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Investigating the role of pectin in carrot cell wall changes during thermal processing: A microscopic approach

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ABSTRACT

Changes in cell wall integrity upon thermal treatment were assessed in carrot cells using novel microscopic approaches using Congo red and different cell wall polysaccharide specific probes (JIM7, LM10, LM11, LM15, LM21, LM22 and CBM3a). Strong thermal processing induced an increased accessibility of cellulose and hemicelluloses by Congo red and the specific probes, except galactomannan, which detection was not affected by the thermal processing. Detection of pectin by JIM7 disappeared upon thermal processing, pointing at the leaching out effect of pectin from cell wall due to β-elimination. Changes observed after thermal processing were moreover similar to changes observed after enzymatic degradation of pectin, and a combination of thermal and pectinases treatments did not cause additional effects. These observations indicated that the presence of native pectin is the main factor governing cell wall polysaccharides accessibility and overall cell wall integrity in carrot, which can be modulated through thermal processing.

Industrial relevance: This work provides new evidences on the specific role of pectin in carrot cell wall integrity, more specifically on how it can be modulated by thermal processing. New light microscopy approaches to assess changes in cell wall integrity are presented. This information is important for food industry since plant cell wall acts as a structural barrier for the release of carotenoids and other micronutrients in plant-based food products.

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1. Introduction

Plant cells are surrounded by a cell wall, a structure which confers mechanical protection and structural support to cells and at the same time enables the interaction between neighbouring cells and with the surrounding medium. The primary cell wall and the middle lamella are basically composed of polysaccharides, but also contain minor amounts of glycoproteins and phenolic compounds (Sandhu, Randhawa, & Dhugga, 2009). Cellulose is a polysaccharide composed of β(1 → 4) linked D-glucoses and is assembled into long microfibrils (Thomas et al., 2013). Other glucosyl polysaccharides, known as hemicelluloses, are characterised by the fact that they are soluble in alkali but cannot be solubilised using chelating agents. The hemicellulose group includes xyloglucan, xylans (arabinoxylan, glucuronoxylan and glucuronoarabinoxylan), mannans (glucomannan, galactomannan and galactoglucomannan) and arabinogalactan (O'Neill & York, 2003). A third group of cell wall polysaccharides are known as pectic polysaccharides or pectin. It is a heterogeneous group of polysaccharides that have α(1 → 4) linked D-galacturonic acid unities in the structure. Homogalacturonan is the major pectic polysaccharide, consisting of linear polymers of galacturonic acid with varying pattern and degree of methyl esterification. Rhamnogalacturonan I is another pectic polysaccharide with a backbone composed of galacturonic acid and rhamnose with linear and branched side chains of galactose and arabinose residues. Other pectic polysaccharides have a backbone of galacturonic acid with side chains containing rhamnose and different other neutral sugars (rhamnogalacturonan II and other substituted galacturonans) (Willats, McCartney, Mackie, & Knox, 2001).

The cell wall has been described as a system of two independent but interacting networks, one formed of cellulose cross-linked with hemicelluloses and another composed of pectin (O'Neill & York, 2003). However, other studies have found evidence of strong interactions between pectin and the other cell wall compounds (Thompson & Fry, 2000; Wang, Zabotina, & Hong, 2012), and propose a new paradigm for the cell wall structure, with a single network of pectin, cellulose and hemicellulose. Anyhow, it is well known that pectic polysaccharides create an intricate structure reinforced with pectin–pectin and ionic interactions (Morris, Ring, MacDougall, & Wilson, 2003). It is believed that cellulose and hemicelluloses, within the cell wall, function as a load-bearing structure, while pectin controls the porosity of the cell wall, preventing the free diffusion of macromolecules such as enzymes. The exact polymer interactions within this network however still

Abbreviations: CBM, carbohydrate-binding module; CR, Congo red; FITC, fluorescein isothiocyanate; HSI, hue, saturation and intensity; MPBS, milk-phosphate buffered saline; NA, numerical aperture; PBS, phosphate buffered saline; PG, polygalacturonanase; PME, pectinmethylesterase.

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remain unclear (Agoda-Tandjawa, Durand, Gaillard, Garnier, & Doublier, 2012).

The chemical fine-structure of the cell wall and the interactions within the polysaccharide network considerably affect the functional properties of vegetable-based food products, such as texture and rheology (De Roeck, Sila, Duvetter, Van Loey, & Hendrickx, 2008), as well as may determine to what extent it acts as a barrier to the release of lipophilic micronutrients from inside cells, such as carotenoids (Morris et al., 2003). Carrot cells contain β-carotene, which has interesting health-promoting properties due to their antioxidant and provitamin A activity (Nishino, Murakoshi, Tokuda, & Satomi, 2009; Rao & Rao, 2007). The barrier properties of the carrot cell wall are therefore an important issue from the nutritional point of view, since carotenoids need to be bioaccessible (i.e., they must be released from the food matrix and incorporated into micelles during digestion before being absorbed) prior to exhibit their benefits for health. The importance of cell walls for the bioaccessibility of carrot carotenoids has been shown in this context (Lemmens, Van Buggenhout, Van Loey, & Hendrickx, 2010; Moelants, Lemmens, Vandebroeck, Van Buggenhout, Van Loeyet al., 2012; Palmero et al., 2013); however, insight into the role of each type of polysaccharide in the structure, integrity and consequently, in its barrier properties and permeability of the cell wall is currently lacking.

By food processing, it is possible to change the microstructure of the cell wall, and food processes may be designed to improve the rheological or nutritional properties of food products (Jolie et al., 2012; Van Buggenhout et al., 2012). Thermal treatment, for instance, is known to affect cell wall integrity and carotenoid bioaccessibility in carrots (Knockaert, Lemmens, Van Buggenhout, Hendrickx, & Van Loey, 2012; Lemmens, Colle, Van Buggenhout, Van Loey, & Hendrickx, 2011). The relation between thermal treatment conditions, cell wall microstructural changes and how these changes are related to changes in the cell wall barrier properties is however still not well understood (Van Buggenhout et al., 2010). Several recently-developed molecular probes that allow specific localization and visualization of certain cell wall polysaccharides (Wallace & Anderson, 2012) could help us to understand these changes. The use of antibodies in combination with fluorescence light microscopy, which is known as immunofluorescence, has been successfully employed to recognise polysaccharides and their exact localization in plant tissue-based preparations (Christiaens et al., 2011; Knox, 1997; Willats et al., 2000). The objective of the present work was to use these probes to investigate the specific role of pectin in the cell wall of carrot tissue, in the context of thermally-induced changes of cell wall integrity. In addition, it was evaluated whether Congo red (CR), a dye traditionally used for cell wall staining (Wood, Fulcher, & Stone, 1983), could provide an alternative for this immunofluorescence approach. Next to thermal treatments, enzyme (pectin methylesterase, PME, and polygalacturonanase, PG) treatments were applied in order to check whether thermally-induced cell wall changes were indeed related to changes in the pectic polymers.

2. Materials and methods

2.1. Sample preparation and thermal treatment

Fresh carrots (*Daucus carota* L. cv. Nerac), purchased in a local retailer, were peeled, cut into pieces and blended in a kitchen blender for 1 min with 1 volume of deionised water. Small cell cluster fractions were isolated from the puree by wet sieving, using a sieve shaker (Retsch, Aartselaar, Belgium) equipped with 40 and 250 μm pore size sieves. Since microscope observations showed that the average carrot cell size is 61.4 (±15.2) μm, the samples prepared for this study contained particles with a limited number of cells (estimated between 1 and 100 cells). A single batch of homogeneous puree obtained from 2 kg of raw carrot was used for the thermal and enzyme treatment, which was performed once.

Carrot samples, consisting of carrot small cell clusters immersed in deionised water as a heating medium, were inserted into stainless steel tubes (3 mm inner diameter) and plunged for 25 min in an oil bath preheated at 95, 105, 115 and 125 °C.

2.2. Enzyme treatment

The applied enzyme treatment consisted of a two-step treatment with two purified pectinases: a first incubation step with pectinmethylesterase (PME, EC 3.1.1.11) from *Aspergillus aculeatus* (Novozymes, Bagsværd, Denmark), and a second incubation with polygalacturonase M2 (PG, EC 3.2.1.15) from *A. aculeatus* (Megazyme, Bray, Ireland). PME catalyses the de-esterification of methylated homogalacturonan, which once de-esterified, is further depolymerised by PG. Carrot samples were incubated in centrifuge tubes containing pH 4.5 100 mM sodium phosphate buffer medium and 7 U PME per g of carrot puree. After 2 h of incubation, the tubes were centrifuged (22,100 ×*g*, 10 min), the supernatant removed and the carrot cell clusters were resuspended in pH 5.5 100 mM sodium phosphate buffer and centrifuged (22,100 ×*g*, 10 min). Samples were further washed in phosphate buffer until pH 5.5 was reached. Samples were thereafter added with 50 U PG per g of carrot puree and incubated overnight. All reactions were performed at room temperature while end-over-end rotated. Non enzyme-treated samples also were incubated under these conditions, but without adding the enzymes. Samples were stored in 70% (v/v) ethanol at 4 °C until microscope analysis, which was within a month.

2.3. Congo red cell wall staining

Samples were incubated for 2 h in 100 $mg \cdot l^{-1}$ CR (Sigma-Aldrich, Diegem, Belgium) at room temperature, were washed three times with deionised water and placed on a slide for light microscopic observation in bright field and fluorescence modes. Samples were examined using an Olympus BX-41 light microscope (Olympus, Tokyo, Japan) equipped with epifluorescence illumination (EXFO, Hants, UK) and an Olympus XC50 CCD camera (Olympus, Tokyo, Japan). Micrographs of parenchyma cell clusters were taken at 40× (NA 0.75). A 530–550 nm band-pass exciter filter, a 575 nm barrier filter and a 570 nm dichroic mirror (Olympus, Tokyo Japan) were used for imaging in fluorescence mode. The microscope and camera settings were kept constant upon comparing different samples. Eight pictures were taken per sample, from which the most representative was selected as image to display.

According to Castleman (1998), 'hue' and 'saturation' are two parameters extracted from the hue, saturation and intensity (HSI) colour space which are normally depending on the light-reactive properties of the dye, while 'intensity' is a third HSI parameter seriously affected by the illumination settings. According to this, the 'sum saturation' value was used to estimate the intensity of the staining in CR experiments using bright field illumination. The measurement was performed in 200 μm^2 squares of ten random cells, clearly not overlaid by other cells. The measurements were done using Cell* v. 2.4 software (Olympus, Tokyo, Japan).

2.4. Cell wall polysaccharide immunolabelling

Samples were washed in 70% ethanol in order to remove carotenoids and minimise autofluorescence prior the immunolabelling. The anti-homogalacturonan antibody JIM7 (Plant Probes, Leeds, UK) was used to label pectin. For hemicellulose immunolabelling, five antibodies kindly donated by Prof. Paul Knox from University of Leeds were employed: LM10 (xylan), LM11 (xylan, arabinoxylan), LM15 (xyloglucan), LM21 (mannan, glucomannan and galactomannan) and LM22 (mannan and glucomannan). Hybridoma supernatants were diluted 1:5 in milk-phosphate-buffered saline (MPBS, 140 mM NaCl − 2.7 mM KCl − 8.0 mM Na_2HPO_4, pH 7.4, containing 3% milk

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