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# Identification of a cytotoxic molecule in heat-modified citrus pectin

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ABSTRACT

Modified forms of citrus pectin possess anticancer properties. However, their mechanism of action and the structural features involved remain unclear. Here, we showed that citrus pectin modified by heat treatment displayed cytotoxic effects in cancer cells. A fractionation approach was used aiming to identify active molecules. Dialysis and ethanol precipitation followed by HPLC analysis evidenced that most of the activity was related to molecules with molecular weight corresponding to low degree of polymerization oligogalacturonic acid. Heat-treatment of galacturonic acid also generated cytotoxic molecules. Furthermore, heat-modified galacturonic acid and heat-fragmented pectin contained the same molecule that induced cell death when isolated by HPLC separation. Mass spectrometry analyses revealed that 4,5-dihydroxy-2-cyclopenten-1-one was one cytotoxic molecule present in heat-treated pectin. Finally, we synthesized the enantiopure (*4R,5R*)-4,5-dihydroxy-2-cyclopenten-1-one and demonstrated that this molecule was cytotoxic and induced a similar pattern of apoptotic-like features than heat-modified pectin.



## 1. Introduction

Despite the development of "smart" or targeted drugs as new therapeutic approach for cancer patients, refractory disease still represents a high hurdle hampering successful chemotherapy. More than 60% of anti-cancer drugs in use nowadays are derived from natural products (Cragg & Newman, 2005, 2013). Vinca-alkaloids, camptothecin, etoposide or taxanes are such examples. Successful remission can be achieved with chemotherapy in most cases but refractory diseases and relapses remain a major obstacle. Indeed, resistance to chemotherapy develops and is still a high hurdle to get over for efficient cancer patient treatment. New molecules are thus needed that could be used alone or in combination with currently used drugs to enhance therapeutic success. Among promising avenues, pectin and pH- or heat-modified pectin have demonstrated chemopreventive and antitumoral activities against some aggressive and recurrent cancers (Leclere, Van Cutsem, & Michiels, 2013).

Pectins are abundant and complex polysaccharides present in the primary cell wall of plants. It is composed of homogalacturonan (HG), substituted galacturonans, rhamnogalacturonan-I (RG-I) and rhamnogalacturonan-II (RG-II). HG is a polymer of α-1,4-linked-galacturonic acid. HG residues can be methyl-esterified at the C-6 carboxyl or acetylated at the O-2 or O-3 depending from the pectin source. Side chains of RG-I and RG-II, whose structure can be very complex, are attached to a backbone of HG (Harholt, Suttangkakul, & Vibe Scheller, 2010; Mohnen, 2008). Citrus pectin consists mainly of HG, lower amount of RG-I and very few RG-II structural domains (Yapo, Lerouge, Thibault, & Ralet, 2007). Acidic pH has been used to modify pectin and generate lower molecular weight fragments able to compete with galectin-3 (Gao et al., 2012). Galectin-3 is a lectin that possesses a conserved carbohydrate-binding domain that specifically recognizes ß-galactoside moieties. It has been described to be overexpressed in metastatic cancers (de Oliveira et al., 2010) and promotes cell migration and survival (Fortuna-Costa, Gomes, Kozlowski, Stelling, & Pavao, 2014). Several works have demonstrated its anti-cancer activities both in vitro and in vivo (Chauhan et al., 2005; Inohara & Raz, 1994; Nangia-Makker et al., 2002).

In another study, pectin can also be modified by heat treatment. Different polysaccharide fractions isolated from ginseng rich

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in HG stopped cell cycle in G2/M phase on colon cancer HT-29 cells. On the other hand, fractions rich in HG and modified by heat treatment exerted a much higher anti-proliferative activity, which was accompanied by caspase-3 activation and apoptosis induction (Cheng et al., 2011). Similarly, rhamnogalacturonan I domain-rich pectin isolated from potato inhibited in vitro HT-29 cell proliferation and provoked a cell cycle arrest in G2/M phase. This inhibition was due to a decrease in cyclin B1 expression and in CDK-1 activity (Cheng et al., 2013). Kang et al. (2006) also produced a citrus pectin-derived oligosaccharide, which was biologically active, but they used irradiation instead of heat treatment. Pectin irradiated with 20 kGy and then dialyzed (molecular weight <10,000) inhibited cancer cell growth.

Jackson et al. (2007) showed that different treatment protocols of pectin can lead to differences in pectin apoptosis-inducing activity and that heat-fragmented pectin has a cytotoxic effect in galectin-3 expressing but also in non-expressing prostate cancer cells. This indicates that the active molecules in pH-modified and heat-treated pectin are not the same. Treatment of heat-modified pectin with pectinmethylesterase to cleave galacturonosyl carboxymethylesters and/or with endopolygalacturonase to remove non-methylesterified HG did not change the pro-apoptotic activity of the modified pectin. On the other hand, mild base treatment that eliminated the ester bounds destroyed the pro-apoptotic activity. Cytotoxic activity thus requires a base-sensitive linkage in oligosaccharides other than a carboxymethylester bound. Size analyses of the active fragments suggested low mass (10–20 kDa) oligosaccharides (Jackson et al., 2007). Recently, we showed that citrus pectin modified by heat treatment induced cell death in HepG2 and A549 cells. The induced cell death differs from classical apoptosis since a different pattern of caspase 3 cleavage was detected. Autophagy was also induced (Leclere et al., 2015).

However, besides these different studies, the nature of the active molecules in both products is not known. In this work, we used a fractionation approach of heat-modified citrus pectin aiming to identify molecules exerting a cytotoxic activity toward cancer cells. We report herein the isolation, synthesis and structural analysis of one such molecule, 4,5-dihydroxy-2-cyclopenten-1-one.

## 2. Material and methods

### 2.1. Fractionation of citrus pectin by heat treatment

Heat fragmented citrus pectin (HFCP) was obtained according to the method described by Jackson et al. (2007). A solution of 0.1% citrus pectin (Sigma P9135) composed (on a dry weight basis) of 74% homopolygalacturonic acid, 8.7% neutral sugars, 6.7% methoxy groups and 2.5% Na, was heated at pH 4.2 for 60 min at 121 °C under a pressure of 1 bar. The solution was then frozen at −80 °C and lyophilized. The dry material was stored at 4°C. Galactose, galacturonic acid, glucose and glucuronic acid monomers (1 g/l, Sigma) were heat-treated at pH 2.9 for 7.5 h.

### 2.2. Cell culture and pectin incubation

HepG2, A549, A431, HeLa, MDA-MB-231 and MCF10A cells were obtained from the American Type Culture Collection HepG2 cells were cultured in DMEM medium (Gibco 31825-023), A549 cells in MEM medium (Gibco 41090-028), A431 cells in DMEM high glucose and pyruvate (Gibco 41966-029), Hela cells in MEM, HEPES, GlutaMAX supplement (Gibco 42360-024) supplemented with sodium pyruvate (100 mM, Gibco 11360-039) and MEM non-essential amino acids solution (100x, Gibco 11140-035) and MDA-MB-231 cells in RPMI 1640 medium (Gibco 21875-034). For routine culture, media were supplemented with 10% fetal bovine serum (Gibco 10270), and the cells were kept at 37 °C in an atmosphere of 95% air and 5% $CO_2$. MCF10A cells were cultured in DMEM/F12 medium (Gibco 11320-074) containing 5% horse serum (Gibco 16050-122), 20 μg/mL EGF, 0.5 μg/mL hydrocortisone, 10 μg/mL insulin and 100 ng/mL cholera toxin. They were maintained in culture in 75 $cm^2$ polystyrene flasks (Corning) with Basal Medium Eagle (BME, Invitrogen), supplemented with L-glutamine (2 mM, supplied by Sigma-Aldrich). For routine culture, both media were supplemented with 10% fetal bovine serum (Gibco 10270) and cells were kept at 37 °C in an atmosphere of 95% air and 5% $CO_2$. For treatment, cells were allowed to adhere for 24 h after seeding. Media were discarded, cells washed twice with PBS (Lonza BE17-516F) and placed in their corresponding medium, without serum, containing the following: 3 mg/mL of filter-sterilized HFCP, 3 mg/mL of filter-sterilized citrus pectin or 50 μM etoposide, used as a positive control. Negative controls were cells incubated in media alone.

### 2.3. Cell viability assay

HepG2 cells were seeded at 50 000 cells/well in 24-well plates before treatments for 24 h. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma M2128) solution was prepared at a concentration of 2.5 mg/mL in phosphate-buffered saline and 500 μL were added per well. After 2 h at 37°C and 5% $CO_2$ atmosphere, media and MTT solution were removed before adding lysis buffer. Optical density was measured 1 h later at 570 nm using a microplate spectrophotometer (Biorad × Mark Microplate spectrophotometer) and *Microplate manager 6* software. The MTT test measures the number of metabolically active (viable) cells.

### 2.4. Cell cytotoxicity assay

HepG2 cells were seeded at 50 000 cells/well in 24-well plates before incubation for 24 or 48 h. For each well, lactate dehydrogenase activity was measured in the supernatant, in the detached cells and in adherent cells after lysis in PBS containing 10% Triton X100 (Merck 9036-19-5). Lactate dehydrogenase activity was detected by colorimetric assay using a cytotoxicity kit (Roche 11644 793 001) and a microplate spectrophotometer. Cytotoxicity percentages were calculated by the ratio of the quantity of LDH present in the supernatant and in detached cells on the total quantity of LDH as in the formula: $100 \times (a+b)/(a+b+c)$ where $a$ = supernatant LDH; $b$ = detached cells LDH; $c$ = adherent cells LDH. 0% LDH release means that all cells are viable while 100% LDH release indicates that all the cells are dead.

### 2.5. Western blot analysis

Cell lysates were prepared in lysis buffer (40 mM Tris; pH 7.5, 150 mM KCl, 1 mM EDTA, 1% Triton X-100) containing a protease inhibitor cocktail (Complete from Roche Molecular Biochemicals; 1 tablet in 2 mL of $H_2O$, added at a 1:25 dilution) and phosphatase inhibitor buffer (25 mM $NaVO_3$, 250 mM PNPP, 250 mM α-glycerophosphate and 125 mM NaF, at a 1:25 dilution). The medium was centrifuged, and pelleted cells were added to cell lysates. The lysates were then centrifuged at 12,000 × $g$ for 5 min, and the supernatants were collected. The proteins (15 μg) were denatured with the addition of LDS sample buffer (Invitrogen NP0007) and heated to 70 °C for 10 min. The proteins were resolved on a 4–12% NuPAGE (Invitrogen) gel and transferred to a low-fluorescence membrane (Millipore IPFL00010). The membranes were kept for 1 h in LiCor blocking solution and probed overnight with either an anti-caspase-3 rabbit antibody (Cell Signaling #9662S) that recognizes the full-length and cleaved forms of caspase-3 at a dilution of 1/500, an anti-PARP mouse antibody (BD Biosciences #551025) at a dilution of 1/1000 or a mouse anti-ubiquitin antibody