Kinetic Compensation and the Role of Cations in Pectinesterase Catalysis

D. Sun and L. Wicker*

Department of Food Science and Technology, University of Georgia, Athens, Georgia 30602

The catalytic rate constant of thermostable pectinesterase (TS-PE) from Marsh grapefruit pulp was determined at pH 7 at temperatures between 25 and 60 °C. TS-PE activity was measured at NaCl concentrations of 0.05, 0.10, 0.15, and 0.20 M and at $CaCl_2$ concentrations of 0.005, 0.010, 0.015, and 0.020 M. For sodium- and calcium-added conditions, the kinetic functions of this reaction agreed with kinetic compensation relations. The isokinetic temperatures for sodium- and calcium-added conditions were 327.8 and 312.4 K, respectively. Different isokinetic temperatures and compensation parameters suggest that sodium and calcium uniquely affect TS-PE catalysis. This is the first demonstration of kinetic compensation in an enzyme-catalyzed reaction.

Keywords: *Activation energy; frequency factor; PE complexes; competitive displacement; citrus juices; clarification*

INTRODUCTION

According to the Eyring theory (Daniels and Alberty, 1975), the rate constant k (s^{-1}) of a unimolecular chemical reaction is estimated by

$$k = (KRT/N_A h) \exp[\Delta S^{\#}/R] \exp[-\Delta H^{\#}/RT] \quad (1)$$

where K, R, N_A, h, $\Delta S^{\#}$, $\Delta H^{\#}$, and T represent transmission coefficient, gas constant [J/(mol·K)], Avogadro's constant (mol^{-1}), Planck's constant (J·s), activation entropy [J/(K·mol)], activation enthalpy (J/mol), and absolute temperature (K), respectively.

The temperature dependence of the rate constant, k, is expressed with the Arrhenius equation as

$$k = A \exp[-E_a/RT] \quad (2)$$

where A and E_a denote frequency factor (s^{-1}) and activation energy (J/mol), respectively.

From eqs 1 and 2, we obtain

$$A = 2.72(RT/N_A h) \exp[\Delta S^{\#}/R] \quad (3)$$

and

$$E_a = \Delta H^{\#} + RT \quad (4)$$

A linear relationship between the natural logarithm of frequency factor ln A and the activation energy E_a and between the activation entropy $\Delta S^{\#}$ and the activation enthalpy $\Delta H^{\#}$ may be observed in a family of related reactions (Rhim et al., 1990):

$$\ln A = \alpha E_a + \beta \quad (5)$$

$$\Delta S^{\#} = \delta \Delta H + \phi \quad (6)$$

* Author to whom correspondence should be addressed [telephone (706) 542-1055; fax (706) 542-1050; e-mail lwicker@arches.uga.edu].

A related family of reactions represents a set of chemical reactions in which only one component is changed. In a family of related reactions, frequency factor and activation energy are interdependent, as well as the activation entropy and activation enthalpy. The constants α, β, δ, and ϕ are compensation parameters, which do not depend on experimental variables such as concentration of reactant, pH, metal ions, or water activity. However, kinetic parameters (A, E_a, $\Delta S^{\#}$, and $\Delta H^{\#}$) depend on these variables (Zsako, 1976). Thus, compensation parameters may better characterize the reaction (Zsako, 1976) and provide insight into the mechanism or classification of the reaction (Barnes et al., 1969). The linear relationship between ln A and E_a and between $\Delta S^{\#}$ and $\Delta H^{\#}$ implies the existence of a unique temperature called the isokinetic temperature (T_c) (Barnes et al., 1969; Pysiak and Sabalski, 1979). At the isokinetic temperature, rate constants of all related family of reactions have the same value. The biological utility of thermodynamic compensation was initially described for viral death (Barnes et al., 1969). It has also been used to study the mechanism of thermal death under various process conditions for *Leuconostoc mesenteroides* and *Bacillus coagulans* (Özilgen et al., 1991) and at different stages of growth for mesophilic yeasts (van Uden and Vidal-Leiria, 1976).

Kinetic compensation parameters have not been investigated for enzyme-catalyzed reactions. Activation of pectinesterase (PE) activity by cations has been described as competitive displacement of PE bound to blocks of carboxylic groups on pectin, which frees PE for further catalysis (Lineweaver and Ballou, 1945; Nari et al., 1991). The inhibition of PE at high cation concentration has been described as competition with PE for free carboxylic groups on pectin (Nari et al., 1991). However, PE shows different magnitudes of activation at the same ionic strength with different cations (Goldberg et al., 1992; Leiting and Wicker, 1997; Snir et al., 1995), suggesting that competitive displacement of PE by cations is not the only mechanism

10.1021/jf980725a CCC: $18.00
Published on Web 03/24/1999

involved in activation and inhibition of PE activity (Wicker, 1996).

If the kinetic compensation parameters can be applied to PE catalysis, further insights into the mechanism of cation activation and inhibition of PE may be possible. The objectives of this study were to determine if kinetic compensation can be used to describe PE catalysis and to investigate the mechanism of the effect of sodium and calcium on PE activity by comparing compensation parameters.

MATERIALS AND METHODS

A thermostable form of pectinesterase (TS-PE) was purified from grapefruit by using protocols described in a previous study (Sun and Wicker, 1996). The specific activity of purified TS-PE was 313 units/mg of protein. The TS-PE catalytic reaction rate k (s^{-1}) was calculated by using the equation $k = V_{max}/[E]_t$, where V_{max} is the maximum reaction velocity and $[E]_t$ is the total enzyme concentration in the reaction mixture. The initial $[E]_t$ of 0.56 nM was estimated under standard assay conditions of 30 °C, 1% pectin, and pH 7.5, using a molecular weight of 53500 (Seymour et al., 1991), for Marsh grapefruit PE. V_{max} was tested by using a pH stat titrator (Brinkman, Westbury, New York) at pH 7.0 in 1% high-methoxyl pectin (Citrus Colloids Ltd., Hereford, U.K.). The pectin was dialyzed against 10 times volume of deionized water for three changes before use. The final NaCl concentration in pectin solution was adjusted to 0.05, 0.10, 0.15, or 0.20 M by adding solid NaCl. The final $CaCl_2$ concentration was adjusted to 0.005, 0.01, 0.015, or 0.02 M by adding solid $CaCl_2$. Higher concentrations of $CaCl_2$ resulted in localized gelation. The temperature of the reaction vessel was controlled to within 0.1 °C by a water bath (Neslab, Newington, NH).

RESULTS AND DISCUSSION

The catalytic rate constants, k, are plotted against $1/T$ with NaCl-added conditions (Figure 1a) and $CaCl_2$-added conditions (Figure 1b). The lines have slopes of $-E_a/R$ and y-axis intercepts of ln A. Activation entropy, $\Delta S^{\#}$, and activation enthalpy, $\Delta H^{\#}$, were calculated from eqs 3 and 4. Although eqs 3 and 4 show that $\Delta S^{\#}$ and $\Delta H^{\#}$ are functions of temperature, numerical values of these parameters varied <2% within a single cation concentration in the temperature range studied. The kinetic parameters are summarized in Table 1.

The natural logarithm of frequency factor (ln A) was plotted against the activation energy (E_a) for sodium-added (Figure 2a) and calcium-added (Figure 2b) conditions. Although ln A and E_a varied at different cation-added conditions, these parameters did not change independently. The increase of ln A was accompanied by an increase of E_a. The compensation relations were also observed between activation entropy ($\Delta S^{\#}$) and activation enthalpy ($\Delta H^{\#}$) (parts a and b of Figure 3, respectively). The compensation parameters α and β in eq 5 were derived from data depicted in Figure 2a,b. The compensation parameters δ and ϕ in eq 6 were derived from data depicted in Figure 3a,b. The isokinetic temperatures under sodium- and calcium-added conditions were calculated from the relationship $T_c = 1/\alpha R$ or $T_c = 1/\delta$ (Rhim et al., 1990). The compensation parameters and isokinetic temperatures are summarized in Table 2. In either cation-added condition, the isokinetic temperatures determined by ln A − E_a compensation and $\Delta S^{\#}$ − $\Delta H^{\#}$ compensation were slightly different. Because eqs 5 and 6 originate from different kinetic theories, the estimated isokinetic temperatures are not interchangeable, nor necessarily the same (Rhim et al., 1990). The isokinetic temperature value was estimated for each cation-added condition by averaging the T_c obtained by both methods. Substituting the average isokinetic temperature into the Arrhenius expression yielded a difference of <10% in the numerical values of ln A.

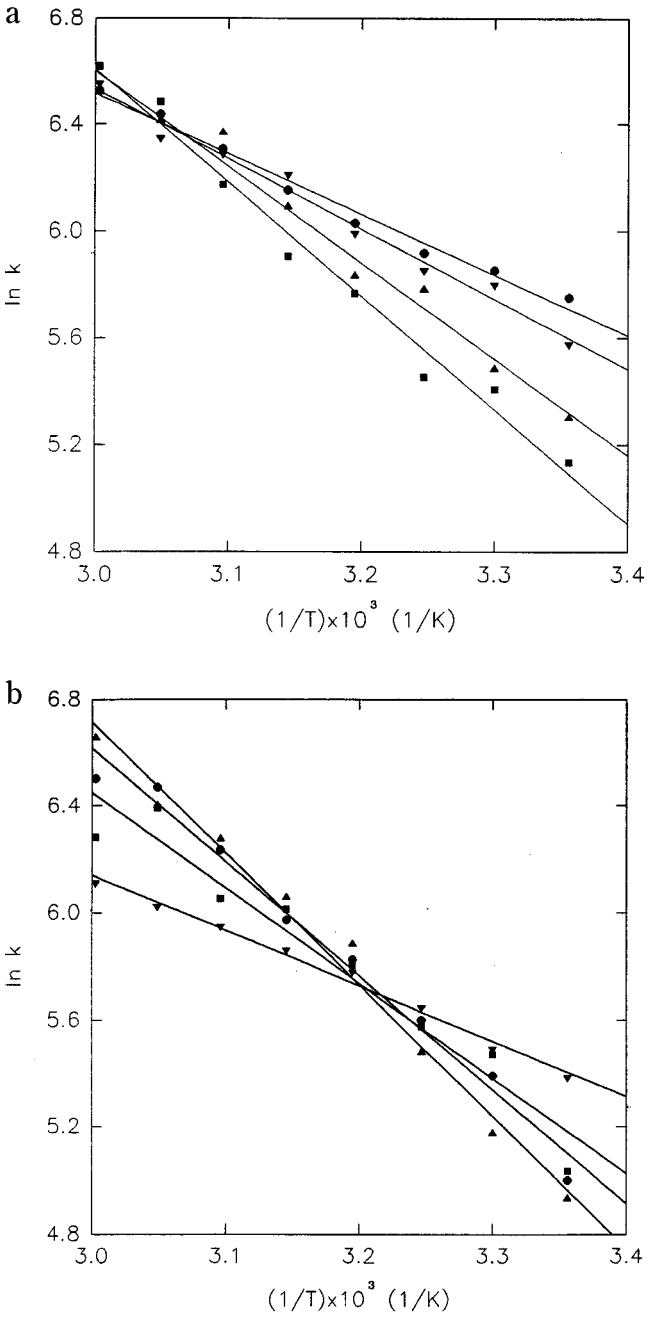

Figure 1. Arrhenius plots for pectin deesterification by thermostable pectinesterase. (a) NaCl added: (■) 0.05 M; (●) 0.10 M; (▲) 0.15 M; (▼) 0.20 M. (b) $CaCl_2$ added: (■) 0.005 M; (●) 0.01 M; (▲) 0.015 M; (▼) 0.020 M.

For sodium-added reaction, T_c was estimated to be 327.8 K. At this temperature, TS-PE activity is the same at different sodium concentrations. The estimated T_c for sodium-added conditions is ~25 K higher than the most commonly used assay temperature or ambient temperatures. At temperatures lower than T_c, the lower E_a (thus the lower the ln A), the higher the k. However, at temperatures higher than T_c, the higher the ln A (thus the higher the E_a), the higher the k. The lowest values for ln A and E_a were observed at 0.1 M NaCl (Table 1),

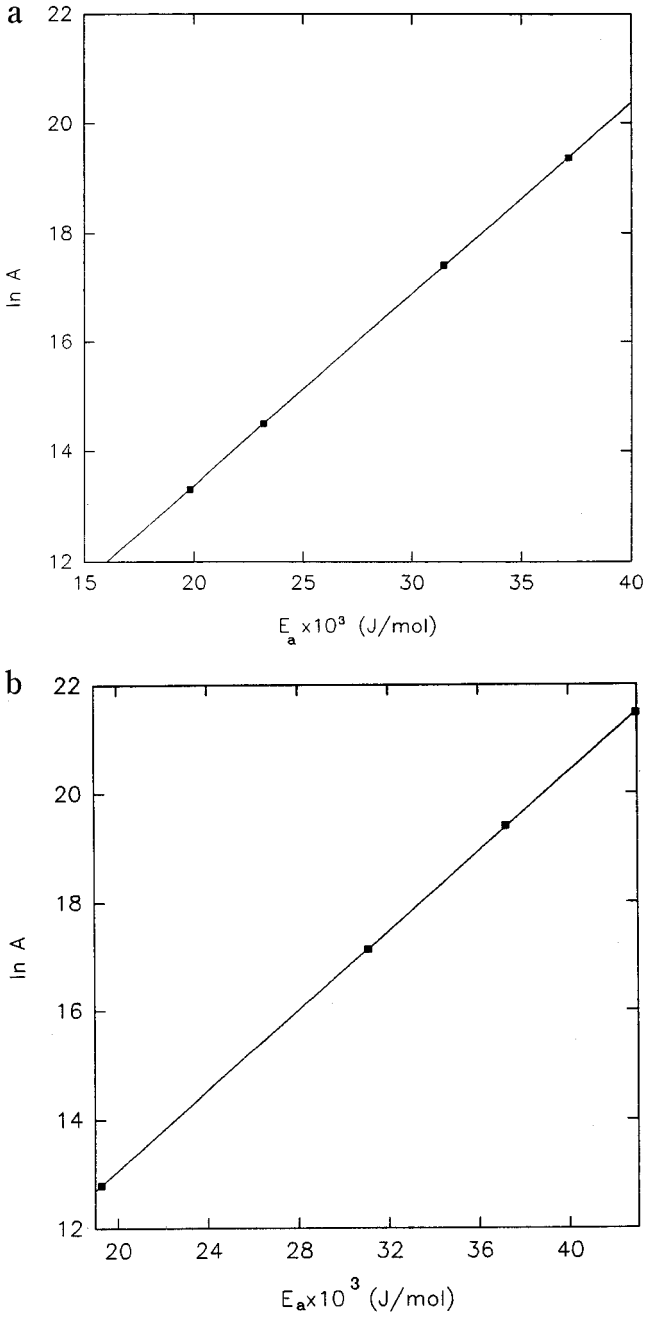

Figure 2. Variation of the frequency factor (ln A) with activation energy (E_a) under different experiment conditions: (a) NaCl added; (b) $CaCl_2$ added.

Table 1. Effect of NaCl and $CaCl_2$ on Kinetic Parameters Describing Deesterification of Pectin by PE, pH 7.0

	R^2	ln A	E_a (kJ/mol)	$\Delta S^{\#}$ (J/mol·K)	$\Delta H^{\#}$ (kJ/mol)
NaCl					
0.05 M (0.05)[a]	0.96	19.38	37.16	−92.76	34.43
0.1 M (0.1)	0.99	13.32	19.81	−145.62	17.08
0.15 M (0.15)	0.97	17.41	31.45	−109.93	28.72
0.2 M (0.2)	0.98	14.50	23.18	−135.33	20.45
$CaCl_2$					
0.005 M (0.015)	0.96	19.40	37.21	−92.55	34.48
0.01 M (0.03)	0.99	12.80	19.28	−150.16	16.54
0.015 M (0.045)	0.97	21.47	42.96	−74.44	40.23
0.02 M (0.06)	0.98	17.13	31.08	−112.40	28.35

[a] Numbers in parentheses are the ionic strength.

which agrees with the results of Lineweaver and Ballou (1945). They observed optimum activity at 0.1 M NaCl

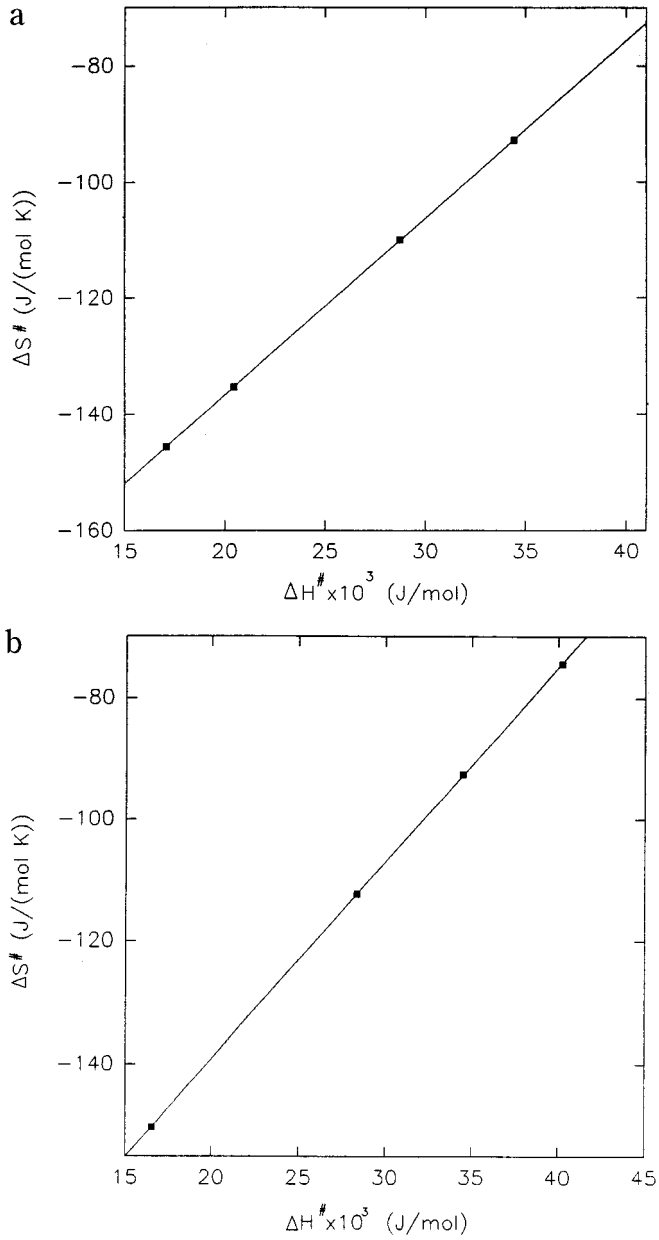

Figure 3. Variation of activation entropy ($\Delta S^{\#}$) with the activation enthalpy ($\Delta H^{\#}$) under different experimental conditions: (a) NaCl added; (b) $CaCl_2$ added.

Table 2. Summary of Compensation Relations and Isokinetic Temperature of PE Catalyzed Reaction under NaCl and $CaCl_2$ Added Conditions

compensation	NaCl added	$CaCl_2$ added
frequency factor−activation energy	ln $A = 0.000349E_a + 6.407$ ($R^2 = 1.00$) $T_c = 327.9$ K	ln $A = 0.000367E_a + 5.735$ ($R^2 = 1.00$) $T_c = 312.4$ K
activation enthalpy−activation entropy	$\Delta S^{\#} = 0.00305\Delta H^{\#} - 197.70$ ($R^2 = 1.00$) $T_c = 327.7$ K	$\Delta S^{\#} = 0.00320\Delta H^{\#} - 203.01$ ($R^2 = 1.00$) $T_c = 312.4$ K

for PE catalysis, at an assay temperature lower than the estimated T_c obtained in this study. Activation entropy−enthalpy compensation can also be used to explain the sodium effect on PE activity in a similar way.

For calcium-added conditions, T_c was estimated to be 312.4 K, which is ~10 K higher than most commonly

used assay temperatures. PE activity was less sensitive to calcium concentration changes above a threshold concentration (Leiting and Wicker, 1997). The PE-catalyzed reaction at 0.01 M $CaCl_2$ showed the lowest E_a (Table 1). This value is within the broad $CaCl_2$ concentration range of 0.002−0.05 M calcium for optimal PE activation previously reported (Leiting and Wicker, 1997). Conversely, Leiting and Wicker (1997) observed PE activation and inhibition over a relatively narrow range of concentration of ferric chloride and lead acetate. The insensitivity of PE activation and inhibition by calcium chloride at 30 °C (303 K) may be because the assay temperature was close to the T_c for calcium-added conditions.

Kinetic compensation has been demonstrated in PE inactivation (Ülgen and Özilgen, 1991). This study demonstrates that there is a kinetic compensation phenomenon in an enzymatically catalyzed reaction. Reaction rates or other kinetic parameters can be predicted by using the compensation relations (Barnes et al., 1969). Sodium-added conditions have different compensation parameters and isokinetic temperature from calcium-added conditions, supporting the hypothesis that their mechanisms of affecting PE activity are different. Furthermore, information on T_c and cation levels in citrus juices may improve the prediction of clarification potential with measured PE activity. Some juices clarify but have similar levels of in vitro PE activity as cloud stable juices. The level of inorganic cations in citrus juices varies depending on source and agricultural practices (Ting and Rouseff, 1986). Calcium concentration ranges from 65 to 120 ppm (Ting and Rouseff, 1986), which is below to close to the amount needed to stimulate PE activity (Leiting and Wicker, 1997). Sodium levels range from 1.5 to 25 ppm (Ting and Rouseff, 1986), well below the amount needed to stimulate PE activity (~5800 ppm). This may help explain the clarification of some juices that have similar levels of in vitro PE activity as cloud stable juices. Juices with similar PE activity measured at 30 °C (303 K) and 0.1 M NaCl have shown markedly different clarification rates, which may be dependent on the calcium level in different juices.

Also noteworthy is that under all conditions, $\Delta S^{\#}$ was negative (Table 1), which is the indication of structural changes that the reactants undergo while forming the activated complex (Daniels and Alberty, 1975). If there is an increase in the rotational and vibrational freedom of the activated complex, $\Delta S^{\#}$ is positive. If there is a decrease in rotational and vibrational freedom of the activated complex, $\Delta S^{\#}$ has a negative value (Daniels and Alberty, 1975). Because $\Delta S^{\#}$ is negative, formation of a PE−pectin activated complex is a process of rotational and vibrational freedom decrease.

Usually, it is accepted that the lower E_a, the higher the reaction rate (Whitaker, 1972; Segel, 1976). The other kinetic parameter, frequency factor (A), has been ignored in this context. In a family of reactions with kinetic compensation, a decrease in E_a is accompanied by a proportional decrease of ln A, which in turn results in a decrease of reaction rate k. However, if we rewrite the Arrhenius equation using eq 5

$$\ln k = (\alpha - 1/(RT))E_a + \beta \qquad (7)$$

is obtained. When $\alpha > 1/(RT)$, that is, $T > 1/(\alpha R)$, k increases as E_a increases; when $\alpha < 1/(RT)$, that is, $T < 1/(\alpha R)$, k increases as E_a decreases; when $\alpha = 1/(RT)$, $T = 1/(\alpha R)$ and k is independent of E_a. Isokinetic temperature $T_c = 1/(\alpha R)$ (Rhim et al., 1990). In a reaction with kinetic compensation, the isokinetic temperature should be considered an important constant and should be referred to when one is discussing the reaction rate's being affected by activation energy or other kinetic parameters. These kinetic parameters are decided by reaction conditions such as cation concentration and pH. In the case of this experiment, we cannot conclude which cation concentration is optimal for the enzyme activity unless the assay temperature is known. At temperatures lower than the isokinetic temperature, a decrease of ln A, E_a, $\Delta S^{\#}$, and $\Delta H^{\#}$ causes an increase of catalytic rate constant, that is, enzyme activation. At temperatures higher than the isokinetic temperature, an increase of ln A, E_a, $\Delta S^{\#}$, and $\Delta H^{\#}$ causes an increase in catalytic rate constant, that is, enzyme activation.

The principle of activation energy−frequency factor and activation entropy−activation enthalpy compensation applies to an enzyme-catalyzed reaction. These compensation relations and isokinetic temperatures have been neither determined nor used in enzyme activation and inhibition studies. Compensation relations can be used to elucidate information on the mechanism of enzyme catalysis. In this study, we have shown that the cations, sodium and calcium, have a unique T_c on PE interaction with pectin and belong to a different family of reactions. If competitive displacement by cations of PE from an inactive pectin−PE complex were the only factor involved in activation and inhibition of PE by cations, then similar effects should be observed with the same ionic strength. The effect of cations on PE activity varied with concentration, ionic strength, and assay pH (Nari et al., 1991; Goldberg et al., 1992; Leiting and Wicker, 1997; Snir et al., 1995). Sodium and calcium have a unique T_c and hence belong to a different family of reactions. Most likely, sodium and calcium uniquely affect PE, pectin, and/or PE−pectin complex formation and dissolution.

ACKNOWLEDGMENT

We thank Citrus Colloids for donation of the pectin used in this study.

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Received for review July 8, 1998. Revised manuscript received February 9, 1999. Accepted February 10, 1999. This research was partially supported by BARD Project US-2222-92R.

JF980725A