JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Alterations in the Activity and Structure of Pectin Methylesterase Treated by High Pressure Carbon Dioxide

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The influence of high pressure carbon dioxide (HPCD) on the activity and structure of pectin methylesterase (PME) from orange was investigated. The pressures were 8−30 MPa, temperature 55 °C and time 10 min. HPCD caused significant inactivation on PME, the lowest residual activity was about 9.3% at 30 MPa. The SDS−PAGE electrophoretic behavior of HPCD-treated PME was not altered, while changes in the secondary and tertiary structures were found. The β-structure fraction in the secondary structure decreased and the fluorescence intensity increased as HPCD pressures were elevated. After 7-day storage at 4 °C, no alteration of its activity and no reversion of its β-structure fraction were observed, while its fluorescence intensity further decreased.

KEYWORDS: Pectin methylesterase; high pressure carbon dioxide; inactivation; structure

INTRODUCTION

In cloudy juice processing, 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) (*1*). Thus, the inactivation of PME is crucial for the cloud stability of juices. In the fruit juice industry, thermal treatment is the most common and least expensive technology that has been used to solve the problem (*2*). Unfortunately, this processing has a negative influence on the juice flavor (*2*). Food consumers increasingly demand food of exceptional quality, that it is minimally processed, more natural and high in the retention of nutritional value. The unique effects of HPCD appear to be able to satisfy these demands. Juice, milk and beer have been processed using HPCD by researchers who reported that organoleptic properties including flavor, color, vitamins and texture are superior to those produced by thermal processing alone (*3*−*5*). To date, the effects of HPCD on microorganisms, enzymes and food qualities have been reported. As a new nonthermal technology, HPCD-induced inactivation of enzymes continues to attract attention. Different plant pectin methylesterases have been determined in primary structure, which are sourced from tobacco, bergamot fruit (*6*), tomato (*7*), and kiwi fruit (*8*). However, the three-dimensional structure of only PME produced by *Erwinia chrysanthemi* (*9*) and carrot PME (*10*) was proposed to be right-handed parallel β-helix. Although HPCD-induced inactivation of PME from orange (*4*, *11*−*13*), carrot (*3*), and apple (*14*) was reported, the effect of HPCD on the structure of PME has not been proposed until presently. The mechanism of HPCD-induced inactivation of enzymes is worth investigating.

The purpose of this work was to investigate the effects of HPCD on the activity and structure of PME from orange and to analyze the relationship between the activity and structure of PME.

MATERIALS AND METHODS

Materials. Commercial PME (Product No. P5400, EC 3.1.1.11) lyophilized powder was purchased from Sigma-Aldrich Co. (Beijing, China). The PME was produced from the peel of Valencia oranges by using a procedure including ammonium sulfate precipitation, size exclusion and DEAE ion-exchange chromatography steps. The PME with an approximate activity of 100 units/mg protein was dissolved in 0.05 M phosphate buffer (pH 7.50) with 0.05 M NaCl to a concentration of 2.37 μM and subjected to HPCD and mild thermal (MT) inactivation. Apple pectin with 70−75% degree of esterification was purchased from Northern Andre Co. (Shandong, China). The purity of CO_2 is 99.5%, which was purchased from Beijing Analytical Apparatus Co. (Beijing, China). All other chemicals in the investigation were of analytical grade.

HPCD Process System. The diagram of the HPCD system was described by Zhi et al. (*14*). 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

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10.1021/jf803501q CCC: $40.75
Published on Web 02/09/2009

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 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 of 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 HPCD 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 PME by HPCD Treatment. To investigate the effect of HPCD on the activity and structure of PME, the PME solutions were subjected to different pressures (8 MPa, 15 MPa, 22 MPa, 30 MPa) at 55 °C for 10 min. Each HPCD treatment was performed in triplicate. 1.0 mL of PME was transferred into a 15 mL plastic tube (Beijing Bomex company, Beijing, China) without the cap, and then the tube was directly placed and enclosed in the HPCD vessel which had been preheated to the experimental temperature, then pressurized by the plunger pump to the required pressure level, and the required pressure was held for the required treatment time. Then 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 HPCD, the PME solution was taken out from the vessel and immediately cooled in an ice bath. Following equilibration to ambient temperature, the PME solution was determined immediately after treatment and after 7-day storage at 4 °C.

Inactivation of PME by MT Treatment. For each experiment, 1.0 mL of PME solution was transferred into a 15 mL plastic tube without the cap and treated by MT in HPCD vessel at 55 °C for 10 min, which was preheated to the experimental temperatures, the vessel pressure being maintained at ambient pressure. The remaining procedures were accorded with HPCD inactivation experiments. MT treatment was performed in triplicate.

PME Assay. The activity of PME was measured at pH 7.5 and 30 °C according to the method proposed by Yeom et al. (*15*), which was based on carboxyl group titration using a digital pH meter (Thermo Orion 868, USA) equipped with a microelectrode (Thermo Orion CHN 060, USA). A 0.05 mL quantity of PME solution was mixed with 20 mL of 1% apple pectin containing 0.1 M NaCl 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] was NaOH concentration (=0.05 N), V_{NaOH} was the volume of NaOH used (=0.05 mL), V_{sample} was the volume of sample used (=0.20 mL of apple PME), and t' was the time (in minutes) needed for pH to return to 7.5 after the addition of NaOH. Analysis was performed in triplicate.

SDS−PAGE Electrophoresis. The molecular weight of protein bands was determined according to the method of Laemmli (*16*). The separating gel was 12% acrylamide; the stacking gel was 4%. The molecular weight of PME was estimated using BioRad (BioRad, Richmond, CA) low molecular weight standards. A constant amount of 15 μL of protein was loaded into sample wells. Gels were stained with Coomassie Brilliant Blue (R250). Electrophoresis was carried out at constant 100 V in a Bio-Rad Mini-PROTEAN 3 Cell (Bio-Rad laboratories Ltd., CA, USA). Analysis was performed in triplicate.

Circular Dichroism (CD) Analysis of PME. The secondary structure of PME was determined by CD according to the method of Liao et al. (*17*). CD spectra were recorded with a PiStar CD Stopped-Flow (Applied Photophysics, Leatherhead, U.K.), using a quartz cuvette of 1 mm optical path length at ambient temperature (25 ± 1 °C). The concentration of PME dissolved in 50 mM sodium phosphate (pH 7.5) was 2.37 μM. CD spectra were scanned in the far UV range (190−250 nm) with three replicates at 50 nm/min, bandwidth = 1 nm. The CD data were expressed in terms of mean residue ellipticity [θ] in deg cm^2 dmol^{-1}, using a mean residual weight (MRW) of 103.8, which was calculated by MRW = $M/(N-1)$, where M is the molecular mass of the polypeptide chain (in Da), and N is the number of amino acids in the chain (*18*). Percentages of secondary structures of PME were calculated by using the online CDSSTR algorithms (*19*, *20*). The CD spectra of all the samples were scanned immediately after treatment and after 7-day storage at 4 °C. All CD spectra measured were corrected with baseline by 50 mM sodium phosphate (pH 7.5) buffer. Analysis was performed in triplicate.

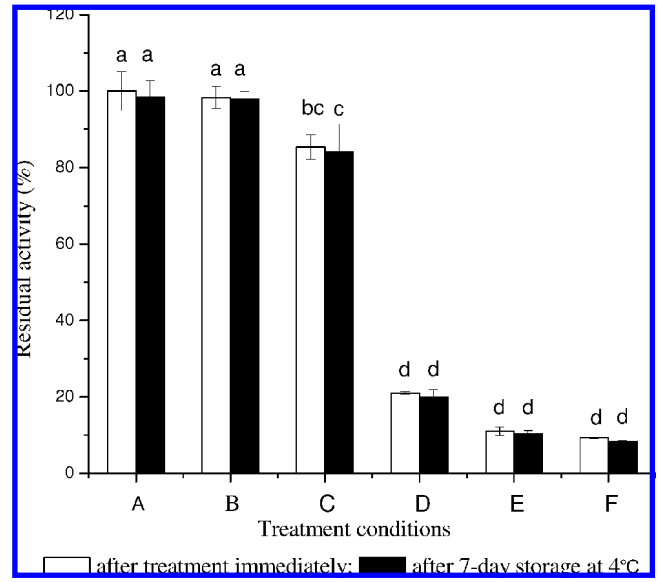

Figure 1. Residual activity of HPCD- and MT-treated PME at 55 °C for 10 min. (A) native; (B) MT; (C) 8 MPa; (D) 15 MPa; (E) 22 MPa; (F) 30 MPa. All data were the means ± SD, $n = 3$.

Tryptophan Fluorescence Spectroscopy Analysis of PME. The fluorescence spectrum was determined according to the method of Liao et al. (*17*). Fluorescence analyses were carried out on a HITACHI F-4500/spectrofluorometer (HITACHI, Japan), using a quartz cuvette of 1 cm optical path length at ambient temperature (25 ± 1 °C). The concentration of PME dissolved in 50 mM sodium phosphate (pH 7.5) was 2.37 μM. The emission spectra from 295 to 600 nm were recorded at the maximum excitation wavelength of 280 nm and represented the mean of three scans. All the samples were observed immediately after treatment and after 7-day storage at 4 °C. Analysis was performed in triplicate.

Statistical Analysis. Analyses of variance (ANOVA) were 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 were performed in triplicate.

RESULT AND DISCUSSION

Inactivation of PME Subjected to HPCD. The inactivation of PME subjected to HPCD and MT is shown in **Figure 1**. Analysis of variance (ANOVA) indicated that HPCD had significant effects ($p < 0.05$) and MT had no significant effect on the residual activity of PME. Similar observations were reported in previous investigations. Balaban et al. (*4*) showed a complete inactivation of PME in orange juice was attained with HPCD. Zhi et al. (*14*) reported that HPCD effectively inactivated PME in buffer and the pressures had a noticeable effect on the residual activity of apple PME. Kincal et al. (*11*) also reported that a minimum residual activity of 44% was observed in orange juice with HPCD at 72 MPa for 10 min. Park et al. (*3*) found

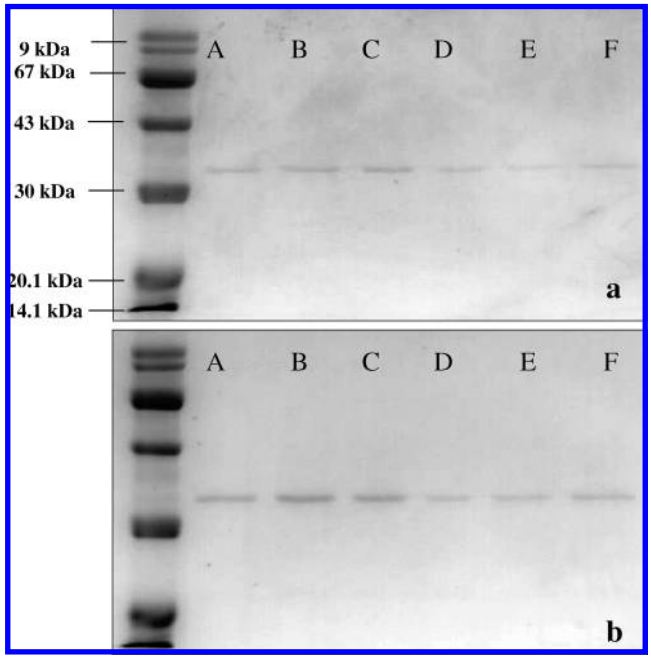

Figure 2. SDS−PAGE profiles of HPCD- and MT-treated PME (**a**, immediately after treatments; **b**, 7-day storage at 4 °C). (A) native; (B) MT; (C) 8 MPa; (D) 15 MPa; (E) 22 MPa; (F) 30 MPa.

that the minimum residual activity of carrot PME was 35.1% after a combined process of HPCD at 4.90 MPa and high hydrostatic pressure at 600 MPa. Corwin et al. (*12*) found that carbon dioxide was a significant factor in further inactivating PME in orange juice beyond that which pressure would achieve alone at 25 °C and at 50 °C. These studies showed that HPCD had effective inactivation on PME, but there were some differences in the inactivation level of different plant PMEs. The stability of plant PMEs depended on the cultivars, pH, isoenzyme forms, total solids level, system, and extraction method (*15*). Thus, it was supposed that the difference in the inactivation level in these studies possibly resulted from the factors influencing the stability of PME, treatment temperature, and HPCD processing conditions. In this study, the least residual activity of PME was about 9.3% at 30 MPa (**Figure 1**), and the discrepancy in the residual activity of PME at 15, 22 and 30 MPa was insignificant, indicating that the pressure between 8 and 15 MPa was probably an inflection for larger inactivation of PME.

The residual activity of HPCD- and MT-treated PME was also monitored after 7-day storage at 4 °C. As shown in **Figure 1**, the residual activity of PME exhibited no significant alteration after 7-day storage, suggesting that HPCD-treated PME could not restore and the inactivation of PME was irreversible. This finding was in agreement with a recent study. Zhi et al. (*14*) showed that the residual activity of HPCD-treated apple PME had no reactivation stored for 4 weeks at 4 °C. However, studies on HPCD-treated polyphenol oxidase (PPO) and horseradish peroxidase (HRP) after 7-day storage at 4 °C showed difference from this study. Gui et al. (*21*) found that inactivation of HPCD-treated PPO could reverse to some extent and the reactivation of PPO was dependent on the pressure level. Gui et al. (*22*) reported that the residual activity of HPCD-treated HPR reactivated significantly after 7-day storage at 4 °C while the pressure applied under 30 MPa, the reversibility of HRP was closely related to the pressure level and treatment time.

Effect of HPCD on the SDS−PAGE of PME. Figure 2 showed SDS−PAGE profile of HPCD- and MT-treated PME. The clear left lane was the marker lane. Obviously, there was only one band in the lane of native PME on SDS−PAGE, since native PME was a well-known enzyme with one subunit (*23*). Rillo et al. (*2*) reported that the purified PME from mandarin fruit consisted of a single polypeptide chain. The molecular weight of native PME in this study was estimated as 33 kDa based on the marker's molecular weight and relative migration (R_m), this estimated value was closer to the values of plant PME's molecular weight in the literature (*2, 23*). As compared with native PME, the SDS−PAGE profiles of HPCD-treated PME were not altered as increasing pressures (**Figure 2a**); there was also one band in each lane of HPCD-treated PME, and all the R_m values in each band on the SDS−PAGE were identical. Thus, the pressures had no effect on the SDS−PAGE electrophoretic behavior of PME and the molecular weight of PME (=33 kDa) was not modified after HPCD, implying that a single peptide chain of PME was not disrupted by HPCD. This finding was in agreement with a recent study. Liao et al. (*17*) reported that the SDS−PAGE electrophoretic behavior of HPCD-treated lipoxygenase (LOX) was not changed. As shown in **Figure 2b**, the SDS−PAGE profiles of all samples after 7-day storage at 4 °C were similar to immediately after HPCD, indicating that the electrophetic behavior of PME did not alter during storage in this study.

CD Spectra Analysis of PME by HPCD. The far-UV CD characterization of HPCD- and MT-treated PME was investigated, and the spectra are shown in **Figure 3**. The far-UV CD spectrum of native PME exhibited a broad negative peak at ~215 nm, which is characteristic of proteins predominantly consisting of β-structure. Johansson et al. (*10*) proposed that PME belonged to the family of parallel β-helix proteins. Goodenough and Carter (*24*) reported that the crystallized mature carrot PME belonged to the family of right-handed parallel β-helix proteins. In this study, the fractions in the secondary structure of native, HPCD- and MT-treated PME were estimated by CDSTTR algorithms; all the normalized root-mean-square deviation (NRMSD) values in this study were less than 0.1, which satisfied the requirements as suggested by CDSSTR algorithms (*20*). As shown in **Table 1**, the fraction of β-structure (including β-sheet and turns) in native PME was up to 63%, and the sum of α-helix and unordered fraction was 37%, confirming that native PME mostly consisted of β-structure as shown in the far-UV CD spectra in **Figure 3**. D'Avino et al. (*7*) also reported that the far UV CD spectrum of tomato PME showed only one negative peak centered at 217 nm indicative of a predominant β-structure, and the percentage of β-structure was 47% estimated according to Yang's method. Dirix et al. (*25*) estimated that the secondary structure of PME deduced from curve-fitting of the deconvoluted amide I′ band, based on the available X-ray diffraction data, was 51% β-helix in carrot and 45% in *Erwinia chrysanthemi*, respectively.

As shown in **Figure 3a**, the spectrum of HPCD-treated PME showed a very slight alteration at 8 MPa, whereas the spectra of HPCD-treated PME at ≥15 MPa exhibited modifications from native PME. The alterations in the far-UV CD spectra of PME as a function of pressures seemed to correspond to those in the residual activity of PME. **Figure 3b** showed the spectra of HPCD-treated and MT-treated PME after 7-day storage at 4 °C. The spectral profiles for all PMEs in the far-UV CD showed no changes after storage, but the negative peak character for β-structure increased. As shown in **Table 1**, HPCD caused a decrease in β-structure fraction, an increase in α-helix fraction, and a fluctuation in unordered fraction of PME secondary structure. Moreover, the β-structure fraction showed a decrease with increasing pressures; meanwhile the activity of HPCD-

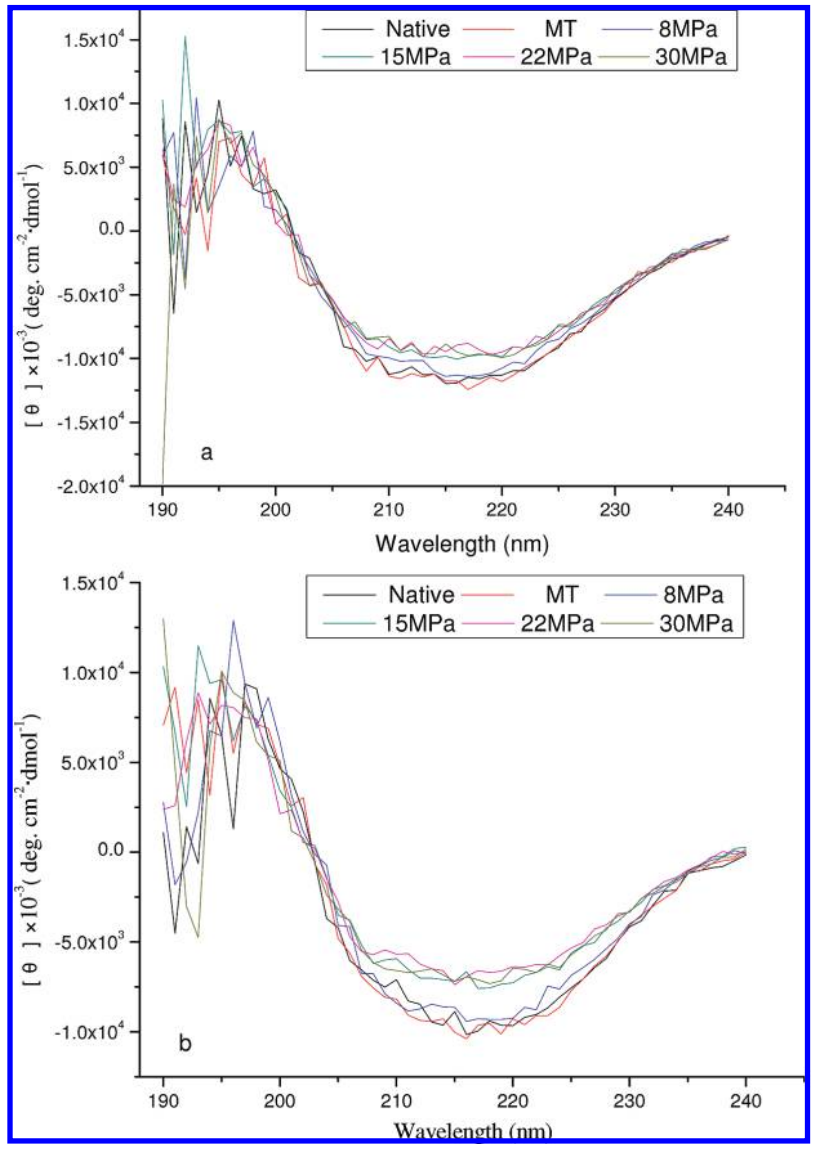

Figure 3. Far-UV CD spectra of HPCD- and MT-treated PME (**a**, immediately after treatments; **b**, after 7-day storage at 4 °C).

Table 1. Estimated Secondary Structural Fractions of PME by HPCD from CD Spectra[a]

treatment condition	secondary structure fraction (%)					
	immediately after treatment			after 7-day storage at 4 °C		
	α-helix	β-structure	unordered	α-helix	β-structure	unordered
native	11 ± 1	63 ± 1	26 ± 2	14 ± 1	59 ± 1	27 ± 1
MT	12 ± 1	59 ± 1	30 ± 1	9 ± 1	63 ± 1	28 ± 1
8 MPa	12 ± 1	60 ± 1	27 ± 5	14 ± 1	59 ± 1	27 ± 1
15 MPa	11 ± 1	59 ± 1	30 ± 5	9 ± 1	59 ± 1	31 ± 1
22 MPa	16 ± 1	55 ± 1	30 ± 1	13 ± 0	55 ± 1	32 ± 1
30 MPa	26 ± 1	53 ± 2	21 ± 2	15 ± 1	52 ± 1	33 ± 2

[a] All data were the means ± SD, $n = 3$.

treated PME decreased. The HPCD-induced disruption in the secondary structure of enzymes was also reported in previous studies. Chen et al. (*26*) reported that HPCD caused noticeable alterations in the composition of α-helix and random coil in the secondary structures of lobster, brown shrimp and potato PPO. Gui et al. (*27*) showed that the α-helix relative content in the secondary structure of HRP decreased with increasing HPCD pressures. Liao et al. (*17*) also found that CD spectra of HPCD-treated LOX were noticeably changed and its α-helix relative content decreased sharply to less than 10%. Moreover, Ishikawa et al. (*28*) reported that the residual activity of glucoamylase and acid protease subjected to HPCD had a good linear relationship with the loss of the α-helix relative content. Meanwhile, the β-structure fraction of HPCD-treated PME was stable after 7-day storage at 4 °C (**Table 1**). However, Gui et al. (*27*) found that the restoration of the α-helix relative content of HPCD-induced HRP inactivation was observed after 7-day storage at 4 °C. The disagreement in the alteration of the secondary structure of two enzymes treated by HPCD was possibly due to their different properties.

Fluorescence Spectra Analysis of PME by HPCD. The fluorescence spectrum was determined chiefly by the polarity of the environment of the tryptophan and tyrosine residues in proteins and by their specific interactions, it provided a sensitive means of characterizing proteins and their conformation, and the quantum yield of fluorescence deceases when the chromophores interact with quenching agents either in a solvent or in the protein itself. The changes in intrinsic tryptophan fluorescence emission correspond to changes in the tertiary structure of the protein. Thus, environmental changes resulting from conformational changes in the tertiary structure of proteins could be measured by intrinsic fluorescence spectroscopy (*29*).

Figure 4 showed alterations in the tryptophan fluorescence intensity and emission maximum wavelength (λ_{max}) of HPCD-

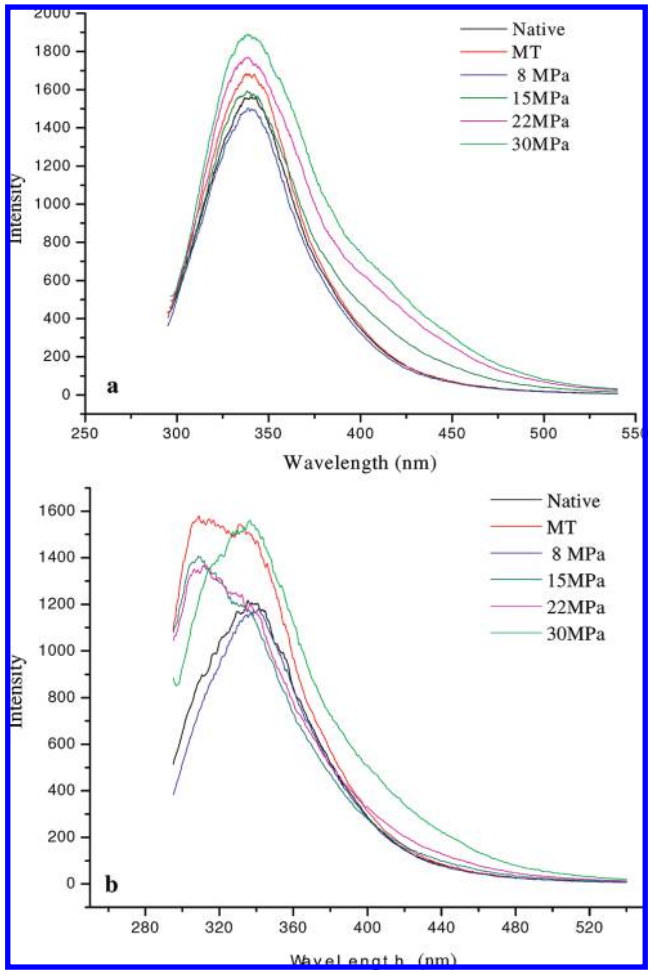

Figure 4. Fluorescence emission spectra of HPCD-treated and heat-treated PME (**a**, immediately after treatments; **b**, after storage for a week at 4 °C).

and MT-treated PME at $\lambda_{ex} = 280$ nm. The λ_{max} of native PME was 340 nm, which was similar to tomato PME reported by D'Avino et al. (*7*). Roughly, the λ_{max} of HPCD- and MT-treated PME remained identical with native PME. The fluorescence intensity of MT-treated PME showed an obvious increase. However, the alterations in the fluorescence intensity of HPCD-treated PME were complicated. The fluorescence intensity of HPCD-treated PME exhibited a decrease at 8 MPa, and was similar to native PME at 15 MPa, thereafter increased with increasing the pressures. These alterations in the fluorescence intensity implied that HPCD induced modification in the tertiary structure of PME. The increase in the fluorescence intensity probably resulted from relocation of tryptophan residues in the three-dimensional structure of PME, the interactions of the tryptophans with quenching groups were weakened and/or the inter-tryptophan distances were longer than that required for energy transfer in the new positions (*30*). Gui et al. (*27*) also found the fluorescence intensity of HRP exposed to HPCD increased with increasing pressure, whereas an opposite observation was obtained using LOX (*17*). A decrease or increase in the fluorescence intensity of HPCD-treated enzymes was closely related to its structure (*17*).The relative fluorescence intensity of PME was estimated as shown in **Figure 5**. The largest relative fluorescence intensity increased only by 20.7% immediately at 30 MPa, and it corresponded to the least residual activity of PME (**Figure 1**), indicating that a slight alteration of the tertiary structure may result in a large change in the residual activity of PME. The fluorescence intensity of HPCD- and MT-treated

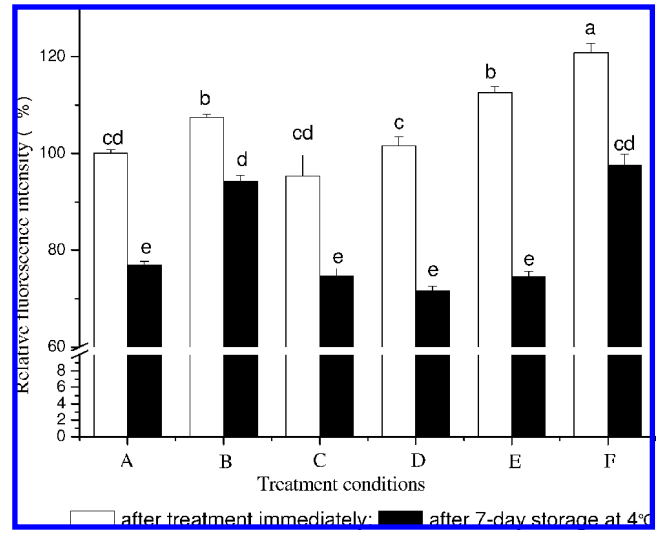

Figure 5. Relative fluorescence intensity of PME immediately after treatments and after 7-day storage at 4 °C. (A) native; (B) MT; (C) 8 MPa; (D) 15 MPa; (E) 22 MPa; (F) 30 MPa. All data were the means ± SD, $n = 3$.

PME after 7-day storage at 4 °C decreased as shown in **Figure 4b**, indicating that the tertiary structure of PME changes during storage. Moreover, there was an approximate 35 nm wide span blue-shift of λ_{max} when PME was subjected to 15, 20 MPa and MT after 7-day storage, indicating that the tryptophan surroundings of PME changed to a less polar environment upon HPCD treatment. A 21 nm red-shift of HPCD-treated HRP λ_m was found (*27*), and no λ_m shift of HPCD-treated LOX was observed (*17*). Presumably, this difference in HPCD-induced λ_m shift was probably attributable to its structural properties.

ACKNOWLEDGMENT

The authors thank Dr. Wallace for his supplying DICHROWEB to use CDSSTR algorithms. This research work is supported by No. 30571297 of the National Natural Science Foundation of China, No. 6062015 of Beijing National Natural Science Foundation, and No. 2006BAD05A02 of the Science and Technology Support in the Eleventh Five Plan of China.

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Received for review November 8, 2008. Revised manuscript received January 3, 2009. Accepted January 4, 2009. This research work is supported by No. 30571297 of the National Natural Science Foundation of China, No. 6062015 of Beijing National Natural Science Foundation, and No. 2006BAD05A02 of the Science and Technology Support in the Eleventh Five Plan of China.

JF803501Q