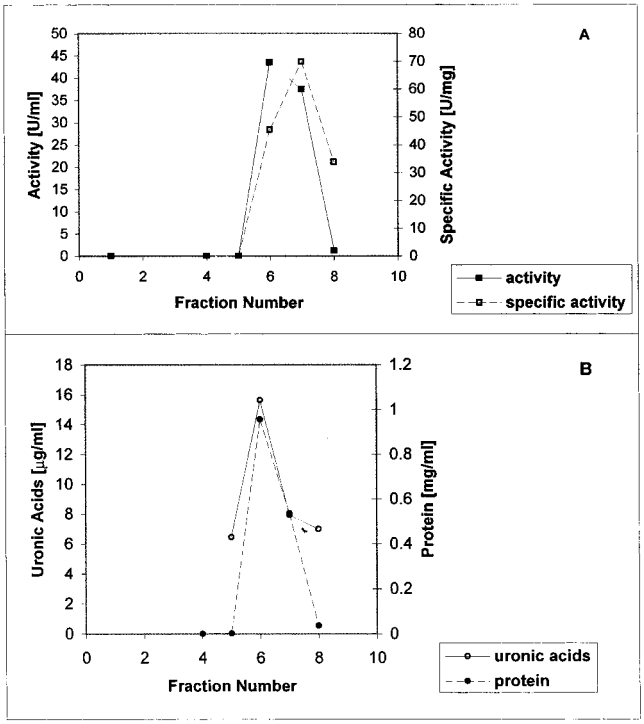

Figure 2. Elution of fractions (from 9 to 11) pooled from Hi-Trap SP chromatography, on Heparin-Sepharose (Pharmacia Biotech). (A) PME activity [U/ml] [left-hand axis] and specific activity [U/mg] [right-hand axis]. (B) Uronic acid content [μg/mL] [left-hand axis] and protein content [mg/mL] [right-hand axis] for the eluted fractions. Values are the average of two determinations.

$SrCl_2$ (Figure 3B) TS-PME increased its activity with increasing temperature, with a maximum at 45 °C. In the presence of spermidine (Figure 3C) TS-PME activity increased with temperature with highest activity at 45 °C at the lower concentrations of spermidine. At 30 °C, no inhibition of TS-PME activity was found up to 10 mM spermidine. This contrasted with results reported in the literature, where crude extracts of PME (thermolabile and thermostable forms) from grapefruit were inhibited by 10 mM spermidine (Leiting and Wicker, 1997).

Figure 4 illustrates representative Arrhenius plots. At all concentrations of $CaCl_2$ and $SrCl_2$, the catalytic rate constant of TS-PME changed linearly with $1/T$ in the temperature range between 25 and 40 °C. In spermidine-containing reactions, the coefficient of the least-squares regression had values >0.96 between 30° and 45 °C. The data in Table 2 is the result the least-squares regression analyses calculated at all concentrations of $CaCl_2$, $SrCl_2$, and spermidine and for the control treatment. The energy of activation (E_a), the preexponential factor (ln A), as well as numerical values for the entropy ($\Delta S^{\ddagger}$) and enthalpy ($\Delta H^{\ddagger}$) of activation were estimated from the Arrhenius data (Table 2). $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ were calculated at 30 °C (standard assay temperature). Figure 5 illustrates the relationship between $\Delta S^{\ddagger}$ and cation concentration for TS-PME reactions containing $CaCl_2$, $SrCl_2$, and spermidine. In agreement with what has already been reported by other authors, TS-PME activity was less sensitive to $CaCl_2$ changes (Leiting and Wicker, 1997; Sun and Wicker, 1999). For $CaCl_2$-containing reactions, in the range of concentration studied, the minimum value of enthalpy and entropy of activation occurred at 0.015 M $CaCl_2$, which is similar to the minimum E_a of 0.01 M $CaCl_2$ reported

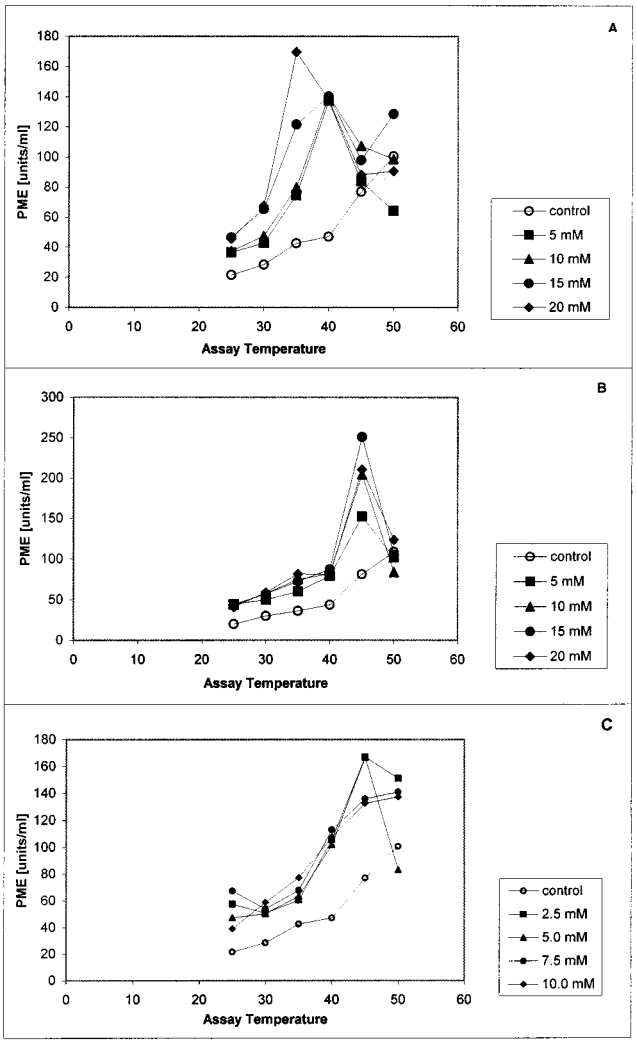

Figure 3. PME activity as a function of assay temperature, for different concentrations of (A) $CaCl_2$; (B) $SrCl_2$; (C) spermidine.

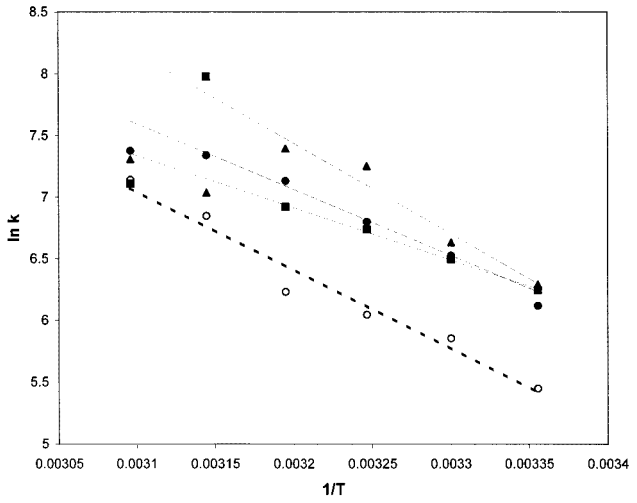

Figure 4. Arrhenius plots for pectin de-esterification by TS-PME. [○], no salt added (control); [■], 15 mM $SrCl_2$; [▲], 15 mM $CaCl_2$; [●], 10 mM spermidine. Least-squares linear regressions are also shown [dotted lines], calculated within the linear range of temperature.

by Sun and Wicker (1999). On the other hand, they reported that the entropy of activation ($\Delta S^{\ddagger}$) of $CaCl_2$-containing reactions had negative values, but in the

Table 2. Effect of $CaCl_2$, $SrCl_2$, and Spermidine on the Kinetic Behavior of TS-PME, As Determined by Titrimetric Measurements at pH 7.5 in 1% High Methoxyl Pectin

	R^2	ln A (intercept)	E_a [kJ/mol]	$\Delta S^{\ddagger a}$ [J/mol]	$\Delta H^{\ddagger a}$ [kJ/mol]
control	0.93	25.4	49.26	−42.3	46.7
$CaCl_2$					
5 mM	0.94	34.0	69.61	29.58	67.1
10 mM	0.97	33.9	69.21	28.75	66.6
15 mM	0.96	30.9	60.9	3.49	58.3
20 mM	0.80	32.9	65.74	20.02	63.2
$SrCl_2$					
5 mM	0.96	18.2	29.69	−102.1	27.2
10 mM	0.96	19.7	33.27	−89.5	30.7
15 mM	0.99	20.5	35.21	−83.7	32.7
20 mM	0.89	21.2	37.18	−76.6	34.7
spermidine					
2.5 mM	0.96	32.5	65.96	16.7	63.4
5.0 mM	0.97	32.1	64.97	13.5	62.4
7.5 mM	0.97	27.3	52.68	−26.0	50.2
10 mM	0.99	24.2	44.40	−52.4	41.9

[a] Calculated at 30 °C (303 K).

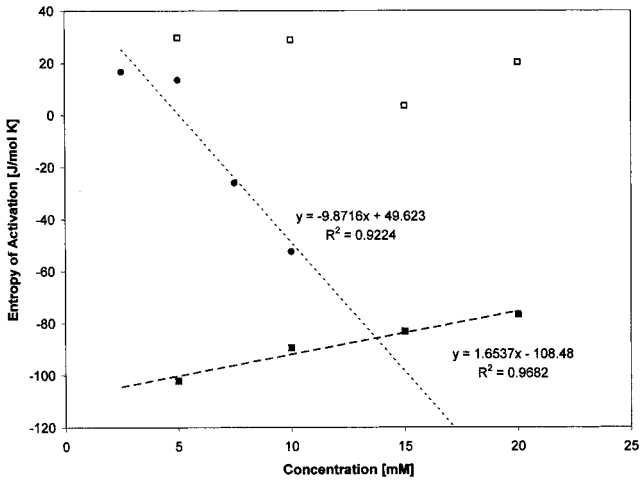

Figure 5. Variation of the entropy of activation (calculated at 303 K) with cation concentration: (□),$CaCl_2$; (■),$SrCl_2$; (●), spermidine. Results of the least-squares linear regression for $SrCl_2$ and spermidine are shown.

present study positive $\Delta S^{\ddagger}$ were obtained. Differences in the purification procedure and in the specific activity of the enzyme preparation (313 U/mg versus 890 U/mg of this study) may explain this difference. In addition Sun and Wicker (1999) did not report the uronic acid content of the enzymatic fraction. Sun and Wicker (1999) determined much lower values of E_a as well as the preexponential factor (ln A) for the TS-PME reactions in the presence of $CaCl_2$. Previous authors have demonstrated that cations affect PME isoenzymes from mung bean, and that cations more strongly stimulate the activity of enzymes which are bound to the cell wall (Goldberg et al., 1992). This might be caused by competitive displacement by the cations of PME bound to pectin, by a different affinity of the different enzymatic forms for the pectin substrate, or different pectin substrates. Data reported in this paper (Figures 1 and

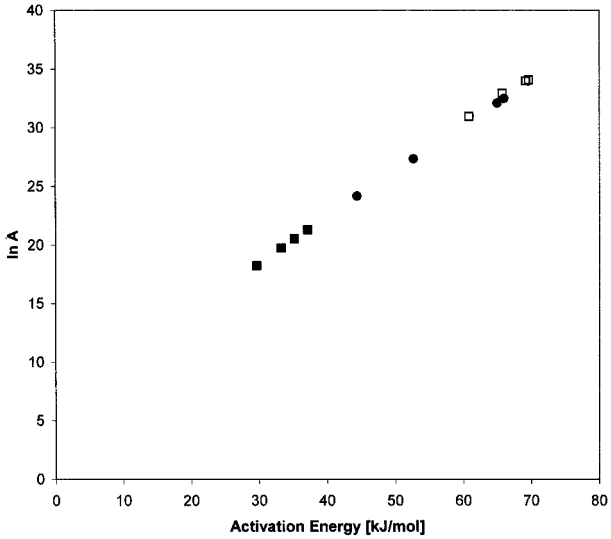

Figure 6. Compensation relation as shown by the variation of the frequency factor (ln A) with the activation energy (E_a [kJ/mol]), under different experimental conditions: (■), $CaCl_2$; (□) $SrCl_2$; (●), spermidine.

2) show that during ion-exchange chromatography, PME coeluted with pectin. The presence of coeluted "components" could modify the effect of cations on the activity of TS-PME, by altering the affinity of the enzyme for the substrate, therefore changing the free energy of activation of the reaction complex. As reported in Table 2 in $SrCl_2$ reactions, the entropy of activation was negative and dependent on concentration (Figure 5). The negative values of $\Delta S^{\ddagger}$ reported for $SrCl_2$ reactions are an indication of restriction of the rotational/vibrational freedom of the enzyme/substrate complex, which causes an increase in the Gibbs free energy of the reaction. This would support an inhibiting effect of strontium, whose atomic radii is similar to that of calcium. On the other hand, $CaCl_2$ reactions had positive values of $\Delta S^{\ddagger}$, an indication of increase of rotational/vibrational freedom of the complex, with decreased Gibbs energy values. These results suggest an activating effect of $CaCl_2$, which is distinct from that attributed to the release of PME from an inactive enzyme−pectin complex. The present data supports the speculation that another mechanism, in addition to competitive displacement of PME from a pectate complex by cations, is involved in PME activity enhancement (Wicker, 1996). TS-PME reactions in the presence of spermidine showed a change in entropy of activation from positive values at low concentration, to negative values at concentrations > 5 mM. These results confirmed previously published data showing that there is a critical concentration of polyamine, which controls the activity of PME (Leiting and Wicker, 1995). Charnay et al. (1992) suggested that the role of polyamines in the activation/inactivation of PME is more complex than that of inorganic cations, because of the possible function of polyamines as regulators of cell-wall growth. Spermidine is naturally present in oranges in a range of

Table 3. Summary of Compensation Relations of TS-PME Reactions under $SrCl_2$, $CaCl_2$ and Spermidine Conditions, Determined at pH 7.5

compensation	$CaCl_2$ reaction	$SrCl_2$ reaction	spermidine reaction
frequency factor vs energy of activation	$\ln A = 0.00036E_a + 9.05$ $R^2 = 0.99$	$\ln A = 0.00041E_a + 6.06$ $R^2 = 0.99$	$\ln A = 0.00038E_a + 7.03$ $R^2 = 0.99$
enthalpy of activation vs. entropy of activation	$\Delta S^{\ddagger} = 0.00299\Delta H^{\ddagger} - 170.4$ $R^2 = 0.99$	$\Delta S^{\ddagger} = 0.00339\Delta H^{\ddagger} - 194.7$ $R^2 = 0.99$	$\Delta S^{\ddagger} = 0.00321\Delta H^{\ddagger} - 186.7$ $R^2 = 0.99$

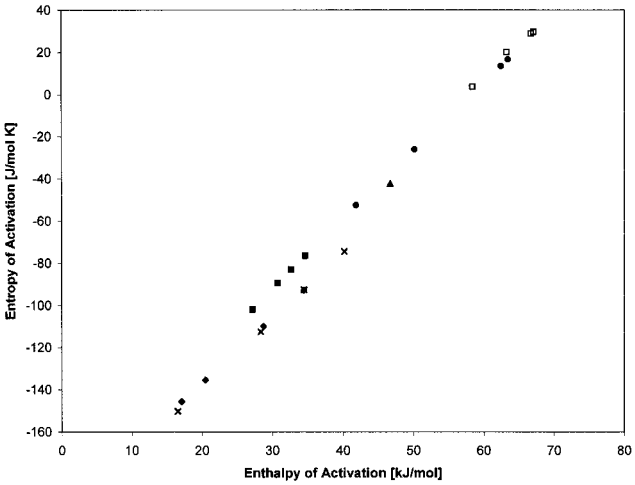

Figure 7. Compensation relation as illustrated by the variation of entropy of activation ($\Delta S^{\ddagger}$ [J/mol K]) with enthalpy of activation ($\Delta H^{\ddagger}$ [kJ/mol]) under different experimental conditions: (▲), control, no salt added; (□), $CaCl_2$; (■), $SrCl_2$; (●), spermidine. Data reported by Sun and Wicker (1999) are also shown: (×), $CaCl_2$; (◆), NaCl.

concentration of 61−67 μM (Bardocz et al., 1993), values lower than those used in this study.

The data in Table 3 summarizes the least-squares regression analyses for the compensation reaction of TS-PME in the presence of cations. In all cases, a compensation effect existed, and compensation parameters were calculated. The principle of activation energy/ln A and activation entropy/enthalpy compensation applied to TS-PME-catalyzed reactions. The compensation effect consists of parallel enthalpy and entropy changes, which offset each other and ultimately result in minor changes in the free energy of the process. Previous work on compensation reactions has shown that the compensation temperature (T_c) is a good indication of differences in the mechanism of reaction occurring with different cations. Sun and Wicker (1999) speculated that increases in E_a, $\Delta S^{\ddagger}$, and $\Delta H^{\ddagger}$ would result in higher enzyme activation above the T_c. They estimated the T_c for sodium added and calcium added to be 327.8 and 312.4 K, respectively. In this work, T_c was calculated at 334, 295, and 311 K for $CaCl_2$, $SrCl_2$, and spermidine added conditions, respectively. Despite the differences in T_c, a compensation relation was valid for all the experimental conditions in TS-PME reactions. A single compensation relation for all cation conditions between ln A and E_a was derived and described by the empirical equation $\ln A = 0.0004E_a + 6.484$ ($R^2 = 0.999$) (Figure 6). A plot of $\Delta S^{\ddagger}$ as a function of $\Delta H^{\ddagger}$ confirmed the analysis (Figure 7). In this case, the compensation relation for TS-PME demethoxylation was described as follows: $\Delta S^{\ddagger} = 0.0033\Delta H^{\ddagger} - 191.02$ ($R^2 = 0.999$). Figure 7 also included values of $\Delta S^{\ddagger}$ and $\Delta H^{\ddagger}$ as reported by Sun and Wicker (1999). In this case as well, a common compensation relation occurred in $CaCl_2$- and NaCl-added reactions. These results did not explain the differences reported in T_c. It may be that temperatures of compensation might not indicate differences in the mechanisms of the reaction. On the other hand, $\Delta S^{\ddagger}$ values clearly showed the effects of cations on the enzymatic activity of TS-PME. Results from the present study and comparisons with those previously published by Sun and Wicker (1999) showed that cations influence the free energy of the pectin/enzyme complex, and different cations affect the TS-PME reaction differently. The same cations might show a different effect depending on the form of PME present, the presence of non-PME proteins, or the presence of pectins.

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