Inactivation of Polyphenoloxidase by Pulsed Light

Lara Manzocco, Agnese Panozzo, and Maria Cristina Nicoli

Abstract: The effect of pulsed light on the inactivation of polyphenoloxidase (PPO) in model solutions was investigated focusing on the effect of enzyme concentration and total energy dose of the treatment. PPO inactivation increased with the dose of the treatment. Complete enzyme inactivation was achieved by pulsed light doses higher than 8.75 J cm^{-2}. At low PPO concentrations (4 to 10 U), the enzyme resulted highly inactivated by pulsed light treatment. Further increase in enzyme units determined a progressive decrease in PPO inactivation. The latter was attributed to protein structural modifications including cleavage and unfolding/aggregation phenomena. PPO amounts higher than 10 U probably favoured enzyme conformations that were less prone to intermolecular rearrangements leading to inactivation.

Keywords: cleavage, polyphenoloxidase, pulsed UV-light, structure, unfolding/aggregation

Practical Application: Pulsed light is very effective in promoting polyphenoloxidase inactivation in model systems. Pulsed light has good potential of successfully application to achieve food enzymatic stability under nonthermal conditions.

Introduction

Pulsed light technology is a powerful germicidal method that has raised a large attention in the last years. It is based on the exploitation of short-duration and high-power pulses of radiation with a spectrum from 100 to 1100 nm, including ultraviolet (UV), visible (VIS), and infrared (IR) light (Oms-Oliu and others 2010a; Falguera and others 2011). Nowadays, pulsed light is mainly used for sterilization purposes of package surfaces. It has also been proposed to decontaminate the surface of shell eggs, fresh-cut vegetables, and ready-to-eat meat products as well as for killing microorganisms in liquids, such as milk, fruit juices, and infant food (Marquenie and others 2003; Hierro and others 2009; Choi and others 2010; Caminiti and others 2011; Hierro and others 2011; Pataro and others 2011; Ramos-Villarroel and others 2011a).

The antimicrobial effects of pulsed light are well known to be primarily mediated by its UV light component. The latter is absorbed by highly conjugated double-bond systems in biomolecules, leading to their chemical modification. Enzymes are among the major targets for photoinduced modifications due to the abundance of endogenous chromophores within their structure. Both amino acid side-chains (for example, thriptophan, tyrosine, phenylalanine, cysteine) and bound prosthetic groups (for example, flavins, heme) may act as efficient chromophores. Enzymatic proteins have the additional ability to bind exogenous chromophores, and rapidly react with other excited state species (Davies and Truscott 2001). The result would thus be a modification in the protein properties due to side-chain oxidation, backbone fragmentation, and/or formation of cross-links and aggregates (Davies 2003).

Polyphenoloxidase (PPO) is one of the most important enzymes involved in food spoilage. It is well known to cause browning in cut fruits and vegetables leading to less attractive appearance and loss in nutritional quality. Although food scientists have largely investigated the possibility to exploit pulsed light to inactivate PPO in fruits and vegetables, still contradictory results are available in the literature. In 1989, Dunn and others (Dunn and others 1989) suggested that pulsed light could inhibit PPO in potato slices and alkaline phosphatase in model systems. More recently, Oms-Oliu and others (2010b) showed that pulsed light promoted an increase in PPO activity in fresh-cut mushrooms. On the other hand, Ramos-Villarroel and others (2011b) observed that pulsed light, combined with antibrowning agents, can prevent enzymatic browning in fresh-cut avocado. Whilst, Charles and others (2013) demonstrated the increase in PPO activity in fresh-cut mango submitted to pulsed light treatment.

Based on these uneven evidences, further research is needed to understand the effect of pulsed light on food enzymes. To this regard, investigations on model systems could provide useful insights into the effects of pulsed light on activity and structure of enzymes.

On the basis of these considerations, the aim of the present research was to study the effect of pulsed light on enzyme activity and structure. PPO was chosen as a typical food enzyme responsible for browning of plant derivatives. To this purpose, an aqueous model system containing increasing units of PPO, simulating actual activity in real foods, was exposed to pulsed light with increasing fluence. The effect of pulsed light was assessed by evaluating enzymatic activity and molecular changes by high-performance liquid chromatography (HPLC) gel-permeation.

Materials and Methods

Polyphenoloxidase solution

Mushroom tyrosinase (PPO) (3933 U/mg, Sigma, St. Louis, Mo., U.S.A.) was used. PPO solutions were prepared diluting increasing amounts of enzyme in 0.1 mol L^{-1} potassium phosphate buffer pH 7 (Sigma). The initial activities of the different PPO solutions on $1.5 \cdot 10^{-3}$ mol L^{-1} L-Dopa (Sigma) in 0.1 mol L^{-1} potassium phosphate buffer pH 7 were 0.0040, 0.0065, 0.01, 0.015, 0.026, 0.046, and 2 Abs min^{-1}.

Aliquots of 100 μL enzyme solutions were introduced into transparent 2 × 3 cm plastic pouches (polycoupled Combiflex PA/PE 090, 20/70, Savonitti, Codroipo, Italy) allowing 80% of

MS 20130400 Submitted 3/20/2013, Accepted 6/5/2013. *Authors are with Dipartimento di Scienze degli Alimenti, Univ. di Udine, via Sondrio 2/A, 33100 Udine, Italy. Direct inquiries to author Manzocco (E-mail: lara.manzocco@uniud.it).*

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the UV radiation to reach the sample, as declared by the producer. The pouches were hermetically sealed (Easy Packer EP400, Orved, VM-16, Musile di Piave, Italy) and submitted to pulsed light treatment.

Pulsed light treatments

Pulsed light treatments were carried out at room temperature by using a pulsed light mobile decontamination unit (Claranor, Rouaine, France) equipped with 4 xenon lamps with emission in the range 200 to 1000 nm (200 to 400 nm: 41%; 400 to 700 nm: 51%; 700 to 1000 nm: 8%). Lamps were positioned at each side of a quartz plaque held in the centre of the cube shaped chamber. PPO solutions packed in plastic pouches as previously described were placed on the quartz plaque. According to the manufacturer's instructions, lamps were set at distances allowing the sample to be exposed to increasing light fluence from 0 to 1.75 J cm^{-2} $pulse^{-1}$. Exposure to higher fluence from 5.25 to 15.75 J cm^{-2} was obtained by delivering to the sample increasing number of pulses (0 to 9), each having a fluence of 1.75 J cm^{-2}. Pulse duration was 50 μs and repetition rate was 0.5 Hz.

Temperature measurements

Sample temperature was measured by a copper-constantan thermocouple probe (Ellab, Denmark) connected to a portable data logger (mod. 502A1, Tersid, Milano, Italy). Measurements were performed within 10 s from the end of the pulsed light treatment.

Polyphenoloxidase activity

The PPO activity was assayed spectrophotometrically (Shimadzu UV-2501PC, Uv-Vis recording spectrophotometer, Shimadzu Corporation, Kyoto, Japan) at 25 °C according to the methodology of Kahn (1985), based on the absorption at 420 nm of the brown polymers formed when L-Dopa is oxidized in the presence of PPO. The reaction was started by the addition of

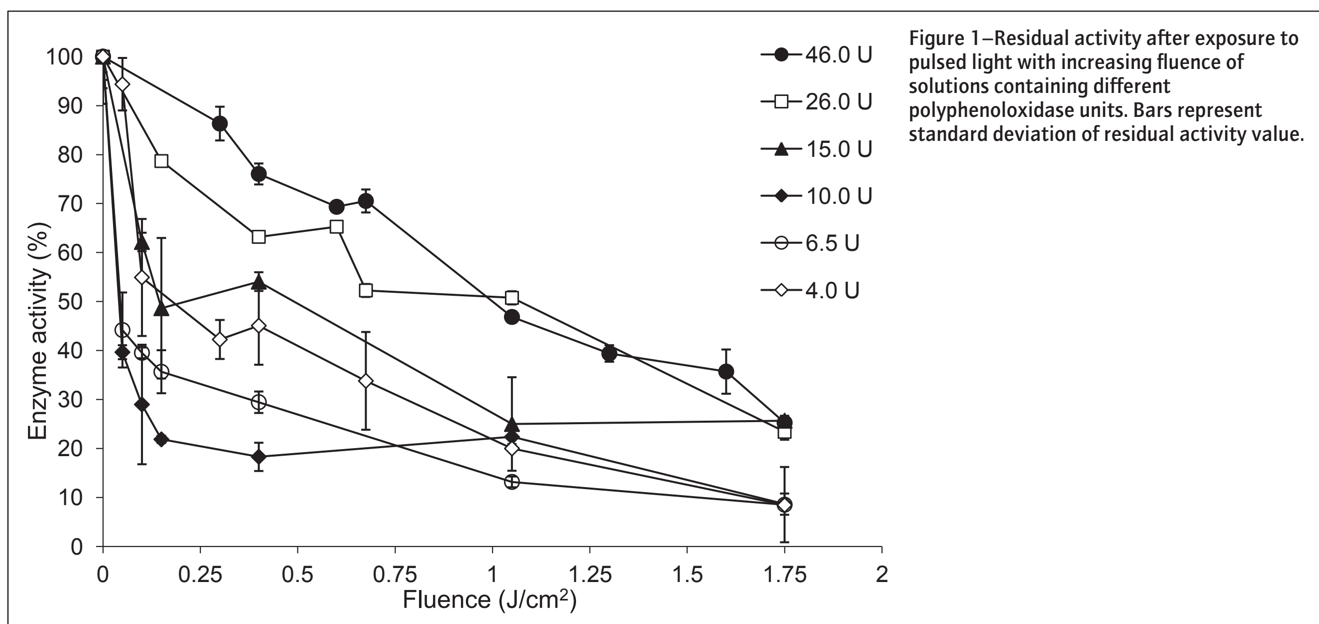

Figure 1–Residual activity after exposure to pulsed light with increasing fluence of solutions containing different polyphenoloxidase units. Bars represent standard deviation of residual activity value.

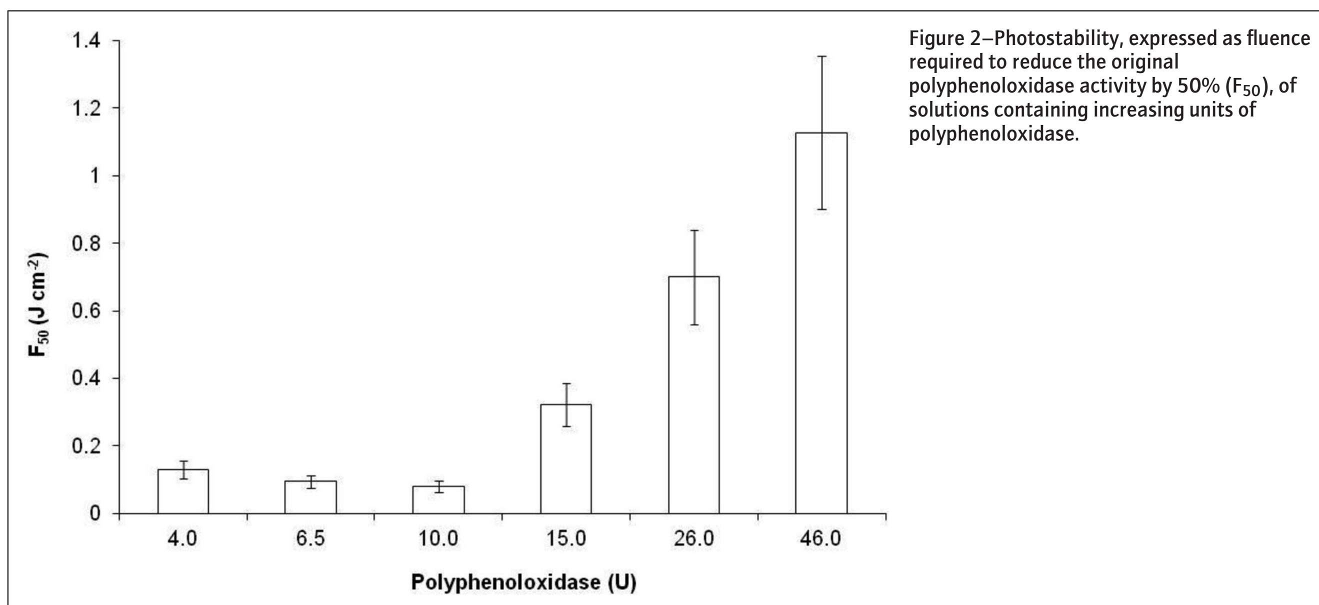

Figure 2–Photostability, expressed as fluence required to reduce the original polyphenoloxidase activity by 50% (F_{50}), of solutions containing increasing units of polyphenoloxidase.

Table 1–Residual activity (±SD) after exposure to increasing number of light pulses with increasing fluence of solutions containing 4 or 30 U of polyphenoloxidase.

Pulse	Fluence pulse^{-1} (J cm^{-2} Pulse^{-1})	Fluence (J cm^{-2})	Activity (%) 4.0 U	Activity (%) 30.0 U
0	0	0.00	100 ± 1.4	100 ± 3.2
1	0.40	0.40	45.1 ± 4.0	63.2 ± 0.8
1	1.75	1.75	7.6 ± 0.6	23.4 ± 1.6
3	1.75	5.25	2.5 ± 2.1	8.2 ± 0.9
5	1.75	8.75	n.d.	n.d.
7	1.75	12.25	n.d.	n.d.
9	1.75	15.75	n.d.	n.d.

n.d. = Not detectable.
$n = 5$.

20 μL of PPO solution to 2 mL of medium containing 0.1 mol L^{-1} potassium phosphate buffer pH 7 and 1.5·10^{-3} mol L^{-1} L-Dopa as substrate. The absorbance at 420 nm was monitored each minute for 15 min. The changes in absorbance per minute were calculated by linear regression applying the pseudo 0 order kinetic model. The eventual final stationary phase was excluded from regression data. One unit of PPO was defined as the amount of enzyme that produces an increase in absorbance at 420 nm of 0.001 per minute under the testing conditions. PPO activity was calculated as the percentage ratio between enzymatic activity of the pulsed light treated solution and of the untreated one.

Linear regression of enzymatic activity as a function of pulsed light fluence was also performed considering the linear part of the curve. The photostability of PPO was calculated as the fluence required to reduce the original PPO activity by 50% (F_{50}) and expressed as J cm^{-2}.

HPLC-gel permeation analysis

HPLC-gel permeation analysis was performed in duplicate by a HPLC system Jasco (model 880–02, Japan Spectroscopic Co., Tokyo, Japan) equipped with a UV/VIS detector. Total of 2 columns were used: BioSep-SEC-S 3000, 30 cm length, 7.80 mm internal diameter and BioSep-SEC-S 2000 30 cm length, 7.80 mm internal diameter, 5 μm granulometry, 125 Å porosity, with separation range among 5 and 150 kDa. Injection volume was 20 μL and the mobile phase, delivered at a flow rate of 0.6 mL min^{-1}, was 0.1 mol L^{-1} potassium phosphate buffer pH 7 in isocratic conditions. The detection wavelength was 220 nm. Bovine Serum Albumin (67 kDa), α-Lactalbumin (15 kDa), and β-Lactoglobulin (18 kDa) from Sigma were used as calibration standards. Peaks integration was performed by CHROM-CARD for Windows software (1.19 version).

Data analysis

At least 5 measurement of enzymatic activity were performed on each sample. Coefficients of variations, expressed as percentage ratio between standard deviation and mean value were lower than 9. HPLC gel permeation analyses were carried out in duplicate. Linear regression analysis was performed using Microsoft Office Excel 2003. Goodness-of-fit was evaluated by means of the determination coefficients (R^2) and the corresponding P-values.

Results and Discussion

To evaluate the effect of pulsed light on PPO activity, aqueous solutions containing increasing units of enzyme (from 4 to 46 U) were prepared and exposed to one pulse of light with different fluence at room temperature (22.1 ± 0.4 °C). PPO activity was assessed by regression analysis of changes in absorbance at 420 nm as a function of time ($R^2 > 0.98$; $P < 0.05$). The temperature of the samples was measured just after the treatment and did not exceed 23.4 ± 0.7 °C. Based on this evidence, PPO inactivation should be mainly attributed to the effect of light exposure.

As expected, the activity of PPO progressively decreased as the fluence of the light pulse increased (Figure 1). The effect of pulsed light on PPO activity strongly depended on the initial amount of the enzyme in the solution. For instance, the activity of the solution containing 10 U of PPO quickly decreased by exposure to low fluence pulses, and reached an almost constant value on further increase in the pulse fluence. By contrast, the solutions containing 26 or 46 U of PPO showed a progressive activity decrease according to a straight line in the entire tested fluence range.

To evidence the relation between the inactivation effect of pulsed light and the amount of enzyme in the aqueous solution, the photostability of PPO was calculated. To this aim, regression analysis of enzymatic activity data reported in Figure 1 was performed as a function of pulsed light fluence considering data relevant to the linear part of the curve ($R^2 > 0.70$; $P < 0.1$). In particular, linear regression was performed in different fluence ranges: (1) 0 to 1.75 J cm^{-2} for the sample containing 46 U; (2) 0 to 1.60 J cm^{-2} for the sample containing 26 U; (3) 0 to 0.15 J cm^{-2} for the sample containing 6.5 to 15 U; (4) 0 to 0.10 J cm^{-2} for the sample containing 4 U.

Regression coefficients data were used to calculate the photostability of PPO (F_{50}, J cm^{-2}). A low value of F_{50} indicates PPO to be easily denatured by pulsed light. By contrast, higher values of F_{50} account for intense resistance of the enzyme to pulsed light. Figure 2 shows the F_{50} values of PPO in aqueous solutions containing increasing units of enzyme.

PPO solutions containing 4, 6.5, and 10 U of enzyme resulted particularly sensitive to pulsed light. In other words, these samples lost 50% of their activity upon exposure to a light fluence approaching 0.1 J cm^{-2}. On the contrary, samples containing higher (15, 26, or 46 U) amounts of enzyme presented higher photostability. In fact, a fluence higher than 0.30 J cm^{-2} was required to produce a 50% decrease in PPO activity. The dependence of PPO photostability on the amount of the enzyme in the solution is in accordance with previous literature data showing that protein concentration can strongly affect their photosensitivity (Manzocco and Nicoli 2012). For instance, no photodegradation of egg white was reported in systems having concentration higher than the critical concentration C^*, which accounts for the quantity of proteins that can be accommodated in a given volume of solution without mutual perturbation (Lapasin and Pricl, 1995). Beyond this specific concentration, the system can be considered crowded. According to the literature (Minton and others 1982; van den Berg and others 1999, 2000; Ellis and Minton 2006), crowding of proteins in water solutions could control protein structure modifications by accelerating their folding and aggregation. It is, thus, possible that macromolecular crowding could favor specific protein conformations, potentially modifying their sensitivity to structural modifications caused by physical stresses. Based on these considerations, it can be inferred that self crowding of PPO, and thus protein vicinity in the aqueous solution, could increase their photostability. However, it is not excluded that in crowded conditions, shadowing effects could play a critical role on protein photosensitivity. In fact, the effect of light is known to be reduced by anything between target object and light source. For instance, microorganisms can be protected from the germicidal effect of light

Table 2–Peak areas (±SD) relevant to HPLC-gel permeation analysis of a 4 U polyphenoloxidase solution as a function of the pulsed light fluence.

Fluence ($J\ cm^{-2}$)	Peak area (arbitrary units × 1000)						
	Peak 1 (tr = 16.8)	Peak 2 (tr = 23.7)	Peak 3 (tr = 26.4)	Peak 4 (tr = 27.9)	Peak 5 (tr = 32.4)	Peak 6 (tr = 34.2)	Peak 7 (tr = 36.6)
0.00	2.6 ± 0.4	32.3 ± 3.1	24.5 ± 4.1	8.9 ± 1.1	19.5 ± 2.8	17.1 ± 2.1	8.1 ± 0.3
0.40	2.2 ± 0.9	33.8 ± 1.4	23.5 ± 0.6	5.7 ± 1.0	19.1 ± 3.2	19.6 ± 2.3	9.7 ± 0.6
1.75	2.3 ± 0.7	28.8 ± 0.8	11.6 ± 3.4	3.0 ± 1.6	20.3 ± 2.2	19.8 ± 1.5	11.2 ± 1.1
5.25	5.0 ± 1.3	22.3 ± 1.1	9.1 ± 1.7	2.7 ± 1.2	22.1 ± 2.7	22.9 ± 2.0	11.7 ± 0.9
8.75	5.6 ± 0.9	14.1 ± 3.7	6.1 ± 1.3	0.4 ± 0.1	31.2 ± 3.8	34.4 ± 2.7	18.3 ± 1.6
12.25	2.2 ± 0.8	8.0 ± 3.0	4.1 ± 1.6	0.1 ± 0.1	29.3 ± 1.4	35.5 ± 3.1	15.8 ± 1.5
15.75	n.d.	6.9 ± 1.7	4.6 ± 1.5	n.d.	20.9 ± 3.9	32.3 ± 2.4	13.8 ± 1.3

n.d. = Not detectable.
$n = 2$.

Table 3–Peak areas (±SD) relevant to HPLC-gel permeation analysis of a 30 U polyphenoloxidase solution as a function of the pulsed light fluence.

Fluence ($J\ cm^{-2}$)	Peak area (arbitrary units × 1000)						
	Peak 1 (tr = 16.8)	Peak 2 (tr = 23.7)	Peak 3 (tr = 26.4)	Peak 4 (tr = 27.9)	Peak 5 (tr = 32.4)	Peak 6 (tr = 34.2)	Peak 7 (tr = 36.6)
0.00	31.6 ± 1.8	304.1 ± 6.6	404.4 ± 1.5	41.6 ± 1.5	12.2 ± 0.2	10.3 ± 1.8	n.d.
0.40	30.3 ± 3.3	303.5 ± 10.4	392.8 ± 9.2	46.9 ± 3.5	16.8 ± 1.3	10.2 ± 0.2	n.d.
1.75	34.7 ± 3.8	261.4 ± 3.8	116.0 ± 5.1	37.4 ± 0.8	14.4 ± 1.1	11.8 ± 0.5	2.4 ± 0.9
5.25	30.6 ± 2.7	201.0 ± 2.2	58.4 ± 2.6	21.3 ± 0.3	12.3 ± 0.2	16.9 ± 2.8	4.6 ± 0.9
8.75	24.2 ± 1.9	155.2 ± 3.4	56.3 ± 1.3	15.9 ± 0.3	10.2 ± 07	17.4 ± 0.2	4.7 ± 1.8
12.25	24.2 ± 2.3	84.1 ± 2.9	57.0 ± 2.1	13.8 ± 0.5	17.6 ± 1.1	34.2 ± 0.5	9.9 ± 0.1
15.75	27.8 ± 2.6	45.4 ± 0.7	54.7 ± 2.4	7.9 ± 0.6	17.7 ± 1.3	34.4 ± 1.5	10.1 ± 0.1

n.d. = Not detectable.
$n = 2$.

by other cells (Cudemos and others 2013). Analogously, proteins could protect each other from light according to local shadowing effects. In this way, molecules placed at the surface of the sample would represent a physical screen for the internal ones, preventing their photoreaction.

To better understand the effect of pulsed light on the structure of PPO, the attention was focused on aqueous solutions containing considerably different amounts of enzyme (4 or 30 U). The solutions were exposed to pulsed light with increasing fluence up to 15.75 J cm^{-2}. Such intense fluencies were chosen to achieve complete enzyme inactivation. Table 1 confirms that PPO activity decreased with the increase in pulsed light fluence. In addition, no residual activity was detected in samples exposed to fluence equal or higher than 8.75 J cm^{-2}. Upon these intense treatments, a slight increase in temperature was detected. However, sample temperature never exceeded 36 ± 0.7 °C, suggesting minor effects of temperature increase on PPO inactivation. Samples showing no residual activity were also stored for 1 wk at 4 °C. Data not shown indicate that the enzyme was unable to recover its activity. These results confirm that intense pulsed light treatments can irreversibly and completely inactivate PPO.

Table 2 shows the evolution of the areas of the peaks detected in the chromatograms of the aqueous solutions containing 4 U of enzyme as a function of the light fluence received during the pulsed light treatment. Total of 7 peaks (peak 1, 2, 3, 4, 5, 6, and 7), having retention times 16.8, 23.7, 26.4, 27.9, 32.4, 34.2, and 36.6 min, were observed. Apparent molecular weights of proteins eluted in peaks 2, 3, and 4 were recognized as the tetrameric, dimeric, and monomeric forms of the quaternary structure of PPO, which have molecular weights of 130, 65, and 32.5 kDa, respectively (Whitaker, 1995). It can be noted that the areas of peaks 2, 3, and 4 progressively decreased with the increase in the pulsed light fluence. The decrease in these active proteins accounts for the loss of enzymatic activity upon pulsed light treatment (Table 1). As shown in Table 2, exposure of PPO to pulsed light also resulted in the modification of the area of peak 1. Such peak was associated to a molecule, reasonably without PPO activity, whose short retention time could be attributed to protein unfolding or aggregation. A certain amount of denatured protein was actually present also in the untreated solution, probably as a consequence of enzyme extraction operations. However, the presence of this protein fraction increased, reaching a maximum value in correspondence of the 8.75 J cm^{-2} treatment. Its further decrease was then observed so that peak 1 became not detectable in the 15.75 J cm^{-2} treated sample. A similar evolution, although with a maximum value at slightly different fluence, was also observed for the areas of peaks 5, 6, and 7. The latter are relevant to molecular species with longer retention time, and an estimated apparent molecular weight around 5 kDa. The peak areas of these protein fragments reached a maximum value in samples treated at about 10 J cm^{-2} fluence. Also in this case, a further increase in fluence promoted the degradation of the protein fragments eluted in these peaks.

It has been suggested that photodegradation of proteins proceeds through aggregation and cleavage phenomena. The former are the result of cross-linking reactions involving aminoacid residues. In fact, upon photo-oxidation, His can easily react with other aminoacid residues to give cross-links (Shen and others 2000). In addition, light would affect the structure of proteins via photoexcitation of Trp, Tyr, Phe, Met, and Cys groups inducing the reduction of disulfide (S-S) bonds (Davies and Truscott 2001). The breakage of disulfide bonds would directly disturb the protein structure causing a loss in its biologic activity. However, the new generated thiol groups could also react to form intermolecular

S-S bonds responsible for aggregation (Wu and others 2008). In addition, unstable free thiols could beget protein radicals that are potential progenitors of backbone fragmentation (Davies and Truscott 2001).

The results acquired by HPLC-gel permeation analysis (Table 2) confirm that pulsed light promotes the modification of the structure of proteins with PPO activity by inducing both unfolding/aggregation phenomena and protein backbone cleavage. However, denatured proteins (peak 1) as well as their fragments (peaks 5, 6 and 7) can further react upon pulsed light exposure. In other words, high fluence treatments could lead to the degradation of the unfolded and cleaved proteins produced at lower fluences.

Protein unfolding and aggregation have been previously reported for PPO exposed to continuous UV light (Manzocco and others 2009). However, in that case, no degradation of the newly formed large protein was detected. It is thus possible that pulsed light could promote PPO degradation *via* reaction pathways other than those characterizing UV treatments. Conversely, it is also possible that similar mechanisms are involved but light spectra and fluence strongly affect the overall extent of protein degradation.

Additional HPLC-gel permeation analysis were also performed on the aqueous solutions containing 30 U of enzyme and exposed to pulsed light (Table 3).

By comparing the peak areas of the untreated samples (0 J cm^{-2}) containing 30 (Table 3) or 4 (Table 2) U of PPO, a marked difference in the relative abundance of the protein eluted in the different peaks can be observed. In fact, while the tetrameric structure of PPO (peak 2) was the most abundant in the 4 U sample, the dimeric one (peak 3) was favored in the 30 U sample. The latter, despite its almost 10-fold higher concentration, also showed a lower amount of small proteins (peaks 5, 6, and 7) than that observed in the solution containing 4 U of enzyme (Table 2). This result supports the hypothesis that self crowding of proteins promotes specific conformational structures, which are reasonably those allowing the excluded volume to be reduced. In other words, crowding conditions favor compact protein structures, while hindering dissociation phenomena. In the crowded environment of the 30 U samples, photodegradation of aggregates eluted in peak 1 was limited and protein fragments moderately increased upon light exposure. It can be inferred that, in this condition, polyphenoloxidase photodegradation mainly occurred through intramolecular reactions with minor contribution of cross-linking among vicinal proteins. However, as stated before, shadowing effects of proteins in a crowded environment cannot be completely neglected.

Conclusions

Results acquired demonstrate that pulsed light allows the inactivation of PPO in model solution as a consequence of protein structure modifications. The PPO sensitivity to pulsed light strongly depended on the enzyme concentration. The vicinity of the enzyme molecules in the solution favors specific protein conformations. At concentrations up to 10 U, PPO would be easily inactivated because proteins could undergo both intramolecular modification and photoreaction with surrounding molecules. By contrast, at higher concentrations, protein vicinity could favor conformations having lower photosensitivity, probably because less prone to intermolecular rearrangements.

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