Food Chemistry 213 (2016) 402–409

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Modification of cell wall polysaccharides during retting of cassava roots

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ARTICLE INFO

Article history:
Received 25 March 2016
Received in revised form 24 June 2016
Accepted 29 June 2016
Available online 30 June 2016

Keywords:
Softening
Pectic polysaccharides
Galactan
Hemicelluloses
Cell wall degrading enzymes
Immunolabelling

ABSTRACT

Retting is an important step in traditional cassava processing that involves tissue softening of the roots to transform the cassava into flour and various food products. The tissue softening that occurs during retting was attributed to the degradation of cell wall pectins through the action of pectin-methylesterase and pectate-lyase that possibly originated from a microbial source or the cassava plant itself. Changes in cell wall composition were investigated during retting using chemical analysis, specific glycanase degradation and immuno-labelling of cell wall polysaccharides. Pectic 1,4-β-D-galactan was the main cell wall polysaccharide affected during the retting of cassava roots. This result suggested that better control of pectic galactan degradation and a better understanding of the degradation mechanism by endogenous *endo*-galactanase and/or exogenous microbial enzymes might contribute to improve the texture properties of cassava products.

1. Introduction

Cassava is one of the most important food crops in tropical countries and is used for human consumption in a large variety of food preparations. Retting of cassava roots is an integral part of cassava transformation for human consumption in sub-Saharan Africa. It is a conventional process that consists of soaking cassava roots in water for a 3–7 day period, during which the roots soften and other chemical modifications occur. Retting of cassava root is characterized by the degradation of endogenous cyanide compounds involved in the development of serious neurological disorders (Cardoso et al., 2005; Tshala-Katumbay et al., 2013). The retting process is also characterized by the production of organic acids and the softening of the root, which are both required to obtain products with acceptable organoleptic properties for consumers.

The softening of the root is an important step for the production of high quality cassava products. Many investigations have focused on the role of starch in determining the cooking time and final texture of cassava roots, but no clear conclusions have been reached (Padonou, Mestres, & Nago, 2005; Safo-Kantanka & Owusu-Nipah, 1992). In the cases of many fruits and vegetables, the softening observed during cooking is influenced by the cell wall of the plant tissues (Waldron, Parker, & Smith, 2003). The final texture is likely to be dependent on the properties of cell wall components, their composition, proportions and interactions in relation to the different levels of structure (Waldron et al., 2003), including the molecular mechanism by which cells adhere to each other. In certain cassava cultivars, root tissues fail to soften even after a prolonged cooking time, which may be related to a higher level of chelator-insoluble pectic polysaccharides (Favaro et al., 2008). The detection of pectinolytic activities during spontaneous retting and the lack of cellulolytic activity supports the hypothesis that pectin might be involved in the root softening process (Ampe & Brauman, 1995). Pectinases detected during retting include three enzymes: pectin methylesterase, pectin/pectate lyase and polygalacturonase. It is unclear if these enzymes have plant and/or bacterial origins (Brauman, Kéléké, Malonga, Miambi, & Ampe, 1996). The use of fermentation starter exhibiting cell wall degrading enzymes activities have been reported to modify the texture of cassava products (Amoa-Awua & Jakobsen, 1995; Amoa-Awua, Frisvad, Sefa-Dedeh, & Jakobsen, 1997; Obilie, Tano-Debrah, & Amoa-Awua, 2003). In addition, different studies have suggested that the enzymatic hydrolysis of cell wall polysaccharides improves the detoxification of cassava roots (Mkpong, Yan,

Abbreviations: GalA, galacturonic acid; Ara, arabinose; Gal, galactose; Rha, rhamnose; HG, homogalacturonan; RGI, rhamnogalacturonan I; CWMs, cell wall materials.

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http://dx.doi.org/10.1016/j.foodchem.2016.06.107
0308-8146/

Chism, & Sayre, 1990; Santana, Vásquez, Matehus, & Aldao, 2002; Sornyotha, Kyu, & Ratanakhanokchai, 2010).

Until now, most of the studies related to the softening of cassava roots have focused on understanding enzyme activities and microbial strains involved in the retting process, but these studies have not precisely linked softening with cell wall component modifications. Precise knowledge of cell wall polysaccharides and cell wall architecture of the roots is essential for understanding the texture modifications occurring during the retting process. In this study, chemical, enzymatic and microscopy methods were combined to analyse the nature of cell wall polysaccharides, identify key polysaccharides, determine their distribution in fresh cassava root, and characterize their modifications in retted root and retted root flour.

2. Material and methods

2.1. Plant material and enzymes

Cassava (*Manihot esculenta*, Crantz) roots were harvested close to Yaounde (Cameroon) from healthy young plants (12 months old) of the bitter cultivar (Ntol) in August 2014. They were soaked in paraffin to preserve them from dehydration and oxidation. They were stored in a cold room at 4 °C.

Cassava flour produced through traditional process of retting, grinding, drying and milling was purchased in Cameroon. The flour was stored in a cold room at 4 °C.

Endo-1,4-β-D-glucanase (Cellulase) from *Trichoderma longibrachiatum* (E-CELTR), *endo*-1,4-β-D-galactanase from *Aspergillus niger* (E-GALN), heat-stable α-amylase from *Bacillus licheniformis* (E-BLAAM) and amyloglucosidase from *Aspergillus niger* (E-AMGDF) were purchased from Megazyme (Bray, Ireland).

2.2. Retting of cassava roots

Fresh cassava roots were peeled and then cut into cubes of 2–3 cm^3 that were immersed in tap water (150 mL/100 g of fresh root) and left until the roots softened at room temperature for 7 days.

2.2.1. Preparation of Alcohol Insoluble Residue (AIR)

The top and bottom ends of fresh roots were removed, and the resulting middle portion was peeled. This material was then used for subsequent analyses or separated into central and peripheral areas, which were analysed separately. Pieces of retted roots were directly analysed.

The samples of fresh or retted roots were treated with boiling alcohol to destroy any enzymatic activity and to eliminate alcohol soluble components, especially carbohydrates with a low degree of polymerization (such as free glucose, sucrose, among others). Pieces of fresh or retted roots were heated at 95 °C for 10 min in an appropriate volume of 96% EtOH to obtain a final EtOH concentration of 70%. The EtOH supernatant was eliminated by filtration, and successive washings with 70% EtOH were applied to the alcohol insoluble residue (AIR) until no remaining carbohydrate was detected in the supernatant using a phenol test (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956). The residue was rinsed with 96% EtOH, then rinsed with acetone and dried overnight in an oven at 40 °C. AIR yields were calculated using the starting mass of the fresh and retted root samples.

2.2.2. Destarching of AIR

AIR was de-starched using conditions derived from a starch assay (McCleary, Gibson, & Mugford, 1997). AIR (50 mg) was suspended in 1 mL of 50 mM sodium MOPS buffer (pH 7) heated at 100 °C, and then 0.2 mL of heat-stable alpha-amylase (60 U) was added and the mixture was incubated at 100 °C for 20 min. After cooling to 50 °C, the pH was adjusted to 4.5 with 0.8 mL of 200 mM acetate buffer (pH 4.5). Then, amyloglucosidase (40 U) was added to the mixture, which was incubated at 50 °C for 60 min. After cooling, 4 volumes of 96% EtOH were added and the resulting slurry was centrifuged (6000*g*, 10 min). The pellet was washed successively with 80% and 96% EtOH and then dried overnight in an oven at 40 °C. De-starched AIR is referred to as cell wall material (CWM).

2.2.3. Treatment of CWM with pure, specific glycosyl-hydrolases

CWM obtained from fresh and retted cassava roots was degraded with different glycosyl-hydrolases to detect the presence of galactan and xyloglucan. CWM (5 mg) was suspended in 1 mL of distilled water containing *endo*-galactanase (20 U) or *endo*-glucanase (20 U). The *endo*-glucanase could hydrolyse xyloglucan as well as cellulose. The suspension was gently agitated using a rotary wheel and digested overnight (16 h) at 40 °C. After centrifugation (20 °C, 20 min, 10,600×*g*), the supernatant was placed in a boiling water bath for 10 min to inactivate enzymes and then was filtered through a 0.45-μm filter and frozen. Samples were diluted 5 times prior to the analysis of degradation products with high performance anion exchange chromatography (HPAEC). The samples (10 μL) were injected into a Carbopac PA-200 (5 × 250 mm) analytical column maintained at 25 °C (Dionex, http://www.dionex.com) and eluted at a flow rate of 0.4 mL min^{-1} using a gradient of ultrapure water (A), 1 M NaOAc (B) and 0.5 M NaOH (C) solutions. The gradient conditions were: 0 min (A, 60%; C, 40%), 30 min (A, 43%; B, 17%; C, 40%), 35 min (A, 35%; B, 25%; C, 40%), 36 min (A, 20%; B, 40% C, 40%), 38 min (A, 20%; B, 40%; C, 40%), and 39–65 min (A, 60%; C, 40%). A pulse amperometric detector (TSP EC2000, http://www.thermo.com) was used for the detection. Oligosaccharide identification was based on retention times compared to reference oligosaccharides obtained from purified xyloglucan that was available in the laboratory collection (Ray, Vigouroux, Quémener, Bonnin, & Lahaye, 2014).

2.3. Determination of the sugar composition of AIR and CWM samples

CWM samples were ultra-ground in liquid nitrogen using a Spex 6700 cryogenic grinder (Spex. Industries, USA).

The neutral sugar compositions were identified, after acid hydrolysis, using gas chromatography of alditol acetates (Englyst & Cummings, 1988). Acid hydrolysis of AIR or CWM was carried out as previously described (Saulnier, Marot, Chanliaud, & Thibault, 1995): the samples were pre-hydrolysed with 72% (w/w; 26 M) sulphuric acid for 30 min at 25 °C and then hydrolysed into monomers at 100 °C for 2 h in 1 M sulphuric acid. Uronic acid content was determined in the acid hydrolysis supernatant using an automated m-phenylphenol method (Thibault, 1979) and galacturonic acid as a standard.

Starch was analysed in AIR according to AOAC method 996.11 (McCleary et al., 1997) with a modification for quantifying glucose using HPAEC instead of an enzyme assay.

2.4. Sample preparation for microscopy

Transverse sections (500 μm thick) were hand-cut from fresh and retted cassava roots. Small cubes of approximately 1 mm^3 were then prepared from root transverse sections using a razor blade under a binocular dissecting microscope. Both the central and edge areas of the roots were sampled. Fragments were then fixed in a mixture of 3% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for one night at 4 °C. After washing, the samples were dehydrated in a graded aqueous