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Molecular interactions between 3,4-dihydroxyphenylglycol and pectin and antioxidant capacity of this complex *in vitro*

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ABSTRACT

This study explored the interaction of pectin with 3,4-dihydroxyphenylglycol (DHPG), a potent phenolic antioxidant naturally found in olive fruit, *via* encapsulation into pectinate beads. MALDI TOF-TOF analysis supported the formation of complexes between DHPG and pectin. A combination of covalent bonds (ester bonds) and non-covalent interactions, mostly hydrogen bonding, were suggested as the cause of DHPG-pectin complex formation. Free radical scavenging assays confirmed that DHPG maintained its antioxidant activity after complexation and after a digestion simulated *in vitro* with gastric and intestinal fluids. Therefore, DHPG-pectin beads could reach the large intestine and contribute to a healthy antioxidant environment.

1. Introduction

Virgin olive oil contains numerous antioxidant phenolic compounds that exert potent anti-inflammatory actions (Muto et al., 2015). In the last few years, several studies have demonstrated that diets supplemented with olive oil and/or olive oil phenolics exert a protective effect on experimental colitis in rodents, which may be mediated by their strongly anti-oxidative potential (Sánchez-Fidalgo et al., 2013; Takashima et al., 2014).

3,4-dihydroxyphenylglycol (DHPG) is a simple phenol present in the olive fruit with the same *ortho*-diphenolic structure as hydroxytyrosol (HT), the major phenolic compound in olive fruit, but with an additional hydroxyl group in the β position. DHPG has higher antioxidant, antiradical, and reducing capacity than HT and prevents lipid peroxidation to a degree that is comparable with vitamin E (Rodríguez, Rodríguez, Fernández-Bolaños, Guillén, & Jiménez, 2007). Furthermore, DHPG is bioavailable, has antioxidant properties in vitamin E-deficient platelet activation and adhesion, and may have anti-inflammatory properties (De Roos et al., 2011).

Pectin is a complex polysaccharide present in the primary cell wall of higher plants. Pectin consists of a homogalacturonan backbone of predominantly α-(1–4)-linked galacturonic acid residues, interrupted by ramified rhamnogalacturonan regions (Schols & Voragen, 1996). The galacturonic acid residues can be methyl-esterified at the carboxyl group, which is essential for the applications of pectins. Pectin, an indigestible soluble fiber, is commonly used in the food and pharmaceutical industries due to its gelling, stabilizer, and thickening properties (Thakur, Singh, & Handa, 1997; Sriamornsak, 2003). Pectin with antimicrobial agent (lysozyme) form composite by electrostatic force and have antibacterial application (Zhang et al., 2015). Pectin also possesses an interesting potential for the delivery of drugs to the colon (Das & Ng, 2010), where it is specifically biodegraded by colonic bacteria and has been found to inhibit both local and systemic inflammation (Markov, Popov, Nikitina, Ovodova, & Ovodov, 2011; Popov et al., 2013).

Our previous study reported the formation of a pectin-HT complex and proposed that HT, DHPG from HT and DHPG-loaded pectinate gel beads, with or without olive oil, reach the colon as agents capable of preventing or improving inflammatory bowel disease (Bermúdez-Oria, Rodríguez-Gutiérrez, Rubio-Senent, Lama-Muñoz, & Fernández-Bolaños, 2017). This study reports the formation of a pectin-DHPG complex and proposes the complex's encapsulation as an efficient system for the delivery of DPHG's antioxidant activity to the colon.

2. Hypotheses

Our previous studies suggested that the formation of a pectin-HT complex promotes a delayed release of HT for a delivery system in the colon region, thus providing a new possibility for treatment of inflammatory disease mediated by oxidative stress.

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The objective of the present investigation is to confirm the presence of a DHPG-pectin complex when the beads based on calcium amidated pectin and calcium pectin-alginate were formed. MALDI TOF-TOF and NMR techniques will study the interactions between pectin and DHPG as well as the efficacy of colon targeting. Also the ability of this phenol-pectin complex to scavenge free radicals, key in the protection against oxidative stress in the colon, will be evaluated by the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay.

3. Material and methods

3.1. Materials

DHPG was isolated and purified with a high degree of purity (≥95%) following procedures protected by patent (Fernández-Bolaños Guillén, Jiménez, Rodríguez, Rodríguez-Gutiérrez, & Lama-Muñoz, 2011; Fernández-Bolaños, Rodríguez-Gutiérrez et al., 2014). Highly methylated citrus pectin (degree of methylation 53%), and sodium alginate from brown algae (*Macrocystis pyrifera,* kelp) with 61% mannuronic acid and 39% guluronic acid were purchased from Sigma-Aldrich (St Louis, MO, USA). Amidated pectin with a low degree of esterification (30%) and a 21% degree of amidation was a generous gift from Herbstreith & Fox KG (Neuenbürg, Germany). Calcium chloride dihydrate ($CaCl_2$) and a mixture of pectinolytic enzymes from Novozyme Corp. were purchased from Sigma-Aldrich (St Louis, MO, USA).

3.2. Preparation of formulations

Calcium pectin-alginate beads and amidated pectin beads were instantaneously produced by the ionotropic gelation method. 100–300 mg of DHPG (95% of purity) in 30 mL of distilled water with 1.0 g of amidated pectin, or 0.8 g of citrus pectin and 0.2 g of sodium alginate, were homogeneously dispersed using a homogenizer (Ultra-turrax ® T50 Basic, IKA, Germany) at a speed of 24,000 rpm for 5 min in an ice-bath to avoid overheating. Air bubbles were removed from the dispersion by sonication using a bath sonicator. The pectin dispersions containing DHPG, pH 5, were dropped into 75 mL of 10% w/v $CaCl_2$ aqueous solution at room temperature using a nozzle with an inner diameter of 1.2 mm. The $CaCl_2$ solution was gently stirred during hydrogel formation. The beads formed were allowed to stand in the solution for 2 min. DHPG-loaded beads gels were separated from the $CaCl_2$ solution by filtration and washed with distilled water. The filtrated $CaCl_2$ solution and wash liquid were saved to measure the free DHPG that was not entrapped within the beads. The separated gel beads were dried at 37 °C for 48 h in an air-circulated oven until a constant weight was obtained.

3.3. Entrapment efficiency and bioactive compound loading

The entrapment efficiency of the bioactive compound (DHPG) was calculated indirectly by determination of the free DHPG content in the aqueous solution after filtration and washes, according to the equation EE% = [(Qt-Qr)/Qt] x 100, where Qt is the bioactive content initially added during the bead loading, and Qr is the sum of content recovered in the aqueous solution after separating and washing the beads.

The amount of bioactive compound (DHPG) present in the beads (bioactive compound loading, BCL) was also determined by an indirect method according to the equation BCL% = [(Qt-Qr)/Wp] x 100, where Qt is the bioactive content initially added during the bead loading, Qr is the sum of content recovered in the aqueous solution after separating and washing the beads, and Wp is the total weight of dry beads recovered per batch. All experiments were performed in triplicate.

3.4. Extraction and analysis of DHPG from beads

Extraction of DHPG from beads was evaluated in aqueous solutions and various organic solvents. The unbound phenolics were extracted as follows: 0.1 g of dry beads in 100 mL of acidified water for 2 h at 37 °C in a shaking water bath. The quantities of DHPG were determined by HPLC according to a previously published method (Rodríguez et al., 2007). The extraction of strongly bound DHPG was also assayed according to the method of Nordkvist, Salomonsson, and Åman, (1984), using 1 M NaOH containing 0.5% sodium borohydride for 20 min. Also the beads were also subjected to acid hydrolysis according to the method of Graciani & Vázquez (1980). 0.1 g of dry beads/emulsion were treated with 10 mL of 3 N HCl in a homogenizer Ultra-Turrax (24,000 rpm for 5 min) and then heated to 100 °C for 10 min and filtered.

Also 0.1 g of the beads were extracted with 25 mL of organic solvent by stirring (30 min), sonication (15 min), and Ultra-Turrax (24,000 rpm for 5 min), using methanol:water (20, 40, 80%) and dimethyl sulfoxide (DMSO): water (10, 30, 60, 90%). All the mixtures were filtered with filter paper. In no case it was possible to quantify the strongly bound DHPG. Also, due to the difficulty of dissolving the dry beads, the DHPG loaded dried beads were broken down by immersion in sodium phosphate buffer (50 mM, pH 6.8) containing 5 mM of EDTA (Ethylenediaminetetraacetic acid). A preliminary acid treatment was necessary to help the erosion of the matrix (Nguyen, Winckler, Loison, & Wache, 2014): Beads (0.1 g) were dispersed in 100 mL of 0.1 M HCl for 2 h at 37 °C in a shaking water bath. After filtration, the beads were further treated by sodium phosphate buffer with EDTA at 37 °C until dissolution. An alkali extraction ($NaHCO_3$, pH 10) at 100 °C for 30 min was also tested.

3.5. In vitro release study: simulated digestion

The DHPG loaded dried hydrogel beads (0.1 g) were immersed in 100 mL at pH 1.2 with 0.1 M HCl solution (simulated gastric juice) at 37 °C with gentle shaking for the first 2 h, and then in a pH 6.8 sodium phosphate buffer solution (simulated intestinal juice) for the following 2–3 h. The samples were withdrawn and assayed for DHPG content by HPLC.

3.6. Purification of 3,4-dihydroxyphenylglycol-pectin complex

Size exclusion chromatography on two-column Superdex Peptide HR 10/30 (30 × 1 cm) (Pharmacia Biotech, Uppsala, Sweden) connected in line and connected to a Jasco LC-Net II/ADC HPLC (Easton, MD, USA) was used to purify the DHPG-pectin complex. Samples of 1.0 g dried DHPG-loaded amidated pectin beads containing 21.6 mg of DHPG were treated with 100 mL of 0.1 M HCl (pH 1.2) at 37 °C for 2 h to remove all the unbound DHPG and to help break down the beads. Then the beads were dissolved in 100 mL of phosphate buffer (50 mM, pH 6.8) containing 5 mM EDTA and a mixture of pectinolytic enzymes (4 μg/mL) – including *endo*- and *exo*-polygalacturonase and pectinesterase (Novo Nordisk, Bagsvaerd, Denmark). The mixture was incubated at 37 °C for 24 h, then 100 μL was applied ten times onto the column and eluted with distilled water at a flow rate of 0.5 mL/min. The peaks were monitored with a Shodex RI-71 Refraction Index detector. Column calibration was performed with galacturonic acid and tri-galacturonic acid (Fluka) as standards for the pectic oligosaccharides.

3.7. Analysis technique of DHPG-pectin complex

The DHPG-loaded and control beads were analyzed by High Resolution Magic Angle Spinning (HR-MAS). ^1H (500.1 MHz) HR-MAS spectra were recorded on a Bruker Avance-500 spectrometer using $CDCl_3$ as solvent.

MALDI TOF-TOF mass analysis of the isolated fractions of Superdex Peptide HR was performed using an UltrafleXtreme Bruker mass spectrometer Smartbeam-II laser. The MALDI TOF-TOF ms spectra were