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Evaluation of pectin nanostructure by atomic force microscopy in blanched carrot

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ABSTRACT

Low-temperature blanching (LTB) is a very attractive method for preserving the texture of fresh vegetables; however, the molecular basis of this treatment remains to be elucidated. The mechanism of textural change in carrots by LTB was investigated. Carrot samples were treated using three kinds of blanching: high-temperature blanching (HTB), LTB, and LTB followed by HTB. Fresh and treated samples were evaluated conventionally for firmness, galacturonic acid content, and the activity of pectin methylesterase. In addition, Raman microscopy was also used to describe the pectin distributions in the samples. However, the Raman maps did not show obvious differences among the samples, but they coincided with the results of galacturonic acid content. Furthermore, the nanostructures of water-soluble pectin (WSP), chelator-soluble pectin (CSP), and diluted alkali-soluble pectin (DASP) fractions were observed by atomic force microscopy. The median length of CSP molecules was shortened from 44.76 nm to 12.57 nm by HTB. On the other hand, LTB induced elongation of CSP molecules (median length: 58.12 nm) and maintaining ability of DASP to form a regular network on mica.

1. Introduction

Frozen vegetables are popular all over the world because of their usefulness for cooking and their high storage stability. To manufacture frozen products, a heat treatment called blanching is conducted at higher than 80 °C prior to freezing. The main objective of this treatment is to inactivate enzymes that cause deterioration of the product during storage (Imaizumi, Tanaka, & Uchino, 2017). However, excessive blanching causes serious degradation of tissue structure due to β-elimination of pectin. Tissue structure is strongly related to texture, and thus an important factor determining consumer opinion of frozen vegetables. Hence, clarification of the textural changes during blanching and other processing steps is important and can only be accomplished by detailed investigation of plant tissue structure.

Pectic polysaccharides exist in the cell wall, especially in the middle lamella, and they contribute to cell–cell adhesion and mechanical strength. Pectins are mainly composed of D-galacturonic acid (GalA) joined in chains by α-(1 → 4) glycosidic linkages. GalAs have free or methylated carboxyl groups and they are mixed in the chain. Highly methylated pectin is more easily depolymerized by β-elimination than pectin with a lower degree of methylation (Sila et al., 2009). In addition, pectin is often cross-linked to neighboring pectin chains via divalent ions such as Ca^{2+}, and the cross-linkages contribute to texture improvement. Recently, many studies have examined low-temperature blanching (LTB), which promotes the activation of pectin methylesterase (PME) (Abu-Ghannam & Crowley, 2006; de Souza; Ni, Lin, & Barrett, 2005; de Souza Silva et al., 2011; Walter, Truong, Simunovic, & McFeeters, 2003). In this treatment, vegetables are immersed in hot water at 50–80 °C. Because pectin polysaccharides are demethylated by PME, LTB enables inhibition of softening during subsequent high-temperature blanching (HTB) and cooking treatment. Additionally, free pectic carboxyl groups provide a greater opportunity to make ionic cross-links (Canet, Alvarez, Luna, Fernández, & Tortosa, 2005; Christiaens et al., 2011b; Wu & Chang,

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2007). Therefore, LTB is expected to improve the texture of frozen vegetables.

Mechanical tests have been used to confirm the effects of LTB on many vegetables, such as potato (Abu-Ghannam & Crowley, 2006), carrot (Lemmens et al., 2009), and broccoli (Christiaens et al., 2011a). Analyzing pectin content, composition, and PME activity can be used to evaluate the effects, and such results have been reported in previous studies (Ando, Hagiwara, & Nabetani, 2017; Christiaens et al., 2011a; Sila, Smout, Vu, & Hendrickx, 2006). From the structural aspect, Imaizumi, Tanaka, Sato, Yoshida, and Uchino (2016, 2015) investigated changes in cell membrane properties and porosity during hot water treatment at 50–70 °C. However, the effects of LTB are still unclear, especially with regard to the structure of the cell wall.

Microscopic technology has developed rapidly over the past few decades, and it has spread to analyzing the distribution of chemical components and the evaluation of nanostructures. To date, Chylińska, Szymańska-Chargot, and Zdunek (2014, 2016) observed the distributions of cell wall components including pectin in apple and tomato tissues by Raman microscopy. Additionally, nanostructures of pectin extracted from pear fruit were also observed by atomic force microscopy (AFM) (Kozioł, Cybulska, Pieczywek, & Zdunek, 2017; Zdunek, Kozioł, Cybulska, Lekka, & Pieczywek, 2016). However, these studies focused on fresh fruit and vegetables. We anticipated that these imaging techniques could contribute to clarify the mechanism of textural change during blanching treatments. Therefore, this study evaluated firmness, pectin content, and PME activity of blanched carrots, and also analyzed pectin distribution and structure using Raman microscopy and AFM.

2. Material and methods

2.1. Sample preparation

The carrot variety 'Nerac' (*Daucus carota* subsp. *sativus*) was used in this study. The carrots were cut into cylinders (15 mm diameter and 10 mm height) using a cork borer and a knife. Fig. 1 shows a schematic diagram of the experimental procedures. The samples were divided into four groups consisting of 40 samples each. One group was used in subsequent investigations without blanching treatment (Fresh). The remaining three groups were processed by HTB (samples were immersed in hot water at 95 °C for 2 min), LTB (samples were immersed in hot water at 60 °C for 60 min), or LTB followed by HTB (LTB + HTB). The heating temperature and time was determined from a preliminary experiment. After each treatment, the samples were immediately cooled in cold water for 2 min.

For Raman microspectroscopy, three samples from each groups were sliced using a vibratome (VT 1000S, LEICA) in the equatorial direction into slices 150 μm thick. The rest were used first for firmness determination and then disrupted for PME assays and the collection of cell wall material.

2.2. Firmness determination

The firmness of the sample was measured using a puncture test with a universal testing machine (Lloyd LRX, Lloyd Instruments) equipped with a 3 mm diameter probe. The sample was penetrated to 5 mm depth at a speed of 10 mm min^{-1}. Firmness was defined as the maximum force value observed in the force-penetration curve. The test was performed on 10 individual samples for each condition.

2.3. PME activity assay

PME extraction and determination of activity were conducted in accordance with the method described by Mierczyńska, Cybulska, Pieczywek, and Zdunek (2015). To extract PME, 3 g of the disrupted sample, which was preliminary frozen in a freezer at −80 °C, was homogenized with cold 8.8% (*w/v*) NaCl solution. Then, the homogenate was centrifuged (6000 rpm, 25 min), and the supernatant was adjusted to pH 7.5 with 1 M NaOH and then used to assay PME activity.

To determine PME activity, 1 mL of crude extract was mixed with 4 mL of 1% (*w/v*) citrus pectin in 0.1 M NaCl and titrated with 0.01 M NaOH to maintain pH 7.5 while incubating at 37 °C for 1 h. One unit of activity was expressed as 1 mmol NaOH g^{-1} min^{-1}. Measurements were performed six times for each condition.

2.4. Extraction of pectin fractions

Pectin fractions were prepared in accordance with Kozioł et al. (2017) with a few modifications. First, cell wall material (CWM) of the samples was collected from 50 g of the disrupted sample using the alcohol insoluble solids methods (Chylińska, Szymańska-Chargot, & Zdunek, 2016; Mierczyńska et al., 2015; Renard, 2005). CWM (0.1 g) was weighed accurately and stirred with 9 mL distilled water overnight at room temperature. The mixture was centrifuged (6000 rpm, 15 min) and the supernatant was collected as the water soluble pectin (WSP) fraction. Next, 10 mL of CDTA (0.05 M, pH 6.5) was added to the residue and stirred overnight at room temperature. The mixture was centrifuged (6000 rpm, 15 min) and the supernatant was collected as the chelator-soluble pectin (CSP) fraction. The residue was then stirred with 10 mL of 0.05 M Na_2CO_3 + 20 mM $NaBH_4$ overnight and centrifuged (6000 rpm, 15 min). The supernatant was collected as the diluted alkali soluble pectin (DASP) fraction.

2.5. Determination of galacturonic acid content

Galacturonic acid (GalA) content in pectin fractions were determined using a San^{++} Continuous Flow Analyzer (Skalar) (Szymańska-Chargot et al., 2016; Zdunek, Kozioł, Pieczywek, & Cybulska, 2014). The sample solution containing pectin was completely decomposed in 96% H_2SO_4 with $Na_2B_4O_7 \cdot 10H_2O$, and the products were transformed to furfural derivatives. The derivatives were reacted with 3-phenyl phenol to form a colored dye, which was measured at 530 nm. The pectin content was calculated as GalA content per CWM weight (mg g^{-1}). The measurement was replicated six times.

2.6. Raman imaging

Raman imaging was conducted using a DXR Raman Microscope (Thermo Scientific), equipped with a diode-pumped, solid state green laser (λ = 532 nm) with a maximum power of 10.0 mW, a diffraction grating of 900 lines per mm, and a pinhole confocal aperture of 25 μm. The Raman light was detected using an air-cooled CCD detector with a spectral resolution of 4 cm^{-1}. A 20×/0.40NA objective was used. Raman maps were recorded with a spatial resolution of 2 μm × 2 μm in the XY plane. The integration time was set as 15 s and fixed for each scan. A single spectrum at each point was recorded for an average of 6 scans. The most prominent Raman marker band for the identification of pectin polysaccharides was centered at 852 cm^{-1}, which is due to vibration of the α-glycosidic bonds in pectin (Szymańska-Chargot et al., 2016). Thus, pectin distribution was expressed as maps of Raman intensity at 852 cm^{-1}.