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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.

Keywords: pulsed light; polygalacturonase; enzyme inactivation; enzyme structure; fluorescence spectroscopy, free sulfhydryl.

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 (Simmons et al., 2012; Müller et al., 2015). 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 (Manzocco et al., 2013a; Janve et al., 2014), it was hypothesized that pulsed light is able to inactivate PG and that the 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 ºC for 10 min. Two mL of 0.1M 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 ºC. 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:

$$\ln \frac{A_F}{A_0} = -kF \qquad (1)$$

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, & 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, & Takai, 1974):

$$\frac{\mu M\ SH}{g} = \frac{73.53\ A_{412}\ D}{C} \qquad (2)$$

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 $10^6/(1.36\ 10^4)$. 10^6 is a conversion factor and $1.36\ 10^4$/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 ºC. 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):

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

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:

$$I_{365} = a + b\ I_{320} \qquad (4)$$

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 (figure 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 ºC), therefore, no photothermal effects can be expected.

Quantitative comparisons of results in PL technology are precluded by the lack of a standardized method for fluence measurement (Gómez-López & Bolton, 2016). However, data coming from the same experimental set up can be compared. We have used the same experimental set up to study the inactivation of POD and PPO. POD inactivation by PL follows a first-order kinetic (k=0.0255 cm^2/J, [enzyme]=0.25-1.25 µg/mL) (Pellicer & Gómez-López, 2017), while PPO inactivation data was best fitted to the Weibull model (Pellicer et al., 2018), yet, the fit to first-order kinetic (k=0.0175 cm^2/J,

[enzyme]=31.5 μg/mL) was also excellent (R^2=0.9925). The comparison of results for these three enzymes indicates that PG is the most PL labile enzyme among them. The only perturbing factor for this comparison, a higher opacity of the treated samples, can be ruled out since PG was by far the most concentrated enzyme in these tests.

There are few studies on the inactivation of purified PG. Fachin. Smout, Ly Nguyen, Van Loey, & Hendrickx (2004) isolated two PG isoenzymes from ripe tomatoes; the inactivation of the heat labile form of the enzyme under heat alone or under combined pressure-temperature inactivation followed first-order inactivation kinetics, as well as the thermal or high-pressure inactivation of PG from a crude avocado extract (Bermejo-Prada et al., 2014); while the inactivation of PG by ultrasound followed a biphasic model (Ma et al., 2015), likely due to the existence of isoenzymes. In foods in general, however, the inactivation of PG by different technologies follows a first-order kinetic, as it has been reported for tomato juice subjected to ohmic heating (Makroo, Rastogi, & Srivastava, 2017), and for beetroot juice during high pressure carbon dioxide treatment (Marszałek, Krzyżanowska, Woźniak, & Skąpska, 2017).

3.2. Free sulfhydryl content

Once the capability of PL to inactivate PG was proved, some studies were conducted in order to gain insight into the structural changes caused by PL to this enzyme, which may help to explain why the loss of activity observed. The most important bond that stabilizes enzyme structure is the disulfide bond, between cysteine residues, because it is covalent. When these bonds get broken, the –SH groups of cysteine residues become free. PG has four disulfide bridges (van Santen et al., 1999). These are very important for the enzyme structure; two of them ensure the "capping" of the core of the β-helix at the N- and C-terminal regions while another connects two adjacent β-helical turns in the middle of the active site cleft (van Santen et al., 1999). The evolution of free sulfhydryl content of PG

during PL treatment is shown in figure 2. A statistically significant ($P<0.05$) increase in free sulfhydryl's in PG was observed with the progress of the PL treatment, leading to a 40 % increase at the end of the treatment in comparison with the native enzyme. The rupture of disulfide bridges destabilizes protein structure and can lead to the unfolding of the enzyme. The reduction of disulfide bridges by PL can proceed by direct absorption of UV photons by cystine or indirectly upon excitation of tryptophan and tyrosine side chains. In the first, even though cystine has a lower extinction coefficient than tryptophan and tyrosine, its photolysis has a high quantum yield (Creed, 1984). In the latter, the photoionization of those aminoacids can generate solvated electrons that can be captured by cystines. The resultant thiol groups can react with other thiol groups creating new disulfide bonds (Neves-Petersen, Petersen, & Gajula, 2012). A higher free sulfhydryl content after PL treatment has also been reported in egg white protein (Manzocco et al., 2013b), gluten (Panozzo, Manzocco, Lippe, & Nicoli, 2016) and whey protein isolate (Siddique et al., 2016, 2017). However, in the case of egg white, the increase of free sulfhydryl content was followed by a decrease, likely due to formation of new S-S bonds from the new generated free thiol groups (Manzocco et al., 2013b). Our results show no evidence of formation of new disulfide bridges. The formation of *de novo* disulfide bonds can have important consequences on the protein structure. If they occur between different molecules can give place to new polymeric forms highly stabilized by disulfide bonds. This phenomenon has been reported as consequence of applying PL to β-lactoglobulin (Elmnasser et al., 2008).

3.3. Steady-state tryptophan fluorescence

The aminoacids that have intrinsic fluorescence properties are tryptophan, tyrosine and phenylalanine. The use of an internal fluorescence probe has the advantage of avoiding potential structural changes that can be caused by external probes (Ioniţă, Stănciuc,

Aprodu, Râpeanu, & Bahrim, 2014a). The wavelength selected in these determinations excites mainly tryptophan. Tryptophan fluorescence is the preferred internal probe in fluorescence spectroscopy of proteins because of its abundance, high absorptivity and high quantum yield, which allows an easy fluorescence detection (Ghisaidoobe & Chung, 2014). Additionally, tryptophan fluorescence is highly sensitive to the local environment, allowing an easy detection of protein unfolding.

A decrease in the intrinsic fluorescence intensity of the PG with the progress of the treatment was observed (figure 3). This implies a loss of tertiary structure upon PL treatment. It has been reported that tryptophan residues in PG are in a hydrophobic environment (Singh and Rao, 2002). The fluorescence of the native PG is high because the tryptophan residues are in a hydrophobic environment, where its quantum yield is high (Ioniţă, Aprodu, Stănciuc, Râpeanu, & Bahrim, 2014b). The decrease in fluorescence with the progress of the PL treatment is consistent with the progressive unfolding of the enzyme, which exposes tryptophan residues to a hydrophilic environment where their quantum yield is lower. A similar result has been found for the inactivation of PG by ultrasound (Ma et al., 2015) and by changing the pH from its optimum pH (4.3) to 7.0 (Jyothi, Singh, & Appu Rao, 2005). A reduction of fluorescence intensity upon PL treatment has also been reported for β-lactoglobulin, sodium caseinate, α-lactalbumin (Elmnasser et al., 2008), and PPO (Pellicer et al., 2018).

3.4. Parameter A

The position of the spectral maximum is together with changes in intrinsic fluorescence intensity one of the indicators of protein unfolding. Spectral shifts are sometimes small and difficult to detect. The spectral parameter A is also a characteristic of the fluorescence spectrum and it is easier to obtain than is the position of the spectral maximum, it can detect shifts of less than 1 nm easily (Turoverov et al., 1976). The parameter A of PG

during PL treatment (figure 4) decreases during the course of PL treatment, confirming the unfolding of the protein. The variation of parameter A with fluence is linear, which is not common in enzyme inactivation processes and may reveal a relatively simple inactivation mechanism. The non-linearity of the change in parameter A is an index used to detect the existence of intermediate states in multi-step inactivation processes (Kuznetsova et al., 2002). For example, the parameter A for the heat inactivation of pectin methylesterase (Nistor, Stănciuc, Aprodu, & Botez, 2014) and PPO (Ioniţă et al., 2014b) has several peaks between 25 and 70-80 ºC; while the parameter A for the effect of pH in PPO increases parabolically (Ioniţă et al., 2014a). Results found for parameter A in this study are highly correlated with the first-order inactivation data (R^2=0.99). This high correlation is not common and may reflect a simple inactivation mechanism. Pellicer et al. (2018) observed a high correlation between the parameter A and residual activity in PPO inactivated by PL, but not with the first-order inactivation data, which was related to the fact that PPO followed a Weibull inactivation model.

3.5. Phase diagram

The phase diagram is a method used to discriminate if the structural changes of a protein subjected to a denaturing treatment is an all-or-none process or a multi-step process. Each straight line of the phase diagram represents one all-or-none transition which edges are populated by different states of the protein. When only one straight line is present there is a single process with corresponding native and inactive proteins; while the presence of different lines represents multiple steps of an inactivation process with different intermediaries (Kuznetsova et al., 2004). Figure 5 shows the phase diagram of the inactivation of PG by PL. It can be observed that the points follow a linear decrease, which indicates that the inactivation is an all-or-none process. This has been reported before for the PL inactivation of enzymes (Pellicer & Gómez-López, 2017; Pellicer et al.

2018) and could be a general mechanism, although more research with other enzymes is advised for confirming this possibility. The simplicity of the process is in harmony with our results found for the parameter A, but it has not been frequently reported for food-related proteins. For example, the thermal treatment of α-lactalbumin at pH's 4.5 and 7.0 (Stănciuc, Râpeanu, Bahrim, & Aprodu, 2012a), β-lactoglobulin (Stănciuc, Aprodu, Râpeanu, & Bahrim, 2012b), PPO (Ioniţă et al., 2014b) and pectin methylesterase (Nistor et al., 2014) have been described as a multi-step process according to phase diagram analysis. Regarding specifically the effects of PL on proteins and according to results from analytical techniques different to the phase diagram, a two-step process has been suggested for the aggregation process of egg white protein where low-fluences promote the rupture of disulfide bonds, and *de novo* intermolecular disulfide bonds are formed after further pulsing (Manzocco et al., 2013b). As commented before, this mechanism is related to an increase of free sulfhydryls at low fluences and a decrease at higher fluences; while our results (figure 1) do not show any decrease, which is consistent with the idea of an only-one step process for the inactivation of PG by PL. The effects of PL on hydrated gluten have been described to occur *via* multiple pathways, including the partial depolymerization of the oligomeric fractions and the unfolding of the monomeric fractions, followed by the formation of fractions of a wide range of molecular weights (Panozzo et al., 2016).

4. Conclusion

In the present study, the inactivation of PG by PL was assayed and some associated structural changes were determined. It was proved that PL is capable to inactivate PG and that the inactivation follows a first-order kinetic. The inactivation is an all-or-none process where disulfide bridges are broken and the enzyme unfolds.

Conflict of interest

The authors have declared no conflict of interest.

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FIGURE CAPTIONS

Figure 1. Inactivation curve of polygalacturonase by pulsed light. Error bars are standard deviation.

Figure 2. Change of free sulfhydryl content of PG during PL treatment. $^{a-c}$: bars with different superscripts are statistically different ($P<0.05$). Error bars are standard deviation.

Figure 3. Intrinsic fluorescence change in polygalacturonase treated with pulsed light at an excitation wavelength of 293 nm and an emission wavelength (λ_{em}) range of 300-450 nm.

Figure 4. Parameter A evolution during the inactivation of PG by PL treatment. Error bars are standard deviation.

Figure 5. Phase diagram of the inactivation of PG by PL.

Figure 1

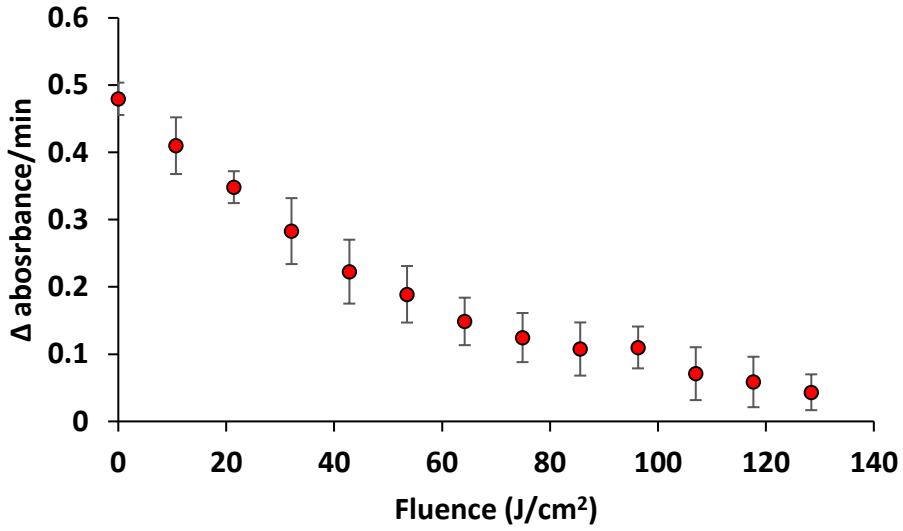


Figure 2

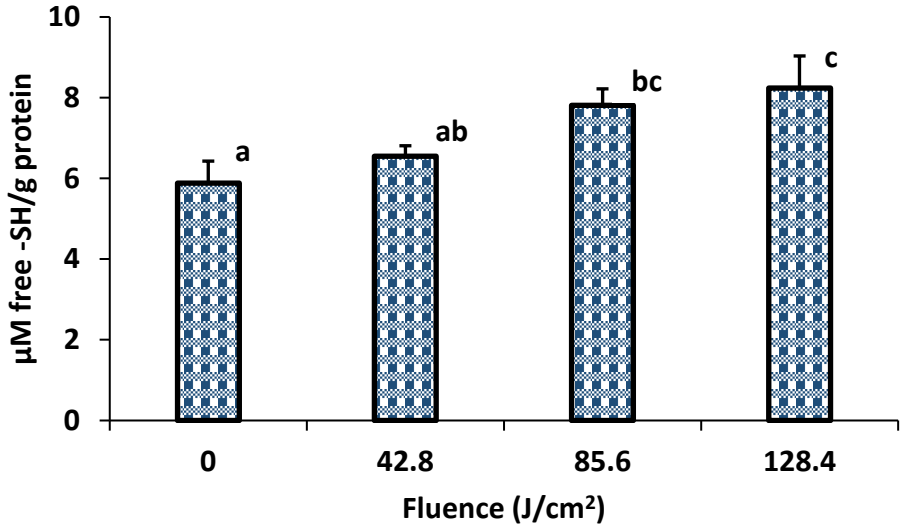


Figure 3

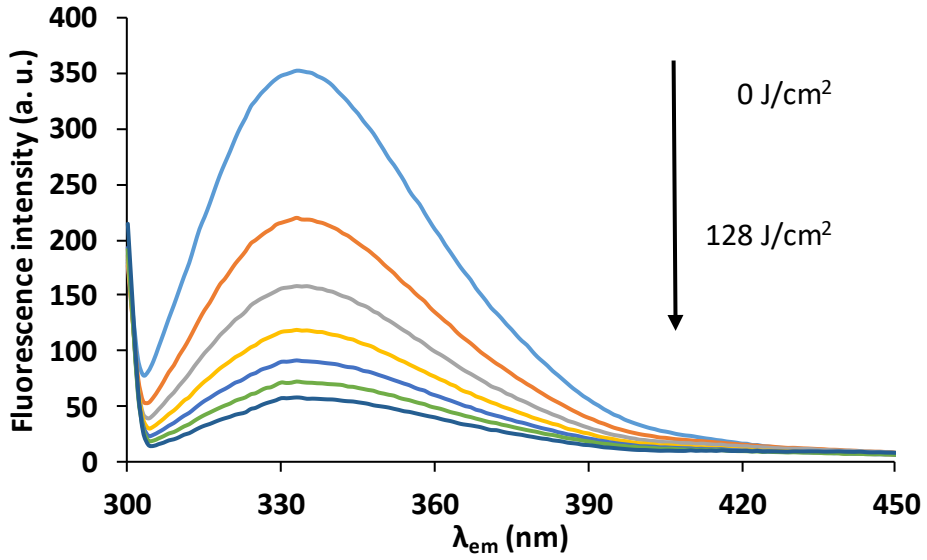


Figure 4

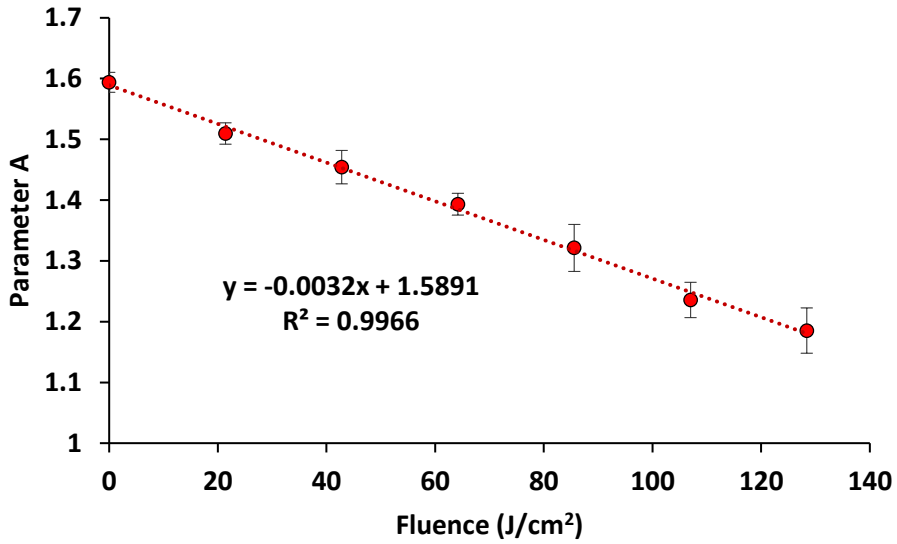
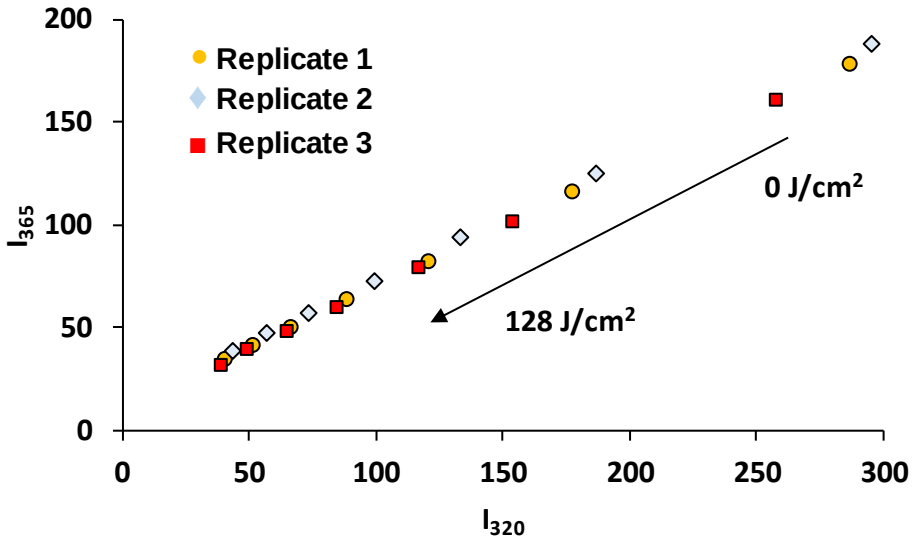


Figure 5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