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Influence of Pure Oxygen on Nanostructure of Water-Soluble Pectin in Pear (*Pyrus bretschneideri* Rehd cv. Huangguan)

Min Lin[1,2], Yun Deng[1,3*], Chenlong Xiao[1], Mingyang Liu[1], Liwei Zhu[1], Wen Luo[1] and **Hongshun Yang[4]**

[1]Department of Food Science and Engineering, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai, 200240, P.R. China
[2]Engineering College, Guangdong Ocean University, Zhanjiang 524025, P.R. China
[3]SJTU-Bor Luh Food Safety Center, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai, 200240, P. R.China
[4]College of Food Science and Technology, Henan University of Technology, 140 South Songshan Road, Zhengzhou, Henan 450052, China
[*]Author for correspondence; e-mail: y_deng@sjtu.edu.cn; Tel: + 86-21-34206613, Fax: + 86-21-34206918

The effects of pure oxygen and storage time on nanostructure degradation of water-soluble pectin (WSP) in pear (*Pyrus bretschneideri* Rehd cv. Huangguan) were examined during 30 d of storage in air (control) and pure oxygen at 2 °C and 95% relative humidity. Qualitative and quantitative information on WSP polymers was determined by atomic force microscopy (AFM) on days 0, 10 and 30. Results showed that the frequency of small width WSP increased in all treatments over storage time, but was higher in the control, indicating pure oxygen treatment inhibited the degradation of WSP molecules and the aggregate separation. Widths of WSP chains consisted of three basic units with widths of 15.625, 23.438 and 58.594 nm obtained from AFM determination. Data obtained in this study suggest that the fundamental structure of the WSP molecule consists of parallel linkages or intertwists between the basic units.

Key Words: atomic force microscopy, nanostructure, pear, pectin, pure oxygen

Abbreviations: AFM – atomic force microscopy, WSP – water-soluble pectin

INTRODUCTION

Pectin is a complex polysaccharide depicted as a triad component encompassing homogalacturonan (HG), rhamnogalacturonan-I (RGI) and rhamnogalacturonan-II (RGII) (Walter 1991). Pectic substances are the major components of the cell wall and middle lamella of plant tissues that undergo structural changes during the ripening and processing of fruits, resulting in loss of firmness and facilitating the attack of pathogens, thereby increasing fruit postharvest decay and decreasing the quality of fresh fruits (Ahmed and Labavitch 1980; Murayama et al. 2002; Deng et al. 2005).

Pear *(Pyrus bretschneideri* Rehd cv. Huangguan) fruit has attracted great interest in China because of its nutritional and organoleptic properties (Xiao et al. 2010). Changes in the composition of polysaccharides in the cell wall of pear during ripening and processing have been extensively described in the literature (Ahmed and Labavitch 1980; Yoshioka et al. 1992; Murayama et al. 1998, 2002; Wakayabashi et al. 2000) but only a few structural studies have been reported. Tao et al. (2009) found that the cell wall of pears was typically divided into four layers by light microscopy, scanning electron microscopy and transmission electron microscopy: compound middle lamella, secondary wall 1, outer secondary wall 2 and secondary wall 2. Angeles et al. (2004) observed the cell wall surfaces and a three-

dimensional view of the elaborate cell interconnections of pears by confocal laser scanning microscopy. Transmission electron microscopy showed that, at the ultrastructural level, the cell walls consisted of a loose network of cellulose microfibrils in pears (Habibi et al. 2009). There was a greater extent of cell separation with the accumulation of pectic materials in the intercellular space of severely watercored tissues in 'Akibae' than in 'Housui' pears (Chun et al. 2003). Nevertheless, there is little information about the nanostructures of polysaccharides in pear fruits. For a better understanding of texture changes and quality properties of pear fruits during storage, further studies involving structural modifications at the molecular level are necessary.

Atomic force microscopy (AFM) has been one of the most powerful and useful tools for determining the structure of native biomolecules at subnanometer resolution. AFM measures the forces acting between a fine tip of probe and a sample surface, and the movement of the probe to stay at the same probe-sample distance is taken to be the sample topography (Yang et al. 2007). AFM images of individual pectin molecules and polymers were reported in the cell wall material of peach (Yang et al. 2005, 2009), unripe tomatoes (Round et al. 1997, 2001), and tomato and sugar beet (Kirby et al. 2008). The information obtained from AFM can be used to analyze the structural and functional properties of pectin at the submolecular level, and to explain the heterogeneity of a polymer at the molecular level while complementing other established techniques.

Storage at high O_2 levels has recently been suggested as a method to retard senescence and maintain the quality of some fruits and vegetables (Kader and Ben-Yehoshua 2000; Wszelaki and Mitcham 2000; Deng et al. 2005, 2007). The effect of high O_2 level on fruit quality depends not only on the gas composition, but also on storage temperature, variety, tissue structure and fruit aging. Our previous studies have reported changes in firmness, cell wall composition and cell wall hydrolases of grapes stored in 80% O_2 (Deng et al. 2005); however, these results failed to illustrate the structural changes in the composition of the fruit cell wall at the nanoscale level. A knowledge of the structure of the cell wall polysaccharide on the nanoscale level is important to have a better understanding of how polysaccharides assemble or depolymerize during high O_2 storage and how to determine texture and quality attributes of postharvest fruits and vegetables.

There are few studies in the literature concerning alterations in the ultrastructure of 'Huangguan' pear fruit during pure oxygen storage. Also, pear fruits have high quantities of water-soluble pectin (WSP) compared with Na_2CO_3-soluble pectin (SSP) and chelator-soluble pectin (CSP), and there was a high correlation between flesh firmness and water-soluble pectin content (Ahmed and Labavitch 1980; Murayama et al. 2002). It is for these reasons that pear fruits have attracted our attention and, accordingly, the water-soluble pectins were chosen for AFM observation.

The objective of this study was to investigate the nanostructure of pear water-soluble pectin (WSP) and the effect of pure oxygen treatment on it, and to understand the degradation mechanism of pear pectin during storage.

MATERIALS AND METHODS

Raw Materials

Huangguan pear fruits (*Pyrus bretschneideri* Rehd cv. Huangguan) harvested in Hebei, China were supplied by a local distributor and transferred to the cold-chain laboratory of Shanghai Jiao Tong University. The pears were selected on the basis of uniform color, size (255–265 g), hardness and freedom from visible physical and fungal infection; the pears were stored for 2 d at 2 ± 1 °C in approximately 95% relative humidity prior to processing. All chemicals used in this study, e. g., dimethysulphoxide (DMSO), ethanol, acetone and others, were purchased from Sinopharm Chemical Reagent Co. Ltd., Shanghai, China.

Storage Conditions

The fruits were subjected to two pretreatments in a gas flow system. Two replicates of 40 pear slices were sealed in 3.8 L stainless steel tubular containers and one of the following two atmospheric treatments were applied: air or 100 kPa O_2. The initial concentrations of O_2 and CO_2 in the container were monitored by using a gas analyzer CheckmateII (Pbi Dansensor, Ringsted, Denmark). The internal concentration of CO_2 was maintained at <0.05% through a 20 h continuous gas flow per day during the pretreatment period. All treatments were run at 2 ± 1 °C in approximately 95% relative humidity. Samples for analysis from each treatment were taken at days 10 and 30.

Cell Wall Preparation and Water-Soluble Pectin Extraction

Cell wall material of pear flesh was extracted using a modification of the methods described by Deng et al. (2005). Briefly, approximately 40 g of pear fruit was homogenized with 200 mL 100% (v/v) of ethanol and boiled for 30 min. The homogenate was centrifuged at 2000 × *g* for 10 min in an Anke LXJ-II B centrifuge

(Shanghai Anting Scientific Instrument Factory, China); the residue was washed three times with 100 mL of ethanol and then recovered by filtration. The residue was incubated overnight at 4 °C with 50 mL of dimethysulphoxide DMSO : water (9 : 1) to remove starch by filtration. It was then washed with water and transferred to 200 mL of chloroform: ethanol (2:1) mixture. After 10 min, the solid material was recovered by filtration and washed twice with 200 mL of acetone until total whitening. The residue dried at 40 °C was used to extract the water-soluble pectin (WSP).

A 50 mg aliquot of dried residue was homogenized in 40 mL of distilled water and stirred overnight at 20 °C. The homogenate was centrifuged at 10,000 × *g* for 10 min. The supernatant was collected as WSP. The solid residue was suspended in 40 mL distilled water for 1 h with shaking at 20 °C and then centrifuged at 10,000 × *g* for 10 min. The pool of both supernatants was considered as WSP. The samples were stored at - 60 °C before analysis.

WSP Nanostructure Analysis

The WSP microstructure characterization was carried out using atomic force microscopy (AFM, Vecco Metrology Group, Digital Instruments, USA) according to the method of Yang et al. (2005). Frozen WSP samples were allowed to defrost completely to room temperature. WSP solutions were diluted to a series of concentrations (ca. 0.5–30 mg mL^{-1}). Then, for each dilution, about 20 mL of the diluted solution was pipetted rapidly onto the freshly cleaved mica surface. The mica surface was air-dried (1 h) at room temperature in a dust-free enclosure.

Tapping mode was performed using a multimode NanoScope IIIa atomic force microscope equipped with a Si_3N_4 cantilevered scanner with a 12 × 12 µm scan size, a 4 µm vertical range and with a scan speed of 2 Hz. The scanner was adjusted to select and capture smaller images within the region accessible for scanning. The integrity of the AFM tip was verified by imaging a reference standard before imaging each sample (Reed et al. 1998).

Selected images were analyzed by software version 5.30r3sr3. The high quality images were obtained by reducing noise of the samples with the function of flattening of the software. The bright and dark areas in the image corresponded to high and low areas in the viewed WSP chains, respectively. Different scales were used in the vertical and horizontal axes; the height mode was used for the analysis (Yang et al. 2005). Both qualitative and quantitative information could be obtained. The dimensions (width and height) of the pectin molecules were determined by the function of section analysis before the images were flattened. The width and height of a single strand can be calculated by the horizontal distance (W) and the vertical distance (V), respectively. The number of times the chain widths occurred was recorded as the frequency (Fq) (Yang et al. 2005).

Statistics

At least ten AFM images were analyzed for each treatment. The data analyses were carried out using SAS 8.0 statistical data analytical software (SAS, Cary, NC, USA).

RESULTS AND DISCUSSION

WSP Nanostructure of Fresh Pear

Figure 1 shows the tapping mode AFM height images of WSP chains of fresh pear fruits. The color bar legends at the right of the images denoted the full height of the samples scanned. The images demonstrated certain heights and widths of the WSP chains in the pears (Fig. 1A). However, aggregated polymers and polymers were not clearly shown in the AFM images, which may be due to the very small size that can be visualized with the software. Correspondingly, the 3D image of the chains indicated a periodic height fluctuation along the contour of the chains (Fig. 1B). Figure 1 also indicates that the surfaces of WSP in pears appeared knobbly, similar to WSP from peaches observed by Yang et al. (2009). Cross-section analysis of pectin chains and strands is also presented in Figure 2. For any AFM image (such as Fig. 2C), a cross-sectional line can be drawn across the image at any place and direction; the height profile of the cross-section is shown in Figure 2A. The software allowed a fixed, movable line to be drawn across a section. Figure 2B demonstrates the results of the portion of the cross-section between the two selected cursors. The power spectrum along the cross-section is shown in Figure 2D. Up to three pairs of cursors can be applied on the line section (Fig. 2E).

The quantitative results of WSP are shown in Table 1. W denoted the width of WSP chains. The number of times that a special chain width occurred was recorded as Fq. In the fresh pear fruits, there was a high frequency of appearance for a chain width of 39.063 nm, a moderate frequency for a chain width of 58.594 nm, and a low frequency for widths of 78.125 and 97.656 nm.

Change in Nanostructure of WSP during Pure Oxygen Storage

The WSP quantitative parameters at different storage conditions are shown in Figure 3 and Table 2. Compared with air storage, the frequency of smaller W value chain

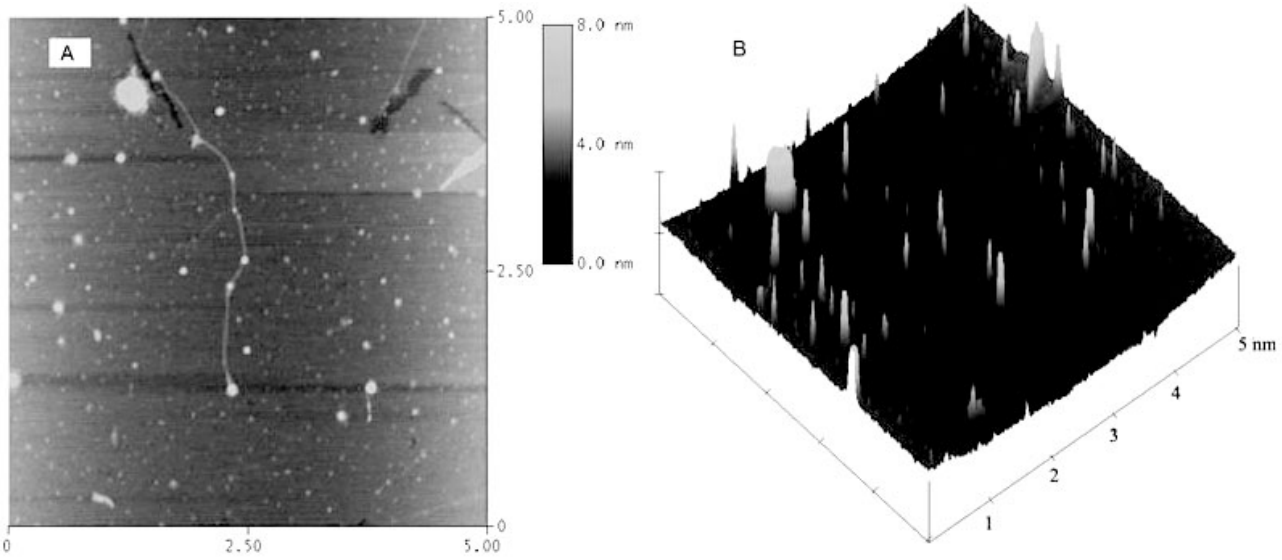

Fig. 1. AFM images of water-soluble pectin (WSP) extracted from fresh pears. (A) WSP image from pears and (B) corresponding 3D image of A.

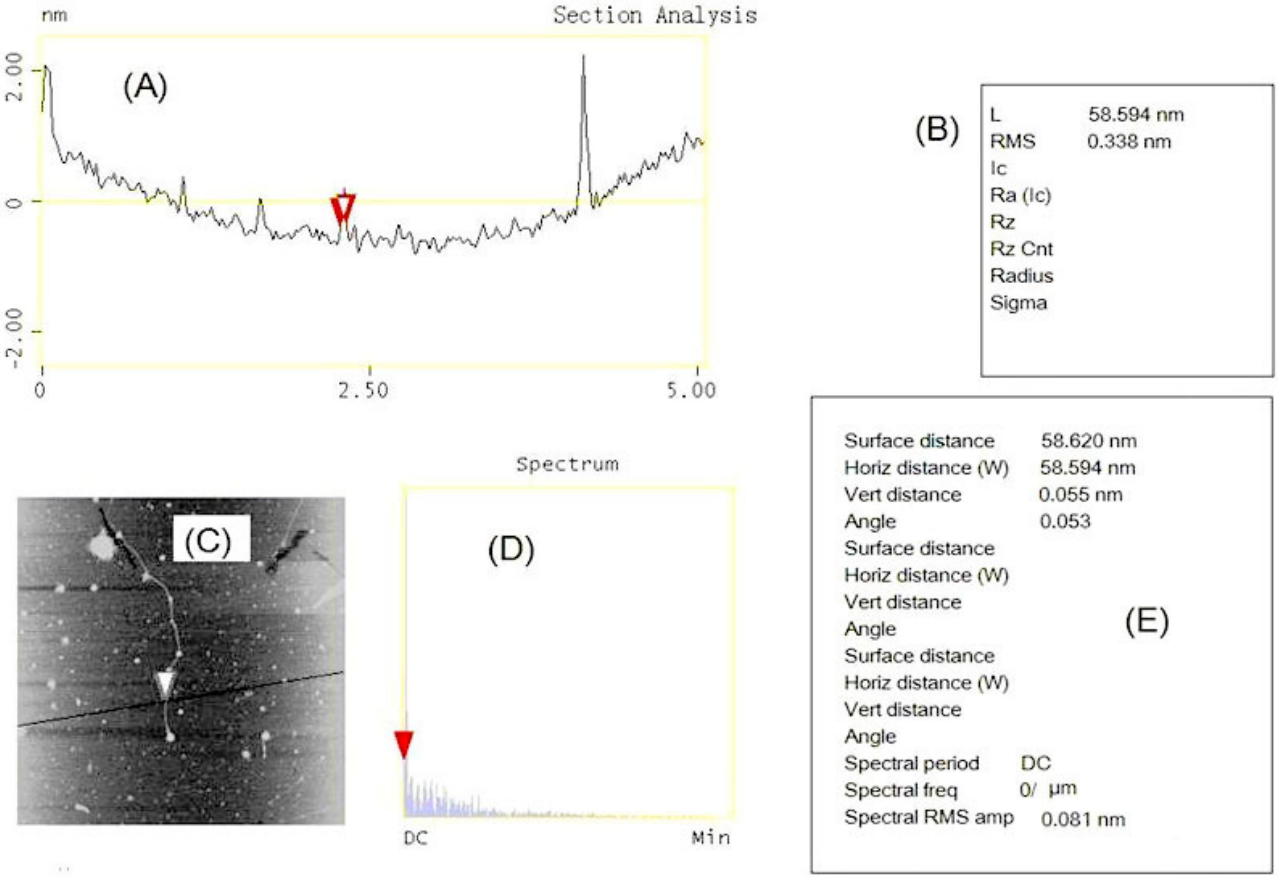

Fig. 2. Cross-section analysis of WSP chains: (A) height profile along a cross-sectional line, (B) dimension measurements of the portion of the cross-section between the two red cursors, (C) a sample of AFM image, (D) power spectrum along the cross-section and (E) dimension measurements of up to three pairs of cursors.

Table 1. Frequency (Fq) and vertical distances (V) of water-soluble pectin (WSP) chain widths (W) in fresh pear flesh[a].

W (nm)	Fq	V(nm)
97.656	1	0.035±0.000
78.125	1	0.183±0.000
58.594	4	0.250±0.100
39.063	7	0.199±0.125

[a]W - peak width of the half height of WSP chains; Fq - number of times particular chain widths were observed; V - height of WSP chains
The heights of WSP chains are expressed as mean ± standard deviation.

widths was lower under pure oxygen storage condition. For example, by day 10, widths of 58.594 and 39.063 nm appeared five times and once, respectively, in pears stored in pure oxygen. However, widths of 39.063, 15.625 and 23.438 nm were presented twice, twice and once, respectively, in air-stored samples. The trends were analogous on day 30. These results suggested that pure oxygen storage delayed the degradation of the WSP molecule and were similar to results on peaches stored under a controlled atmosphere of 2% O_2 + 10% CO_2 (Yang et al. 2005).

Based on section analysis, the chain widths of WSP reflected several basic units: 58.594, 23.438 and 15.625 nm. The other chain widths can be composed of these basic units. For instance, the sum of 58.594 and 39.063

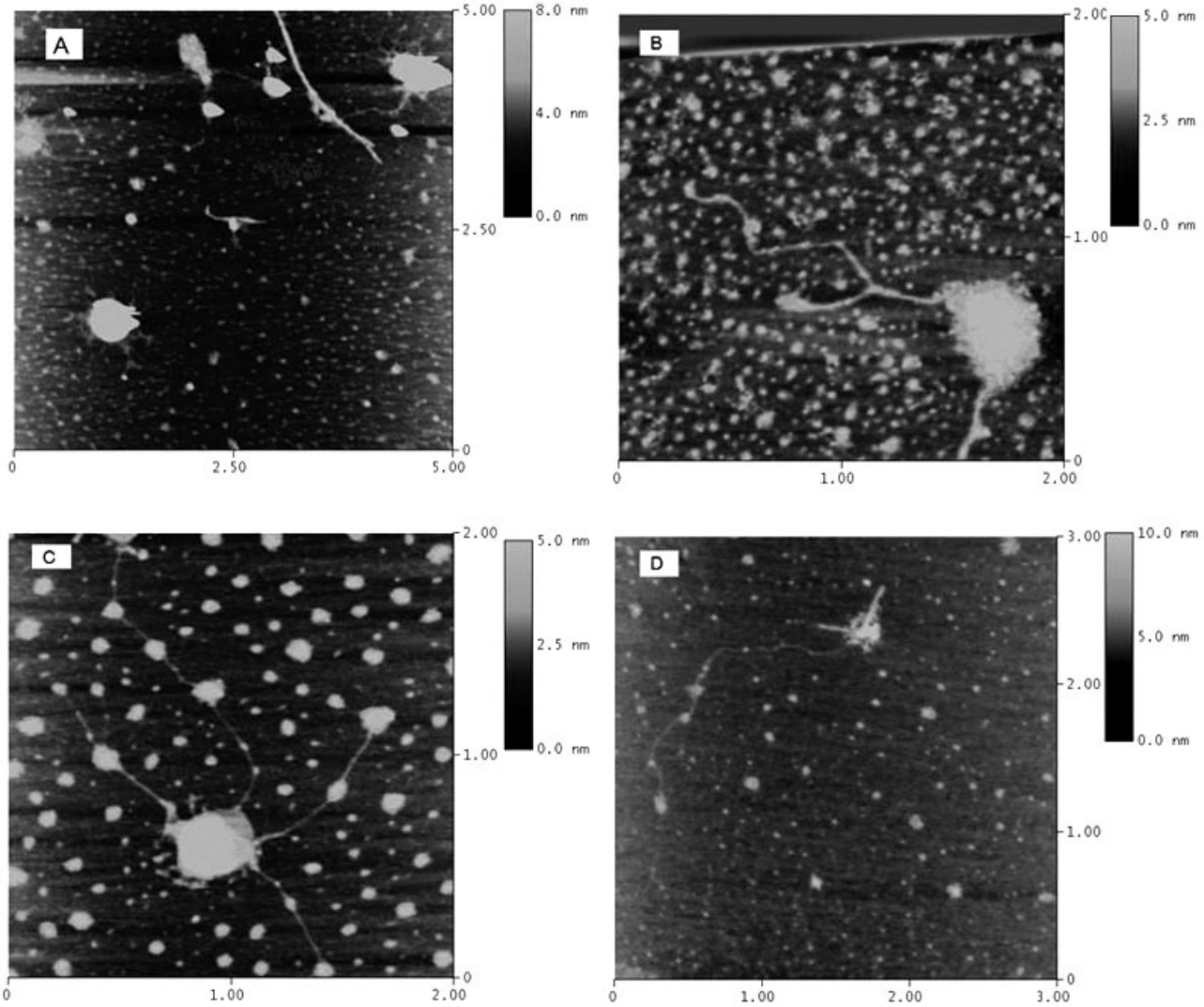

Fig. 3. AFM images of WSP from pears stored at different conditions: (A) WSP from pears stored in air for 10 d, (B) WSP from pears stored in air for 30 d, (C) WSP from pears stored in pure oxygen for 10 d and (D) WSP from pears stored in pure oxygen for 30 d.

Table 2. Frequency (Fq) and vertical distances (V) of WSP chain widths (W) under different storage conditions[a].

	10 d				30 d			
	Pure Oxygen		Air		Pure Oxygen		Air	
W (nm)	Fq	V (nm)	Fq	V (nm)	Fq	V (nm)	Fq	V (nm)
58.594	5	1.150±0.715	-	-	3	0.633±0.483	-	-
39.063	1	1.615±0.000	2	0.215±0.104	2	0.216±0.186	1	3.306±0.000
31.250	-	-	-	-	-	-	1	0.440±0.000
23.438	-	-	1	0.089±0.000	1	0.231±0.000	3	0.282±0.181
15.625	-	-	2	0.151±0.062	-	-		-

[a]W - peak width of the half height of WSP chains; Fq - number of times particular chain widths were observed; V - height of WSP chains.
The heights of WSP chains are expressed as mean ± standard deviation.

nm is approximately 97.656 nm, and the sum of 15.625 and 23.438 nm is 39.063 nm. The widths 78.125 nm and 31.25 nm were approximately double the widths 39.063 nm and 15.625 nm, respectively. The WSP widths of pear fruits obtained in the current study were comparable to those of 'Jinxiu' yellow peaches (Yang et al. 2005). These findings further confirmed that these fruits have similar structural characteristics of pectic substances. In addition, the aggregates were observed even at low concentrations where few single chains were viewed from AFM images due to moisture evaporation during drying and strong intermolecular interactions (Round et al. 1997, 2001; Yang et al. 2009). Therefore, these aggregated polymers and polymers were excluded from the statistical analysis.

Mechanism of Effect of Pure Oxygen on WSP Structure Changes

The current results also showed that the W values of WSP chains of pears generally decreased with an increase in storage time, consistent with previous reports on the storage of peaches (Yang et al. 2005) and the ripening of papaya fruits (Manrique and Laiolo 2004). These results suggest that the reduction in chain width is a general feature of WSP structure changes, which may be related to solubilization and depolymerization of the middle lamella. Pectin is the main polysaccharide constituent of the middle lamella (Manrique and Laiolo 2004). The chain widths of pear WSP are composed of three basic units of 58.594, 23.438 and 15.625 nm, which constitute the structure of the WSP molecule by parallel linkages or intertwists between the basic units (Yang et al. 2005). Generally, changes in pectin structures are accompanied by softening of most of the fruit flesh (Ketsa et al. 1999). A wide range of endogenous and exogenous enzymes such as pectinmethylesterases (PME) and polygalacturonases (PG) synergistically modify and degrade the smooth and hairy regions of pectin (Walter 1991). The degradation of cell wall pectins during fruit ripening and processing is associated with the degradation of the chains by β-elimination and the increased reaction time for the neutral protocol led to increased cleavage (Morris et al. 2002). Sila et al. (2006) also found in carrot pectin isolates that sensitivity to the β-elimination reaction is high in the water-soluble pectin fraction as opposed to chelator- and alkali-soluble fractions. The increase in WSP is usually due to solubilization of the esterified pectin by some other enzymes or by de novo synthesis although there was a low correlation between PME activity and pectin solubilization, serving partial demethylation of HG within the plant cell wall which, in turn, can form a substrate for PG activity (Walter 1991). Deng et al. (2005) observed that the lower level of WSP in 80% O_2-stressed grapes was correlated with delayed softening and inhibited PG activity, reducing the degradation and depolymerization of pectin substances in contrast to air.

Fruit softening was more related to pectin solubilization and depolymerization in comparison with cellulose and hemicellulose catabolism (Rosli et al. 2004; Deng et al. 2006). Furthermore, Wakayabashi et al. (2000) found that the increase in WSP content during avocado ripening was at the expense of decreasing sodium carbonate-soluble fractions. The WSP, SSP and hemicelluloses in table grapes seemed to have major structural roles in maintaining the firmness and preventing the deterioration of fruits stored in high O_2 (Deng et al. 2005). These facts may indicate that pectin degradation alone could be insufficient to cause fruit softening until other cell wall components were partially degraded. Therefore,

further research is required to investigate the effects of pure oxygen on the compositional alterations of cell wall materials and the nanostructural changes of chelate-soluble pectin, sodium carbonate-soluble pectin, cellulose and hemicellulose in pear fruits.

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