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Modification of chitosan to deliver grapes proanthocyanidins: Physicochemical and biological evaluation

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ABSTRACT

Chitosan (CS) and lauryl succinyl chitosan (LSC) particles were synthesized by ionic gelation to encapsulate high molecular weight proanthocyanidins (PAs) extracted from grape seed in an attempt to improve the cellular transport and delivery of the PAs. The extracts and particles were subjected to simulated gastrointestinal assays, cytotoxicity studies and cell permeation studies. The LSC and CS physicochemical properties were also evaluated and compared. The LSC particle sizes were 3640 ± 33 nm and the CS particles were 458 ± 11 nm; both were loaded with seed extract. The LSC released a lower PAs amount than the CS, protecting the encapsulated extract from the stomach pH. The cytotoxicity studies on HEK-293 cells showed that the half maxima inhibitory concentration (IC_{50}) of the raw extract was 0.006 mg/mL, and with the stabilization (on LSC or CS) this value increased to 1.7 mg/mL. The encapsulation of the extracts decreased their toxicity, allowing higher concentrations to be used. The transport studies through Caco-2 monolayer cells indicated the effective release of PAs from the particles. The phenols released by the LSC particles were significantly higher than that released by the native CS during the Caco- 2 cell permeation study.

1. Introduction

Proanthocyanidins (PAs) are phenolic compounds present in vegetables and fruits. Their chemical structures are based on a flavan-3-ol molecule, with modified substitutions dependent on the natural source from which they are extracted, with the origins being several types of catechins (El Gharras, 2009). The PAs isolated from grape skin have a higher mean degree of polymerization (mDP) and a higher quantity of galloylated units than the PAs from grape seed (Eriz, Sanhueza, Roeckel, & Fernandez, 2011; Souquet, 1996).

The characterization of PAs from seeds and grapes showed that PAs with a high mean degree of polymerization (mDP) (over 3 flavan-3-ol units) produce higher bioactivities compared with PAs with lower mDPs, which was associated with the existence of a higher number of hydroxyl groups and with the presence of epigallocatechin in the PA molecular chain (Godoy, Roeckel, & Fernandez, 2012). In grapes, the PA molecular weight distribution is broad, ranging from 300 to 20000 Da for monomers to polymers, respectively, and this results in only partial absorption in the intestine therefore limiting their effectiveness. Additionally, there are other factors causing PAs to become unstable, such as the presence of oxygen and changes in pH, temperature, and light. In this context, the encapsulation of catechins is emerging as a useful strategy to protect these bioactive compounds, thus retaining their structural integrity until the time of consumption or administration (Khushnud & Mousa, 2013). Moreover, this approach provides carriers that have the ability to prevent degradation during digestion, subsequently enhancing their bioactivity and bioavailability, promoting a controlled release as well as a targeted delivery (Sarvaiya & Agrawal, 2015; Summerlin et al., 2015).

Chitosan (CS) has been widely used to encapsulate compounds due to its biodegradability, biocompatibility, low toxicity and mucoadhesivity (Artursson, Lindmark, Davis, & Illum, 1994; Tobio, Gref, Sanchez, Langer, & Alonso, 1998). CS is a polycation, it interacts electrostatically with anionic molecules during the synthesis of NPs. The polyanion tripolyphosphate (TPP) interacts with

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CS to form spherical CS particles that make it possible to trap other molecules such as PAs. The mucoadhesiveness of the CS allows the interaction, passing and absorption of NPs in the gastrointestinal system. However, the intestinal absorption is limited by the instability of CS in acidic environments. To overcome this problem, the use of a lauryl succinyl chitosan (LSC) derivative has been proposed because its properties as a stable carrier in acidic media have been demonstrated in the administration of insulin (Rekha & Sharma, 2009).

Absorption through the epithelial cell layer of the PAs is affected by their high molecular weight; molecules over 1740 g/mol are poorly absorbed compared to the monomers, dimers or trimers of these molecules (Deprez, Mila, Huneau, Tome, & Scalbert, 2001). This is because PAs form complexes with membrane proteins, causing much stronger cell-cell junctions. Therefore, encapsulation should increase the PAs intestinal uptake. In this study, CS and LSC were evaluated as encapsulants to develop particles that improve the delivery and the cellular transport of PAs from grape seed. The extracts and the particles were subjected to simulated gastrointestinal assays, cytotoxicity studies on HEK-293 cells, and studies of cell permeation on caco-2 cells. The LSC and CS physicochemical properties were also evaluated and compared.

2. Materials and methods

2.1. Materials

The (+)-catechin (C), (−)-epicatechin (EC), phloroglucinol, ascorbic acid, polystyrene PSS (Polymer Standards Service), chitosan (low molecular weight), tripolyphosphate (TPP, technical grade) and Tris buffer were purchased from Sigma Aldrich (St. Louis, MO, USA). Chromatographic grade acetone, acetonitrile, acetic acid, acetic anhydride, pyridine, tetrahydrofuran (THF), 2-dodecyl succinic-1-yl anhydride (LSA), dimethylformamide (DMF), *n*-hexane, and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). Solvents of analytical grade acetone, methanol, ethanol and 37% hydrochloric acid (HCl) were also purchased from Merck (Darmstadt, Germany). Dulbecco's modified Eagle medium (DMEM) and penicillin/streptomycin were purchased from Corning (Corning, NY, USA). Fetal bovine serum (FBS) was purchased from Biological Industries (Cromwell, CT, USA). The bidistilled water used in all solutions was purified to HPLC grade using a Millipore Milli-Q fast water system (Bedford, MA, USA). Toyopearl HW-40F column packing material was obtained from Supelco (St Louis, MO, USA).

2.2. Proanthocyanidins (PAs) extraction

Grape (*Vitis vinífera* L.) samples were collected from Quillón in the Itata Valley, Bio Region, Chile. The seeds of 400 grapes were manually separated, and the phenolic compounds were extracted in Erlenmeyer flasks using a solution of acetone:MQ water (2:1, v/v). The solution was agitated for 15 h at 35 °C at 250 rpm (New Brunswick Scientific Co, Inc., G24 Environmental Incubator Shaker, USA) in the absence of light and was bubbled with N_2 (Kennedy & Jones, 2001). Later, the extracts were purified through size exclusion chromatography using Toyopearl HW-40F resin packed in an Omnifit column (Tosoh Corporation bioscience division, Japan) (Kennedy & Taylor, 2003), with modifications. Three volumes of ethanol/water (55:45% v/v) were used instead of methanol to remove the sugars and phenolic acids.

2.3. Chemical characterization of the PAs

The PAs obtained in section 2.2 were characterized through acid-catalyzed depolymerization with phloroglucinol (Cerpa-Calderon & Kennedy, 2008; Kennedy & Taylor, 2003). The resulting solution was filtered with a 0.45 μm politetrafluoroetileno (PTFE) filter and analyzed by high precision liquid chromatography (HPLC) (Hitachi Merck LaChrom L7000 series, Japan). Two columns 100 × 4.6 mm (Chromolith RP-18e, Merck, Germany) connected in series and protected with a pre-column of 5 × 4.6 mm (Chromolith RT 18, Merck, Germany) were used. A binary gradient containing a mobile phase A (1%v/v of aqueous acetic acid) and a mobile phase B (1% v/v of acetic acid/acetonitrile) were used. The elution peaks were detected at 280 nm, and the injection volume was 20 μL at a flow rate of 3 mL/min. The mDPs were calculated using an external catechin standard (100 ppm).

The extracts were also characterized by gel permeation chromatography (GPC) (YL HPLC Young Lin instrument, Japan) (Williams, Porter, & Hemingway, 1983), with modifications. Ten milligrams of seed extract were treated with pyridine and acetic anhydride (2 mL, 1:1, v/v) overnight at room temperature in order to acetylate the extract. The mixture was then poured into 5 mL ethanol, agitated and subsequently evaporated at room temperature. The resulting solutes were dissolved in tetrahydrofuran (THF) (2–5 mg/mL) and then filtered with a 13 mm filter (PTFE). The flow rate used was 1 mL/min at a 940 psi and 23 °C; THF was the solvent, the injection volume was 20 μL, and the samples were analyzed at 254 nm. Different molecular weights of the standard polystyrene PSS (Polymer Standards Service) were dissolved in THF to prepare the calibration curve.

2.4. Synthesis of lauryl succinyl chitosan

The chitosan derivative was synthesized according to a process developed by Rekha and Sharma (2009). A solution of 2% w/v CS was prepared in aqueous acetic acid 2% v/v and vacuum filtered (Whatman nº1). To a known volume of the CS solution, methanol was added at four times the known volume and stirred. Subsequently, 2% v/v of 2-dodecyl succinic-1-yl anhydride (LSA) was added. This solution was stirred for 15 min and allowed to stand overnight to allow the reaction to proceed. The gel formed by the reaction was dissolved in a volume of HCl/dimethylformamide (DMF), which was added under stirring until the gel completely dissolved. The new solution was dialyzed with a cellulose acetate membrane of 14–16 kDa against distilled water for 48 h, under constant stirring and with 4 changes of water. Finally from the dialysis step, a precipitated solid polymer sample of LSC was obtained by lyophilization. The derivative was characterized by FT-IR (Nicolet Impact 410 Spectrometer) in the form of a KBr pellet for solid samples with an 8 mm IR beam diameter (Ferracane & Greener, 1984).

2.5. Encapsulation of the extracts

Particles were synthesized using LSC with 2% LSA and native CS, and they were loaded with grape seed extract. A solution of sodium tripolyphosphate (TPP) was added dropwise into the polymer solution. For the synthesis of the LSC particles, 0.2% w/v LSC was dissolved in a 0.2 N HCl/DMF solution, and for the CS particles, a solution of 0.2% w/v was prepared in aqueous 0.175% v/v acetic acid. The 0.1% w/v TPP solution was added to both solutions separately (at one drop/second) in a polymer:TPP ratio of 6:1; the seed extract was previously added to the TPP solution in a polymer:TPP ratio of 8:1. The particles formed were collected by centrifugation at 10,000 rpm for 10 min. The LSC particle pellet was washed with MQ water and re-suspended in 5 mL of a 10% w/v aqueous sucrose solution. The new solution was lyophilized (Labconco, Freezer Dry System, USA) to a dry powder for further analysis and was stored at