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Pectin methylesterase inactivation by High Pressure Carbon Dioxide (HPCD)

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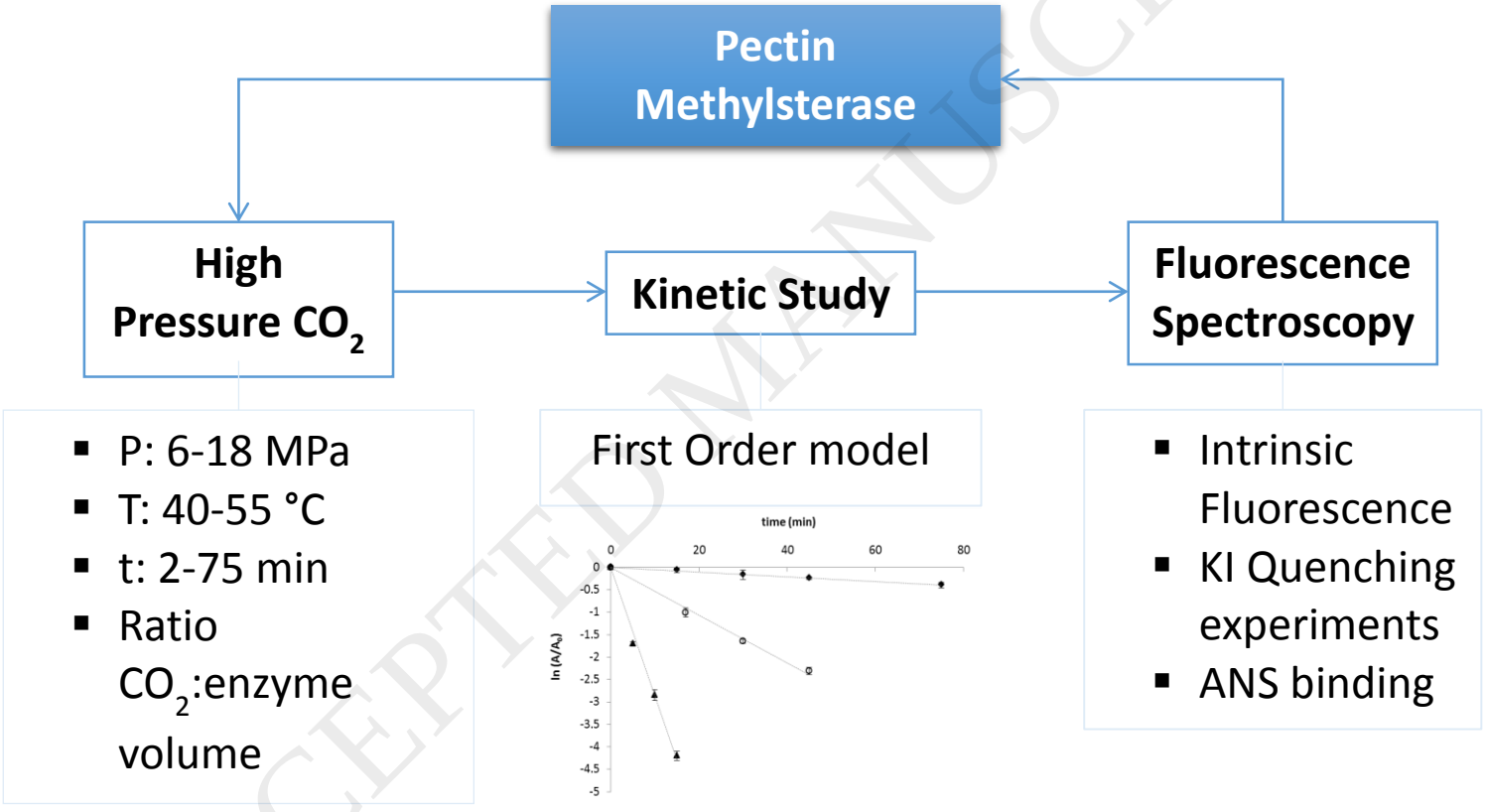
Graphical abstract



Highlights:

- Supercritical CO_2 successfully inactivated Pectin Methyl Esterase (PME)
- The ratio CO_2/enzyme was found to be a critical parameter
- A complete inactivation kinetic study was done using the first order model
- Supercritical CO_2 induced dramatic changes in the enzyme tertiary structure

Abstract

In the present work the effect of High Pressure Carbon Dioxide (HPCD) on the activity and structure of a commercial pectin methylesterase (PME) was studied and the results were compared to the conventional thermal inactivation technology. The use of supercritical CO_2 (pressure 6-18 MPa, temperature 40-55 °C and time up to 75 minutes) increased dramatically the PME inactivation rate. The experimental data fitted the first order model and the inactivation kinetic study of PME was completed with the calculations of the activation energy and volume of activation.

HPCD treatment induced important changes in the tertiary structure of the enzyme, as revealed the intrinsic fluorescence, KI quenching and ANS binding analyses. They showed that HPCD treatment rearranged the structure of the enzyme. These changes induced by HPCD were significantly different from those induced by mild thermal treatment. Finally, the structural changes detected correlated with the enzyme activity losses observed.

Keywords: PME, HPCD, enzyme inactivation, CO_2, juice

1 Introduction

Esterases are key enzymes for the food industry, since they hydrolyze water soluble esters or short-chain fatty acid triacylglycerols (TAG) [1]. More specifically pectin methyl esterases (PME; EC code 3.1.1.11) cause pectin demethylation, which produces the clarification and turbidity loss of juices [2]. Despite the efficiency of the thermal treatments to inactivate these endogenous enzymes [3], they produce changes in the organoleptic properties of the product. Therefore, it is necessary to use alternative procedures to inactivate those enzymes in order to preserve the nutritional properties of the juice, understanding that nowadays costumers demand products that are as less processed as possible.

HPCD satisfies that demand since temperatures are usually below 50 °C [4], whereas pressures can be as high as 60 MPa [5,6]. The exposure time is highly dependent on the enzyme, being necessary exposure times up to 60 minutes in some cases. HPCD has been widely used to inactivate PME in juices from many different fruits and vegetables: apple [4,7,8]; carrot [3,9]; peach [3,10]; orange [11,12] and beetroot [2]. A review of the experimental conditions used by those researchers reveals the significant differences between the experimental conditions (pressure, temperature, time, ratio CO_2 to volume of enzyme dissolution) and the experimental results achieved. In the literature, there is not a uniform criteria to describe the inactivation kinetics of the PME: some authors have described the PME inactivation using the two fraction model [7,11], others have used the fractional conversion model [3] and even the Weibull model has been successfully used [4]. It seems that the inactivation kinetics is strongly affected by the source of the enzyme and the way of processing it. Consequently, it is difficult to compare results and to draw conclusions regarding the inactivation mechanism of the PME when treated by HPCD.

The structure of the PME depends on the nature of the enzyme, existing slight differences between the PMEs with a vegetal origin or a bacterial one [13]. PME folds into a right handed parallel β-helix structure [14] consisting of three parallel β-sheets named PB1, PB2 and PB3 [15]. The active site of the enzyme is located in the PB3 [13] where two aspartic acid and one arginine residues are present [14]. The aromatic residues are lined up in the binding cleft [15] close to the active site. Although there are several PME isoenzymes as a function of their source, all of them share structural similarities. It is thought that the changes in the cleft of the catalytic site determine the way it interacts with the substrate [14]. Another difference is the molecular weight of the enzyme, in fact PMEs that have a bacterial or fungi origin have a molecular weight in the range 25 to 45 kDa, according to Kholi et al. [13]. D'avino et al. [14] reported a MW of 36.9 kDa for the PME from *E. chrysanthemi* and 34.5 kDa for tomato PME. These differences in the structure might affect the way PME interacts with the supercritical carbon dioxide.

In order to overcome the limitations derived from the interference of other species present in the juice, and in an attempt to understand the inactivation mechanism induced by HPCD, pure PME will be used in this work. The aim of the present work is to understand the inactivation mechanism of commercial PME treated under different conditions of CO_2, below and above the critical point. A complete kinetic study is proposed: three different temperatures (40, 48 and 55 °C) and three different pressures (6, 12 and 18 MPa; that led to gas and supercritical CO_2) and experimental times from 2 to 75 minutes. The experimental results fitted the first order kinetic

model and were used to calculate the kinetic parameters, such as the decimal reduction time, decimal reduction parameters (Z_P and Z_T), the activation energy and the volume of activation. Finally the possible changes induced in the tertiary structure of the PME will be evaluated by means of fluorescence spectroscopy.

2 Material and Methods

2.1 Enzyme and chemicals

Pectin methylesterase (Biopectinasa PME Ultra, origin *Aspergillus niger*) was purchased from Biocon Española, S.A. (Barcelona, Spain). This enzyme presented an initial activity higher than 6000 U/g. Prior to the HPCD experiments, the enzyme was diluted in distilled water according to the instructions of the manufacturer. The enzyme was diluted one hundred times in order to have a PME solution with a theoretical activity of 60 U/g. The pH of the enzymatic solution was then measured resulting to be 4.8.

Carbon dioxide (99.9%) was supplied by Air Liquide S.A. (Spain). The physical properties of CO_2 were taken from the NIST Chemistry Webbook.

All other chemicals used in this work to prepare dissolutions were analytical grade purchased from Sigma Aldrich. Pectin (citrus), used in the PME activity assay, was purchased from Alfa Aesar.

2.2 Experimental Set-up

All the experiments were carried out in a stainless steel high-pressure batch stirred reactor with an internal volume of, approximately, 80 mL which was submerged in a thermostatic water bath. The experimental set up was a slight modification of the set up described by Melgosa et al [16]. A schematic representation of the experimental set up is shown in Figure 1.

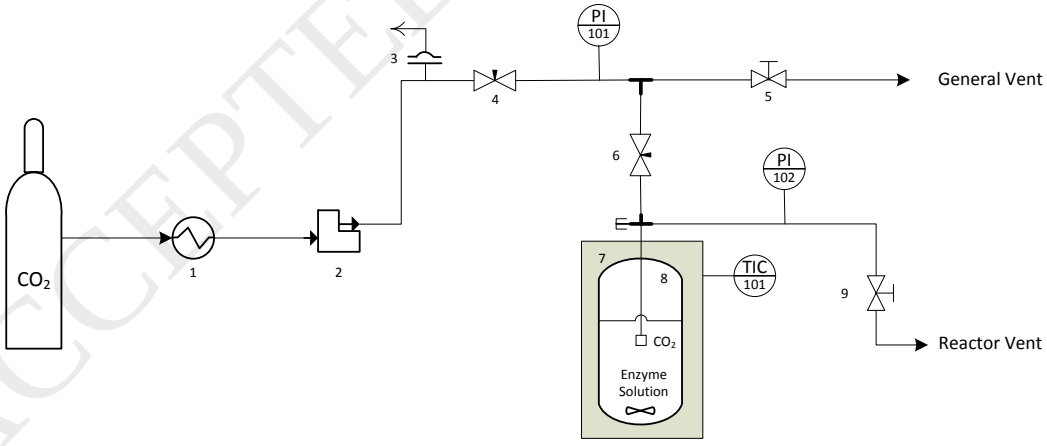


Figure 1. Experimental set-up of HPCD for inactivation of enzymes. 1: CO_2 cryostat; 2: Syringe pump (260D Teledyne ISCO); 3: bursting disk; 4: general process valve; 5: general vent valve; 6: individual inlet valve; 7: water bath; 8: high pressure batch reactor with magnetic stirring; 9: depressurization valve. PI-101: general pressure gauge; PI-102: reactor pressure gauge; TIC-101 temperature controller of water bath

In a typical experiment, the desired volume of PME solution was loaded into the reactor, which

was tightly closed. Subsequently it was placed in the water bath previously set at the desired temperature. Afterwards CO_2 was bubbled directly into the enzyme solution (through a sintered stainless steel micro-filter with a pore size of 10 μm) in a sufficient amount to reach the desired working pressure. Experiments were done in a temperature and pressure range commonly used in non-thermal inactivation HPCD systems: temperature ranged from 40 to 55 °C and pressure from 6 to 18 MPa. Once the enzyme was kept at the desired pressure and temperature for the desired time, the reactor was depressurized and the enzyme solution was taken out. All the samples were frozen at -20 °C until the moment of being analyzed in order to evaluate the residual activity of the enzyme after a treatment.

2.3 Experimental Work

The types of experiments were done: thermal inactivation experiments (no CO_2 was used) and HPCD experiments.

2.3.1 Thermal treatment inactivation

Experiments at atmospheric pressure served as control, in order to evaluate the pure effect of temperature on the enzyme stability. The experiments done using CO_2 were compared with those obtained at atmospheric pressure and then the effect of CO_2 was evaluated. In the atmospheric pressure experiments, a reactor containing 25 mL of PME solution was placed in a water bath at temperatures in the range 40-60 °C. Samples were taken periodically (total time 180 minutes) and kept frozen until analysis.

2.3.2 HPCD experimental work

2.3.2.1 CO_2/enzyme ratio

In a second stage of the experimental work, the volume of enzyme solution loaded into the reactor was changed. As it was described in a previous work [17], the CO_2/enzyme ratio is a critical parameter from both the enzyme inactivation performance and the economic point of view. It is known that the physical properties of CO_2 (such as density) change dramatically when changing pressure and temperature. Therefore, total mass of CO_2 loaded in the reactor significantly changes depending on the working conditions, leading to experiments done at different pressures, temperatures and masses of CO_2 to inactivate the enzyme.

In order to evaluate the influence that the CO_2:enzyme ratio (g/mL) has on the PME inactivation performance, some experiments using different amounts of enzyme solution loaded in the reactor were done, whereas temperature and pressure were kept constant at 48 °C and 18 MPa, respectively. This led to ratios grams of CO_2: volume of enzyme solution in the range 1-7 g/mL. The mass of CO_2 added into the reactor was calculated considering the free volume of the reactor (after having loaded a volume of enzymatic solution, and considering that the total volume of the reactor is 80 mL) and the CO_2 density.

The stirring rate was set at 600 rpm, and all the experiments lasted 15 minutes in a first set of experiments and 45 minutes in a second set.

2.3.2.2 HPCD treatment: kinetic study

Three different temperatures (40, 48 and 55 °C) and pressures (6, 12 and 18 MPa) were used and experiments lasted from 2 to 75 minutes. In all the experiments done in this section, the same volume of enzymatic solution was used: 25 mL. Once the inactivation rate was measured, the experimental data were analyzed and fitted to a first order kinetic model, as described in section 2.5.

2.4 Analytical procedures

2.4.1 Enzymatic Activity Evaluation

The enzyme activity was assayed following the Resolution OIV-OENO 363-2012 for the *"Determination of pectin methylesterase activity in enzymatic preparations (complement to resolution 9-2008)"*. The activity of the enzyme PME on pectin solutions results in the appearance of free carboxylic groups. To determine the activity of PME, those carboxyl groups can be titrated during the enzymatic hydrolysis with sodium hydroxide solution at constant temperature and constant pH-value.

In this procedure, 50 mL of 1% pectin solution were placed in a beaker at 40 °C. The pH of the pectin solution was adjusted to a value of 4.0 using a 0.1 M NaOH solution and subsequently 0.1 mL of the PME solution were added. At this point the analysis is started, being titrated up to pH 4.0 with a solution of 0.01M NaOH for 10 minutes at 40 °C. After 10 minutes, the analysis was stopped and the consumption on 0.01 M NaOH was read off.

The activity of the sample (expressed in U/mg) is calculated according to [1], were *"n"* is the consumption of 0.01 M NaOH expressed in μmol; *"t"* is the assay time expressed in minutes; *"V"* is the volume of sample used in the assay expressed in mL and *"c"* is the concentration of the enzymatic solution expressed in g/L.

$$\text{PME activity } ({}^{\text{U}}/_{\text{mg}}) = \frac{\text{n}}{\text{t}\cdot\text{V}\cdot\text{c}} \qquad [1]$$

The residual activity (RA) was calculated as the ratio of the measured activity after a treatment (A) and the activity before treatment (A_0), according to the equation 1:

$$\text{RA} = {}^{\text{A}}/_{\text{A}_0} \qquad [2]$$

2.4.2 Fluorescence Spectroscopy

2.4.2.1 Intrinsic fluorescence experiments

The tertiary structure of the PME was determined by fluorescence spectroscopy using the FLS980 photoluminescence spectrometer (Edinburgh Instruments, Livingstone, UK). The sample was excited at a λ_{em}=280 nm and the emission spectra was recorded in the range from 290 to 450 nm. A 1 cm path length cell was used and the emission and excitation slits were set at 2 and 1 nm, respectively.

2.4.2.2 Quenching experiments

A stock solution of KI (2 M) containing 0.1 M sodium thiosulfate was used as a quencher. In the experiments aliquots of the quencher solution were added to the PME solutions to achieve a quencher concentration in the range from 0.1 to 1 M. Excitation was set at 280 nm and the emission and excitation slits were set at 2 and 1 nm, respectively. The emission spectra were recorded in the range 290-450 nm. The decrease in fluorescence intensity was analyzed using the Stern-Volmer equation:

$$I_0/I = 1 + K_{SV}Q \qquad [3]$$

In equation (3) I_0 and I represent the fluorescence intensity in the absence and in the presence of a given concentration Q of quencher, respectively. K_{SV} is the Stern-Volmer constant, which describes the accessibility of the quencher to the fluorophore (tryptophan). The addition of externally added quenchers such as iodide tends to quench tryptophan fluorescence. If the tryptophan residue is located on the protein surface, the emission will be strongly affected by the addition of the quencher; on the other hand if the tryptophan residue is buried, its emission will be less affected after the addition of the quencher [18]. Changes in the PME structure induced by HPCD will be evaluated using KI quencher. In all the cases, the effect of the dilution provided by the successive addition of KI solution was considered in the calculations.

2.4.2.3 ANS binding measurements

ANS (8-Anilino-1-naphthalenesulfonic acid) is an extrinsic fluorescence probe that usually binds to loosely packed solvent-accessible hydrophobic core. The exposure of the hydrophobic areas on protein surfaces, which is observed by the increase in the fluorescence intensity at 480 nm, has been frequently studied.

A stock solution of ANS (1 mM) was prepared in distilled water. In the ANS binding experiments, 0.1 mL of ANS were added to 2.4 mL of the protein solution. The excitation wavelength was set at 380nm and the emission spectra were recorded in the range of 400–650 nm. The excitation and emission slits were set at 2 and 2 nm, respectively.

2.5 Inactivation kinetics

The first order kinetic model is commonly used to describe the inactivation of enzymes. This model assumes that the logarithm of the residual activity decreases linearly with the time. The equation 3 describes mathematically this model:

$$\ln\left(A/A_0\right) = -kt \qquad [3]$$

A is the activity of the enzyme after treatment, A_0 the initial activity of the enzyme, *k* is the inactivation rate constant (kinetic constant) at given pressure and temperature conditions and *t* is the time the enzyme is exposed to the pressure and temperature conditions. From the kinetic constant, it is possible to calculate the decimal reduction time (D), defined as the treatment time needed for a 90% inactivation of the initial enzyme activity in a given condition, as presented in equation [4]:

$$D = \frac{\ln(10)}{k} \qquad [4]$$

It was also calculated the pressure and temperature increase needed for a 90% reduction of the D value, defined as Z_P (MPa) and Z_T (°C), respectively (equations 4 and 5). The values of Z_P and Z_T are obtained as the negative reciprocal slope of the regression line that represents the log D versus pressure and temperature relationships, respectively.

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

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

Finally, two more parameters were calculated: the activation volume (V_a, cm^3/mol) was calculated using the Eyring equation (eq 7) to evaluate the kinetic constant dependence with pressure; and the activation energy (E_a, kJ/mol) was calculated by means of the Arrhenius equation (eq 8) in order to evaluate the kinetic constant dependence with temperature. Both V_a and E_a were calculated from linear regression of *ln k* versus *P* or *(1/T)*, respectively.

$$\ln\left[\frac{k_1}{k_2}\right] = -\frac{V_A}{RT}[P_2 - P_1] \qquad [7]$$

$$\ln\left[\frac{k_1}{k_2}\right] = \frac{E_A}{R}\left[\frac{1}{T_2} - \frac{1}{T_1}\right] \qquad [8]$$

2.6 Statistical analysis

All the statistical analysis was done using the software Statgraphics X64. This software was also used to perform the non linear regression of the experimental data to obtain the kinetic constants of the first order kinetic model presented in section 2.5.

3 Results and Discussion

3.1 Thermal Inactivation experiments

The inactivation of PME at temperatures of 45, 50, 55 and 60 °C under atmospheric pressure conditions is shown in Figure 2. In this figure, it is possible to see that the inactivation of PME under atmospheric pressure fitted the first order kinetic model. The kinetic constants were calculated and are presented in Table 1.

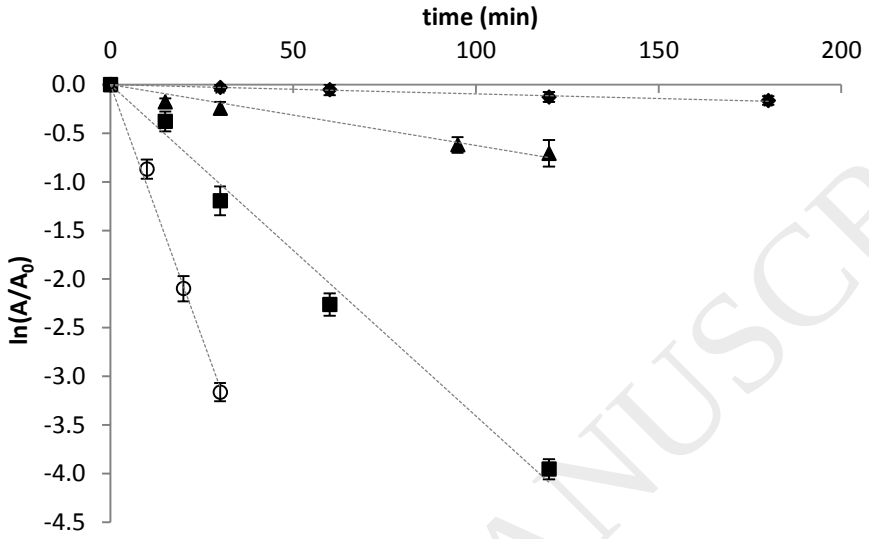


Figure 2. Inactivation of PME under atmospheric conditions at different temperatures: 45 °C (◆), *50 °C* (▲), *55 °C* (■) *and 60 °C* (○).*The inactivation curves were modelled using a first-order model (dotted lines).*

It can be clearly seen that temperature plays an important role in the inactivation of PME since the higher the temperature the faster the inactivation of PME. From the kinetic constants presented in Table 1, it was possible to calculate the kinetic parameters Z_T and the activation energy (equations 6 and 8, respectively). Z_T resulted in a value of 7.3±0.6 °C (which indicates that the activity of this enzyme is very sensitive to temperature changes) and the activation energy was 278.5±19.4 kJ/mol.

Table 1. Calculated kinetic constants of PME under atmospheric conditions in the range 45 to 60°C

T (°C)	k (min^{-1})	D (min)	R^2
45	0.00095±0.00006	2423.8±67.5	0.988
50	0.00626±0.00048	367.8±12.3	0.965
55	0.03409±0.00202	67.5±1.7	0.989
60	0.10397±0.00469	22.1±0.4	0.995

The results presented in this work are in agreement with the results presented in the literature by other authors who used commercial PME: Willińska et al. [19] used a PME obtained from *Aspergillus niger* and treated at temperatures in the range 52-66 °C, dissolved in a buffer or added to different fruit juices. The PME behaved in a similar way regardless the media in which

it was dissolved, being described its inactivation kinetics by the first order model. The activation energy was in the range 285-309 kJ/mol, being 300±5 kJ/mol when it was dissolved in an acetate buffer (0.1 M, pH 3.6) and the Z_T value was in the range 6.9-7.4 °C. Duvetter et al. [20] studied the thermal inactivation of PME from *Aspergillus aculeatus* in the range 46-56 °C; these authors observed that the enzyme inactivation followed a first order reaction. A noticeable effect of the pH was observed: at values around 3 the Z_T value was 7.3±0.2 °C and when pH was increased up to 4.5, the Z_T value decreased to 5.7±0.1 °C. Other authors used PME obtained from vegetables, as it has been summarized in Table 2:

Table 2. Kinetic parameters obtained by several authors when studying the inactivation of PME obtained from different vegetables

Authors	**Source**	**Sample type**	**T (°C)**	**Z_T (°C)**	**E_a (kJ/mol)**	**Model**
Castro et al. [21]	pepper	Purified extract	56-64	5.7±0.1	371.6±7.3	Fractional conversion
Ando et al. [22]	Japanese radish	Purified extract	50-70	9.6	190.3	Fractional conversion
Ly-Nguyen et al.[23]	carrot	Purified extract	48-60	7.5±0.2	274.5±5.8	Fractional conversion
Jolie et al. [24]	carrot	Purified extract	52.5-65	5.8±0.3	323.3±3.2	First order
Balaban et al. [12]	orange	Juice	40-60	8.8	166.6	First order

From Table 2 it can be concluded that Z_T and activation energy values for mild heat treatments are very close despite the source of the PME. The small discrepancies can be attributed either to the differences in the experimental methodologies followed by the researchers or to the slight structural changes PMEs have as a function of the source [14].

3.2 HPCD experiments

3.2.1 Ratio CO_2/enzyme

Results regarding the different ratios CO_2/enzyme (g/mL) are presented in Figure 3, where ratios from 1 to 7 were tried in different experimental conditions (48 °C and 18 MPa for 15 and 45 minutes). In a previous work [17] we noticed the lack of systematic studies dealing with the effect of this process parameter on the enzyme inactivation. Moreover, in most of the cases, extremely high CO_2 to volume of enzymatic solution ratios were used. For instance, Manzocco et al. [25] used ratios in the range 9.8 to 12.6 g/mL (as a function of the pressures and temperatures selected). Other authors such as Zhang et al. 2010 and 2011 [26] and [27] used very small volumes of sample (1-3 mL) placed in large reactors up to 850 mL, which means extremely high ratios of CO_2 per milliliter of sample.

In our previous work [17], it was found that there is a critical ratio that controls the inactivation performance: bellow that critical value the inactivation of the enzyme strongly depends on pressure and temperature and above it the addition of further amounts of CO_2 does not improve the results, being a waste of CO_2 from the economic point of view. This critical value for the PME inactivation is 3, according to Figure 3. When increasing the CO_2:volume of enzyme ratio above 3 no further decrease in the residual activity was achieved: in experiments that lasted 45 minutes the minimum residual activity value was 0.15±0.02 whereas in the 15 minutes

experiments this minimum value was 0.24±0.02. Figure 3 shows that it is possible to decrease the working time by increasing the amount of CO_2 loaded in the reactor. For instance if a 25% of residual activity is desired it is possible to work using a ratio 1:1 for 45 minutes (lower consumption of CO_2 in exchange for a higher working time) or it is possible to increase the ratio CO_2:volume of enzyme up to 3 and reduce the working time to 15 minutes. The selection of the working conditions should be made not only according to economic criteria, but also on the product quality criteria, considering how the long exposure of the juice to the mild temperature affects it or how higher ratios of CO_2 affect its properties.

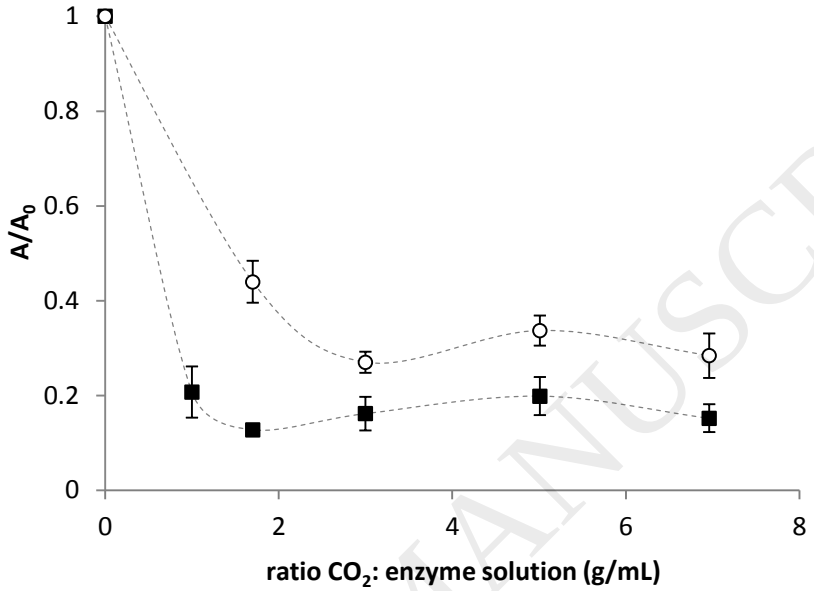


Figure 3. Residual activity of PME treated at 48 °C and 18 MPa for 15 (○) *and 45* (■) *minutes using different ratios CO_2:volume of enzyme solution.*

The explanation for the above mentioned results might be related to the changes induced to the PME solution by the addition of CO_2: on one hand the density and the volume are increased (up to 5% and up to 6%, respectively, being pressure dependent [28]). On the other hand CO_2 might induce a decrease in pH as a consequence of carbonic acid formation, which might explain the dramatic reduction in the residual activity achieved when high ratios of CO_2:volume of enzymatic solution were used. The solubility of CO_2 in pure water at 45 °C has been measured by Illera et al. [29] in the range 4.95-5.94 g CO_2/100 g of liquid, when pressure was shifted from 8 to 20 MPa, data that can be useful to estimate the pH change.

3.2.2 Kinetic experiments of inactivation by HPCD

The addition of CO_2 did not change significantly the inactivation pattern of the PME compared to the atmospheric pressure experiments: a linear inactivation with time was observed according to the first order model, as shown in Figure 4. However, the addition of CO_2 led to a much faster inactivation of the PME enzyme. Experiments done at 55 °C led to a complete inactivation in only 15 minutes, whereas at 40 °C more than 75 minutes were needed to reduce the residual activity by only 20%.

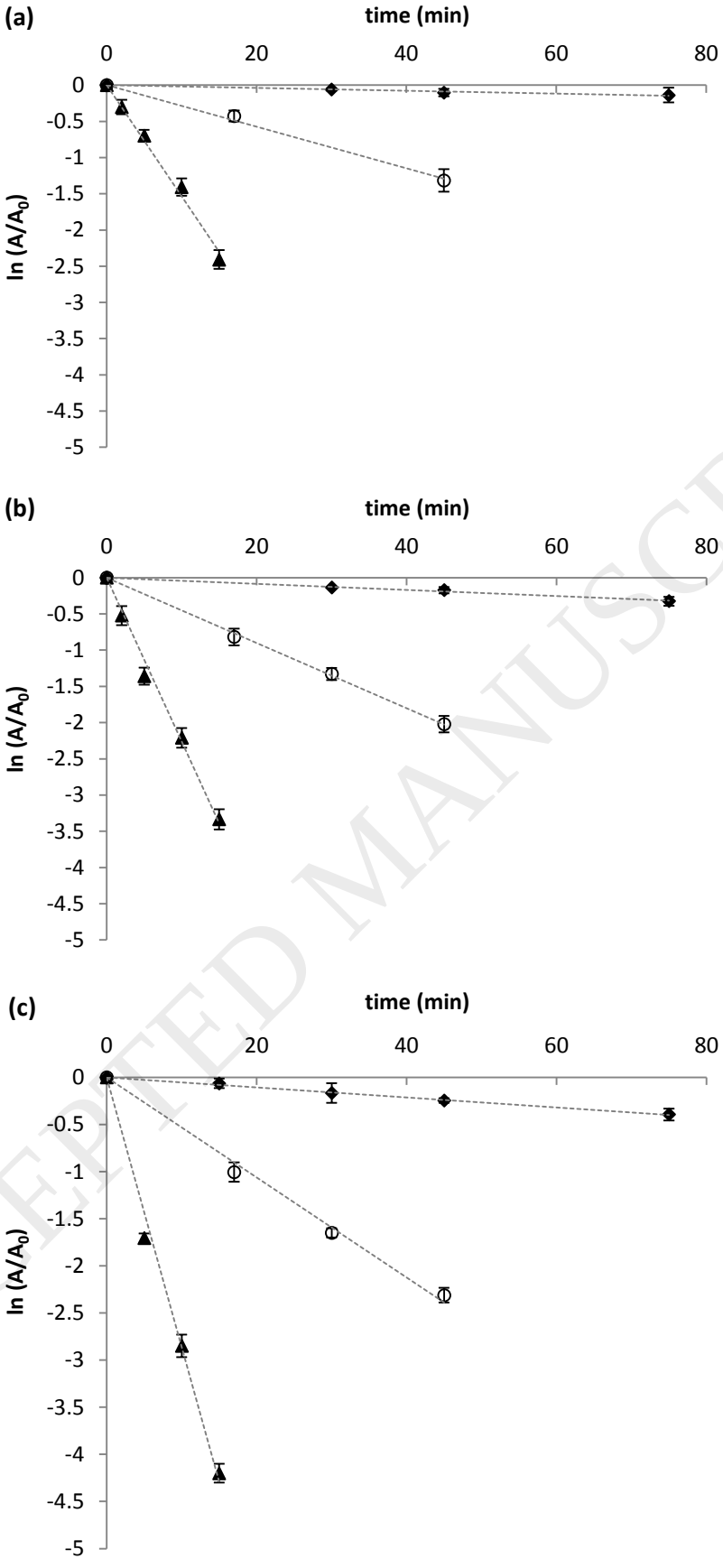


Figure 4. PME residual activity as a function of the exposure time to HPCD at three different

temperatures: 40 °C (◆), 48 °C (○) and 55 °C (▲). In Figure a) experiments were done at 6 MPa; in b) at 12 MPa and in c) at 18 MPa. Dotted lines represent the first order kinetic model.

PME inactivation resulted to be temperature and pressure dependent. High temperatures led to a much faster inactivation performance. It was observed that pressure had more limited effect, especially under supercritical conditions: moving from the atmospheric pressure to HPCD (6 MPa) involved a great improvement in the PME inactivation, the same was observed when increasing the pressure from 6 to 12 MPa (which involves the phase change of CO_2 from gas to supercritical state). However further increases of pressure from 12 to 18 MPa (no phase change, only a slight increase in the CO_2 density) had a very limited effect on the enzyme inactivation. Changes in pressure and temperature induce changes in the physical state of CO_2: at 40 °C density rises dramatically from 149.3 kg/m^3 at 6 MPa to 717.8 kg/m^3 at 12 MPa, changing the state from gas to supercritical state. Another small increase in the density up to 819.5 kg/m^3 is observed when increasing the pressure up to 18 MPa. At 55 °C density moves from 129.7 kg/m^3 at 6 MPa to 504.5 kg/m^3 at 12 MPa (from gas to supercritical state) and 723.1 kg/m^3 at 18 MPa. On one hand, supercritical CO_2 and water are barely miscible and on the other hand this increase in pressure involves more CO_2 used per mL of enzyme solution, which as has been previously explained, has an important effect on the inactivation of PME and of PPO in a previous work.

Besides temperature and pressure, the pH lowering effect induced by CO_2 has been considered as another parameter affecting the inactivation performance of the HPCD processes [3]. The pH lowering effect is associated with the solubility of CO_2 in the enzyme solution [9]. However, as has been reported above, CO_2 in the supercritical state is barely miscible with water (solubility is lower than 1%). This drawback can be partially overcome by improving the way water and CO_2 are put in contact. A closer contact between the two phases can enhance the miscibility rate. In the experimental set up used in this work, CO_2 is bubbled directly into the enzymatic solution in the form of fine bubbles. This way of working was found to greatly improve the inactivation performance [11]. The pH of some samples after the HPCD experiments was measured and compared to the pH of the initial PME solution (4.8). After the HPCD treatments, no significant changes in the pH were observed, being around 5.0 in the experiments done at 12 MPa, regardless the temperature after 15 minutes (A/A_0 was 0.95 at 40 °C, 0.44 at 48 °C and 0.04 at 55 °C). Since different inactivation rates were achieved it seems clear that the inactivation mechanism of the PME under CO_2 is not controlled by the pH decrease, which is in agreement with Zhi et al. [7]. It seems more admissible that the turbulence generated by the fine bubbles and the stirring in the reactor improve the contact between the two phases and promote the molecular effects of the CO_2 on the enzyme that would lead to its inactivation.

To the best of the authors' knowledge, there is only one work in the literature that deals with the inactivation of a commercial pure PME enzyme by HPCD. Zhou et al 2009 [30] used a commercial PME from peel of Valencia oranges and reported a residual activity of 0.09 at 30 MPa and 55 °C after 10 minutes. A pressure decrease down to 22 MPa led to a residual activity slightly higher. No significant differences in the residual activity were registered when pressure was shifted from 15 to 30 MPa; however, the increase of pressure from 8 to 15 MPa affected significantly the residual activity (from RA 80 to 20%). This is in agreement with our experimental results. Unfortunately, these authors did not report any other temperature or a deeper kinetic study.

3.2.3 Modelling the HPCD inactivation kinetics of PME using a first order model

The decimal reduction times, calculated using the first order kinetic constants, as well as the kinetic parameters calculated from them, are reported in Table 3. The experimental data fitted the first order model, having regression coefficients higher than 0.991 in all the cases. The decimal reduction time varied from 1181.5 minutes (40 °C, 6 MPa) to 8.1 min (55 °C, 18 MPa).

Table 3. Decimal reduction time, Z-values, activation energy and activation volume of commercial PME treated by HPCD at different pressures and temperatures calculated using the first order model

	D (min)				
T (°C) / P (MPa)	**40**	**48**	**55**	**Z_T (°C)**	**E_a (kJ/mol)**
6	1181.5±59.9^{C,d}	80.2±2.1^{D,e}	15.0±0.3^{E,h}	7.9±0.8^{a}	249.7±20.4^{b}
12	543.8±15.6^{F,c}	51.1±0.6^{G,f}	10.2±0.2^{H,i}	8.7±0.6^{a}	227.2±12.9^{b}
18	433.4±6.4 ^{I,c}	43.4±1.0^{J,g}	8.1±0.2^{K,j}	8.7±0.4^{a}	227.4±8.5^{b}
Z_P (MPa)	27.6±6.7^{B}	45.0±12.1^{A}	44.4±6.3^{A}		
V_a (cm^3/mol)	-217.6±60.8^{C}	-136.5±36.7^{D}	-141.5±20.0^{D}		

Different small letters in columns and capital letters in rows indicate significantly different values ≤ 0.05.

Some other researchers have studied the HPCD inactivation of PME from different sources. The first order kinetic model was also used by Balaban et al. [12] and Xu et al. [8] to describe the inactivation of PME from orange juice and apple juice by HPCD, respectively. The former researcher found a complete inactivation of the PME at 55 °C and 75 minutes, whereas at 60 °C the time to complete inactivation decreased down to 45 minutes at 31 MPa. A reduction of pressure (down to 13.7 MPa) led to similar results. It was reported that the higher the pressure, temperature and experimental time, the lower the residual activity. However a close view of the experimental results reported reveals the limited effect the pressure had on PME inactivation performance: in this sense when working at 60 °C doubling the pressure (from 13.7 to 31 MPa) involved a reduction of the D value of less than two minutes (from 12.1 to 10.6 minutes), in agreement with our results. The latter researcher observed a linear decrease of the residual activity when working at 60 °C and 22 MPa in the range 3 to 10 minutes, to a minimum activity around 45%. These authors also noticed the important role that temperature has on the PME inactivation.

Other researchers have reported completely different trends in the PME inactivation by HPCD. For instance Zhou et al. used the fractional conversion model to describe the inactivation of PME from carrot and peach [3]. These authors found that carrot PME was more sensitive to the HPCD treatment than peach, probably because of the pH drop induced by HPCD; they reported a decrease in the D value from 6.9 to 0.5 minutes with a pressure increase from 8 to 15 MPa, using purified extracts of carrot PME treated at 55 °C. On the other hand Zhi et al. [7] used the two fraction model to describe the inactivation of the PME from apple. Briongos et al. [11] used different kinetic models to describe the PME from orange inactivation kinetics, two fraction, fractional conversion and Weibull. In any case, a linear decrease was detected.

3.2.3.1 Pressure effect: Z_P and Activation volume of PME solutions treated by HPCD

The pressure sensitivity parameter (Z_P) changed in a great extent with temperature, from 27.6±6.7 to 44.4±6.3 MPa, which indicates that, at higher temperatures, higher pressure is required to decrease the decimal reduction time by 90%. This indicated that pressure had less effect on PME inactivation than temperature. The statistical analysis of the results revealed that there were no statistically significant differences between the values of Z_P at 48 and 55 °C, but the value of Z_P at 40 °C was statistically different. Zhou et al. 2009 [3] reported Z_P values, calculated at 55 °C, for two different substrates: when using carrot as PME source, 5.8 MPa was reported whereas the Z_P value was 48.3 MPa for PME obtained from peach. Zhou et al. 2014 [31] reported a Z_P value of 16.4 MPa for the PME obtained from peach (55 °C). The different results obtained by these authors, despite using the same raw material, can be due to the peach variety or to the purification process used to obtain the PME solution. Zhi et al. [7] used peach as raw material using the two fraction model; Z_P was 21.8 MPa for the labile fraction, calculated at 55 °C in the pressure range 8-30 MPa. No data were reported for the stable fraction.

The pressure dependence of the kinetic rate was represented by the Eyring equation (equation 7). Figure 5 shows this dependence for the PME experiments of HPCD. A reasonably good regression coefficient was obtained (0.96 for the experiments at 40 °C; 0.93 at 48 °C and 0.98 for those at 55 °C). The activation volume was in the range -217cm^3/mol at 40°C to - 141cm^3/mol at 55 °C. This increases in the activation volume with temperature shows the lower effect of pressure on the enzyme inactivation when temperature is increased. A limited effect of pressure can be also observed at temperatures above 48 °C, as indicated by the similar activation volumes obtained at 48 and 55 °C (see Table 3). Zhou et al. [3] reported an activation volume of -1079 cm^3/mol at 55 °C (in the pressure range 8 to 15 MPa) for carrot juice. The same authors reported a peach juice activation volume equal to -130 cm^3/mol. In other work, Zhou et al. [10] reported an activation volume of -383 cm^3/mol for peach juice at 55 °C; which is a substantial difference with their previous work [3], and reveals the importance of the source of PME.

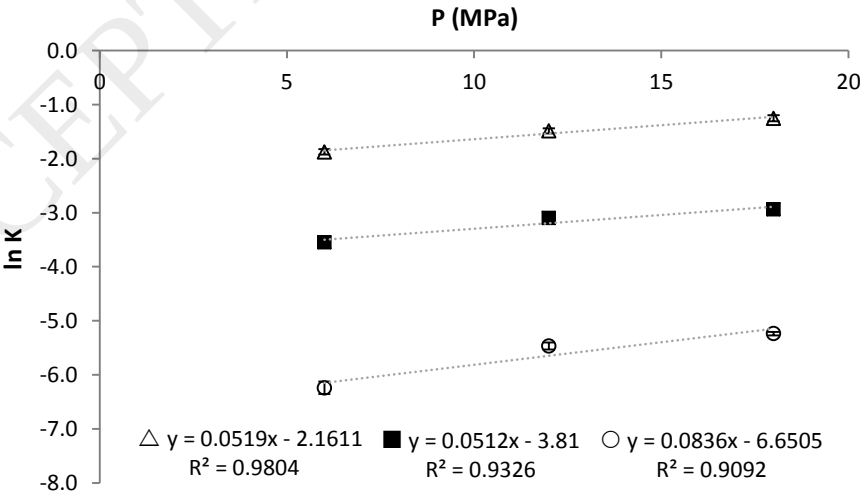


Figure 5. Pressure dependence of the kinetic constant used to calculate the activation volume at different temperatures: ○ 40 °C; ■ 48 °C and △ 55 °C.

3.2.3.2 Temperature effect: Z_T and Activation energy (E_a) of PME solutions treated by HPCD

The temperature sensitivity parameter (Z_T) did not change significantly (statistical analysis revealed it, at a confidence level of 95%, as it is shown in Table 2), being around 8.7 °C throughout the pressure range studied, which, again, indicates that PME inactivation by HPCD is not significantly affected by pressure. At atmospheric pressure, this value was slightly lower, 7.3 ± 0.6 °C, which indicates that, at atmospheric conditions, without the presence of CO_2, PME is slightly more susceptible to temperature changes, since only an increase of 7.3 °C would induce a reduction of 90% of the decimal reduction time. Zhi et al. [7] used peach as raw material using the two fraction model; Z_T for the labile fraction was 17.2 °C, and 22.3 °C for the stable one, proving the higher sensitivity to temperature of the labile fraction (pressure 30 MPa, temperature range 35-55 °C). Zhou et al.[31] reported a Z_T value of 13.3 °C (at 15 MPa) for the same raw material.

Figure 6 represents the dependence of the rate constant with temperature, according to the Arrhenius equation (eq. 8). E_a from the slopes of Figure 6 have been evaluated revealing a good correlation in all the studied pressures, including the results of the experiments done at atmospheric pressure. In all the studied cases, the regression coefficient was above 0.99. Figure 6 also shows that there is no effect of pressure, resulting in parallel lines with similar slope. The activation energy reported at atmospheric pressure was 278.5±19.4 kJ/mol, whereas, in the HPCD experiments, it decreases to values in the range 227-249 kJ/mol; this was a slight decrease that showed the very limited effect of pressure on the inactivation performance of the PME enzyme.

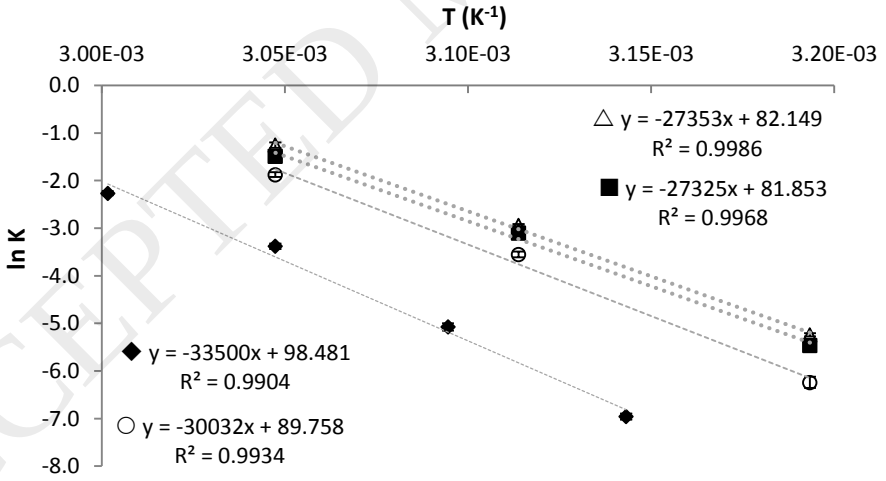


Figure 6. Temperature dependence of the kinetic constant used to calculate the activation energy at different pressures: ◆ atmospheric pressure; ○ 6 MPa; ■ 12 MPa and △ 18 MPa.

The values of Z_T and E_a reported by Zhou et al. [10] for experiments done at 15 MPa when treating PME from peach juice were 13.3 °C and 1845.9 kJ/mol respectively. Balaban et al. [12] reported a significant decrease in the activation energy when using HPCD to treat orange juice, moving from 166.6 kJ/mol at atmospheric pressure to 97.4 kJ/mol at 31 MPa. The Z_T parameter

was also reduced, indicating an increase in the sensitivity of the enzyme to temperature under supercritical CO_2 conditions (from 8.8 to 5.2 °C) contrary to our results.

3.3 Conformational changes in the PME enzyme

The changes in the tertiary structure of the protein were studied by means of fluorescence spectroscopy. The main results of the fluorescence emission experiments are summarized in Table 4, where results corresponding to PME treated by HPCD under different conditions are compared to results of PME treated thermally (at 90 °C for 10 minutes, which guaranteed the denaturalization of the enzyme, and at 55 °C for 10 minutes). Non-treated PME served as control (referred as *"native PME"*). The results of the different test runs are depicted in the following sections.

Table 4. Experimental results of the fluorescence emission study of samples treated by HPCD and thermally

					Fluorescence Spectra		KI Quenching		ANS Binding	
	T (°C)	P (MPa)	t (min)	A/A_0	I/I_0	λ_{max} (nm)	f_a	K_{SV}	I/I_0	λ_{max} (nm)
	Native PME			1	1.00	338	0.49±0.01	16.2±1.2	1.00	535
HPCD	40	6	45	0.900	1.00	338	$0.86±0.01^{b}$	$4.9±0.1^{B}$	1.07	513
	40	12	45	0.842	0.88	338	$0.68±0.04^{a}$	$10.4±1.1^{A}$	1.27	488
	40	18	45	0.780	0.97	338	$0.86±0.08^{b}$	$5.1±0.8^{B}$	1.26	506
	48	6	45	0.268	0.50	337	$0.60±0.03^{c}$	$7.8±1.2^{C}$	1.66	482
	48	12	45	0.132	0.30	334	$0.68±0.03^{c,d}$	$5.4±0.9^{C,D}$	1.09	503
	48	18	45	0.099	0.33	336	$0.78±0.05^{d}$	$4.7±0.4^{D}$	1.38	490
	55	6	10	0.245	0.53	338	$0.97±0.15^{f}$	$2.0±0.4^{E}$	1.58	485
	55	12	10	0.110	0.40	334	$0.88±0.04^{e,f}$	$2.5±0.2^{E}$	2.00	471
	55	18	10	0.058	0.26	338	$0.66±0.03^{e}$	$3.6±0.4^{F}$	1.34	475
Thermal	55	0.1	10	0.715	0.96	339	$0.55±0.02^{g}$	$10.6±1.1^{G}$	1.21	508
	90	0.1	10	0.000	0.75	331	$0.52±0.20^{g}$	$0.8±0.3^{H}$	10.3	467

HPCD experiments: different small letters indicate significant differences in the value of f_a, at different pressures at a given temperature. Different capital letters indicate significant differences in the value of K_{SV}, at different pressures at a given temperature. Confidence level was set at 95%.

Thermal experiments: different small letters indicate significant differences in the value of f_a, at different temperatures. Different capital letters indicate significant differences in the value of K_{SV}, at different temperatures. Confidence level was set at 95%.

3.3.1 Fluorescence Intensity results

Changes in the protein structure can be evaluated by following the intrinsic fluorescence of some residues (tryptophan –TRP-, tyrosine –TYR- and phenylalanine –PHE-) [32]. These changes involve the reallocation of the residues which affect the emission spectra of those residues [33]. The comparison of the emission spectra before and after an inactivation treatment can provide information regarding the structural changes induced by the treatment. Tryptophan is a hydrophobic residue whose emission wavelength is very sensitive to the polarity of the surrounding environment; for instance, in a non-polar environment, the maximum emission wavelength is around 305 nm, red shifting up to 350 nm when it is exposed to very polar media

[34]. This exposure to the polar environment involves a transference of the observed energy to the solvent, which results in a higher emission wave length (340-350 nm) and lower intensity of the emission signal (quenching effect of water) [32].

Native PME had the maximum emission at 338 nm; the addition of HPCD did not change dramatically the maximum emission wavelength, only a slight blue shifted in the experiments done at 12 MPa at 48 and 55 °C. In the experiments done at 40 °C the maximum emission intensity did not change significantly. However, when temperature was increased up to 48 °C, a decrease of 70% in the emission intensity was observed in the experiments where CO_2 was in the supercritical state (12 and 18 MPa). This reduction in the emission can be attributed to the low polarity of the supercritical CO_2, and its ability to interact with the non-polar residues of the enzyme. Because of this interaction, these residues tend to be exposed to the polar environment (water) that quenches the emission energy, resulting in a decrease of the emission intensity. At 55 °C the same pattern was observed. Zhou et al. 2009 [30] reported opposite results for samples treated under similar conditions (temperature 55 °C, pressure range 8-30 MPa, treatment time 10 minutes). These authors have reported increases in the emission intensity in a commercial PME obtained from orange peel. Significant differences were observed between the way that CO_2 was put in contact with the enzymatic solution: in our case it was bubbled directly into the PME solution, whereas they placed a 15 mL plastic tube containing 1 mL of sample in 850 mL vessel, which was filled with CO_2 and pressurized. In that case, the contact between the phases is expected to be poor, and hence the interaction between CO_2 and the enzyme weaker.

The thermal inactivation experiments revealed that at 55 °C the emission intensity was 96% of the emission of the native PME. As can be seen in Table 4, the presence of CO_2 decreases dramatically the emission of the PME revealing that CO_2 induces changes in the structure of the PME. At 90 °C the maximum intensity is 75% of the native intensity, but the enzyme is completely inactivated. This proves that CO_2 induces changes in the structure that lead to the inactivation of the enzyme but these changes are different compared to those induced in the thermal inactivation experiments.

Changes in fluorescence intensity with exposure time to HPCD, at 55 °C and 12 MPa, are shown in Figure 7. It can be seen that long times exposed to the CO_2 tend to provide lower emission intensities, which can be related with the decrease in the residual activity of the enzyme. Similar results were obtained for other pressures.

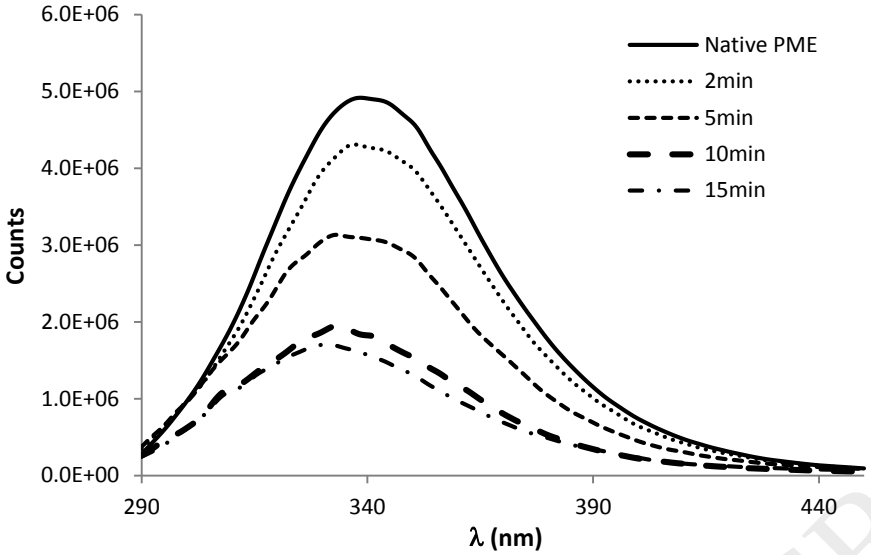


Figure 7. Fluorescence emission spectra for PME treated by HPCD (55 °C, 12 MPa) for different times (from 2 to 15 minutes)

3.3.2 Quenching Experiments

KI was used as quencher agent in order to get information about conformational changes suffered by the enzyme due to the HPCD treatment. KI has a charged and strongly hydrated character which allows it to extinguish the fluorescence emitted by fluorophores located near the surface of the protein [35].

The Stern-Volmer equation (Eq. 3) provides useful information regarding the position of the fluorophores, since the greater exposure of the fluorophores to the quencher the higher the K_{SV} constant, whereas buried fluorophores provided lower values for the K_{SV} constants [35]. It is known that PMEs possess 5 different tryptophan residues [35], two of them exposed to the solvent, another two partially exposed to the solvent and finally one completely buried in the protein structure. D'Avino et al. [14] also studied the solvent accessibility to the TRP residues. These authors concluded that 3 of the five TRP residues were buried in the protein (accessibility surface lower than 14%) and the remaining two were lying on the cleft so they were more exposed to the solvent (accessible surface of 28%).

The plot of our experimental data showed a downward curvature of the Stern-Volmer equation, there was a non-linear response of the changes in the maximum fluorescence intensity versus the concentration of the quencher. It was then used a modified Stern-Volmer equation [9], which includes a factor, f_a, that represents the fraction of fluorophores accessible to the quencher [14].

$$\frac{I_0}{(I_0 - I)} = \frac{1}{f_a} + \frac{1}{f_a K_{SV} Q} \qquad [9]$$

Using equation [9], the native PME solution provided a K_{SV} value of 16.2±1.2 M^{-1}. The samples treated at 40 °C for 45 minutes showed a value of K_{SV} of 4.9±0.1 M^{-1} at 6 MPa, 10.4±1.1 M^{-1} at

12 MPa and 5.1±0.8 M^{-1} at 18 MPa; the differences detected between experiments done at 6 and 18 MPa were not statistically significant. At 48 °C a decrease with pressure was observed, since pressure significantly affected the values of the K_{SV}: at 6 MPa it was 7.8±1.2 M^{-1}, decreasing to 5.4±0.9 M^{-1} at 12 MPa and finally it was 4.7±0.4 M^{-1} at 18 MPa, which indicates that pressure has effect on the structure of the protein. This has been proved by the statistical analysis performed. However, in the experiments done at 55 °C, the K_{SV} constant value obtained was slightly lower and in the range 2.0 to 3.6 M^{-1}, (in the pressure range studied) observing a slight increase of the K_{SV} with the pressure, but statistically significant differences were only observed at 18 MPa. The changes in the K_{SV} values under different conditions of pressure and temperature reveal that the structure of the PME is affected by the CO_2, changing the exposure of the fluorophores to the quencher, which might also be related to the activity losses detected.

According to Table 4, the f_a of the native PME is 0.49 (which indicates that around half of the fluorophores are exposed to the quencher); for HPCD treated samples f_a is increasing but the value of the K_{SV} decreases. This result indicates that the structure of the protein has changed: it is more open and the fluorophores, besides transferring the fluorescence to the quencher, might be transferring energy to the water surrounding them. This can be explained by the fact that higher accessibility of the fluorophore to the quencher does not mean a higher K_{SV} constant and the decreases in the intensity observed in the intrinsic fluorescence experiments.

The thermal inactivation experiments showed that at 90 °C the K_{SV} had a value of 0.8±0.3 M^{-1} with a f_a of 0.52 whereas at 55 °C K_{SV} was 10.6±1.1 M^{-1} and f_a 0.55. It seems that at 90 °C the structure of the enzyme has dramatically changed: K_{SV} value was almost 0 and significantly lower than HPCD at 55 °C, 18 MPa and 10 minutes (K_{SV}=3.6 M^{-1}), with similar residual activities for both (almost 0). The value of f_a was very close to that of the native enzyme, which means that almost the same fraction of the fluorophores was accessible to the quencher; however, the HPCD treatment led to slightly higher values. At 55 °C there is a slight decrease in the activity (around 25%) and slight decrease in the K_{SV}, but the fraction of accessible fluorophores remains constant.

3.3.3 ANS binding experiments

ANS is a molecule that only becomes fluorescent when bound to a hydrophobic region of a protein. In general, proteins in their native state (perfectly folded) tend to hide their hydrophobic residues inside them. The presence of CO_2 (which is hydrophobic in supercritical state) can alter the structure of the enzyme, making the hydrophobic residues more open to the polar environment. Proteins may expose some of their hydrophobic residues on their surface in the denatured state. It is known that some proteins have intermediate states during folding processes of the denaturation process. By monitoring the exposure rate of the hydrophobic sites on the surface of the folding or denaturing intermediates, it is possible to detect the degree of folding or denaturing process of proteins. If a protein is fully denatured, it does not have any organized structure of having both charged region and hydrophobic region in a complementary manner.

When ANS is bound to the protein, the emission intensity is increased and the emission maximum is blue shifted. If there are more hydrophobes exposed to the surface of the protein there will be more biding effects, which result in an increase of the intensity; simultaneously

more blue shift of the emission maximum wavelength will be seen when ANS is bound to more hydrophobic regions.

In the present work slight changes in the intensity where detected when ANS was added. Native PME provided the maximum emission intensity at 535 nm. When the PME was exposed to HPCD at 40 °C for 45 minutes, a clear blue shift of the maximum emission was observed; changing to 513 nm when CO_2 at 6 MPa was added (I/I_0 was 1.07) further decreasing to 488 nm when CO_2 was pressurized to 12 MPa (supercritical state) and back to 506 nm at 18 MPa. It seems that at 40 °C the pressure plays an important role on the PME structure, exposing the hydrophobic zone to the polar environment. The low polarity of the supercritical CO_2 can affect and trigger the opening process. At 48 °C the addition of CO_2 (pressure 6 MPa) led to a dramatic blue shift of the maximum emission intensity to 482 nm. Further increases of pressure buried the hydrophobic core of the protein compared to the results at 6 MPa, as probed the red shift of the signal up to 503 nm. High pressure contributes to the collapse of the three dimensional structure of the enzyme.

At 55 °C, the effect of pressure and time on the maximum ANS emission intensity was also clearly observed. These results, combined with the low residual activities detected in those conditions, lead to the complete inactivation of the protein and to the loss of the tridimensional structure. In this sense at 55 °C and 10 minutes, the maximum intensity clearly blue shifted: 485 nm at 6 MPa, 471 nm at 12 MPa and 475 nm at 18 MPa. The intensities compared to the initials were 1.58, 2.00 and 1.34, at 6, 12 and 18 MPa, respectively. This is indicating dramatic changes in the structure of the enzyme that match with the low residual activities detected, which were 0.24, 0.11 and 0.06 respectively. Similar trends were observed for other temperatures studied.

From the above results, it seems that CO_2 at 6 MPa begins to open the structure of the enzyme exposing the hydrophobic core: CO_2 is in the gas state and can easily diffuse in the enzyme solution. When pressure is increased up to 12 MPa CO_2 turns into supercritical state, and a dramatic increase in the density is observed (water and supercritical CO_2 are barely miscible), which contributes to a reallocation of the residues, now buried and hidden from the polar environment (water).

In the thermal inactivation experiments, clear differences were observed: at 90 °C it was observed a blue shift of the emission to 467 nm and a dramatic increase of the signal (10.3 higher than the native enzyme), which proved that the hydrophobic core of the enzyme was completely open. Temperature induced the denaturalization of the enzyme and dramatic changes in its structure. Thermal inactivation at 55 °C also provided a blue shift of the signal to 508 nm and the maximum intensity resulted to be only 20% higher than that of the native enzyme. In any case, CO_2 at 55 °C induced more changes in the enzyme: there was a more pronounced blue shift of the signal to the range 471-485 nm and the maximum emission was from 34 to 100% higher. These results prove that CO_2 tend to change and open the structure of the enzyme, promoting the loss of the enzymatic activity.

4 Conclusions

HPCD was found to be a useful tool to inactivate commercial PME. Compared to thermal inactivation treatments, CO_2 led to dramatic reductions in the experimental time required to

completely inactivate the enzyme. The systematic study carried out at different pressures (60-18 MPa) and temperatures (40-55 °C) allow to conclude that temperature resulted to be the key parameter in the inactivation of PME, since PME complete inactivation was only observed at temperatures of 48 °C or higher regardless the pressure used. The pressure and temperature sensitive parameters (Z_P and Z_T) were in the range 27.6-45.0 MPa and 7.9-8.7 °C, respectively, confirming the observed results. The experimental data fitted the first order kinetic model, serving to characterize the PME inactivation process in terms of activation energy and activation volume. It was also observed that the ratio CO_2 enzymatic solution used is critical and has to be carefully selected. Finally, the modifications in the PME structure induced by the HPCD treatment were determined by fluorescence assays. It was observed that the inactivation pattern induced by HPCD was different from that achieved only by high temperature.

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