



# Effect of pulsed light on activity and structural changes of horseradish peroxidase



Bei Wang<sup>a,b</sup>, Yanyan Zhang<sup>a,d</sup>, Chandrasekar Venkitasamy<sup>b</sup>, Bengang Wu<sup>a</sup>, Zhongli Pan<sup>b,c,\*</sup>, Haile Ma<sup>a,\*</sup>

<sup>a</sup> School of Food and Biological Engineering, Jiangsu University, 301 Xuefu Road Zhenjiang, Jiangsu 212013, China

<sup>b</sup> Department of Biological and Agricultural Engineering, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA

<sup>c</sup> Healthy Processed Foods Research Unit, USDA-ARS-WRRC, 800 Buchanan Street, Albany, CA 94710, USA

<sup>d</sup> School of Food and Biological Engineering, Zhengzhou University of Light Industry, Dongfeng Road 5, Zhengzhou, Henan 450002, China

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## ABSTRACT

The objective of this research was to investigate the effects of pulsed light on the activity and structure of horseradish peroxidase in buffer solution. Enzyme residual activities were measured. Surface topography, secondary, and tertiary structures of horseradish peroxidase were determined using atomic force microscopy (AFM), Raman spectroscopy, and fluorescence spectroscopy, respectively. Results showed that a complete inactivation of horseradish peroxidase was achieved by application of 10 pulses of pulsed light treatment at an intensity of 500 J/pulse. The AFM analysis revealed that the aggregation of enzyme protein increased and surface roughness decreased with the increase in the treatment time. Fluorescence and Raman spectroscopy analysis exhibited that pulsed light destroyed the tertiary and secondary protein structures. The  $\beta$ -sheet composition was decreased while  $\beta$ -turn and random coils were increased. Pulsed light could effectively inactivate horseradish peroxidase by destroying the secondary and tertiary structures of protein in the active center of the enzyme.

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## 1. Introduction

Peroxidase (POD) is a group of enzymes which widely exist in fruits and vegetables. Presence of POD in foods is not desirable to food processing industries as it causes enzymatic browning of foods during processing, and unpleasant flavor and reduced storability or shelf-life of food products (Lopez et al., 1994). Therefore, the extent of POD inactivation is a major index in assessing the effectiveness of food processing and quality. Since enzymatic browning could result in a serious negative impact on food quality, preventing or inhibiting enzymatic browning during food processing and storage has become an important goal for high quality foods.

Heating of foods is a widely used method to inactivate enzymes. Although POD can easily be inactivated by heating, it could negatively affect the food properties such as flavor, color and nutritional values (Ercan & Soysal, 2011). Alternative methods to conventional heating, including vacuum degassing, pulsed electric field, ultra-

sound, and adding antioxidant or decolorization additives have been investigated in recent years to inactivate enzymes (Jang & Moon, 2011; Zhong, Hu, Zhao, Chen, & Liao, 2005). However, none of the above methods were entirely successful as some processes were very complex and expensive and others could not eliminate either browning adequately or caused loss of nutrients.

Pulsed light (PL) is a FDA-approved, “non-thermal” technology (FDA, 1996) with the potential to be used as an alternative method to inactivate POD. During PL irradiation, high intensity and broad spectrum light (100 to 1100 nm) is delivered in duration pulses. Photo-thermal and photo-chemical effects generated by the pulses were shown to inactivate microbes effectively (Wang et al., 2015). This new technique attracted the attention of researchers studying food decontamination due to its high disinfection efficiency, however, studies related to enzyme inactivation were rarely reported. Gómez-López, Ragaert, Debevere, and Devlieghere (2007) mentioned that PL could lower the enzymatic activity due to broad spectrum pulsed light. Dunn et al. (1989) claimed in their patent that the browning of potato slices due to enzymatic activity could be inhibited after 2 to 5 flashes of PL at a fluence of 3 J/cm<sup>2</sup>. The polyphenol oxidase extracted from the PL treated potato slices had less activity than that extracted from the untreated ones. They also reported that similar effects have been demonstrated for banana and apple slices. Luse and McLaren (1963) found that the

\* Corresponding authors at: Department of Biological and Agricultural Engineering, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA (Z. Pan).

E-mail addresses: [zhongli.pan@ars.usda.gov](mailto:zhongli.pan@ars.usda.gov), [zlpn@ucdavis.edu](mailto:zlpn@ucdavis.edu) (Z. Pan), [mhl@ujs.edu.cn](mailto:mhl@ujs.edu.cn) (H. Ma).

enzymes, including chymotrypsin, lysozyme, ribonuclease and trypsin, could be inactivated at wavelength of 253.7 nm, because the light was absorbed by a particular amino acid residue and caused the rupture of the chemical bonds. Hollósy (2002) explained that ultraviolet (UV) was the main reason that led to the enzyme inactivation, because the protein had a strong absorption in the waveband of UV. When the conventional UV operated in a continuous mode, enzyme inactivation was achieved by the use of a low-pressure mercury lamp to generate energy at 254 nm. Since PL has intense broad spectrum light pulses with rich UV, it has a potential to be applied in POD inactivation.

Horseradish peroxidase (HRP) is a typical and widely studied representative enzyme of peroxidase. The biochemical properties and structure of HRP have been well studied (Chattopadhyay & Mazumdar, 2000). The isoenzyme of HRP is a monomeric glycoprotein with a molecular mass of ~44 kDa and dimension of 6 nm (Lopes et al., 2015; Rennke & Venkatachalam, 1979). Microscopic images could directly display the morphology change of HRP after denaturation. Atomic force microscopy (AFM) can provide more detailed information of a single protein particle, such as three-dimensional height, particle size and surface roughness (Jin et al., 2016). HRP contains a single tryptophan which has been used to investigate the conformational changes by using fluorescence techniques. Conformational changes and denaturation increase the distance between the tryptophan residue and the heme group, which lowers the heme quenching effect of tryptophan emission and increases the fluorescence intensity (Chattopadhyay & Mazumdar, 2000; Pina et al., 2001). Raman spectroscopy has an advantage of being used to investigate secondary structural changes of protein during denaturation, aggregation, or gelation of both solid and liquid samples (Ikeda & Li-Chan, 2004; Liu, Zhao, Xie, & Xiong, 2011). These effective methods were also used in this study.

The objective of this work was to investigate the effect of PL on the activity and structural change of HRP. For this purpose, HRP was used as a model enzyme and the influence of PL treatment on the residual enzyme activity and conformation was investigated using AFM, fluorescence, and Raman spectroscopic techniques.

## 2. Materials and methods

### 2.1. Materials and reagents

Horseradish peroxidase (HRP) enzyme (BR, RZ = 3.0, enzyme activity  $\geq 250$  U/mg) was purchased from Aladdin Industrial Co. Ltd. (Shanghai, China). All chemical reagents used in this study were obtained from Sigma-Aldrich-Fluka Inc. (Shanghai, China).

### 2.2. Pulsed light (PL) treatment on HRP

The HRP inactivation experiment was conducted by a PL system (ZW-SY-2D, Zhongwu Guangdian Co. Ltd., Ningbo, China). The PL system consists of a treatment chamber with 2 linear xenon flash lamps, a high power charging/discharging component, a cooling component and a control module. The system generated 0.5 Hz or 1 Hz pulse frequencies of polychromatic light in the wavelength range of 200–1100 nm. Energy produced by single pulse ranged from 100 to 500 J under 2 kW power.

The HRP solution was prepared by dissolving 50 mg of powder in 100 ml of phosphate buffer (0.1 mol/L, pH 7.0). A volume of 5 ml HRP solution with concentration of 0.5 mg/ml was placed in a glass petri dish with diameter of 9 cm. The petri dish was placed at 10 cm vertically below the xenon lamp with lid removed and irradiated by PL. The treatments were conducted at each of the three levels of intensities of 100, 300 and 500 J/pulse with 1, 2, 4, 6, 8

and 10 pulses for studying the activity of residual enzyme, and at the intensity of 500 J/pulse with 5, 10, 20, 40 and 60 pulses for determining the changes in enzyme structure. The temperature of HRP solution was controlled below 30 °C by using the cooling fan set in the chamber during the treatments.

### 2.3. Residual activity measurement

HRP activity was determined by a colorimetric method described by Lopes et al. (2015) with some modifications. The assay was performed at 25 °C in a 1 cm path length glass cuvette using UV/Vis spectrophotometer (T6-1650F, Persee Co. Ltd., Beijing, China) at 470 nm. The reaction mixture consisted of 2 ml phosphate buffer (0.1 mol/L, pH 7.0), 0.1 ml (0.5 mg/ml) HRP, 0.9 ml guaiacol (0.05 mol/L in water) and 2 ml hydrogen peroxide (0.5% w/v in water). The increase in absorbance was followed for a total reaction time of 3 min by recording the absorbance. A mixture of 2.1 ml of phosphate buffer (0.1 mol/L, pH 7.0), 0.9 ml guaiacol (0.05 mol/L in water) and 2 ml hydrogen peroxide (0.5% w/v in water) was used as blank solution. One unit of HRP activity was expressed as an increase of 0.001 unit of absorbance per minute.

The residual activity (RA) of HRP was calculated by the following Eq. (1)

$$RA (\%) = \frac{\text{Activity of HRP after PL treatment}}{\text{Activity of HRP before PL treatment}} \times 100\% \quad (1)$$

### 2.4. Atomic force microscopy (AFM) scanning

AFM measurement was performed according to the method described by Zhang et al. (2016) with some modifications. HRP solution with a concentration of 1.0 mg/ml in phosphate buffer (pH 7.0) was prepared. A drop of 5  $\mu$ l suspension was deposited onto a freshly peeled mica substrate and dried in a laminar flow hood for 1 h at ambient temperature (25 °C). The surface topography images and nano-mechanical properties of samples were obtained by an AFM with NanoScope V controller electronics (Bruker Corporation, Karlsruhe, Germany) in Peak Force QNM mode. All of the images were scanned in air using a standard peak force-model silicon cantilever with a force constant of 12.715 N/m. The radius of curvature of probe tip was 8.53 nm. Surface topography images, height distribution, and surface roughness were obtained using the offline software NanoScope Analysis V1.4 (Bruker Corporation, Karlsruhe, Germany). The surface roughness was further used for the topographic analysis. Mean roughness ( $R_a$ ) and root-mean-square roughness ( $R_q$ ) are defined as follows (Mendez-Vilas, Bruque, & González-Martín, 2007):

$$R_a = \frac{1}{N} \sum_{i=1}^N |Z_i - \bar{Z}| \quad (2)$$

$$R_q = \sqrt{\frac{\sum_{i=1}^N (Z_i - \bar{Z})^2}{N}} \quad (3)$$

where  $Z_i$  is the height of the  $i$ th point with respect to the lowest one in the image and  $N$  is the total number of points comprised in the image.

### 2.5. Fluorescence spectroscopy analysis of HRP

Intrinsic fluorescence spectra of HRP (0.5 mg/ml in phosphate buffer, pH 7.0) were measured at ambient temperature (25 °C) using a Cary Eclipse spectrophotometer (Varian Inc., Palo Alto, CA, USA) equipped with a 1 cm path length cell. The excitation wavelength of 280 nm (slit = 5 nm), emission wavelength of 290 to 400 nm (slit = 5 nm) and scanning speed of 10 nm/s were used.

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