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Pulsed light inactivation of polygalacturonase

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ABSTRACT

Pulsed light is a non-thermal technology capable to inactivate enzymes. This study investigated the effect of pulsed light on the activity of polygalacturonase, and on the structure of the enzyme by means of fluorescence emission spectra, free sulphydryl detection and analysis of changes in parameter A and phase-diagram. The results showed that pulsed light is able to inactivate polygalacturonase in buffer, with > 90% reduction of enzyme activity after applying 128 J/cm^2 and a first-order kinetic constant of 0.0426 cm^2/J under the experimental conditions. The free sulfhydryl detection revealed the rupture of sulfhydryl bridges. Fluorescence spectra analysis showed that the tertiary structure of polygalacturonase was changed. Phase diagram analysis shows the existence of only two populated states. It is suggested that the inactivation of polygalacturonase by pulsed light is an all-or-none process where disulfide bridges are broken and the enzyme is unfolded.

1. Introduction

Pulsed light (PL) is a non-thermal method for food preservation based in the application of flashes of a high-intensity wide-spectrum light encompassing from infrared to UV light (Gómez-López, Ragaert, Debevere, & Devlieghere, 2007). It was initially investigated for use in microbial inactivation, but its application field has become broader in the last years, including enzyme inactivation. Enzyme inactivation by this technology is possible because PL systems emit a significant amount of UV light, which is absorbed by proteins. Tryptophan, tyrosine, phenylalanine and cystine are the specific aminoacids responsible for the photochemical inactivation of proteins. Enzyme are inactivated by the disturbance of its natural conformation and/or the transformation of its active site due to the photoionization of aromatic aminoacids and rupture of disulfide bridges (Vladimirov, Roshchupkin, & Fesenko, 1970).

Polygalacturonase (PG) (EC 3.2.1.15) is together with pectin methyl esterase one of the most important enzymes related to the quality of fruits, vegetables and their products. Both enzymes are responsible for the degradation of pectin. In the fruit juice industry, induced changes by PG can be desirable (better clarification or increased juice extraction) or must be prevented (to ensure cloudy juices) (Duvetter et al., 2009). While it is known that UV light penetration decreases with turbidity, the use of suitable reactor designs overcome this limitation (Müller et al., 2015; Simmons et al., 2012). PG hydrolyzes the α-glycosidic bonds in the homogalacturonan region of pectin (van Pouderoyen, Snijder, Benen, & Dijkstra, 2003). The structure of PG is composed mainly of β-sheets folded in a helical shape called β-helix and it is stabilized by four disulfide bridges (van Santen et al., 1999).

The last decade has undergone an increasing interest for the inactivation of enzymes by PL and for the study of associated structural changes. The inactivation of polyphenol oxidase (PPO) (Manzocco, Panozzo, & Nicoli, 2013a; Pellicer, Navarro, & Gómez-López, 2018), peroxidase (POD) (Pellicer & Gómez-López, 2017), lipoxygenase (Janve, Yang, Marshall, Reyes-De-Corcuera, & Rababah, 2014) among others has been achieved and the induced structural changes related to the loss of activity have been reported in several studies with different level of detail. The study of enzyme inactivation by PL can be framed in the wider application field of PL protein modification, which has led to interesting outcomes such as allergenicity abatement of protein allergens (Shriver & Yang, 2011) and modification of functional properties of proteins (Manzocco, Panozzo, & Nicoli, 2013b; Siddique, Maresca, Pataro, & Ferrari, 2016). Diverse structural modifications of proteins subjected to PL treatment have been described, such as aminoacid oxidation (Fernández, Ganan, Guerra, & Hierro, 2014; Siddique et al., 2016), ejection of the prostetic group of an enzyme (Pellicer & Gómez-López, 2017), protein unfolding (Manzocco et al., 2013a) and protein aggregation (Elmnasser et al., 2008).

Based upon the fundamental knowledge of the photochemistry of proteins (Vladimirov et al., 1970) and on previous studies on inactivation of enzymes (Janve et al., 2014; Manzocco et al., 2013a), it was hypothesized that pulsed light is able to inactivate PG and that the

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loss of activity can be related to changes in its structure. Therefore, the aim of this research was to study the effect of pulsed light on polygalacturonase activity and to gain insight on the associated structural changes that may help to explain its loss of activity. The information generated in this research can be useful to expand the application field of PL and increase the understanding of the mechanisms underlying the effects of this technology on enzymes and proteins in general.

2. Material and methods

2.1. Materials

A commercial polygalacturonase (Sigma-Aldrich, St. Louis, United States) was used in this study. TRIS and HCl were purchased from Scharlau (Barcelona, Spain). The rest of the reagents were from Sigma-Aldrich.

2.2. Treatments

The enzyme preparation (0.5 mg/mL, 20 mL) was placed in a Petri dish without cover and treated with PL in a XeMaticA-Basic-1L unit (Steribeam, Kehl, Germany). This system can be operated at different voltages. In this case, a discharge voltage of 2.5 kV was used, which generates a light with an energy of 500 J/pulse and 21% of UV component. The flash has a pulse width of 200 μs and a characteristic polychromatic emission spectrum similar to one previously reported (Cudemos, Izquier, Medina-Martinez, & Gomez-Lopez, 2013). Homogenization of the samples between pulses was carried out by a stirrer (Topolino, IKA, Staufen, Germany). The fluence impinging on sample surface was 2.14 J/cm^2 per pulse according to oscilloscope measurements (PC-Lab 2000 LT PC, Velleman Instruments, Gavere, Belgium) taken with an in-built photodiode, which were converted to fluence units using manufacturer performance charts. Different fluences were supplied to enzyme preparations by using different number of pulses up to 60. The temperature of the samples during treatment was measured by a ScanTemp 410 infrared thermometer (TFA Dostmann, Wertheim, Germany). Samples from the enzyme preparation were withdrawn at different fluence intervals to build the inactivation curve and for analytical determinations. The concentration of the enzyme was the same for all tests.

2.3. Enzymatic activity

The PG activity assay was based on the release of reducing groups produced by PG and measured by spectrophotometry (UV–Vis spectrophotomer, Shimadzu model UV-1603, Japan) (Aguiló-Aguayo, Soliva-Fortuny, & Martín-Belloso, 2008). 100 μL of the enzyme extract was mixed with 300 μL of 0.2% polygalacturonic acid and incubated at 35 °C for 10 min. Two mL of 0.1 M borate buffer, pH 9.0 and 400 μL of 1% cyanoacetamide were added to stop the reaction, and boiled for 10 min. After cooling, the absorbance was measured at 276 nm and 22 °C. A blank was prepared in the same way without adding the enzyme. Enzymatic activity was measured every five pulses.

Results were fitted to a first-order inactivation kinetics according to:

$$\ln\frac{A_F}{A_0} = -kF \tag{1}$$

where A_F is the enzymatic activity at fluence F (J/cm^2), A_0 is the enzymatic activity before treatment and k is the first-order inactivation rate (cm^2/J).

2.4. Free sulfhydryl content

Free sulfhydryl content was determined by the method of Ellman (Ellman, 1959) as modified by Siddique, Maresca, Pataro, and Ferrari (2017), but with an enzyme concentration of 0.5 mg/mL. In brief, 2.75 mL of enzyme solution was mixed with 0.25 mL of a 1 g/L of Ellman's reagent in 50 mM Tris-HCl buffer. The absorbance of the solution after 30 min of incubation in darkness at room temperature was measured at 412 nm by an UV–Vis spectrophotomer (Shimadzu). Samples were measured every 20 pulses.

Free sulfhydryl concentration was calculated according to Beveridge, Toma, and Nakai (1974):

$$\frac{\mu MSH}{g} = \frac{73.53 A_{412} D}{C} \tag{2}$$

where A_{412} is the absorbance at 412 nm, D is the dilution factor, C is the sample concentration (mg enzyme/mL) and 73.53 is derived from $10^6/(1.36\cdot10^4)$. 10^6 is a conversion factor and $1.36\cdot10^4$/M cm is the molar absorptivity.

2.5. Steady-state intrinsic fluorescence

Intrinsic tryptophan fluorescence was measured in the steady-state mode at an excitation wavelength (λ_{ex}) of 293 nm and an emission wavelength (λ_{em}) range of 300–450 nm, at 1 nm interval with 5 nm slits in a spectrofluorimeter (RF-Shimadzu, Japan) with a quartz cuvette of 1 cm optical path at 25 °C. Samples were measured every 10 light pulses up to 60 pulses and fluorescence spectra are reported.

2.6. Parameter A

The changes in fluorescence were also analyzed by using the parameter A (Turoverov, Haitlina, & Pinaev, 1976):

$$Parameter\ A = \left(\frac{I_{320}}{I_{365}}\right)_{293} \tag{3}$$

where I_{320} and I_{365} are fluorescence intensities at λ_{em} = 320 and 365 nm respectively for an λ_{ex} = 293 nm.

2.7. Phase diagram

The phase diagram was used to detect the potential existence of intermediate conformations of the enzyme during the inactivation process. The following equation was used for the phase diagram method:

$$I_{365} = a + b\ I_{320} \tag{4}$$

where I_{365} and I_{320} are the fluorescence intensities at wavelengths 365 and 325 nm when using λ_{ex} = 293 nm, under different fluences; and a and b are the intercept and the slope respectively of the I_{365} versus I_{320} plot (Kuznetsova, Turoverov, & Uversky, 2004).

2.8. Data analysis

Data was processed using Microsoft Excel 2016. Statistical analysis was carried out by IBM Statgraphics 24 using one-way ANOVA and Tukey test with P = 0.05. Results are the mean ± standard deviation of three experiments carried out in different days.

3. Results and discussion

3.1. Inactivation kinetics

The first aim of this work was to test the capability of PL to inactivate PG. Results show (Fig. 1) that PL indeed inactivates PG. The inactivation is higher than 90% after application of 128 J/cm^2 and follows a first-order kinetic with k = −0.0426 cm^2/J (R^2 = 0.9959) under our experimental conditions. The inactivation seems to be purely a photochemical process since no significant temperature rise was recorded (< 3 °C), therefore, no photothermal effects can be expected.