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COMMUNICATION

Ozone Treatment Induces Changes in Antioxidative Defense System in Blueberry Fruit During Storage

Tomasz Piechowiak[1] · Bartosz Skóra[2] · Maciej Balawejder[1]

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Abstract

The major aim of this study was to investigate the effect of ozonation process on the level of oxidative stress markers in blueberry fruit during cold storage (4 °C). Blueberry (*Vaccinum corymbosum* L.) fruit was ozonated with an ozone concentration of 15 ppm for 30 min, every 12 h for 28 days of storage at 4 °C. The results indicated that ozone treatment activated a defense mechanism against oxidative stress in blueberry fruit. Ozonated fruit was characterized by higher activity of antioxidant enzymes i.e. superoxide dismutase, glutathione peroxidase, and phenylalanine ammonia-lyase than non-ozonated fruit, over the first 21 days of storage. In turn, the level of superoxide anion radical and hydrogen peroxide in ozonated fruit was significantly lower compared with the untreated material. However, after 21 days of storage, ozone treatment contributed to the oxidative modification of protein which could be a reason of decreasing enzymes activity, involved in cell protection against oxidative stress.

Keywords Antioxidants · Blueberries · Cold storage · Enzymes · Glutathione · Ozone

Introduction

Ozone is an agent with strong oxidative properties. Many studies have suggested that ozone can be effectively applied in extending a commercial value of berry fruit including the assurance of microbiological safety as well as a high nutrition and processing value of stored product (Alexandre et al. 2011; Contigiani et al. 2018; Jaramillo-Sánchez et al. 2019; Carbone and Mencarelli 2015). Our previous research has shown that ozonation process with an ozone concentration of 15 ppm for 30 min, every 12 h, for 28 days of storage in cold conditions (4 °C) significantly decreased the growth of fungi and aerobic bacteria in blueberry fruit. After 28 days of storage, the contamination with gray mold (*Botrytis cinerea*) for control fruit amounted to 27.5%, while the absence of symptoms was observed in ozonated sample. Furthermore, ozonation inhibited the loss of antioxidant compounds during storage, such as ascorbic acid and flavonoids. For instance, after 7 days of storage, total content of flavonoids and anthocyanins in ozonated fruit did not change significantly, while in control fruit it decreased by ~20% and 32%, respectively (Piechowiak et al. 2019).

However, ozone in aqueous media decomposes rapidly into the reactive oxygen species (ROS) such as superoxide anion radical, hydrogen peroxide, and hydroxyl radical, which excess is toxic for plant. ROS as a very reactive individual cause the oxidation of biomolecues, which are important for the proper functioning of the cells, i.e., proteins, lipids, and DNA. Oxidative modifications of proteins lead to the loss of their biological properties, while increased peroxidation of unsaturated fatty acids in lipids membrane causes a damage of its structure, and consequently, decrease in cell turgor and death of the cell (Cai and Yan 2013; Moller et al. 2007). Moreover, the high level of ROS in plant cells is closely correlated with the activity of polyphenol oxidase, which is responsible for enzymatic browning and the loss of polyphenol compounds in fruit during storage (Sachadyn-Król et al. 2016). Finally, in context of fruit processing and storage, it leads to inferior sensory attributes, nutritional value, and firmness as well as an increased sensitivity of the fruit to mechanical damage and microbial contamination (Giongo et al. 2013; Jin et al. 2017). In view of the above, in order to fully explain

✉ Tomasz Piechowiak
tpiechowiak@ur.edu.pl

[1] Department of Chemistry and Food Toxicology, Institute of Food Technology and Nutrition, University of Rzeszow, St. Cwiklinskiej 1a, 35-601 Rzeszow, Poland

[2] Department Lifestyle Disorders Regenerative Medicine, Faculty of Medicine, University of Information Technology and Management in Rzeszow, St. Sucharskiego 2, 35-225 Rzeszow, Poland

the role of ozone treatment in shaping the quality of blueberry fruit during storage, it is necessary to determine the effect of ozonation on the level of oxidative stress markers in fruit. Therefore, the level of reactive oxygen species (ROS), the activity of antioxidant enzymes, and the level of oxidative damage of protein in ozonated and non-ozonated blueberry fruit during storage were investigated in this study.

Materials and Methods

Ozonation Process in Laboratory Scale: Storage Experiment

The research material was fresh blueberry (*Vaccinum corymbosum* L.) fruit of the Bluecrop variety, purchased from the local plantation. Blueberries were fully colored, ready for consumption, without signs of mold infestation. Before starting the experiment, blueberries were screened for uniform size and absence of mechanical damage.

Blueberries were ozonated using gaseous ozone at the concentration of 15 ppm (gas flow rate, 4 m s^{-1}) for 30 min, at 12-h intervals during whole period of storage according to the procedure presented by Piechowiak et al. (2019). Ozonation was conducted using ozone generator (CSI, Ekotech, Poland) and ozone analyzer UV-106 M (2B Technologies, USA). Blueberries (control and ozonated samples) were stored in a cooling chamber at 4 °C (90–95% relative humidity) for 28 days. The storage experiment was performed in triplicate.

Samples for analysis (ozonated and control fruit) were collected after 0, 7, 14, 21, and 28 days of storage and kept at − 67 °C.

Biochemical Analysis

Antiradical Activity Assay

Blueberries (5 g) were homogenized with 25 mL of 50% methanol solution. The homogenate was shaken for 30 min (150 rpm) and clarified by centrifugation at 7500*g* for 10 min. The supernatant obtained was used to determine the antiradical activity using ABTS radicals which was assayed according to the spectrophotometric method presented by Biskup et al. 2013. The results were expressed as mg of quercetin equivalent per 1 g of dry matter of blueberries.

Determination of Glutathione Content

Frozen blueberry tissue (5 g, − 67 °C) was ground and homogenized in ice bath with 15 mL of chilled 100 mM sodium phosphate buffer (pH 8.0) containing 5 mM EDTA. The homogenate was centrifuged at 15,000*g* for 15 min, at 4 °C. The supernatant obtained was used for glutathione (G-SH) content assay using fluorimetric method with *o*-phthalaldehyde. The results were expressed as a μmol of G-SH per 1 g of dry mass of blueberry tissue (Bartosz 2006).

Determination of Reactive Oxygen Species Level in Fruit

Blueberry fruit (5 g) was homogenized with 20 mL of chilled 100 mM sodium phosphate buffer (pH 7.4). Homogenate was centrifuged at 15,000*g* for 15 min at 4 °C, and the supernatant was used for ROS level assay.

The superoxide anion radical ($O_2^{-\bullet}$) level was determined on the basis of the ability to reduction of nitro blue tetrazolium (NBT) by $O_2^{-\bullet}$ and spectrophotometric detection of formazan produced ($\Delta A\ min^{-1}\ g^{-1}$).

The hydrogen peroxide (H_2O_2) level was estimated with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA). H_2DCF-DA is hydrolyzed by intracellular esterase forming 2′,7′-dichlorodihydrofluorescein which is oxidized by H_2O_2 to fluorescent 2′,7′-dichlorofluorescein (DCF). The results were expressed as an increase in fluorescence within 1 min ($\Delta F\ min^{-1}\ g^{-1}$) (Bartosz 2006).

Enzymatic Activity Assay

Frozen sample of blueberries (− 67 °C, 2.5 g) was ground and homogenized with 10 mL of 0.9% NaCl containing 0.05% (w/v) triton X-100 (Sigma-Aldrich) and 1 mM of phenylmethylsulfonyl fluoride (Sigma-Aldrich). The homogenate was centrifuged at 15,000*g* for 30 min, at 4 °C. Phenolic compounds from supernatant were removed using PVPP filtration (Ranatunge et al. 2017).

The superoxide dismutase (SOD) activity was assayed using colorimetric method which is based on the measurement of the level of inhibition of epinephrine autoxidation by SOD. One unit of SOD activity is the amount of enzyme required to inhibit the epinephrine autoxidation by 50% within 1 min (Piechowiak and Balawejder 2019a).

The glutathione peroxidase (GPx) activity was analyzed using methodology presented by Piechowiak and Balawejder (2019b). One unit of GPx was defined as the amount of G-SH (μmol) oxidized within 1 min, per 1 mg of protein.

The phenylalanine ammonia-lyase (PAL) activity in fruit was assayed using spectrophotometric method described by Zhou et al. 2014. One unit of PAL activity was defined as the amount of enzyme needed to catalyze the formation of 1 μmol trans-cinnamic acid within 1 h. The Bradford method was used for the measurement of total protein concentration in the samples (Kruger 1994).

Determination of Thiol Groups in Blueberry Proteins

Frozen blueberry tissue (5 g) was homogenized in ice bath with 15 mL of chilled 50 mM Tris-HCl buffer (pH 8.0). Homogenate was shaken for 10 min (150 rpm) and then centrifuged at 7500*g*, for 15 min at 4 °C. Supernatant was purified from phenolic compounds using PVPP filtration and mixed with cold acetone (1:4, v/v). The mixture was incubated at − 20 °C for 2 h and pelleted by centrifugation at 14,000*g*, for 20 min. The pellet was resuspended in 50 mM Tris-HCl buffer (pH 8.0) and the level of -SH group in protein sample was determined using Ellman's method. The results were expressed as mmol of -SH groups per 1 g of protein in the sample (Bartosz 2006).

Statistical Analysis

Research results were presented as mean ± SD from three storage experiments, including two repetitions of fruit biochemical analysis ($n = 6$). The significance of differences was performed using one-way ANOVA and the Tukey test ($\alpha = 0.05$) with STATISTICA 13.0.

Results and Discussion

The Activity of Antioxidant Enzymes and Phenylalanine Ammonia-Lyase

Antioxidant enzymes, such as superoxide dismutase (SOD, EC 1.15.1.1) and glutathione peroxidase (GPx, 1.11.1.9), are the first defense line against occurrence of oxidative stress in eukaryotic cells. SOD catalyzes the scavenging of the superoxide anion radical leading to formation of hydrogen peroxide and molecular oxygen, while glutathione peroxidase accelerated the reduction of hydrogen peroxide to water in the presence of reduced glutathione (Cai and Yan 2013). As shown in Fig. 1A and B, the activities of SOD and GPx in ozonated fruit increased sharply after 7 days and remained at a high level to the 21st day of storage ($p < 0.05$) Then, the activities of these enzymes decreased significantly. Similar relationships were also noticed by Boonkorn et al. (2012). The authors determined the effect of ozone treatment (ozone conc., 400 μL L^{-1}) for 2, 4, and 6 h, on the microbial contamination and antioxidant status of tangerine fruit before 3 days of storage at 25 °C. They found that the activities of SOD and enzymes involved in the scavenging of hydrogen peroxide (catalase and ascorbate peroxidase) increased after ozonation and remained markedly higher than control, through whole period of storage. Probably, the reactive oxygen species produced during ozone decay in water activated the enzymes responsible for ROS neutralization. Therefore, the increased level of superoxide anion radical enhances the superoxide dismutase activity, while higher concentration of hydrogen peroxide provokes the enzymes responsible for H_2O_2 neutralization to higher activity.

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.24.) accelerates the deamination of L-phenylalanine to *trans*-cinnamic acid, which can be transformed into phenolic acids, flavonoids, and tannins. These compounds belong to the group of low-molecular weight antioxidants which maintain the cellular redox homeostasis by directly reacting with ROS and oxidation products of cell component. Research showed that the activity of PAL is induced by many biotic and abiotic stress factors and is closely correlated with the level of phenolic compounds in plant (Ortega-García and Peragón, 2009). In this research, we observed an increase in PAL activity in blueberries in ozonated fruit over the first 21 days of storage (Fig. 1C). It is reasonable to assume that increased accumulation of polyphenols including flavonoids and anthocyanins in ozonated fruit which was noticed in our previous research could be caused by higher activity of this enzyme (Piechowiak et al. 2019). This observation is consistent with the research conducted by Chen et al. (2019). Based on the proteomic analysis, they found that the higher level of phenolic compounds in ozonated strawberry fruit (ozone conc., 5 ppm, time of process: 10 h) was associated with the improved expression of proteins involved in phenylpropanoids biosynthesis. In contrast, Sachadyn-Król et al. (2016) found that PAL activity in ozonated pepper fruit showed a decreasing tendency after 20 days of storage. Probably, this difference may have been related with too strong process conditions, which led to the occurrence of oxidative stress in plant.

The Level of Reactive Oxygen Species and Antiradical Activity

In this study, data clearly showed that ozonated fruit was characterized by lower level of ROS than untreated material during whole period of storage (Fig. 2A, B). It can be explained by higher ability to neutralization of ROS created in cells due to their enzymatic apparatus and a higher level of low-molecular weight antioxidants. Moreover, antiradical activity tested using $ABTS^{\bullet+}$ confirmed the higher ability of ozonated fruit to free radical neutralization (Fig. 2C) This observation is consistent with Xu et al. (2019). The authors used the ozone treatment with gaseous ozone at the concentration of 0.68 mg L^{-1} for 10 min to improve the postharvest quality of coriander prior to storage at room temperature. They indicated that ozonation inhibited the accumulation of $O_2^{\bullet-}$ and H_2O_2 in coriander which was associated with higher activity of peroxidase, catalase, and ascorbate peroxidase. However, in our research, the ROS level in treated material after 21 days of storage was high enough to reduce natural defense mechanisms protecting plant from ROS action. After this period of time, we observed a significant decrease in SOD, GPX, and

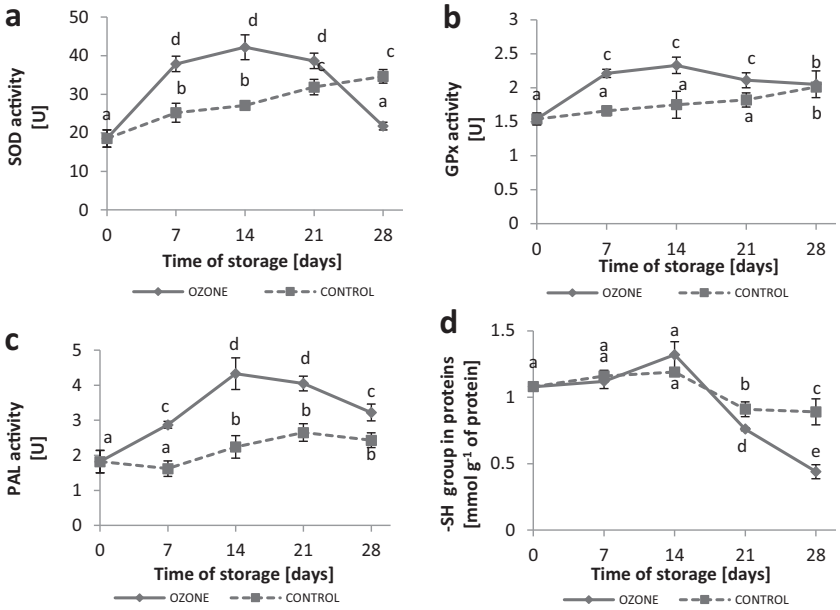

Fig. 1 The effect of ozonation process on the activity of superoxide dismutase (A), glutathione peroxidase (B), phenylalanine ammonia-lyase (C), and the level of thiol groups in proteins (D) in blueberry fruit during storage. Mean values ($n = 6$) with standard deviations (error bars) with the same lower case are not significantly different to each other according to the T-Tukey test ($\alpha = 0.05$)

PAL activity, as well as the reduction in concentration of low-molecular weight antioxidants reacting with $ABTS^{\bullet+}$. Moller et al. (2007) report that one of the negative consequences of oxidative stress is oxidative modifications of protein, including enzymes and changing its properties. In this research, the level of thiol groups in proteins isolated from the fruit was chosen as a marker of oxidative damage of protein. We noticed that the level of thiol groups in proteins extracted from the ozonated and control fruit showed no significant changes over the first 21 days of storage (Fig. 1D). However, after this time, the level of –SH group was significantly lower in ozonated fruit protein than in control sample.

The level of –SH group in proteins was closely related with the amount of reduced glutathione (G-SH). Glutathione is a tripeptide, which the base duty is to maintain thiol protein groups in reduced form by creating disulfides complex: protein-S-S-glutathione. Moreover, it is a cofactor for the glutathione peroxidase and direct participate in reaction with pro-oxidants and free radicals (Choudhury et al. 2017). In our research, we observed a lower level of G-SH content in ozonated fruit after 14 days of storage than in control sample (Fig. 2D). Probably, this effect was caused by increased activity of GPx and direct glutathione reaction with ozone or free radicals.

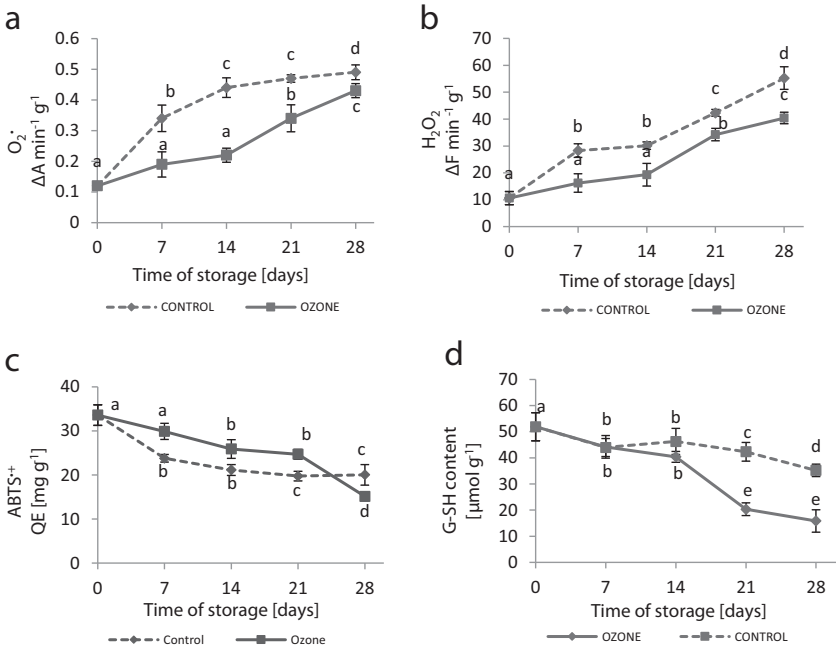

Fig. 2 The effect of ozonation process on the level of superoxide anion radical (A), hydrogen peroxide (B), antioxidant activity against ABTS radical (C), and the level of glutathione (D) in blueberry fruit during storage. Mean values ($n = 6$) with standard deviations (error bars) with the same lower case are not significantly different to each other according to the T-Tukey test ($\alpha = 0.05$). QE quercetin equivalent

Conclusions

The presented study demonstrated the relationships between the level of reactive oxygen species and natural defense mechanism against oxidative stress in blueberry fruit after storage in ozone-enriched atmosphere. Ozonated fruit was characterized by higher activity of antioxidant enzymes i.e. superoxide dismutase, glutathione peroxidase, and phenylalanine ammonia-lyase than non-ozonated fruit, over the first 21 days of storage. In turn, the level of superoxide anion radical and hydrogen peroxide in ozonated fruit was significantly lower compared with the untreated material due to improved activity of ROS detoxification systems. However, after 21 days of storage, ozone treatment contributed to the oxidative modifications of protein which could be a reason of decreasing enzyme activity, involved in cell protection against oxidative stress. The obtained results complement a knowledge in the field of food science concerning the use of ozonation technology in prolonging the postharvest shelf-life of soft fruit.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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