Pulsed light inactivation of horseradish peroxidase and associated structural changes

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ABSTRACT

Pulsed light (PL) is a non-thermal preservation method in which foods are subjected to one or several intense pulses of wide-spectrum light. Peroxidase (POD) is an enzyme that needs to be inactivated or inhibited because of its deleterious effects on the quality of fruits and vegetables. The feasibility of using PL to inactivate POD was tested and results explained based on measurements of UV–vis spectrum, far-UV circular dichroism and tryptophan fluorescence, and the phase-diagram method. PL reduced the activity of POD by more than 95% after applying 128 J cm⁻²/C₀². There was observed a decrease in the Reinheitszahl value and ellipticity and an increase in tryptophan fluorescence at incremental fluences, as well as linear phase diagrams. The study indicates that the inactivation of POD by PL is an all-or-none process related to loss of helical structure, weak unfolding and ejection of the prosthetic group.

1. Introduction

Pulsed light (PL) is a green technology for food processing based on the application of short pulses of high-intensity polychromatic light including UV light (Gómez-López, Ragaert, Debevere, & Devlieghere, 2007). The applicability of PL to foods has been mainly studied for microbial inactivation (Gómez-López, Devlieghere, Bonduelle, & Debevere, 2005) and other potential uses have been recently revised (Gómez-López, 2015). PL is part of the group of the so-called non-thermal technologies intended to replace as far as possible thermal treatments, which impair quality attributes of foods. Typical PL treatments consist of flashes of high fluence (about 1 J cm⁻¹) and short duration (0.2–0.4 ms) supplied at high repetition rates (0.5–3 Hz). The electromagnetic spectrum is continuous from 200 to 1100 nm. This range covers UV, visible and infrared wavelengths.

One of the novel applications of PL is the inactivation of enzymes. The effect of PL on enzymes relates to its UV portion because proteins absorb in the UV range. The peptide bond absorbs at 180–230 nm, while the aromatic residues have peak absorbance between 258 and 280 nm and disulfide bonds absorb near to 260 nm (Schmid, 2001). Heme groups are also excellent UV absorbers and also absorb visible light. Protein photoreaction is generally reported to occur via two major mechanisms: 1) direct photo-oxidation or 2) indirect protein oxidation by singlet oxygen generated by energy transfer (Manzocco, 2015).

The first report on enzyme inactivation by PL is a patent (Dunn et al., 1989). The holders of this patent claimed the inactivation of polyphenol oxidase (PPO) in potato slices and of alkaline phosphatase in vitro. In the peer-reviewed literature, the first report on inactivation of enzymes by PL in vitro was on PPO (Manzocco, Panozzo, & Nicoli, 2013), followed by alkaline phosphatase (Innocente et al., 2014) and lipoygenase (LOX) (Janve, Yang, Marshall, Reyes-De-Corcuera, & Rababah, 2014). The successful use of PL to inactivate food enzymes in these studies has been accompanied by some investigations on the associated structural changes. The loss of PPO activity has been related to unfolding/aggregation phenomena and protein backbone cleavage after HPLC-gel permeation analysis (Manzocco et al., 2013) and LOX inactivation was accounted for by protein fragmentation (Janve et al., 2014).

Peroxidase (E.C. 1.11.1.7) (POD) is one of the main enzymes that must be inactivated during processing fruit derivatives because it catalyses several undesirable reactions such as those leading to browning (Falguera, Moulin, Thevenet, & Ibarz, 2013). This enzyme consists of 308 aminoacid residues including one tryptophane and four disulphide bridges in a single polypeptide chain. It has eight neutral carbohydrate side-chains, a hemin prosthetic group named ferriprotoporphyrin IX and two Ca²⁺ (Welinder, 1979). Thermal processing has been classically used for the inactivation of POD...
in the food industry. This process leads to unwanted side effects such as losses of sensory and nutritional value and associated problems of marketability (Surowsky, Fischer, Schlueter, & Knorr, 2013). For these reasons, alternative non-thermal technologies for the inactivation of POD and other food enzymes are sought, being PL one of the potential methods to achieve this goal. One of the main drawbacks of PL technology is the poor penetration of UV light. However, the inactivation of POD by continuous UV light treatments has been proved in apple juice (Falguera, Pagán, & Ibarz, 2011), grape must (Falguera, Garvín, Garza, Pagán, & Ibarz, 2013) and pear juice (Falguera, Garvín, Garza, Pagán, & Ibarz, 2014).

Nowadays, there are different analytical techniques that allow the characterization of the structural changes that enzymes suffer during their inactivation. They have been used to explain enzyme inactivation by classical and novel technologies such as supercritical carbon dioxide (SCCO₂) (Gui et al., 2006), pulsed electric field (PEF) (Zhong, Hu, Zhao, Cheng, & Liao, 2005; Zhong et al., 2007), continuous UV light (Neves-Petersen et al., 2007), cold plasma (CP) (Surowsky et al., 2013), heat (Stancicu, Aprodou, Ionița, Bahrim, & Răpeanu, 2015) and microwaves (MW) (Lopes et al., 2015). We believe that these techniques should be used to gain deeper insight on the effects of PL on enzymes. The results found for this enzyme of concern will also become a starting point to better understand the effects of PL on enzymes in general.

As a consequence, our goals have been testing the potential of PL to inactivate POD as well as to explain it based on the structural changes that the enzyme undergoes during PL treatment.

2. Materials and methods

2.1. Reagents

The horseradish POD enzyme type VI-A, 2,2’-azinobis (3-ethylbenzothiazolinesulfonic acid) (ABTS) and hydrogen peroxide (H₂O₂) were purchased from Sigma-Aldrich (Madrid, Spain). H₂O₂ solutions were freshly prepared every day, and their concentrations were calculated using ε₄₄₃ = 39.4 M⁻¹ cm⁻¹.

2.2. Pulsed light system

PL treatment was performed in a XeMaticA-Basic-1L system (Steribeam, Kehl, Germany). The system produces pulses of 200 μs of broad-spectrum pulsed light ranging from infrared to ultraviolet light with 21% of UV content. A stirrer (Topolino, IKA, Staufen, Germany) was incorporated to the chamber for interpulse sample homogenization. The system is equipped with one 19-cm long xenon lamp placed at the top of the treatment chamber. It was operated at a discharge voltage of 2.5 kV, which generates 500 J pulse⁻¹. The corresponding emission spectrum has been reported in Cudemos, Izquier, Medina-Martínez, and Gómez-López (2013). Samples received an incident fluence of 2.14 J cm⁻² pulse⁻¹. Incident fluence was determined by analysis of in-built photodiode readings using a PC-Lab 2000 LT PC oscilloscope (Velleman Instruments, Gavere, Belgium), and manufacturer performance charts. Different fluences were obtained by increasing the number of pulses.

2.3. Treatment

A Petri dish without cover with 20 ml of sample was placed on top of the stirrer below the centre of the lamp. The distance between the lamp and the sample surface was 6.7 cm. A total of 60 pulses were applied, which amounts a cumulative fluence of 128 J cm⁻² for the highest energy treatment. Samples were withdrawn at different fluence intervals for analytical measurements.

2.4. Enzymatic activity

POD solutions were prepared diluting increasing amounts of enzyme in 100 mM sodium acetate buffer pH 5. The POD activity was followed spectrophotometrically in a Shimadzu model UV-1603 spectrophotometer at the absorption maximum of the ABTS radical cation of 414 nm (ε₄₄₃ = 31.1 mM⁻¹ cm⁻¹). One unit of enzyme (U) was defined as the amount of enzyme that produced 1 μmol of ABTS radical per minute (Fortea et al., 2012). The enzymatic activity was determined by measuring the activity between 0 and 60 light pulses at five light pulses steps. All experiments were carried out in triplicate and further analysis were evaluated in duplicate. The F₅₀, the fluence necessary to decrease the POD activity by 50%, was used as index of photostability. F₅₀ was calculated by linear regression from the inactivation curves considering the linear part of the curve (Manzocco et al., 2013).

The standard reaction medium contained 100 mM sodium acetate buffer (pH 5.0), increasing concentrations of POD (0.0504, 0.252, 0.504 and 1.26 μg ml⁻¹), 3 mM ABTS, 2 mM H₂O₂ in a final volume of 1 ml. Those POD concentrations corresponded respectively to 15, 59, 112 and 251 U.

2.5. Temperature measurements

Sample temperature was measured by using an infrared thermometer (ScanTemp 410, TFA, Germany). A standardized procedure was set up in order to perform every measurement in the same way. This was necessary because the thermometer could not be inserted in the PL system without perturbing the light transfer pattern. Also because the door chamber is automatically locked during pulses. The infrared beam of the thermometer was directed to the centre of the sample at a thermometer-sample distance of 10 cm and an angle of 45° with respect to the horizontal. Measurements were taken immediately before starting treatments and one second after treatments; the latter because it takes time to open the door of the treatment chamber. Measurements were performed immediately after the end of the PL treatment.

2.6. UV–visible spectrum

The absorption spectroscopy measurements were performed in the range between 200 and 800 nm using a UV-spectrophotometer (Shimadzu UV-1603, Japan) at 25 °C. A 1 cm path-length cuvette was used and enzyme solution was replaced by buffer for the blank.

2.7. Far-UV circular dichroism analysis

Far-UV circular dichroism (CD) spectra were recorded in a PiStar-180 Spectrometer (Applied Photophysics, Leatherhead, United Kingdom), with 1 mm path-length rectangular quartz cuvette at 20 °C. CD spectra were scanned in the UV range (250–200 nm) with three replicates. The number of samples per wavelength was automatically determined by the equipment by adaptive sampling with a signal-to-noise ratio of 0.01; bandwidth = 1 nm.

All CD spectra measured were baseline corrected with the respective buffer spectrum. The ellipticity values were obtained in millidegrees directly from the instrument and converted to the mean residue ellipticity ([θ]) expressed in deg cm² dmol⁻¹, using the following equation:

\[
[\theta] = \frac{\text{MRW}}{10^3} \frac{\Theta}{C}
\]  
(1)
where [θ] is the measured ellipticity in degrees, c is the protein concentration (g ml⁻¹), d is the path-length (cm) and MRW is the mean residue weight for the peptide bond calculated from:

\[ MRW = \frac{M}{N-1} \]  

(2)

Where M is the molecular mass of the peptide chain (Da) and N is the number of amino acids in the chain (Kelly, Jess, & Price, 2005). M and N values were taken from Welinder (1979). POD concentration was 7.44 μM.

The secondary structure fractions were calculated using the software K2D3 (Louis-Jeune, Andrade-Navarro, & Pérez-Iratxeta, 2012) with the data allowed by this program, which is in the range 200–240 nm.

2.8. Steady-state tryptophan fluorescence spectroscopy

Tryptophan fluorescence spectra were measured with a spectrophotometer model RF-Shimadzu 5301 PC (Shimadzu, Japan), using a quartz cuvette of 1 cm optical path-length at room temperature (25 °C). All samples were observed after 0, 10, 20, 30, 40, 50 and 60 light pulses. Determinations were carried out with a sampling interval of 1 nm and slits of 5 nm. POD concentration was 7.44 μM. The emission spectra (λem from 300 to 450 nm) were obtained at the maximum excitation wavelength λex = 293 nm (Gui et al., 2006) and represent the average value of three measurements.

2.9. Phase diagrams

Phase diagrams were constructed to probe if the structural transformation of the POD from native to inactive form follows an all-or-none or multi-step process. The phase diagram method is based on the equation:

\[ I(λ_1) = a + bI(λ_2) \]  

(3)

where \( I(λ_1) \) and \( I(λ_2) \) are the spectral intensities at wavelengths \( λ_1 \) and \( λ_2 \) under different fluences and \( a \) and \( b \) are the intercept and the slope respectively, of the \( I(λ_1) \) versus \( I(λ_2) \) plot (Kuznetsova, Turoverov, & Uversky, 2004).

2.10. Data analysis

The data were processed using Excel 2006 (Microsoft, USA) except for ANOVA and Tukey post hoc test, which were performed by using SPSS 17.0 (IBM, NY, USA), with \( p \leq 0.05 \).

3. Results and discussion

3.1. Inactivation kinetics

The inactivation of POD by PL is shown in Fig. 1. Different concentrations of enzyme were assayed to assess its possible effect on the inactivation rate, given the evidence presented by Manzocco et al. (2013). These authors studied the inactivation of PPO by PL, and observed that high concentrations of enzyme decreases inactivation. They attributed the finding to the crowding effect. Our results show no evidence of crowding effect, likely because this effect occurs at much higher protein concentrations. For example, increased resistance of egg white proteins to UV-C light takes place above a limit concentration of 2200 μg ml⁻¹ (Manzocco & Nicoli, 2012). That concentration is 1000 times higher than the enzyme concentrations used in the present work, however, more reference values are missing from the literature. POD inactivation was statistically slower (\( p < 0.05 \)) at the lowest assayed POD concentration (15 U). On the other hand, there was not statistical difference (\( p > 0.05 \)) in inactivation rates at the rest of the concentrations (59–251 U). Actually, the first order inactivation plots show a complete overlapping of the inactivation curves for this range of concentrations. The decreased inactivation at low enzyme concentration is likely due to a lower probability of contact between photons and enzyme with decreasing protein concentration (McDonald et al., 2000). Since the result for 15 U was an exception, the analysis was focused on the results obtained in the 59–251 U range. After applying 128 J cm⁻², the residual POD activity was 4% (Fig. 1). The inactivation of POD by PL follows a first order kinetic with a rate equal to 0.0255 cm² J⁻¹ under the assay conditions: this rate was calculated by pooling the inactivation rates found for the range 59–251 U. The F50 value was 27.0 J cm⁻². F50 values have been reported for the inactivation of other enzymes by PL (Innocente et al., 2014; Manzocco et al., 2013), but results cannot be compared due to differences in experimental set-ups. One of the most important parameters in the characterization of PL treatments is the measurement of fluence, which is fundamental to compare inter-laboratory results. Bench-top PL systems are the most common device used by researchers worldwide. To the date, there is not a standard protocol to measure fluence in bench-top PL systems. Only recently an approach to a standard protocol has been proposed (Gómez-López & Bolton, 2016).

PL is considered a non-thermal method because it is not based on heat. However, sample heating can be an unwanted side effect derived from the infrared portion of the lamp emission. Therefore, temperature must be measured during treatments to assess a possible contribution of heat to the observed effects. Sample temperature during tests changed from 20 to 27 °C. This temperature is too low for provoking any thermal effect. POD is considered the most heat resistant enzyme in fruits and vegetables. Its inactivation is used since long time ago by the food industry as indicator of the efficiency of the blanching process of fruits and vegetables. As an example of its thermostability, Lopes et al. (2015) did not observe inactivation of horseradish POD when incubated at 30–45 °C up to 48 h. Those treatment conditions are far harsher conditions that those experimented by POD during PL treatment in the current study. Therefore, the POD inactivation by PL can be considered exclusively a photochemical process. As any other photochemical process, the reaction rate of POD inactivation can be affected by temperature, yet the inactivation will still follow a photochemical mechanism. However, the slight increase of temperature has not significantly affected the inactivation curves, as it can be deducted from the linearity of the first order inactivation plots (\( R^2 > 0.99 \)).
Once the inactivation of POD by PL was demonstrated, different analytical techniques were used in order to explain the effect of PL on this enzyme.

3.2. Absorption spectra

The evolution of the absorption spectrum of POD was used to evaluate structural changes. The spectra show the characteristic peaks of POD (Fig. 2), one in the UV range corresponding to the absorption of the peptide groups of the protein main chain (Schmid, 2001). Other features of the spectra are the Soret band due to heme absorption and some absorbance between 250 and 300 nm due to aromatic and cysteine residues (Schmid, 2001). The change of the Reinheitzahl value ($R_z$), defined by the ratio between the Soret absorbance band ($\lambda = 403$ nm) and the absorbance at 280 nm upon different fluences is shown in Fig. 3. A decrease in the $R_z$ value is considered an indicator of the ejection of the heme group (Neves-Petersen et al., 2007). The $R_z$ value declines linearly with fluence due to decrease in the Soret band and increase of absorbance at 280 nm. This indicates that an alteration of the heme group has occurred, which might be the ejection of this moiety from the active site. Under such premise, approximately 50% of the heme group was released at the end of the treatment compared to the native protein. Similar results were reported by Neves-Petersen et al. (2007), where after 16 h of illumination with 296-nm UV light, a residual activity of 3% was detected while the $R_z$ value only decreased by 69%. Heme ejection has been reported for horseradish POD treated with guanidinium chloride (Pappa & Cass, 1993), pH (Carvalho et al., 2003), heat (Carvalho et al., 2003; Chattopadhyay & Mazumdar, 2000) and UV light (Neves-Petersen et al., 2007).

3.3. Far-UV circular dichroism

Far-UV CD was used to study the effect of PL on the secondary structure of POD. Fig. 4a shows the spectra of untreated and treated POD at increasing fluences. The spectral range used (200–250 nm) corresponds principally to the peptide bond (Kelly et al., 2005). The spectra include two typical negative peaks at 208 nm and 222 nm characteristic of proteins with high percentage of helical structure. The decrease in ellipticity at those wavelengths indicates a progressive loss of helical structure with fluence. The proportion of $\alpha$-helix and $\beta$-sheet of the native enzyme together with their change throughout the inactivation process is shown in Fig. 4b. The percentages of $\alpha$-helix (28%) and $\beta$-sheet (19%) of the native POD are consistent with studies about its structure. It is known that the proportion of $\alpha$-helix of POD is higher than that of $\beta$-sheet as it has been established by using different methods such as circular dichroism (Strickland, Kay, Shannon, & Horwitz, 1968), Fourier transform infrared spectroscopy (Holzbaur, English, & Ismail, 1996) and crystallography (Gajhede, Schuller, Henriksen, Smith, & Poulos, 1997). However, a previous study on POD inactivation by PL reports a higher percentage of $\beta$-sheet with respect to $\alpha$-helix (Wang et al., 2017). The proportion of $\alpha$-helix of the native enzyme decreases from 28 to 16% at the end of the treatment, while the proportion of $\beta$-sheet increases from 19 to 26% (Fig. 4b). A loss of helical structure has also been found to explain the inactivation of POD by other non-thermal treatments such as SCCO$_2$ (Gui et al., 2006), PEF (Zhong et al., 2005, 2007) and CP (Surowsky et al., 2013). Additionally, an increase of $\beta$-sheet structure has been reported for the inactivation of POD by acidification (Hamaguchi, Ikeda, Yoshida, & Morita, 1959) and CP processing (Surowsky et al., 2013).

![Fig. 2. Absorption spectrum of native and pulsed light treated peroxidase at different fluences.](image-url)

![Fig. 3. Change in Reinheitzahl value of native and pulsed light treated peroxidase at different fluences.](image-url)
3.4. Tryptophan intrinsic fluorescence

Fluorescence techniques are useful to study potential changes in the tertiary structure of the enzymes. The fluorescence of tryptophan was used as an internal probe in this study. Protein fluorescence is mainly caused by residues of tyrosine and tryptophan. An excitation wavelength of 293 nm was used in order to maximize the dominance of tryptophan fluorescence to the observed signal. The distance between the tryptophan in horseradish POD and the nearest haem edge is 1.3 nm. This causes that the tryptophan fluorescence intensity is quenched by the haem by energy transfer (Pappa & Cass, 1993). A conformational change near the heme cavity of the enzyme can increase the distance between tryptophan and heme and the quenching effect is less pronounced (Carvalho et al., 2003). The treatment of POD with PL caused a progressive increase in fluorescence during processing (Fig. 5). This indicates that the distance between the tryptophan residue and the heme group increases during treatment. This result can be due to the unfolding of the protein that decreases tryptophan quenching or that heme group has been ejected.

The fluorescence peak was located at 329 nm and no shifts were observed with increasing fluence. This indicates that the conformational change of POD due to PL is relatively weak and the protein do not completely unfold since a fluorescence red-shift is expected when tryptophan buried in an enzyme interior become solvent exposed as the result of unfolding. The increase in fluorescence intensity could be due to a local relaxation of the chain where the tryptophan residue is located rather than a gross conformational change (Tsaprailis, Sze, & English, 1998).

A plot of fluorescence intensity at 329 nm vs fluence (not shown) shows a regular pattern that can be described by the equation $y = -0.005x^2 + 1.337x + 93.899$ ($R^2 = 0.997$). The plot does not show transitions that could indicate formation of intermediates (Tsaprailis et al., 1998).

A decrease in fluorescence intensity with the progress of the treatment has also been reported for the inactivation of POD by other non-thermal methods such as SCCO$_2$ (Gui et al., 2006), PEF (Zhong et al., 2005) and CP (Surowsky et al., 2013).

3.5. Phase diagrams

The complete unfolding of an enzyme is not always a one-step process where the protein completely unfolds. It can also be a process that can include several steps that give rise to the appearance of different partially unfolded intermediates. The existence of intermediates can be revealed by building phase diagrams (Kuznetsova et al., 2004). In order to probe if the structural transition that lead to POD inactivation is an all-or-none or a multistep process, phase diagrams were built by plotting data on CD and tryptophan fluorescence intensity. A linear relationship has been postulated to indicate that the unfolding process proceeds as an all-or-none transition. In contrast, a non-linear relationship would indicate that POD inactivation is a multi-step process with different intermediates that are unfolded in a sequential process (Stojanovski, Breydo, & Uversky, 2016). Fig. 6a shows a phase diagram built with CD data where a linear relationship is observed. A phase diagram of tryptophan fluorescence (Fig. 6b) also shows a linear relationship. These results, in accordance with the no evidence of intermediate states previously discussed, are consistent with the hypothesis that the inactivation process of POD by PL can be considered an all-or-none transition. This kind of process is best described as follows:

$$N \rightarrow I$$  \hspace{1cm} (4)

where N represents the native POD and I the inactivated enzyme. This representation does not consider a potential refolding, which was out of the scope of this study. The finding does not imply that the process for complete unfolding of POD by PL follows this pathway; it only implies that the pathway from native to inactive enzyme is a one-step process. This pathway could lead to a partially folded enzymatically inactive intermediate. Indeed, the results from tryptophan fluorescence supports the idea of a partial unfolding of POD. It has been described that the denaturation of horseradish POD by pH and heat also follows an all-or-none process (Stanciuc et al., 2015).

![Fig. 5. Tryptophan fluorescence emission spectra of native and pulsed light treated POD at different fluences.](image)

![Fig. 6. Phase diagrams from (a) circular dichroism spectra data and (b) tryptophan relative fluorescence data of native and pulsed light treated POD at different fluences (J cm$^{-2}$).](image)
4. Conclusion

The inactivation of POD by PL requires 128 J cm$^{-2}$ to cause more than 95% loss of activity under the assay conditions. It follows a first-order kinetic with no evidence of crowding effect. The lack of evidence of intermediates according to the plot of peak tryptophan fluorescence intensity vs fluence together with the linear change of horseradish peroxidase treated with supercritical carbon dioxide. Food Chemistry, 97, 480–485.


Falguera, V., Garza, S., Pagán, J., Garvin, A., & Ibarz, A. (2013). Effect of UV-vis irradiation on enzymatic activities and physicochemical properties of four grape musts from different varieties. Food and Bioprocess Technology, 6, 2223–2229.


