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Effects of Chitosan and Salicylic Acid with Mallow and *Aloe vera* Extracts on Enzyme Activities in *Citrus unshiu* Tangerines

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ABSTRACT

Products without synthetic chemicals are of global interest due to the presence of active compounds such as phenolic substances and their antioxidant properties. This research applied various concentrations of chitosan, salicylic acid, *Aloe vera* gel, and mallow mucilage treatments on *Citrus unshiu* tangerines. The research aim was to find their best concentrations, leading to optimal fruit quality maintenance. The experiments were in a factorial design and laid out as a completely randomized block in a research orchard at Islamic Azad University, Sari Branch. The experimental treatments were chitosan (0.5, 1, and 1.5%), salicylic acid (SA: 1, 1.5, and 2 mM), *A. vera* gel (15, 30, and 45%), and mallow mucilage (15, 30, and 45%). Treatment applications were applied in immersion form, followed by storage at four storage periods (0, 15, 30, and 45 days) for 45 days. Total protein content and superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) activities were investigated in the experiment. The results showed that chitosan 1.5%, *A. vera* gel (30% and 45%), and 2 mM SA prevented total protein degradation and improved POD activities during 45 days of storage. However, they had no significant effect on SOD and CAT activity.

Introduction

Citrus trees produce subtropical fruits with a high economic value in Brazil, USA, and China. Multiple species exist in the citrus family that comprise the citrus industry worldwide. *C. unshiu* tangerines have good marketability in Iran for their palatability and marketing time. This variety is suitable for cool subtropical regions and has a wide distribution in the temperate zone, Japan, China, Spain, and countries with appropriate climatic conditions (Qazvini et al., 2016). Most fresh fruits and vegetables are known for their therapeutic value and health-promoting activities. Therefore, products without synthetic chemicals are of global interest due to their naturalness, antioxidant properties, and active compounds, such as phenolic substances (Suleria et al., 2015). In this regard, healthy, natural, and biodegradable compounds can improve the shelf life of horticultural products and increase health indicators in society. Compounds compatible with the environment and humans comprise healthy methods for better controlling postharvest diseases (Asghari et al., 2012). Chitosan is a non-toxic, biodegradable, functional, and bio-structured compound with antimicrobial and antifungal activities that effectively control fruit rot. It can form a coating on fruits and vegetables and reduce their respiration rates by regulating

carbon dioxide and oxygen permeability.

Antimicrobial properties of chitosan molecules result from positively charged amino groups (Hosseini et al., 2009). *A. vera* plant gel is transparent, odorless, and non-viscous. It has high absorbability, a suitable alternative to chemical coatings for postharvest crops. *A. vera* gel contains many compounds, the most important of which are vitamins, amino acids, enzymes, salicylic acid, anthraquinones, and saponins. Meanwhile, fungicidal properties in salicylic acid (SA) and saponins inhibit the growth and reproduction of fungi to control fungal growth (Choi et al., 2001).

Mallow has nutritional and medicinal applications, and the essential active ingredients of its flowers include mucilage, flavonoid, tannin, phenolic compounds, and anthocyanins, e.g., malvin, delphinidin, and malvidin (Dehkordi et al., 2003). The topical application of SA protects plants against direct oxidative damage (Horvath et al., 2007). SA treatment effectively reduced the respiration of harvested fruits in a concentration-dependent manner (Mo et al., 2008; Srivastava et al., 2000). In addition, high concentrations of SA affect the width of stomata and cause stomata closure.

Fresh weight and respiration rates correlated with stomatal pore width (Manthe et al., 1992). In relevant research, Qaisarbigi et al. (2015) investigated chitosan effects on disease resistance and radical oxygen species levels in Washington navel oranges (*C. sinensis* L. Osbeck). They reported that chitosan treatment with a concentration of 2% reduced disease incidence and rot diameter compared to the control. The chitosan treatment (2%) increased superoxide dismutase (SOD), peroxidase (POD), hydrogen peroxide (H2O2), and glutathione (GSH) activities. However, it decreased catalase (CAT) and ascorbate (AsA) activities during storage. Ascorbate peroxidase (APX) activity slightly increased in fruits treated with chitosan during storage for 2-3 weeks. These results showed that treatment with chitosan stimulated disease resistance in fruits by regulating H2O2 levels, antioxidant enzyme activity, and ascorbate-glutathione cycle (Zeng et al., 2010).

Considering the high importance of citrus fruit storage and improving the export status of these fruits from their origin in Iran, the country's northern climate, this research aimed to assist in storing *C. unshiu* tangerines using biodegradable compounds compatible with human health.

Materials and Methods

C. unshiu tangerines were harvested at physiological maturity when the fruits had developed full color. They were harvested from a commercial orchard in the Miandorud district, Sari City, in late November 2019. The fruits were properly sorted and immediately transferred to the laboratory. After washing the fruits with tap water, damaged fruits were discarded. Healthy and uniform fruits were selected in terms of size and color without physical damage or disease symptoms. This factorial experiment was conducted as a completely randomized block and involved investigating the effects of chitosan, SA, *A. vera* gel, and mallow mucilage treatments on the quality factors of *C. unshiu* tangerines. Their best concentrations were selected for further application. The experimental treatments were chitosan (0.5, 1, and 1.5%), SA (1, 1.5, and 2 mM), *A. vera* gel (15, 30, and 45%), and mallow mucilage (15, 30, and 45%). The fruits were immersed in these solutions for 3 min. The fruits were then spread on a net to dry. Dried fruits were transferred to a cold storage facility (7 °C) at 90% relative humidity. The fruits were stored for 45 days and sampled at four storage periods (0, 15, 30, and 45 days).

Measured traits

To measure the activities of SOD, CAT, and POD enzymes, 5 g of healthy mesocarp tissue was sampled from tangerine fruits and placed in frozen liquid nitrogen until enzyme assays, followed by storage at −80 °C (Peng et al., 2009).

Enzyme extract extraction

To obtain enzyme extracts, 1 g of tissue sample was ground with 3 mL of sodium phosphate buffer (pH 6.7) in a cold mortar to form a uniform mixture. The resulting mixture was immediately centrifuged at 18,000 RPM (4 °C) for 15 min. After complete precipitation, the supernatant was used for measuring enzyme activity (Mac-Adam et al., 1992).

SOD activity

After preparing the enzyme extracts, SOD activity was determined by measuring the ability of each extract to inhibit the photochemical reduction reaction of nitrobluetetrazolium (NBT). The reaction solution for SOD activity measurement consisted of 50 mM phosphate buffer (pH 7.5), 13 mM methionine, 75 mM NBT, 0.1 mM EDTA, and 2 mM riboflavin. The enzyme reaction solution was maintained in complete darkness. The SOD reaction medium (2.5 mL) with 200 µL of the enzyme extract was placed under fluorescent light for 10 min. After preparing two sets of tubes containing 2.5 mL of the reaction medium and

200 µL of the extraction buffer, one series was placed in the dark and the other was placed under a fluorescent lamp for 10 min. After 10 min, all samples were transferred to the dark, and the absorbance of each sample was measured at 560 nm with a spectrophotometer (Shimidazucarry 50). The device was set to zero with a tube containing the extraction buffer (as a blank) without the enzyme extract and was exposed to the dark. Then, absorbance values of light-exposed tubes containing the reaction medium and extraction buffer were measured against a blank. The absorbance of each sample (containing the enzyme extract) was then measured at 560 nm (Giannopolitis et al., 1977).

Differences between absorbance in the samples containing the enzyme extract and that of the control sample indicated inhibition percentages of spontaneous reactions of Formosan formation by SOD. Therefore, the absorbance reduction percentage was calculated per sample. One unit of SOD activity was defined as an enzyme concentration that causes 50% inhibition of NBT reduction. This method is based on the conversion of NBT into Formosan in the presence of light and color appearance. If SOD is present in the environment reaction, it prevents this reaction and reduces dye formation and color appearance (Giannopolitis et al., 1977). Finally, SOD activity was calculated based on enzyme unit per gram of protein (U g^{-1} protein) for all samples.

CAT activity

CAT activity was measured through H_2O_2 reduction at 240 nm min^{-1} according to Pereira et al. (2012). The reaction mixture comprised 0.1 M phosphate buffer (pH 7) and 240 mM H_2O_2. The process started by adding 0.1 mL of enzyme extract. The enzyme activity was expressed as an enzyme unit per gram of protein (U g^{-1} protein).

POD activity

To measure POD activity, 3 mL of a 0.1 M sodium phosphate buffer solution, 50 µL of pure guaiacol liquid (C7H8O2), and 50 µL of 3% H_2O_2 were added to the enzyme extract. Optical absorbance changes were immediately recorded at 436 nm wavelength using a spectrophotometer at 15-second intervals for 3 min. After adding H_2O_2 and guaiacol, the solution turned brownish red. To calculate POD activity, the last absorbance value was subtracted from the first read absorbance value and divided by three (Mac-Adam et al., 1992).

Statistical analysis

The statistical analysis of the obtained data and the comparison of means were performed using SAS 9.1 and MSTAT-C software. Graphs were drawn using Microsoft Excel. The results were tested with the GLM analysis of variance, and mean values were compared with Duncan's multi-range test.

Results

The variance analysis describes the studied treatments and their effects on the measured traits (Table 1). Accordingly, the storage period significantly affected total protein and SOD contents ($P \leq 0.05$), with CAT and POD levels ($P \leq 0.01$). The studied treatments significantly influenced total protein and POD contents ($P \leq 0.01$). The interaction effect of treatment and storage periods was significant only on POD ($P \leq 0.05$). Observations were consistent with the research hypothesis that chitosan, salicylic acid, mallow mucilage, and *A. vera* gel can effectively enhance the storability of tangerines, as observed in the current research during the 45 days of storage.

Table 1. Analysis of variance regarding the effects of salicylic acid (SA), chitosan (Chi), mallow mucilage (MM), and *A. vera* gel (AG) treatments on the enzyme factors of *C. unshiu* tangerines during the cold storage period at 7 °C for 45 days.

		Mean squares			
Source of variation	df	Total protein	SOD	CAT	POD
Replication (R)	3	1300.8*	0.241*	0.217**	438.4**
Treatment (T)	12	1206.8**	0.063^{ns}	0.044^{ns}	269.2**
T × R	36	534.17	0.05^{ns}	0.027^{ns}	146.75*
Test error	104	455.64	0.068	0.035	98.21
Coefficient of variation	-	31.29	13.19	15.15	34.44

* and ** respectively indicate significant differences at 0.5 and 1% levels. ns: no differences.

Total protein content of fruits

The total protein content of fruits was measured by the Bradford Protein Assay using spectrophotometry. The studied treatments affected the total protein content of *C. unshiu* tangerines during the storage period (Fig. 1). The trend of total protein changes in the measurement periods under the influence of different treatments was complex, but it had

positive effects. The highest protein content in each period was obtained with 15% A. vera, 30% mallow mucilage, and 2 mM SA treatments. The average total protein on the 15th day was more than on the 0th day, but it decreased noticeably on the 30th day and increased again on the 45th day. At the end of storage, the highest average total fruit protein content (99.32 mg g^{-1} of fresh weight) occurred in the 15% *A. vera* gel treatment, which was significantly different from the other treatments.

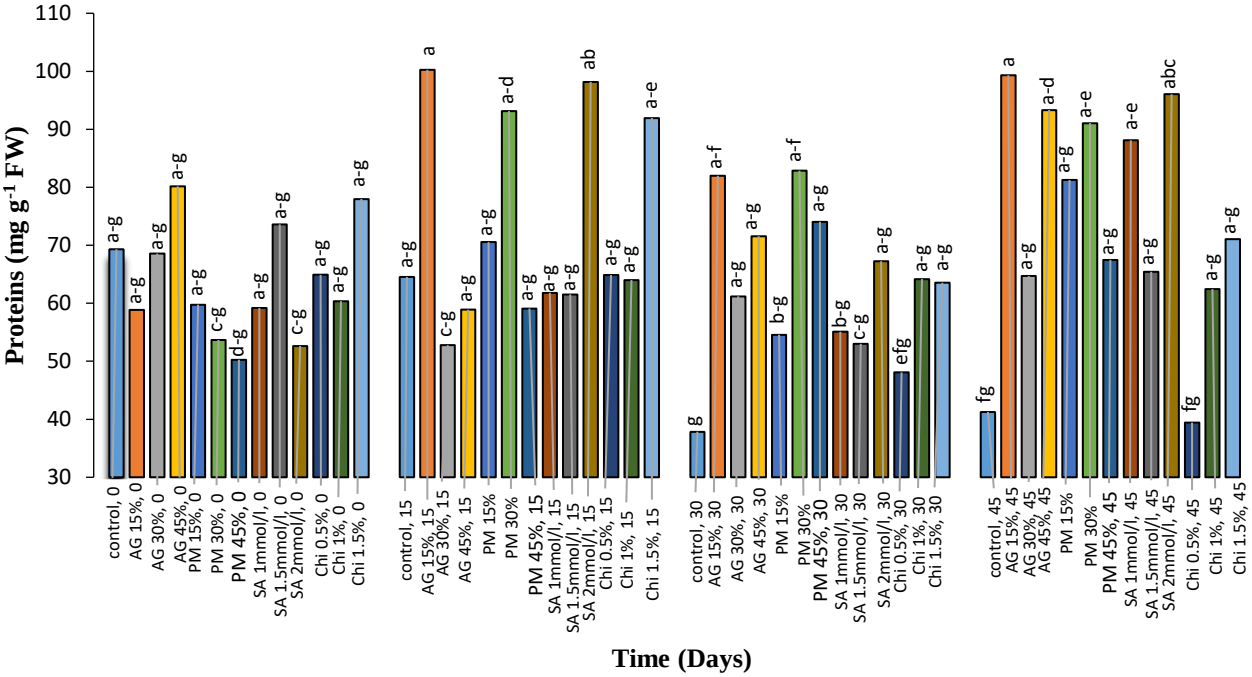

Fig. 1. Effects of salicylic acid (SA), chitosan (Chi), mallow mucilage (MM), and *Aloe vera* gel (AG) treatments on the total protein content of *C. unshiu* tangerines during cold storage at 7 °C for 45 days. Columns with similar letters are not statistically different.

SOD and CAT activity

SOD patterns changed in *C. unshiu* tangerines during cold storage at 7 °C for 45 days (Fig. 2). Accordingly, the enzyme activity in the fruits increased on the 15th day compared to the beginning of storage, but showed a decrease from the 15th day onward and finally reached the lowest level (1.88 U g^{-1} protein) on the last day of storage. The gradual changes in SOD and CAT activities resulted from the fact that the treatments and coatings had a protective role on the fruit skin and prevented rapid changes in enzymatic activities in fruit tissues.

According to Figure 2, CAT activity increased from day 0 (the beginning of storage) until day 30 when it reached the highest level (0.455 U g^{-1} protein), but it decreased slightly at the end of storage. The lowest level of CAT activity (0.276 U g^{-1} protein) was observed at the beginning of storage.

POD activity

The studied treatments affected the POD activity of *C. unshiu* tangerines during the storage period, indicating a trend of increase in POD activity, parallel to longer storage. The POD activity reached its maximum on days 30 and 45 (Fig. 3). The POD activity under the influence of treatments was measured from day 0, but none of the treatments led to a significant difference in the activity of this enzyme during this period. The differences were observed from day 15 and became more noticeable on days 30 and 45. The highest average POD activity (49.6 U g^{-1} protein) was achieved on day 30 in 0.5% chitosan treatment, which was not significantly different from the other treatments, except for A. vera (15% and 45%), mallow mucilage (30% and 45%), and 2 mM SA treatments (Fig. 3).

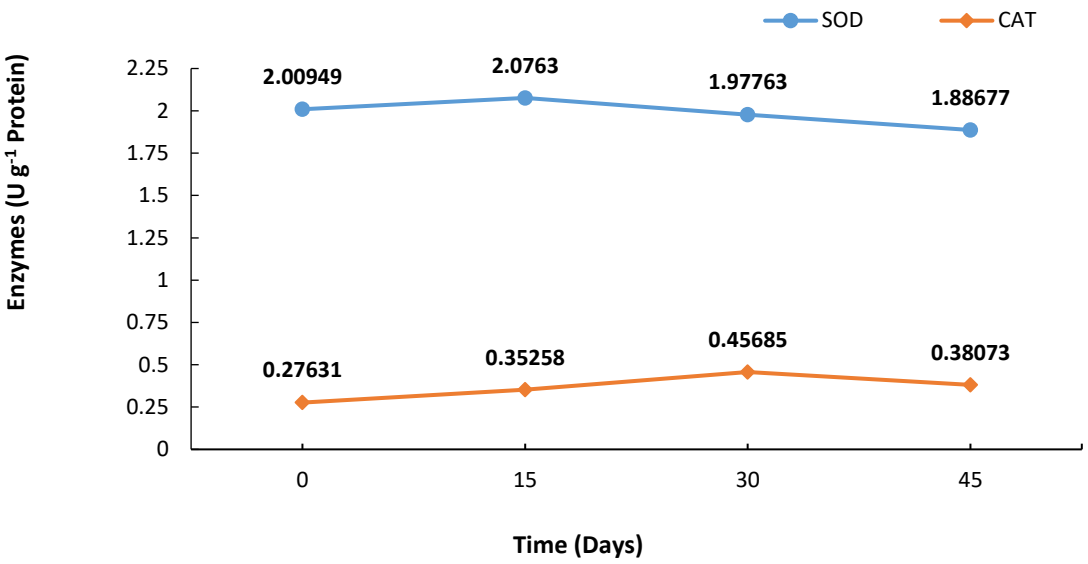

Fig. 2. Changes in SOD and CAT of *C. unshiu* tangerines during cold storage at 7 °C for 45 days. Columns with similar letters are not statistically different.

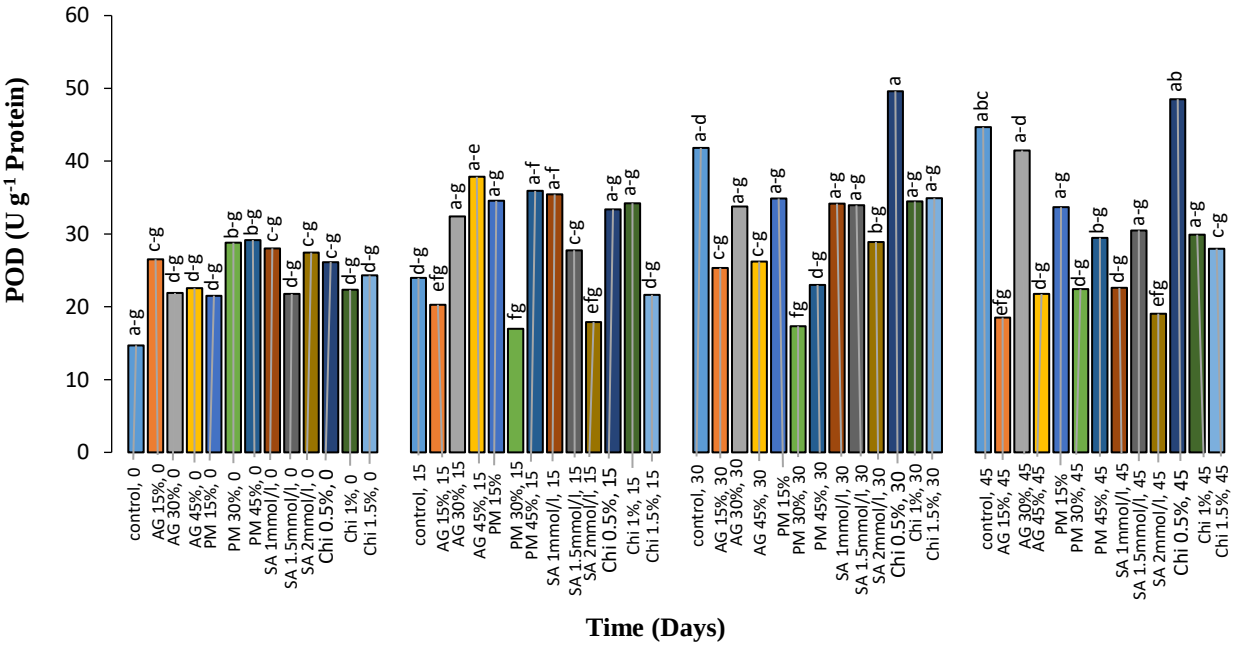

Fig. 3. Effects of salicylic acid (SA), chitosan (Chi), mallow mucilage (MM), and aloe vera gel (AG) treatments on the peroxidase activity of *C. unshiu* tangerines during cold storage at 7 °C for 45 days. Columns with similar letters are not statistically different.

Discussion

Chitosan is a non-toxic, biodegradable, functional, and bio-structured compound with antimicrobial and antifungal activities that effectively control fruit rot. It can form a coating on fruits and vegetables and reduce their respiration rates by regulating carbon dioxide and oxygen permeability. This antimicrobial property results from positively charged amino groups (Hosseini et al., 2009). The number of studies on edible coating application on fresh products has increased considerably in recent years, demonstrating the scientific community's interest in the subject.

Edible coatings were prepared from naturally occurring renewable sources such as

polysaccharides, proteins, lipids, and extracts of various plants rich in bioactive compounds. Edible coatings are thin layers (usually less than 0.3 mm) that cover the surface of fresh fruits and vegetables and can be eaten while remaining undetectable on the tongue. Previous studies on chitosan as a coating agent represent around half of the total investigations on storability efforts aimed at fruits or vegetables, most of which confirm the current findings on chitosan on tangerine storability. Other effective coatings are pectin, *A. vera*, cassava starch, shellac, carnauba wax, and hydroxypropyl methylcellulose. Different methods can help researchers to apply edible coatings on fruits and vegetables, such as dipping, spraying, and brushing followed by air-drying. *A. vera* plant gel is transparent, odorless, and non-viscous, and has high absorbability, which is a very suitable alternative to chemical coatings for postharvest crops. The application of *A. vera* gel significantly enhanced tangerine storability in this research because it contains many compounds, the most important of which are vitamins, amino acids, enzymes, salicylic acid, anthraquinones, and saponins. Meanwhile, the fungicidal properties of salicylic acid (SA) and saponins inhibit the growth and reproduction of fungi to control fungal growth (Choi et al., 2001).

Mallow has a historic application as a nutritional and medicinal agent. Active ingredients in its flowers include mucilage, flavonoid, tannin, phenolic compounds, and anthocyanins (e.g., malvin, delphinidin, and malvidin) (Dehkordi et al., 2003). The current research showed the substantial effect of mallow extract on tangerine storability. This observation can be explained by the molecular interactions between mallow extract substances and fruit skin on the tangerine fruits.

Salicylic acid induces the production of resistance proteins and transmits resistance signals through its effect on the expression of various genes (Durner et al., 1997; Zhang et al., 2008). It also activates antioxidant systems and the ascorbate-glutathione cycle, which induces resistance to cold damage (Wang et al., 2006). Salicylic acid increases the activity of peroxidase and polyphenol oxidase enzymes. Also, it increases the amount of total phenolic compounds while not causing a significant change in the level of soluble solids, vitamin C, titratable acid, firmness, and total weight loss (Yang et al., 2012). Previous studies showed that salicylic acid treatments effectively inhibit blue mold in peach fruits. In addition, the results of this experiment showed that a combination of SA and ultrasound controls a significant amount of decay caused by blue mold in peach fruits, thus significantly affecting the results compared to the treatment with salicylic acid alone. PAL, PPO, and POD enzymes are involved in the phenylpropanoid pathway, which are secondary metabolism substances and cause structural resistance (Schlumbaum et al., 1986; Wallace and Fry, 1999).

Frost damage induced PPO and POD. Salicylic acid causes an increase in polyamines and leads to a reduction in cold damage (Luo et al., 2011). The topical application of SA protects plants against direct oxidative damage (Horvath et al., 2007). SA treatment effectively reduced the respiration of harvested fruits in a concentration-dependent manner (Mo et al., 2008; Srivastava et al., 2000). In addition, high concentrations of SA affect the width of stomata and cause stomata closure. Fresh weight and respiration rate correlated with stomata pore width (Manthe et al., 1992).

Salicylic acid reduces the production of ethylene by preventing the aging process and has a positive effect on the pH of the fruit (Huang et al., 2008). For this reason, it improved the activity of enzymes in this research, and the best treatment was 2 mM salicylic acid.

In relevant research, Qaisarbigi et al. (2015) investigated chitosan effects on disease resistance and radical oxygen species levels in Washington navel oranges (*C. sinensis* L. Osbeck). They reported that chitosan treatment with a concentration of 2% reduced disease incidence and rot diameter compared to the control. The chitosan treatment (2%) increased superoxide dismutase (SOD), peroxidase (POD), hydrogen peroxide (H2O2), and glutathione (GSH) activities. However, it decreased catalase (CAT) and ascorbate (AsA) activities during storage. Ascorbate peroxidase (APX) activity was slightly stimulated in fruits treated with chitosan during storage for 2-3 weeks. These results showed that treatment with chitosan stimulated disease resistance in fruits by regulating H2O2 levels, antioxidant enzyme activity, and the ascorbate-glutathione cycle (Zeng et al., 2010).

The trend of total protein changes obtained in this study is consistent with changes reported in similar research (Baghali et al., 2011). Both showed a reduction in total protein degradation in the treatment process. In a study by Ying et al. (2022), the activity of SOD and CAT enzymes increased during the treatment and storage process, while in this study, there was no significant change in the activity of these enzymes during the treatment. The peroxidase enzyme activity in this study increased and had significant changes during the treatment and storage process, similar to the cases reported earlier (Huang et al., 2008).

C. unshiu tangerines have good marketability in

Iran because of their palatability and marketing time. This variety is suitable for cool subtropical regions and has a wide distribution in temperate zones, Japan, China, Spain, and countries with appropriate climatic conditions (Qazvini et al., 2016). Most fresh fruits and vegetables are known for their therapeutic value and health-promoting activities. Therefore, products without synthetic chemicals are of global interest for having active compounds such as phenolic substances and their antioxidant properties (Suleria et al., 2015). In this regard, healthy, natural, and biodegradable compounds can improve the shelf life of horticultural products and increase health indicators. Compounds compatible with the environment and humans can follow healthy methods for better controlling postharvest diseases (Asghari et al., 2012).

Conclusion

The results showed that applying 2 mM SA was the best treatment for increasing the shelf life of *C. unshiu* tangerines during 45 days of storage, which was more effective than the other treatments. The highest protein content in each period occurred in response to the *A. vera* (15%), mallow mucilage (30%), and 2 mM SA treatments. SOD and CAT activities increased in the fruits on day 15 compared to the beginning of storage. However, this trend decreased from day 15 onward, reaching the lowest level (88.1 U g-1 protein) on the last day of the investigation. A trend of increase occurred in POD activity during the measurement periods, parallel to prolonged storage time. The highest POD activity (mean protein 49.6 U g-1) appeared on day 30 in response to the 0.5% chitosan treatment.

Conflict of Interest

The authors indicate no conflict of interest in this work.

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