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Inactivation of Apple Pectin Methylesterase Induced by Dense Phase Carbon Dioxide

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The inactivation of apple pectin methylesterase (PME) with dense phase carbon dioxide (DPCD) combined with temperatures (35−55 °C) is investigated. DPCD increases the susceptibility of apple PME to the temperatures and the pressures have a noticeable effect on apple PME activity. A labile and stable fraction of apple PME is present and the inactivation kinetics of apple PME by DPCD is adequately described by a two-fraction model. The kinetic rate constants k_L and k_S of labile and stable fractions are 0.890 and 0.039 min^{-1}, and the decimal reduction times D_L and D_S are 2.59 and 58.70 min at 30 MPa and 55 °C. Z_T representing temperature increase needed for a 90% reduction of the D value and the activation energy E_a of the labile fraction at 30 MPa is 22.32 °C and 86.88 kJ /mol, its Z_P representing pressure increase needed for a 90% reduction of the D value and the activation volume V_a at 55 °C is 21.75 MPa and −288.38 cm^3/mol. The residual activity of apple PME after DPCD exhibits no reduction or reactivation for 4 weeks at 4 °C.

KEYWORDS: Pectin methylesterase; dense phase carbon dioxide; inactivation; apple

INTRODUCTION

Apple juice is commonly drunk as a clear juice, but there has been a growing market for natural cloudy apple juice over the past few years (*1*). However, cloud loss is a major quality defect occurring in cloudy fruit and vegetable juices. Yamasaki et al. (*2*) reported that suspended substance in a simplified model of apple juice was a protein−carbohydrate complex that was surrounded by negatively charged protective pectin. While undesired, clarification is strongly influenced by demethylation of pectin by endogenous pectin methylesterase (PME, EC 3.1.1.11) yielding acidic pectin with a lower degree of esterification, which can cross-link with polyvalent cations such as Ca^{2+} to form insoluble pectate precipitates, or becomes a target for pectin-degrading polygalacturonases (PG) (*3–5*). Thus, the control of PME activity is crucial for the cloud stability of juices (*6*). In the fruit juice industry, thermal treatment (e.g., 90 °C, 1 min for citrus juices) is the most common and least expensive technology that has been used to solve the problem (*6*). Unfortunately, this processing has a negative influence on the juice flavor (*4, 7*). In recent years, nonthermal technologies (irradiation, ultrahigh pressure, pulsed electric fields, pulsed magnetic fields, and dense phase carbon dioxide) are attracting interest and gaining acceptance as potential alternative or complementary processes to traditional thermal methods in the food industry (*5, 8*).

Several nonthermal methods have been proposed to inactivate PME. Plant PMEs have been isolated, purified, and studied in terms of pressure−thermal processing stability (*4, 9, 10*). The high-pressure technique has indeed attracted a larger number of researchers, although the main limitation of this method is the difficulty of controlling and managing the operating pressure in such an extreme range of values, and it is hard to inactivate some pressure-resistant enzymes (e.g., PME, PG) at lower pressure (<400 MPa) (*1, 11*), thus its widespread use in industry appears limited (*12*).

As a new nonthermal technology, the use of DPCD for inactivation of enzymes continues to attract attention. It is clear that CO_2 is, in general, able to substantially reduce the microbial activity of any microorganism at relatively mild operating conditions and can inactivate enzymes at temperatures where thermal inactivation is not effective (*7*). Moreover, it is nontoxic and easily removed by simple depressurization and outgassing (*12*). Most researches show that longer treatment time, higher pressure, and higher temperature increase the inactivation effect. However, PMEs from different sources have different characteristics, and multiple isoenzymes existing in the same source

Table 1. Effects of Mild Heat on the Activity of Apple PME under Atmospheric Pressure

	PME residual activity (%)[a,b]		
treatment time (min)	35 °C	45 °C	55 °C
0	100.00 ± 3.58 a	100.00 ± 3.58 a	100.00 ± 3.58 a
5	113.66 ± 5.93 a	107.28 ± 3.26 a	95.50 ± 5.16 a
15	108.03 ± 8.05 a	111.42 ± 9.10 a	90.67 ± 2.78 ab
30	109.79 ± 6.81 a	104.59 ± 3.09 a	88.31 ± 2.21 ab
45	104.59 ± 3.09 a	100.36 ± 1.71 a	86.28 ± 4.33 ab
60	100.36 ± 1.71 a	100.86 ± 2.99 a	81.11 ± 2.18 b

[a] All data represent means ± SD, $n = 3$. [b] Within the same column, the letters a and b mean the values are not significantly different at $p < 0.05$.

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with different molecular weight, isoelectric points, and/or kinetic properties (*3, 4, 13–15*). Furthermore, Chen et al. (*14*) in their critical review pointed out that the first-order model did not give the best fit for predicting heat inactivation of multiple PMEs in citrus juices and proposed a two-component first-order model as an alternative.

The inactivation kinetics of apple PME induced by DPCD has not been described before today, thus it is worth reporting. The purpose of this study is to investigate the effects of DPCD on inactivation of apple PME and to model the inactivation kinetics of apple PME by DPCD.

MATERIALS AND METHODS

PME Extraction. The extraction of apple PME was performed by the method described by Balogh et al. (*9*) with a little modification. Fifteen kilograms of apples (*Fuji*, Shandong, China) were purchased at commercial maturity from a local supermarket and stored at 0 ± 1 °C in a cold warehouse until processing. The pH and total soluble solid were 3.8−4.0 and 11.5 °Brix. Apples were washed, sliced, and juiced with a juice extractor (ZHJ-308A1, Fushan Ouke Electric Appliance Co., Guangdong, China). To avoid undesirable enzymatic browning during the processing, 0.10% (w/v) of L-ascorbic acid (Beijing Chemicals Co. Beijing, China) as antibrowning agent was added to apple slices before juicing. After being dejuiced, apple PME was extracted from the apple pomace by using 0.2 M Tris-chloride buffer (pH 8.0) with 1 M NaCl (1:2 w/v) for 18 h at 4 °C. The mixture was filtrated by using two layers of cheese cloth and the filtrate was precipitated with 30% saturated ammonium sulfate, while it was stirred for 20 min and centrifuged (13 000 × g, 20 min) after standing for 1 h. The supernatant was precipitated again with 80% saturated ammonium sulfate, stirred for 20 min, and centrifuged (13 000 × g, 20 min) after standing for 2 h. The precipitate was dissolved in a minimum volume of 20 mM Tris-chloride buffer pH 7.5 and dialyzed against the same buffer overnight with at least three changes of buffer before use. This apple crude PME obtained was quickly frozen with liquid nitrogen and stored at −18 °C. All extraction procedures were performed at 4 °C.

DPCD Process System. The diagram of the DPCD system was described by Liao et al. (*16*). The stainless steel pressure vessel with a volume of 850 mL was designed to withstand a pressure of 50 MPa. The vessel temperature was maintained by a THYS-15 thermostatic bath (Ningbo Tianheng Instrument Factory, Zhejiang, China). An XMTA-7512 temperature controller (Yuyao Temperature Meter Factory, Zhejiang, China) was used to monitor the temperature with two thermocouples. One thermocouple was fixed in the vessel lid to monitor the CO_2 temperature in the upper part of the vessel and the other was placed at the middle wall of the vessel to monitor the temperature of the middle part of the vessel, which was used as process temperature. A 2TD plunger pump (Huaan Supercritical Fluids Extraction Co. Ltd., Jiangsu, China) with a maximum pressure of 50 MPa and a maximum flow rate of 50 L/h was used to pressurize the vessel. A DBY-300 pressure transducer (Shanxi Qingming Electronic Group Corporation, Shanxi, China) was fixed in the vessel lid to monitor the vessel pressure. All the data for temperature and pressure were displayed on a control panel. All parts of the system exposed to high pressure were made of stainless steel. The vessel had gastight connections to the gas inlet and outlet, and the fluid sample inlet and outlet. The vessel lid could be sealed by screws during DPCD processing. A 2XZ-4 vacuum pump (Huangyan Qiujing Vacuum Pump Factory, Zhejiang, China) was connected to the vessel for evacuating the air in the vessel and building the vacuum state of the vessel.

Inactivation of Apple PME with DPCD. To investigate the effect of DPCD on the inactivation of apple PME, the apple PME buffers were subjected to different pressure levels (8−30 MPa) combined with moderate temperatures (35−55 °C) for various times (5−60 min).

For each experiment, 1.5 mL of apple PME was transferred into a 15 mL test tube (Beijing Bomex company, Beijing, China) without the cap and then the tube was directly placed in the DPCD vessel, which had been preheated to the experimental temperature, and then pressurized by the plunger pump to the required pressure level, then the required pressure was held for the required treatment time. Next the depressurization was performed by opening the pressure relief valve for 1−5 min, the depressurization time was closely related to the applied treatment pressures. After DPCD, apple PME was taken out from the vessel and immediately cooled in an ice bath. Following equilibration to ambient temperature, the pH value of the sample was determined, then the sample was frozen with liquid nitrogen and stored in −18 °C for activity determination.

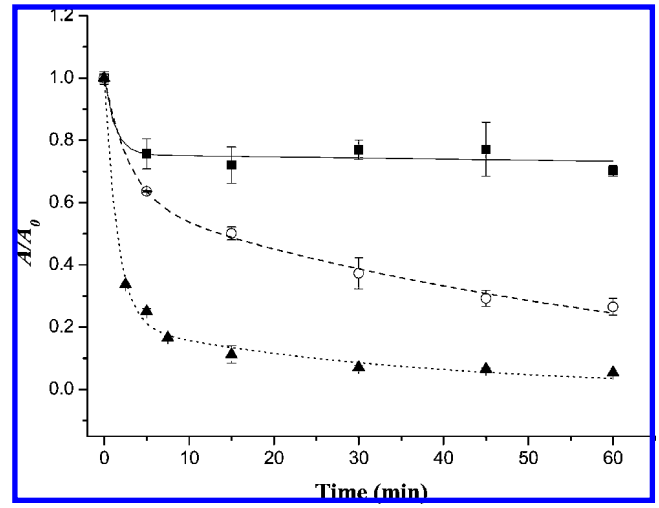

Figure 1. Effect of temperatures of dense phase carbon dioxide on the inactivation of apple PME as a function of treatment time at 30 MPa. The modeled curves at 35 (■), 45 (○), and 55 °C (▲) based onthe two-fraction model $A = A_L \exp(-k_L t) + A_S \exp(-k_S t)$. All data are the means ± SD, $n = 3$. Data point without error bars have a smaller standard deviation than the data symbol.

The pH of apple PME buffer was measured with a digital pH meter (Thermo Orion 868, USA) equipped with a microelectrode (Thermo Orion CHN 060, USA).

Inactivation of Apple PME by Mild Heat Treatment. For each experiment, 1.5 mL of apple PME buffer was transferred into a 15 mL test tube without the cap and treated by mild heat under atmospheric pressure in a DPCD vessel, which was preheated to the experimental temperatures. The remaining procedures were in accord with that of DPCD inactivation experiments.

PME Assay. The activity of PME was measured at pH 7.5 and 30 °C according to the method proposed by Kimball (*17*), which was based on carboxyl group titration. A 0.20 mL sample of apple PME tempered at 30 °C was mixed with 20 mL of 1% apple pectin (DE 70−75%, Andre Co., Shandong, China) containing 0.1 M NaCl (at 30 °C) and incubated at 30 °C. The solution was adjusted to pH 7.0 with 2.0 N NaOH, and then the pH of the solution was readjusted to pH 7.5 with 0.05 N NaOH. After the pH reached 7.5, 0.05 mL of 0.05 N NaOH was added. The time required for the solution's pH to return to 7.5 was measured. PME activity (A) expressed in pectin methylesterase units (PMEU) was calculated by the following (eq 1).

$$A = \frac{[\mathrm{NaOH}]V_{\mathrm{NaOH}}}{V_{\mathrm{sample}}t'} \quad (1)$$

where [NaOH] is NaOH concentration (=0.05 N), V_{NaOH} is the volume of NaOH used (=0.05 mL), V_{sample} is the volume of sample used (=0.20 mL of apple PME), and t' is the time (in minutes) needed for pH to return to 7.5 after the addition of NaOH.

Modeling and Analysis of Kinetics of PME Inactivation. A first-order kinetic model could often describe the inactivation of enzymes, whereby enzyme activity decreased linearly as a function of time (*18*).

$$\ln(A/A_0) = -kt \quad (2)$$

where A_0 is the mean initial activity of the enzyme, A the mean residual activity after treatment, k (min^{-1}) the inactivation rate constant at a given temperature and/or pressure, and t (min) the holding time.

When a biphasic inactivation pattern was observed, the experimental data were analyzed by using a two-fraction model (*19–22*). This model took into account the existence of several isoenzymes which could often be grouped into two fractions (i.e., labile and stable fraction), one more resistant to temperature and/or pressure than the other. Both were considered to be inactivated according to first-order kinetics, independently of each other. There was a fast inactivation period followed by a decelerated decay, the total activity decay was therefore described by eq 3

$$A = A_L \exp(-k_L t) + A_S \exp(-k_S t) \quad (3)$$

where A_L and A_S refer, respectively, to the activity of the labile and stable fractions in relation to total enzyme inactivation; k_L and k_S are the inactivation rate constants of both labile and stable fraction (min^{-1}), respectively.

The decimal reduction time (D value) defined as the treatment time needed for 90% inactivation of initial activity of the enzyme at a given condition was obtained.

$$D = \frac{\ln(10)}{k} \quad (4)$$

The pressure or temperature increase needed for a 90% reduction of the D value was reflected by Z_P (MPa) or Z_T (°C), respectively. Mathematically, they followed the equations.

$$\log\left[\frac{D_1}{D_2}\right] = \frac{P_2 - P_1}{Z_P} \quad (5)$$

$$\log\left[\frac{D_1}{D_2}\right] = \frac{T_2 - T_1}{Z_T} \quad (6)$$

The pressure and temperature dependence of k could be expressed by the activation volume (V_a, cm^3/mol) and activation energy (E_a, kJ/mol), as can be seen in the Eyring (eq 7) and Arrhenius (eq 8) equations, respectively (*7, 8, 21, 22*).

$$\ln\left[\frac{k_1}{k_2}\right] = \frac{V_a}{R_p T}[P_2 - P_1] \quad (7)$$

$$\ln\left[\frac{k_1}{k_2}\right] = \frac{E_a}{R_T}\left[\frac{1}{T_2} - \frac{1}{T_1}\right] \quad (8)$$

where P_2 and P_1, T_2 and T_1 are pressures and temperatures corresponding to the decimal reduction times D_1 and D_2 or constants k_1 and k_2, respectively. R (=8.314 J mol^{-1} K^{-1}) is the universal gas constant, T is the absolute temperature (K). The values of Z_P and Z_T are obtained as the negative reciprocal slope of the regression line representing log D versus P and T relationship, respectively. Both V_a and E_a are estimated from linear regression of ln k versus P or ($1/T$), respectively.

Statistical analysis. Analysis of variance (ANOVA) was carried out by using the software Microcal Origin 7.5 (Microcal Software, Inc., Northampton, USA). ANOVA tests were performed to determine the significance at 95% confidence. All experiments are performed in triplicates.

RESULTS AND DISCUSSION

Effect of Temperatures on the Residual Activity of PME under Atmospheric Pressure. As shown in **Table 1**, the residual activity of apple PME under atmospheric pressure exhibits some fluctuations after mild heat at 35, 45, and 55 °C. It seems that apple PME is activated at 35 or 45 °C, but ANOVA analysis indicates that the two temperatures have no significant effects on the residual activity of apple PME ($p > 0.05$) as compared to the control sample. Although the residual activity of apple PME is reduced at 55 °C as the treatment time increases, and a significant reduction of the residual activity of apple PME is obtained after 30 min ($p < 0.05$), the maximum reduction of apple PME activity is still less than 20% for 60 min, indicating that apple PME is rather stable in this study.

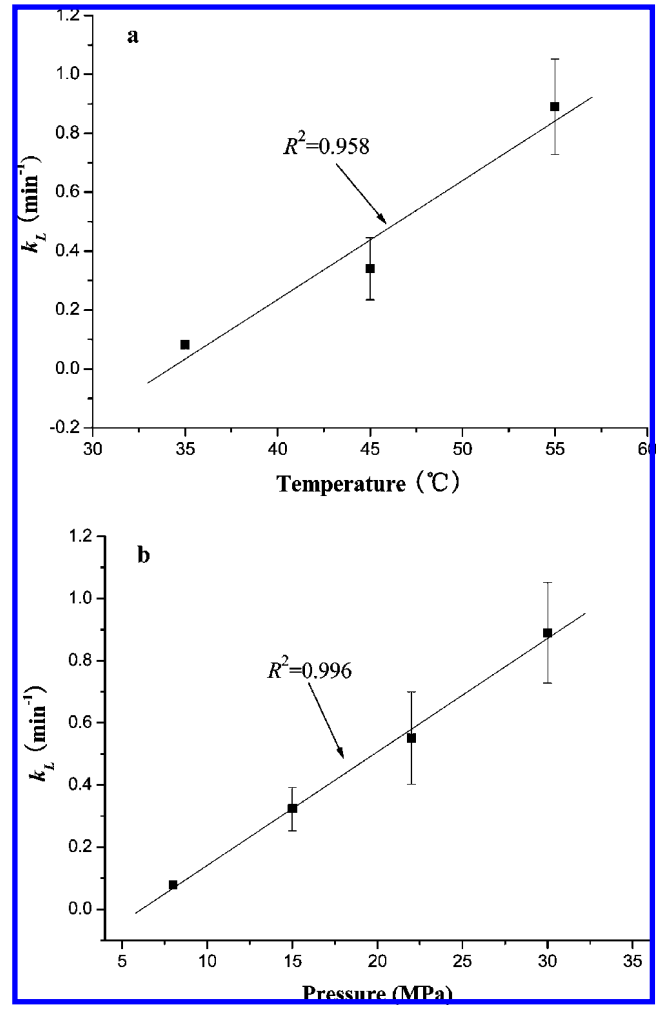

Figure 2. A linear correlation of the inactivation rate constant k_L of the labile fraction in apple PME with temperatures at 30 MPa (a) or pressures at 55 °C (b) under dense phase carbon dioxide. All data are the means ± SD, $n = 3$. Data points without error bars have a smaller standard deviation than the data symbol.

Some similar results were reported. Vera et al. (*23*) described that about 80% of the activity of extracted PME from hawthorn fruit still remained after heating at 55 °C for 40 min. Assis et al. (*24*) suggested that the crude PME from acerola (borate−acetate buffer, 50 mM, pH 8.3) was very stable at 50 °C with 10% loss of activity for 100 min and needed 110 min for its inactivation at 98 °C. Leite et al. (*25*) found the concentrated PME of guava (borate−acetate buffer, 50 mM, pH 8.3) was very stable as a function of time (1−60 min) of incubation at various temperatures (50−98 °C). However, Denès et al. (*26*) found that 90% of reduction in purified apple PME activity (citrate−phosphate buffer, 6 mM, pH 4) was observed at 55 °C for 2.6 min and the heat inactivation of PME in the range of 45−59 °C within 15 min could well be described by the classical first-order model. This contradiction is possibly attributed to the lower pH of the PME buffer solution and purified PME in Denès's study and the higher pH of the PME buffer solution and crude PME of this study, because lower pH in buffer solution and purification destabilizes the PME subjected to heat.

Effect of Temperatures on the Residual Activity of Apple PME Exposed to DPCD Treatment. The effect of temperatures on the residual activity of apple PME exposed to DPCD at 30 MPa is illustrated in **Figure 1**. The inactivation behavior of

Table 2. Estimation of the Kinetic Parameters for Apple PME Inactivation Exposed to Dense Phase Carbon Dioxide Based on the Two-Fraction Model[a]

T (°C)	P (MPa)	A_L (%)	A_S (%)	k_L (min^{-1})	k_S (min^{-1})	D_L (min)	D_S (min)	R^2 ($p < 0.05$)
35	30	24.63 ± 6.70	75.37 ± 5.22	0.082 ± 0.008	0.002 ± 0.000	28.08	1151.50	0.940
45	30	38.94 ± 4.96	61.04 ± 4.45	0.340 ± 0.015	0.015 ± 0.002	6.77	153.53	0.997
55	30	72.81 ± 4.54	25.90 ± 3.73	0.890 ± 0.013	0.039 ± 0.009	2.59	58.70	0.993
55	22	62.09 ± 7.96	35.36 ± 6.16	0.551 ± 0.039	0.037 ± 0.005	4.18	61.45	0.991
55	15	56.51 ± 5.65	40.81 ± 5.40	0.323 ± 0.072	0.034 ± 0.006	7.12	67.62	0.996
55	8	39.51 ± 3.61	63.69 ± 4.73	0.079 ± 0.022	0.053 ± 0.017	29.06	43.31	0.984

[a] Two-fraction model: $A = A_L \exp(-k_L t) + A_S \exp(-k_S t)$. A_L and A_S: activity of the labile and stable fractions in relation to total enzyme inactivation; k_L and k_S: inactivation rate constants of both labile and stable fraction; D_L and D_S: decimal reduction times of both labile and stable fraction which are estimated by eq 4 based on the respective mean values of k. Data represent means ± SD, $n = 3$.

apple PME exposed to DPCD is very different from that of apple PME under the atmospheric conditions, the residual activity of apple PME decreases significantly after DPCD. Moreover, the maximum reduction of the residual activity of apple PME exposed to DPCD is 94.57% at 55 °C for 60 min, which is far greater than that of the activity of apple PME at 55 °C under atmospheric pressure, suggesting that apple PME is more susceptible to the temperatures under DPCD conditions. Due to pH reduction of apple PME buffer from 7.5 to about 5.0 after DPCD (data not shown), the effect of pH reduction alone on PME activity is further examined. The residual activity of apple PME not subjected to DPCD still retains 88.80% of its original activity after pH adjustment from 7.5 to 5.0 with hydrochloric acid, indicating that pH reduction alone does not inactivate PME to a greater extent in this study. However, a greater reduction was observed by Denès et al. (*26*) that the activity of purified PME (citrate−phosphate buffer, 6 mM) from apples (*Golden Delicious*) at pH 4.0 represented only 1% of the optimum activity at pH 7.5. This discrepancy may result from the different apple cultivars and purified PME, which was more susceptible. Therefore, this increase in the susceptibility of apple PME to temperature is possibly attributed to an interaction of pressure and pH reduction with DPCD. The inactivation of apple PME is probably due to its structural change by DPCD, because Gui et al. (*27*) reported that DPCD caused some changes in the secondary and tertiary structures of horseradish peroxidase.

The residual activity of apple PME seems to conform to a biphasic kinetics (**Figure 1**) since the first-order model (eq 2) does not provide a better fit for the inactivation of apple PME with DPCD at 35 or 55 °C ($R^2 \leq 0.679$). When a biphasic inactivation pattern was observed, the experimental data were analyzed by using a two-fraction model (*18, 19, 22, 26*). Therefore, it is assumed that labile and stable fractions of apple PME are present and they result in the biphasic inactivation pattern. All the inactivation curves in **Figure 1** are well fitted to a two-fraction first-order model with higher regression coefficients ($R^2 \geq 0.940$). In earlier investigations on PME inactivation by heat or ultrahigh pressure, the presence of labile and stable fractions has been reported in other PMEs purified from several fruits and vegetables, such as oranges (*28, 29*), potatoes and carrots (*30*), white grapefruits (*15*), acerola (*24*), sweet cherries (*31*), persimmon (*32*) plums (*21*) and pepper (*22*). However, no similar studies of PME inactivation by DPCD were previously reported with the two-fraction model.

The biphasic kinetic parameters of inactivation rate constants k_L and k_S, decimal values D_L and D_S, are summarized in **Table 2**. It can be seen that k_L is 20−40 times more than k_S, and each D_L is also far smaller than its corresponding D_S, respectively, confirming that the labile and stable fractions are present in apple PME. The D values of the heat-labile and heat-stable fractions at 90 °C were estimated to be 0.225 and 32 s by Wicker et al.

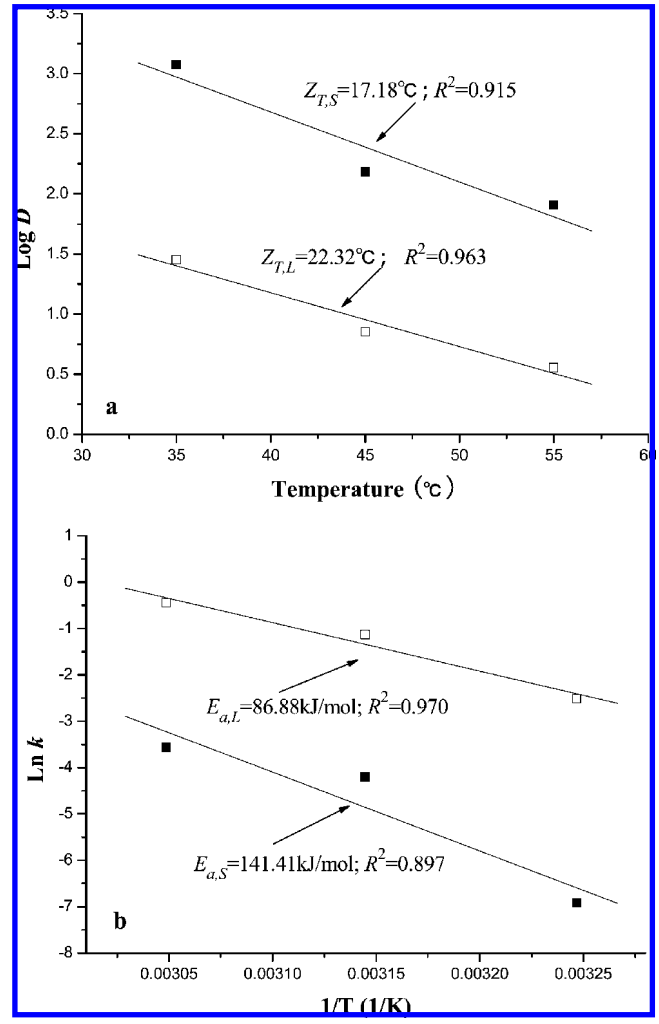

Figure 3. Z_T (a) and E_a (b) for temperature-dependence inactivation of the labile fraction (□) and the stable fraction (■) for apple PME with use of dense phase carbon dioxide at 30 MPa.

(*28*). However, Balaban et al. (*7*) obtained the D value of PME was 20.9 min in orange juice inactivated with DPCD at 31 MPa and 55 °C, and the inactivation kinetics was in agreement with first order. Moreover, k_L in this study is linearly correlated to temperatures with a regression coefficient $R^2 = 0.958$ in **Figure 2a**.

The Z_T and E_a values of the two fractions at 30 MPa, calculated by eqs 6 and 8, are displayed in **Figure 3a**,b. The labile fraction in apple PME exhibits higher Z_T (22.32 °C) and lower E_a (86.88 kJ /mol) than those ($Z_T = 17.18$ °C and $E_a =$ 141.41 kJ /mol) of the stable fraction. Balaban et al. (*7*) determined the Z_T and E_a values of PME were 5.2 °C and 97.4 kJ/mol, respectively, in orange juice inactivated with DPCD at 31 MPa. Wicker et al. (*28*) reported that the Z_T values for the

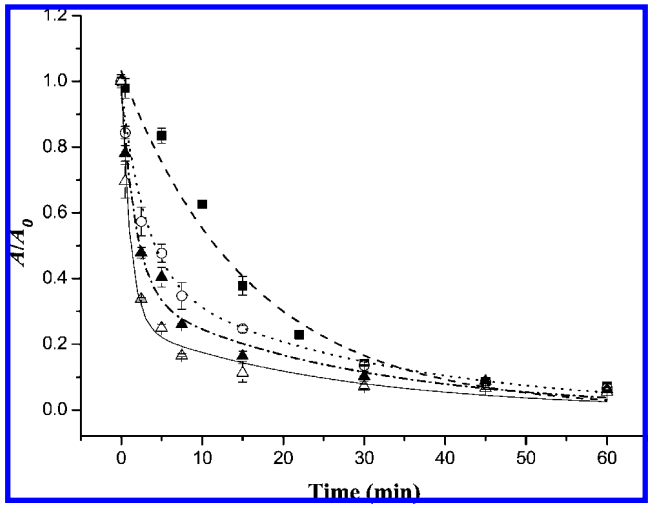

Figure 4. Effect of pressures of dense phase carbon dioxide on the inactivation of apple PME as a function of treatment time at 55 °C. The modeled curves at 8 (■), 15 (○), 22 (▲), and 30 MPa (Δ) are based on the two-fraction model $A = A_L \exp(-k_L t) + A_S \exp(-k_S t)$. All data are the means ± SD, $n = 3$. Data points without error bars have a smaller standard deviation than the data symbol.

labile and stable fractions were 10.8 and 6.5 °C from thermal inactivation of citrus PME in orange juice pulp while Tajchakavit et al. (*34*) found that the Z_T values for the labile and stable fractions were 17.6 and 31.1 °C in orange juice. It is hard to compare these data due to different sources and different experimental conditions. Guiavarc'h et al. (*15*) found that an E_a less than 97.3 kJ/mol was only obtained using pressure higher than 600 MPa when the purified heat-labile PME of white grapefruit (in 20 mM, Tris Buffer, pH 7.0) was submitted to combined thermal (10−62 °C) and high pressure (100−750 MPa) treatment. Similarly, the E_a was estimated as 109 kJ/mol at 600 MPa for PME in orange juice under high hydrostatic pressure combined with moderate temperature (30−60 °C) (*34*). The E_a values of the labile fraction and the stable fraction for thermal inactivation of purified plum PME (in 20 mM, Tris Buffer, pH 7.5) at 60 °C were 273.9 and 354.3 kJ/mol (*20*), which were far greater than that in this study. As compared to these previous investigations, it seems to suggest that DPCD treatment with lower pressure is possibly more effective in inactivating PME than high hydrostatic pressure or thermal treatment.

Effect of Pressures on the Residual Activity of Apple PME Exposed to DPCD. Figure 4 shows the effect of pressures on the residual activity of apple PME exposed to DPCD at 55 °C. As the pressure increases from 8 to 30 MPa, the residual activity of apple PME is significantly reduced to 97.86%, 84.38%, 78.09%, and 69.61% after 0.5 min ($p < 0.05$), respectively, and it takes 22.0, 15.0, 7.5, and 5.0 min to reach a similar residual activity (22.92%, 24.67%, 25.99%, and 25.02%) of PME at the corresponding pressures. Moreover, the maximum reduction of PME residual activity attained 92.75%, 93.39%, 93.77%, and 94.57% after 60 min, respectively, and no significant difference of the residual activity of PME under these conditions has been observed ($p > 0.05$). These data reflect that pressures have a noticeable effect on the residual activity of PME at the beginning of DPCD.

The inactivation curves of apple PME with increasing pressures at 15, 20, and 30 MPa are not well fitted to the first-order kinetic model (eq 2) due to the poor reliability with lower coefficients ($R^2 \leq 0.892$). Thus, the two-fraction model (eq 3) is also introduced with a good fit ($R^2 \geq 0.984$), suggesting the

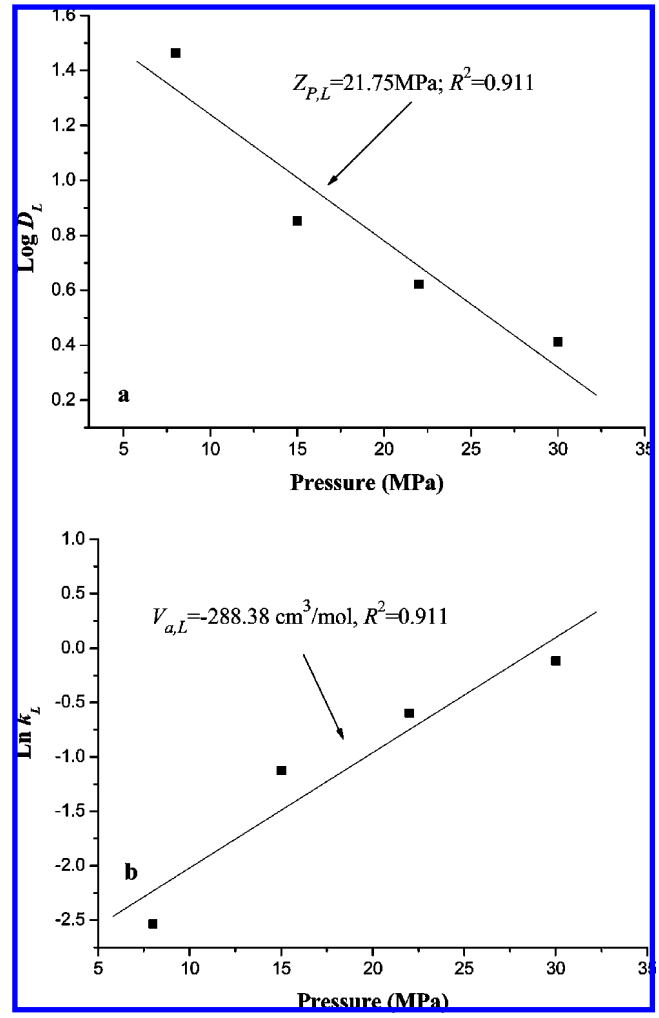

Figure 5. Z_P (a) and V_a (b) for pressure-dependence inactivation of the labile fraction for apple PME with use of dense phase carbon dioxide at 55 °C.

presence of a labile and a stable fraction of apple PME, as previously described in this study.

As shown in **Table 2**, A_L increases with increasing temperatures at 30 MPa or increasing pressures at 55 °C. As previously reported, A_L was not estimated and the relationship of A_L with temperature or pressure was not given (*18, 19, 22*). Only a few investigations provided the value of A_L. The A_L of purified plum PME at 60 °C was 59.1% (*21*). Tribess and Tadini (*33*) also reported that A_L of PME in orange juice increased from 89% to 99.9% depending on temperatures (82.5−87.5 °C). It appeared from these references that A_L also increased with increasing temperatures. As compared to these values, A_L in this study is smaller since treatment temperatures (≤55 °C) are lower. The drastic variation of A_L is possibly due to the increase in the susceptibility of apple PME to temperature or pressure under DPCD as previously shown in this study.

The biphasic kinetic parameters of the inactivation rates k and the decimal values D of PME at different pressure levels are also summarized in **Table 2**. For the labile fraction, k_L and D_L values are closely related to the pressure levels. Higher pressures result in higher k_L values (lower D_L) at 55 °C, and k_L is linearly related to pressures with a regression coefficient $R^2 = 0.996$ in **Figure 2b**. The D_L value of 2.59 min for the labile fraction is minimized at 30 MPa and 55 °C, which is lower than the D value of 10 min for PME in orange juice inactivated with DPCD at 31 MPa and 60 °C (*7*), indicating that the labile

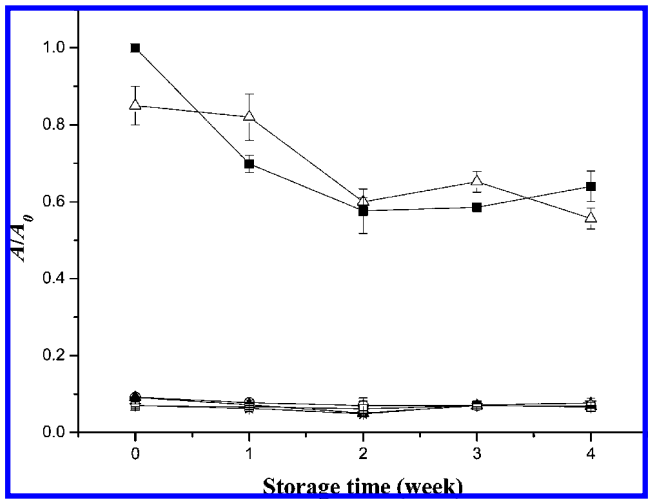

Figure 6. Residual activity of apple PME after 4-week storage at 4 °C. Apple PME is heated at 55 °C for 60 min under atmospheric pressure (Δ), and treated with dense phase carbon dioxide at 55 °C for 60 min under different pressures: 8 (○), 15 (▲), 22 (☆), or 30 MPa (□). Untreated apple PME (■) is used as a control. All data are the means ± SD, $n = 3$. Data points without error bars have a smaller standard deviation than the data symbol.

fraction of apple PME is more susceptible to DPCD than PME in orange juice. The k_S or D_S values of the stable fraction are far smaller or larger than that of the labile fraction in **Table 2**, confirming that the stable fraction is more resistant to the pressure than the labile fraction.

As the labile fraction is susceptible to the pressure, its Z_P (=21.75 MPa) and V_a (=−288.38 cm^3/mol) at 55 °C calculated by eqs 5 and 7 are shown in **Figure 5a**,b. The V_a of PMEs was contradictorily reported in previous investigations due to different sources and different experimental conditions. Ly-Nguyen et al. (*4*) obtained the V_a (=−39.4 cm^3/mol) of purified carrot PME (in 20 mM Tris-HCl buffer, pH 7.0) at 54 °C during high-pressure treatment (400−825 MPa). Castro et al. (*22*) obtained the V_a (−19.01 cm^3/mol) of the labile fraction of purified pepper PME (20 mM citrate buffer, pH 5.6) at 54 °C during ultrahigh pressure treatment (300−800 MPa). Guiavarc'h et al. (*15*) reported the V_a around −30 cm^3/mol at any temperatures (10−58 °C) of purified heat-labile PME from white grape (in 20 mM, Tris Buffer, pH 7.0) subjected to combined thermal and high-pressure inactivation experiments in the ranges 10−62 °C and 0.1−800 MPa. Polydera et al. (*35*) studied the V_a of endogenous PME in freshly squeezed orange juice was estimated as −19.76 cm^3/mol at 50 °C under high hydrostatic pressure (100−800 MPa) combined with moderate temperature (30−60 °C).

Change of the Residual Activity of Apple PME after DPCD. The residual activity of apple PME exposed to DPCD is measured for 4-week storage at 4 °C (**Figure 6**). The residual activity of the untreated PME and PME heated at 55 °C under atmospheric pressure for 60 min exhibited a 42.40% and 40.00% reduction ($p < 0.05$) after 2-week storage, respectively, while there is no significant change during the remaining 2 weeks. The residual activity of apple PME exposed to DPCD at 8 or 15 or 22 or 30 MPa and 55 °C for 60 min does not show reduction or reactivation for 4 weeks at 4 °C, indicating that apple PME can be irreversibly inactivated by DPCD in this study. However, Gui et al. (*8*) found that the reactivation of horseradish peroxidase (HRP) activity occurred after DPCD and suggested that the inactivation of HRP was reversible and dependent on the pressure level and treatment time.

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