

POLYPHENOL OXIDASE (PPO) AND PECTIN METHYLESTERASE (PME) INACTIVATION BY MEANS OF HIGH PRESSURE CARBON DIOXIDE (HPCD)

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Abstract. HPCD is a promising technology to inactivate the enzymes responsible for the juice spoilage, such as PPO and PME. In order to understand the mechanism that induces this inactivation, a study using two commercial enzymes (PPO from mushroom and PME from *Aspergillus niger*) was carried out. The effect of pressure, temperature, exposure time and ratio CO₂/enzyme ratio loaded in the reactor were studied. The experimental results (residual activity) were fitted to a kinetic model that served to develop a complete kinetic study: the kinetic constants, activation volume and activation energy were calculated, as well as the pressure and temperature sensitivity parameters (Z_P and Z_T , respectively). The changes in the tertiary structure of the enzymes after different treatments were analyzed by fluorescence spectroscopy running different tests: intrinsic fluorescence measurement, KI quenching and ANS binding experiments.

In the case of PPO, the experimental results revealed that this enzyme inactivation kinetics fitted the two fraction model, which indicates the presence of labile and stable isoenzymes. Exposure time (2 to 15 minutes), temperature (25 to 45 °C) and pressure (50 to 200 bar) were the studied experimental conditions that led to different physical states for the CO₂ (gas, liquid and supercritical). Despite the different experimental combinations of pressure and temperature tried, a similar inactivation pattern was observed: a sudden decrease in activity (more than 75% of the total activity loss was observed within the first 2 minutes) was followed by a slowed decay. At constant temperature, higher inactivation rates were observed the higher the pressure, obtaining an almost complete inactivation at 200 bar after 5 minutes regardless the temperature. When temperature was increased, much faster inactivation rates were observed. Z_P and Z_T were in the range 69-78 bar and 27-40°C, respectively.

In the case of the commercial PME, the use of supercritical CO₂ (pressure 60-180 bar, temperature 40-55 °C and times up to 75 minutes) increased dramatically the PME inactivation rate, showing that pressure had a limited effect on PME inactivation but temperature had an important effect. The pressure and temperature sensitivity parameters (Z_P and Z_T) confirmed that trend, being in the range from 276 to 450 bar and 8.7 °C, respectively. The experimental data fitted the first order model and the inactivation kinetics study of PME was completed with the calculations of the activation energy and volume of activation.

The ratio CO₂/volume of enzyme (g/mL) loaded in the reactor was found to be critical for both enzymes. It was seen that ratios higher than 3 did not improve the inactivation kinetics, being a waste of CO₂ from the economic point of view. Below that critical value, the inactivation of the enzyme strongly depended on pressure and temperature. In both cases the structure of the enzyme was dramatically affected after exposure to HPCD, as revealed by the fluorescence spectroscopy analysis that showed significant changes in the tertiary structure of the enzyme, which were compatible with the losses in activity observed.

Key-words. PPO, PME, juice, Supercritical Carbon Dioxide, green process, food technology

INTRODUCTION

Polyphenol oxidase (PPO) and Pectin Methyltransferase (PME) are two of the main enzymes responsible for the quality loss in freshly squeezed juices. PPO causes the browning of the juice due to the oxidation of polyphenols and PME causes juice clarification, being both effects unaccepted by the consumers. In order to prevent this undesirable effect, it is necessary to inactivate those enzymes. Conventional processes are based on thermal inactivation of the enzymes: the juice is exposed at high temperatures for short periods of time, being affected the organoleptic properties of the juice as a consequence. As an alternative treatment, HPCD (High Pressure Carbon Dioxide) seems to be promising. This technique uses CO₂ in conditions of pressure and temperature usually above the critical point (73.8 bar and 30.9 °C). Typical operating values are pressures up to 300 bar and temperatures in the range 20 to 50 °C¹.

In this work, two commercial enzymes (PPO and PME) were exposed to HPCD at different combinations of pressure, temperature, time and CO₂/volume of enzyme ratios in order to study the inactivation kinetics of the enzymes exposed to HPCD. In the literature, it is possible to find different works that use HPCD to inactivate enzymes from juices, but the inactivation mechanism or the proper effect of the CO₂ on the enzyme is not often explained. Moreover, juices are very complex and it is difficult to draw conclusions about the effect of the CO₂ on the enzymes. The aim of this work is to shed light on this aspect. The changes induced by HPCD in the tertiary structure of the enzyme were studied by fluorescence spectroscopy.

MATERIALS AND METHODS

Enzymes and Chemicals

Mushroom tyrosinase (polyphenol oxidase -PPO- EC 1.14.18.1) (reference T3824, 25 KU) was purchased from Sigma Aldrich (St. Louis, MO) and dissolved in a phosphate buffer solution (50 mM, pH 6.5) in order to have a 25 U/mL concentration. Pectin methylesterase (Biopectinasa PME Ultra, origin *Aspergillus niger*, EC 3.1.1.11) was purchased from Biocon Española, S.A. (Barcelona, Spain) and diluted in distilled water to have a theoretical activity of 60 U/mL.

Carbon dioxide (99.9%) was supplied by Air Liquide S.A. (Spain). The physical properties of CO₂ were taken from the NIST Database.

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. A schematic representation of the experimental set up is shown in Figure 1.

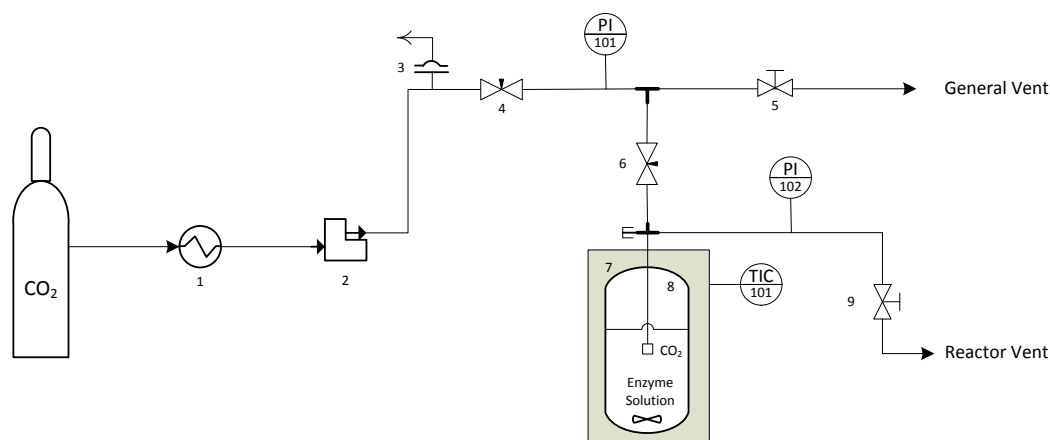


Figure 1. Experimental set-up for HPCD enzyme treatment. 1: CO₂ cryostat; 2: syringe pump; 3: bursting disk; 4: general process valve; 5: general vent valve; 6: individual inlet pump; 7: water bath; 8: high pressure batch reactor with magnetic stirring; 9: depressurization valve. PI-101: general pressure gauge; PI-102: reactor pressure gauge; TIC-101 temperature controller of water bath

In a typical experiment, the desired volume of the enzyme solution was loaded into the reactor (in order to reach the desired CO₂/enzyme dissolution volume desired, as a function of the pressure and temperature selected), which was then tightly closed. Subsequently it was placed in the water bath previously set at the desired temperature. Afterwards CO₂ was bubbled directly into the enzyme solution (through a sintered stainless steel micro-filter with a pore size of 10µm) in a sufficient amount to reach the desired working pressure. Pressure and temperature were held the desired operating time. Experiments were done in a temperature and pressure range commonly used in non-thermal inactivation HPCD systems: temperature from 40 to 55 °C and pressure from 60 to 180bar in the case of the experiments using PME and from 25 to 45 °C and 60 to 200 bar in the case of PPO. After different inactivation times, 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 to evaluate the residual activity of the enzyme after treatment.

Chemical Analysis

The PPO enzyme activity was assayed spectrophotometrically (Jasco V-750, Japan) using catechol as substrate and following a slightly modified protocol from Baltacıoğlu et al². The PME 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 tertiary structure of the enzymes was determined by fluorescence spectroscopy using the FLS980

photoluminescence spectrometer (Edinburgh Instruments, Livingstone, UK). The sample was excited at a $\lambda_{em}=280\text{nm}$ and the emission spectra were recorded in the range from 290 to 450 nm. A 1cm path length cell was used and the emission and excitation slits were set at 2 and 1nm, respectively.

Kinetic Models

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 time. Equation 1 describes mathematically this model:

$$\ln\left(\frac{A}{A_0}\right) = -kt \quad (1)$$

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. The two fraction kinetic model takes into account the existence of several isoenzymes, grouped into two fractions, a labile (L) fraction and a stable (S) one. Both enzymes are considered to be inactivated according to 1st order kinetics, but independently of each other, according to equation 2:

$$A = A_L \exp(-k_L t) + (1 - A_L) \exp(-k_S t) \quad (2)$$

where A_L and A_S ($A_S=1-A_L$) are the activity of the labile and stable fractions respectively and k_L and k_S the inactivation rate constants of both the labile and stable fractions respectively.

EXPERIMENTAL RESULTS

PPO inactivation performance was described using the two fraction kinetic model, whereas PME inactivation followed a first order kinetics. In PPO experiments a sudden activity decrease (more than 75% of the total activity loss was observed within the first 2 minutes) was followed by a slowed decay. At constant temperature, higher inactivation rates were observed the higher the pressure, obtaining an almost complete inactivation at 200 bar after 5 minutes regardless the temperature. In general, and compared to the PPO, PME has been observed to be more resistant to the HPCD treatment: longer times and higher temperatures are required to achieve significant inactivation (more than 40 °C, preferably above 50 °C). The temperature sensitivity parameter was significantly lower for PME (7.9-8.7 °C) compared to PPO (27.0-40.7 °C). In turn, it was observed that pressure increases did not increase the inactivation rate of the PME (Z_p values for PME were in the range 276-450 bar, whereas for PPO were from 69.5 to 78.7 bar). The activation volume for the PPO labile fraction was in the range -94 to -69 cm³/mol and for PME was in the range -218 to -142 cm³/mol, increasing when increasing pressure. The activation energies were in the range 16-25 kJ/mol PPO and 227-250 kJ/mol for PME. They were calculated using the Eyring and Arrhenius equations, respectively, and can be found in Illera et al.¹

These changes in the enzymes behavior when exposed to HPCD treatment can be attributed to the differences in the enzymes structure, which is more complex for the PPO (3 or 4 subunits with a MW around 130 kDa) whereas PME is formed by only one subunit with a MW around 35 kDa. In general, the more complex the structure the more susceptible is to changes in the environment³. This also can explain the differences in the inactivation kinetics: PME followed the simplest model, the first order one; in turn, the PPO followed a two fraction model, which revealed the presence of two isoenzymes.

It was clearly seen that the amount of CO₂ used in the experiment affects dramatically the inactivation performance. As far as the authors' knowledge, the ratio CO₂ versus volume of enzyme solution has not been systematically studied in the literature, not having been found papers reporting this parameter. For instance Manzocco et al.⁴ used 10 mL of enzyme solution in a 150 mL reactor, which led to ratios in the range 9.8-12.6 g/mL depending on the pressure and temperature selected; another example is Liu et al.⁵, who used red beet as raw material loading 5 mL of sample in a 10 mL vial, which was placed in a 300 mL reactor. The amount of CO₂ used was not reported in any of these works, but it can be intuited that ratios CO₂:volume of solution are very high. Similar results were observed for PME. Our work clearly shows that the ratio CO₂/volume of enzyme (g/mL) loaded in the reactor was critical. It was observed that ratios higher than 3 did not improve the inactivation kinetics, being a waste of CO₂ from the economic point of view. Below that critical value the inactivation of the enzyme strongly depends on the pressure and the temperature, as can be seen in Figure 2.

Finally in Figure 3 it is possible to see how the intrinsic fluorescence of PME changed with time at 55 °C and 120 bar. The residual activity after 15 minutes was lower than 10%. The KI quenching and ANS binding experiments supported the idea of structural changes in the enzyme after HPCD treatment.

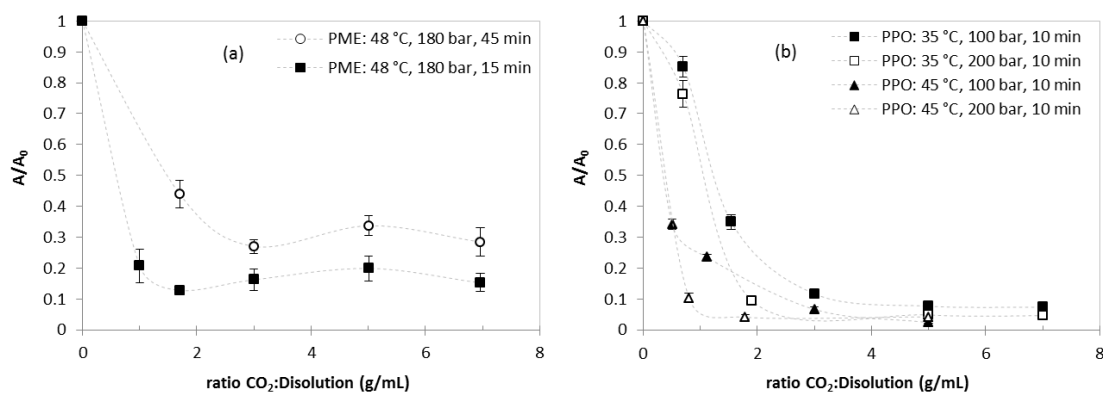


Figure 2. Residual activity of PME (a) and PPO (b) treated using different ratios CO₂:volume of enzyme solution under different pressure, temperature and time conditions.

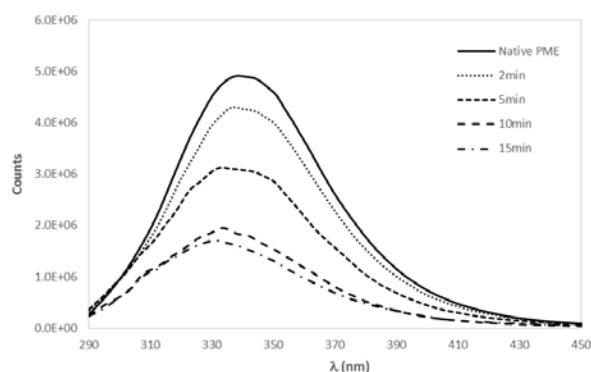


Figure 3. Fluorescence emission spectra for PME treated by HPCD (55 °C, 120 bar) for different times.

CONCLUSIONS

PPO and PME activity is significantly affected by HPCD, but in different ways: PPO is affected by both pressure and temperature, while PME is only affected by temperature. It is critical the ratio CO₂/amount of enzyme loaded in the reactor since ratios higher than three times more CO₂ than enzyme guarantee a complete inactivation regardless of pressure and temperature. PPO and PME have completely different inactivation kinetics: PPO presents a two biexponential kinetics while PME exhibits a first order kinetics. The analysis of the kinetic parameters reveals a higher stability of PME to the HPCD treatment. Both enzymes suffer dramatic changes after the HPCD treatment, as the fluorescence spectroscopy analysis revealed.

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