Research Article

Plasma Affects Sugar Conversion during Storage of *Citrus sinensis* Osbeck: Melatonin Is a Key Factor

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Received: July 28, 2023 Revised: September 10, 2023 Accepted: September 21, 2023 Published: November 16, 2023

Abstract

Background: Atmospheric pressure plasma as a green preservation technology for fruits and vegetables can effectively reduce the number of microorganisms on the surface of fruits and vegetables to achieve the effect of reducing the decay rate and prolonging the freshness period. In addition, plasma can affect the quality of fruits and vegetables and delay the aging of fruits and vegetables. However, the intrinsic mechanism by which plasma affects the quality of fruits and vegetables is unclear.

Objective: The aim of this work is to observe the quality changes of *Citrus sinensis* Osbecks led by plasma treatment during storage period and to analyze the intrinsic mechanism behind it, for the purpose of providing a theoretical reference or the application of plasma in fruit preservation and quality enhancement.

Methods: Postharvest *Citrus sinensis* Osbecks were treated with dielectric barrier discharge (DBD) for 5 min, and then the total soluble solids, titratable acids and solid-acid ratios of fruit pulp during storage before and after treatment were measured using a hand-held digital refractometer, and an acid-base burette. Changes in melatonin, sucrose converting enzyme, and simple sugar content of fruit were measured with an ultraviolet-visible spectrophotometer.

Results: It was found that the soluble solids and solid-acid ratio of *Citrus sinensis* Osbecks by DBD treated were significantly increased during storage, while titratable acid did not change significantly. Melatonin and sucrose converting enzyme measurements revealed that plasma treatment significantly increased melatonin content and sucrose converting enzyme activity in the pulp. Measurements of monosaccharides showed that the glucose and fructose contents of the treated *Citrus sinensis* Osbeck pulp increased, while the sucrose content decreased.

Conclusion: The reactive oxygen and nitrogen species generated with DBD plasma can effectively increase the melatonin content in the pulp of *Citrus sinensis* Osbecks, thereby improving citrus quality.
including promoting sugar conversion and enhancing flavor.

Keywords: plasma, Citrus sinensis Osbeck, fruit quality, melatonin, sugar conversion


1 INTRODUCTION

Low temperature plasma can produce a variety of reactive oxygen and nitrogen oxygen species, such as O₃, 'O₂, NO and NO₂⁻, which are widely used in many fields such as biomedicine and agriculture[1-5]. In agricultural production, the active nitrogen and active oxygen produced by low-temperature plasma affect the physiological response of agricultural products and do not cause thermal damage to agricultural products during the treatment process[6-9]. Therefore, research on the application of low-temperature plasma in agricultural products is gradually gaining attention. For example, Mee et al.[10] found that low-temperature plasma could increase the antioxidant capacity and phenolic content of citrus peels. Chaitanya et al.[11] also reported an increase in the total phenolic and flavonoid content of blueberries after 1min of low-temperature plasma treatment. This is due to the reactive radicals in the plasma reacting with the phenolic derivatives, thereby increasing the phenolic content. It has also been explained that the plasma increased the activity of phenol-producing enzymes such as phenylalanine deaminase[12]. In addition, certain antioxidant enzyme activities are increased in agricultural products due to oxidative stress to reduce the adverse oxidative reactions caused by active substances which are produced by low temperature plasma. For example, plasma increases superoxide dismutase (SOD) enzyme activity during storage of shiitake mushrooms. The SOD enzyme activity of blueberries treated with low-temperature plasma was significantly increased compared with the control group[13,14].

Although studies on the physiological effects of plasma on agricultural products are gradually being reported, the reactive nitrogen and reactive oxygen produced by plasma is a complex reaction system. And there are still many unknown effects and mechanisms of action of plasma on the physiological responses of agricultural products due to the complex physiological and biochemical properties of most agricultural products. With the current development of plasma diagnostics, the types and patterns of reactive nitrogen and reactive oxygen produced are gradually being clarified, and there are more and more possibilities for their effects on agricultural production when applied in agriculture[15-18]. Several studies have reported that low-temperature plasma was able to keep total soluble solids in blueberries, fresh pears, and eggplants at high levels, which possibly due to the effect of plasma on sugar metabolism[19-21]. Melatonin, one of the plant hormones, is closely related to sugar metabolism in plants with extremely strong scavenging function of oxygen/nitrogen free radicals[22-24]. When low-temperature plasma treats agricultural products, melatonin production may be promoted by the stimulation of reactive radicals such as singlet oxygen 'O₂, nitric oxide NO and nitrite NO₂⁻ generated by the plasma. In addition, NO is involved in the production of melatonin synthesis precursors, which will also indirectly promote melatonin production[25]. In conclusion, low-temperature plasma may affect melatonin synthesis and thus regulate the conversion of sugars. However, this opinion has not been verified by relevant research. Carrying out research on melatonin and sugar conversion in fruits and vegetables by low-temperature plasma is of great significance for the application of low-temperature plasma in the field of fruit and vegetable preservation. In addition, as the duration of low-temperature plasma treatment increases, the reactive free radicals produced alter the amino acid side chains in the aromatic rings of cysteine, phenylalanine, tyrosine, and tryptophan, leading to enzyme inactivation[26,27]. For example, Misra et al.[28] and Silvia et al.[29] found that peroxidase and polyphenol oxidase activities in strawberries and apples progressively decreased with increasing treatment time. Attention should be paid to the appropriate treatment time to avoid the deterioration of the quality of fruits and vegetables caused by too long treatment time in the application of low-temperature plasma in the preservation of fruits and vegetables.

Therefore, a dielectric blocking discharge was used to treat Citrus sinensis Osbeck for a short period of time after harvesting in this study. The effects of dielectric barrier discharge (DBD) on postharvest melatonin content and sugar conversion of Citrus sinensis Osbeck were investigated by measuring melatonin content, sugar content and sugar conversion-related enzyme activities in the pulp to provide a reference basis for the application of low-temperature plasma in agriculture.

2 MATERIALS AND METHODS

2.1 Citrus Preparation

Citrus sinensis Osbeck used in the study was harvested in June 2021. The fruits with physical scars were removed
after cutting the stems and leaves, and intact citrus of uniform size (about 50g) were selected for use.

2.2 DBD Discharge Apparatus

The schematic diagram of the plasma reactor was shown in Figure 1. The DBD reactor consisted of a stainless-steel column rod, two quartz tubes, water and several support components. A stainless-steel cylindrical rod with a diameter of 29mm and a length of 250mm was used as a high voltage electrode, which was coaxially connected to the inner quartz tube through a polytetrafluoroethylene joint. The inner quartz tube was 2mm thick and serves as a dielectric barrier between the electrodes. The outer quartz tube was 4mm thick and was supported at both ends by stainless steel flange assemblies. The outer diameters of the two quartz tubes are 34 and 50mm respectively. Water flowing between the inner and outer quartz tubes contacts the flange assembly and serves as a grounding electrode. A plasma electric field was generated between the stainless-steel cylindrical rod of the DBD generator and the coaxial quartz tube. The air flow rate injected into the DBD reactor was controlled by a mass flow controller (Sevenstar, D07-7). The DBD reactor was powered by an internally built AC power supply providing a variable peak-to-peak voltage of 16-26kV and a fixed frequency of 16.5kHz.

The DBD plasma gas flow rate condition in this device was 67cm³/min and the voltage was fixed at 24kV, the gaseous product has a maximum temperature of 28°C. 120 selected fruits were placed at the bottom of the treatment chamber and treated for 5min. Then, both treatment and control citrus groups were placed in storage at 4°C and above 85% relative humidity, and 12 fruits from each group were sampled at 24h intervals for measurement.

2.3 Quality Analysis of Citrus

Citrus pulp was manually juiced with a juicer and the juice was collected and total soluble solids in the juice was measured using a handheld digital refractometer. Titratable acidity was determined by titrating 20mL of juice to the endpoint of pH 8.1 with 0.1mol/L NaOH and expressed as a percentage of citric acid. The solid-acid ratio is the ratio of soluble solids to titratable acid and is one of the indicators to evaluate the flavor and ripeness of the fruit.

2.4 Quantification of Melatonin

After careful weighing of 2g of citrus fruit pulp, 10mL of phosphate buffer solution was added, and the pulp was well homogenized manually and then centrifuged at 30min for 20min. The supernatant was collected and the melatonin content was measured using a plant melatonin ELISA kit (provided by Wuhan Purity Biological Co., Ltd.).

2.5 Sugar Content and Sucrose Metabolism-related Enzyme Analysis

Glucose, fructose and sucrose were determined by anthrone-sulfuric acid method. Take 5g of citrus pulp sample, add 75% ethanol and then collect the solution in a water bath at 80°C for 15min and then cool to 20°C, centrifuged at 10,000rpm for 10min. The supernatant was collected and add deionized water to 20mL. 10mL of the supernatant solution was taken in a test tube, add 2mL of 0.05mol/L KOH solution in a boiling water bath for 10min, cool and then add 6mL of anthranilic sulfate solution on ice. After cooling, 6mL of anthranilic sulfate solution was added on ice and shaken well and heated in a boiling water bath for 3.5min to measure sucrose. 1mL of supernatant solution and 1 mL of deionized water were added to the test tube. Shake well with 6mL of anthranilic sulfate solution on ice, then heat in a boiling water bath for 3.5min for glucose measurement and in 50°C water bath for 3.5min for fructose measurement. The absorbance was measured at 620nm after cooling.
Sucrose convertase activity was determined according to the method of Lowell et al\textsuperscript{[32]}. Approximately 2g sample of citrus fruit pulp was weighed, carefully ground in liquid nitrogen, and 10mL of extraction buffer (200mmol/L potassium phosphate buffer, 5mmol/L MgCl\textsubscript{2}, 0.1% β-mercaptoethanol, 0.05% Triton-X 100, 0.05% BSA, 2% PVPP) was mixed and centrifuged at 12,000rpm for 30min. The supernatant was added with (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} to 80% saturation and centrifuged at 12,000rpm for 20min. The supernatant was removed and was buffered with 200mmol/L potassium phosphate buffer, 0.25mmol/L MgCl\textsubscript{2}, 0.01% β-mercaptoethanol, 0.05% BSA, pH 7.5 for dialysis desalting to obtain the enzyme extract. The enzyme extract was extracted from 500µl of the reaction system containing 80mmol/L potassium acetate-phosphate (pH 4.5 for sucrose acid converting enzyme and pH 7.5 for neutral converting enzyme) and 100mmol/L sucrose. The reaction was carried out at 37°C for 30min and terminated by adding 490µL of DNS reagent. The reaction was terminated by adding 490µL of DNS reagent at 37°C for 30min, and the absorbance at 540nm was measured after cooling in a boiling water bath for 5min. The inactivated enzyme extract was used as a control to calculate the sucrose convertase activity.

2.6 Statistical Analysis

One-way ANOVA was performed using GraphPad prims software (version 8 GraphPad software, Inc, La Jolla, CA, United States) for measurements of soluble solids, titratable acid, solid-acid ratio, melatonin, sugar content, and enzyme activities related to sucrose metabolism, using Tukey’s HSD multiple comparisons with a significance level of 0.05.

3 RESULTS

3.1 Effect of DBD on Total Soluble Solids Accumulation in Citrus sinensis Osbeck

To understand the effect of reactive oxygen and nitrogen species (RONS) produced by DBD on the quality of Citrus sinensis Osbeck during storage, some quality traits of Citrus sinensis Osbeck during storage after 5min of DBD treatment after harvesting were measured in this study. The results of citrus quality measurement were shown in Figure 2. The soluble solids content of Citrus sinensis Osbeck treated with DBD for 5min was significantly higher than that of the control group after five days of storage. The daily changes of total soluble solids data were analyzed and it was found that the total soluble solids content of treated Citrus sinensis Osbeck was significantly higher than that of the control group at the beginning of the second day of storage, which was stable with the increase of storage time. The results of titratable acid determination showed that there was no significant difference in titratable acid content between the treated and control groups after five days of storage as well as on daily variation. In addition, the ratio of soluble solids to titratable acid content (solid-acid ratio) was commonly used to evaluate fruit flavor. The solid-acid ratio was an important indicator of fruit quality as the higher the ratio, the better the fruit flavor. It was found that the solid-acid ratio of treated Citrus sinensis Osbeck was significantly higher than that of the control group by calculating the solid-acid ratio with significant differences from the first day storage. The solid-acid ratio was mainly affected by total soluble solids and titratable acid, so it was inferred that RONS produced by DBD mainly affected the solid-acid ratio by increasing the total soluble solids content in the pulp of Citrus sinensis Osbeck.

3.2 Changes in Melatonin Levels in Fruit Pulp

To investigate whether the changes in soluble solids were because of plasma on melatonin levels in the pulp, melatonin levels were measured in Citrus sinensis Osbeck after 5min of DBD treatment and during a 5-day storage period. Figure 3 showed the results of melatonin measurement. The melatonin level in the treated Citrus sinensis Osbeck increased from 8.7ng/g to 10.7ng/g after 5 days of storage compared with the control group, and the melatonin level in the treated group increased gradually compared with the control group from the first day of storage to a maximum of 11.37ng/g on the third day of storage, after which the melatonin level remained stable. This indicated that the treatment of Citrus sinensis Osbeck with DBD for 5min could effectively increase melatonin content. The increased melatonin might be attributed to the increased production of NO by DBD through the induction of the expression of genes related to melatonin synthesis. Moreover, melatonin as an antioxidant which might lead to increased melatonin synthesis in citrus due to oxidative stress as a result of ROS such as oxidative produced by DBD\textsuperscript{[33,34]}. Melatonin was also an anti-aging hormone, which affected the respiratory metabolism of citrus to improve quality traits such as soluble solids and sugar metabolism in fruits.

3.3 Sucrose Converting Enzyme Activity

Changes in melatonin might affect the monosaccharide content of the pulp by affecting the activity of sucrose-converting enzymes and thus alter the taste of the fruit. Therefore, we measured the sucrose acidic and neutral converting enzyme activities in Citrus sinensis Osbeck after plasma treatment for 5 days of storage in this study shown in Figure 4. Treatment with free radicals generated by air discharge for 5min significantly increased the activity of acid and neutral converting enzymes in Citrus sinensis Osbeck during 5 days of storage. The acid converting enzymes of Citrus sinensis Osbeck were significantly increased on the first day of storage after the discharge treatment, and then showed decreasing trend with the increase of storage time. INVINV protein inhibits converting enzyme activity and thus sucrose catabolism. Treatment of citrus with DBD increased the synthesis of melatonin, which promoted sucrose-converting enzyme activity by inhibiting the

https://doi.org/10.53964/mltp.2023006
Figure 2. Effect of RONS generated by dielectric barrier discharge on citrus quality. A: Total soluble solid; B: Titratable acid; C: Solid-acid ratio. Values are means of at least five replications ± SE. *t-test significant difference. ***P<0.001.

Figure 3. Effect of plasma on citrus melatonin levels. Values are means of at least five replications ± SE. *t-test and ANOVA significant difference. ***P<0.001.
expression of the INVINH gene.\textsuperscript{[35]}

### 3.4 Sucrose, Glucose, Fructose Content

The results of sucrose, glucose and fructose measurements were shown in Figure 5. The sucrose content of Citrus sinensis Osbeck stored for 5 days after discharge treatment decreased significantly, while the fructose and glucose content increased significantly. In addition, plasma treatment led to a significant difference in the elevation of fructose and glucose in Citrus sinensis Osbeck on the third day of storage, thus indicated that plasma elevation of fructose and glucose in Citrus sinensis Osbeck is an accumulative process, and the difference in sugar content becomes more pronounced as the storage time increases.

### 4 DISCUSSION

The reactive oxygen and reactive nitrogen species (RNS) were produced with air as treatment gas when DBD was used, such as $^1$O$_2$, NO and NO$_2$.\textsuperscript{[36,37]} NO-based RNS was shown to be involved in melatonin biosynthesis, where NO had an important role in the production of melatonin synthetic precursors and an increase in NO concentration may stimulate melatonin production\textsuperscript{[38]} as shown in Figure 6 in previous studies. However, whether it could ultimately affect melatonin content still lacks experimental validation. Our results confirmed that RNS produced using DBD could increase melatonin content in Citrus sinensis Osbeck pulp which might be mainly attributed to the action of NO-based reactive nitrogen radicals. Meanwhile, singlet oxygen acted as reactive oxygen radicals with oxidative effects in organisms, and melatonin, as a reactive oxygen scavenger, might promote melatonin synthesis in response to the stimulation of singlet oxygen.\textsuperscript{[39,40]} The increase of melatonin in the pulp elevated the activity of sucrose-converting enzymes, which in turn promoted the conversion of sucrose to glucose and fructose.

### 5 CONCLUSION

In this study, we investigated the effects of reactive oxygen and RNS generated by DBD on mass and sugar conversion during storage in Citrus sinensis Osbeck. RONS produced by using DBD low temperature plasma could effectively increase the melatonin content, which in turn improved the quality of citrus, promoted sugar conversion and enhanced the taste. In addition, it has

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Figure 4. Plasma enhances citrus sucrose convertase activity. A: Activity of acid invertase; B: Activity of neutral invertase. Values are means of at least five replications ± SE. *-t-test and ANOVA significant difference. ***P<0.001.
Figure 5. Plasma promotes the conversion of sucrose to fructose and glucose in citrus fruit pulp. A: Sucrose; B: Fructose; C: Glucose. Values are means of at least five replications ± SE. *-test and ANOVA significant difference. ***P<0.001.

Figure 6. Chemical and biological mechanisms by which plasma affects fruit quality and sugar conversion. Plasma-generated active substances affect citrus quality and sugar conversion by stimulating melatonin synthesis processes and reactive oxygen species scavenging mechanisms in citrus fruit pulp.
also been shown that prolonged low-temperature plasma treatment can cause damage to blueberry anthocyanins, which might be caused by excessive oxidation of the fruit by RONS species with oxidative properties generated by low-temperature plasma. Moreover, in the field of fruit and vegetable preservation, although NO had an important role in delaying plant aging, high concentrations of NO could cause oxidative damage to plants. Therefore, when low-temperature plasma was used for the preservation of fruits and vegetables, attention should be paid to the appropriate treatment time and the concentration of the RONS species generated. This study revealed the mechanism of ozone-free NO production by DBD to improve the quality of Citrus sinensis Osbeck, which provided an important theoretical reference for the application of low-temperature plasma in fruit preservation and quality enhancement.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant No. 51877021, 61864001).

Conflicts of Interest

The authors declared no conflict of interest.

Author Contribution

Ma X and Liu K made key contributions to the conception or design of the study and to the drafting and revision of the article; Ran C was involved in obtaining and analyzing the data for the interpretation of the study; and Zhou X was involved in the revision of the key contents of the article.

Abbreviation List

DBD, Dielectric barrier discharge
RONS, Reactive oxygen and nitrogen species
RNS, Reactive nitrogen species
SOD, Superoxide dismutase

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https://doi.org/10.53964/mltp.2023006


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