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Suppression of the *tert*-butylhydroquinone toxicity by its grafting onto chitosan and further cross-linking to agavin toward a novel antioxidant and prebiotic material

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ABSTRACT

The enzyme-mediated grafting of *tert*-butylhydroquinone (TBHQ) onto chitosan and further crosslinking to agave inulin (agavin) has been successfully achieved in a mild and non-toxic two-step route. The resulting products were characterized by nuclear magnetic resonance (NMR) and Infra-red spectroscopies to assess the molecular structure. The study of acute oral toxicity in mice revealed no adverse short-term effects of consumption in the synthesized materials with non-toxicity evidence until 2000 mg/kg through an oral acute administration. Importantly, this study proves that the compound maintains the radical scavenging capacity of the phenolic antioxidant upon ferric-reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) assays with a measured half-maximal inhibitory concentration (IC_{50}) for the best case of 1.54 g/L based on inhibition of 2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid diammonium salt (ABTS). Additionally, the novel compound presented high prebiotic activities as ascertained in the presence of lactic acid bacteria (LAB).

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1. Introduction

The use of oxidant retardants as additives, such as phenolic antioxidants, in commercial food products is extensively used to preserve quality standards. However, some are regarded as toxic with minimal toxic dosage or even median lethal doses (LD_{50}) as pointed out by regulatory and health organizations (Shahidi, Janitha, & Wanasundara, 1992). In this regard, the synthetic phenol derivatives are a group of highly effective antioxidants, such as the *tert*-butylhydroquinone (TBHQ) which is widely used for unsaturated vegetable oils or many edible animal fats. The Federal Drug Administration (FDA) in USA sets an upper limit for this antioxidant of 0.02% of the oil or fat content in food, but at increasing doses it has some negative health effects on lab animals, thus producing precursors to stomach tumors and damage to DNA (Van Esch, 1986). Despite of these facts, TBHQ is extensively used owing to economic factors versus less toxic alternatives. Currently, the food and pharmaceutical industries as well as the areas of biomedicine and biotechnology are in search of new compounds that are multifunctional, biocompatible and safe (Barone & Medynets, 2007). This has increased the interest for non-toxic natural antioxidants although these approaches are often limited by the low cost-effective output compared to the use of synthetic additives.

On the other hand and following the same principle of reducing potential toxicities in food additives, the use of biopolymers as the case of chitosan, cellulose derivatives or fructans, which are regarded as harmless to human health, has been pointed out. Chitosan is obtained by the *N*-deacetylation of chitin (Vishakha, Kishor, & Sudha, 2012), present as structural component in the exoskeletons of insects and crustaceans, and found use in many

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applications such as components for biomedical scaffolds and healing sutures or for removal of metal ions in waste water treatment, as well as it use as matrices for cell immobilization (Vishakha et al., 2012). On the other hand, fructans, a family of biopolymers having mostly fructose units in the backbone that play the role of energetic reserve in many plants, found applications in food as rheological agents, sweeteners, fat substituent as well as prebiotics (Pan, Rezaei, & Soor, 2011; Vijn & Smeekens, 1999). The agave inulin, or agavin, is a type of fructan extracted from agave plants abundant in Mexico that presents a β(2–1) fructosyl–fructose linkages with additional branches of β(2–6) fructose structures, in addition to internal glucose units (López, Mancilla-Margalli, & Mendoza-Díaz, 2003). Noteworthy, despite of many studies related to other fructans, such as the inulins from Jerusalem artichoke or chicory, which are linear polymers of fructose with terminal glucose units (Flamma, Glinsmannb, Kritchevskyc, Proskyd, & Roberfroid, 2001; Roberfroid, 1993), there is little work on the study and application of the branched agavin.

Generally, polymer modification promotes diversification and acquisition of characteristics to these substances, one of the features that can be added, especially for natural polymers in food applications, is the crosslinking that allows generating network type structures which might enhance the properties inherent in their precursors (Bhattacharya, Rawlins, & Ray, 2009). Additionally, these modifications might also promote radical scavenging capacities by the grafting of antioxidant adducts, which in turn might decrease the absorption of such in the digestive tract (Kikugawa, Kunugi, & Kurechi, 1990), especially those regarded as toxic, thus avoiding accumulation and fast track release from human body.

This paper aims to report the production of a novel non-toxic and antioxidant compound with prebiotic capacities and dietary fiber throughout a mild and non-toxic two-step route with an initial enzyme-mediated grafting of TBHQ onto chitosan, and subsequent crosslinking to agavin with citric acid (CA). Acute toxicity study in mice proved the safeness of this composite and others proofs showed the antioxidant capacity and the enhanced prebiotic characteristics of this novel compound.

2. Materials and methods

2.1. Materials

TBHQ (PubChem CID:16043) was purchased from Aldrich (USA) and used as supplied. Sample of agavin (Metlin®) extracted from *Agave tequilana* Weber blue variety was a kind gift from Nekutli SA de CV (Guadalajara, Mexico). Agavin number average molecular weight (M_n) was 2271 Da with polydispersity index of 1.4 as determined by Size Exclusion Chromatography in our laboratory using pullulan standards calibration curve, which is equivalent to approximately an average of 14 repeat units per chain. Chitosan (PubChem CID:21896651) medium molecular weight (200,000 cps) was purchased from Sigma–Aldrich. Chitosan viscosimetric molecular weight (M_v) was 486.33 KDa as measured in our laboratory using an Oswald viscosimeter and the Mark–Houwink–Sakurada equation (Barone & Medynets, 2007). Horseradish peroxidase (HRP) type II was supplied by Sigma–Aldrich (USA). HRP specific activity was 482 U per mg of protein following the methodology reported by Kessey (1987), where one activity unit (U) is defined as the amount of enzyme that oxidize 1.0 μmol of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS, Sigma–Aldrich) per minute at pH 5 and at 25 °C as measured spectrophotometrically at 405 nm. Hydrogen peroxide (H_2O_2) (PubChem CID:784) 50%, 2,2′-azobis-(2-methylpropionamidine) dihydrochloride (AAPH) (PubChem CID:76344), fluorescein (PubChem CID:16850) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) (PubChem CID:40634) were supplied by Sigma–Aldrich (USA). Citric acid (CA) monohydrate (PubChem CID:22230), monobasic sodium phosphate (NaH_2PO_4) (PubChem CID:23672064), sodium hydroxide (NaOH) (PubChem CID:14798) and hydrochloric acid (HCl) (PubChem CID:313), all reagent grade, were obtained from JT Baker (Mexico). Absolute ethanol (PubChem CID:702) was acquired from Química Barsa (Mexico). LAB strains were a kind gift by the Faculty of Chemistry collection at the UNAM.

2.2. Enzymatic grafting of TBHQ onto chitosan

Chitosan was added to HCl solution (0.1 M) until a concentration of 0.3% wt/vol. At the same time two ethanolic solutions of TBHQ (40 and 80 mM) were prepared (Itzincab-Mejía, López-Luna, Gimeno, Shirai, & Bárzana, 2013). The chitosan and each ethanol-TBHQ solutions were mixed in an 80/20 vol/vol mixture in an amber round-bottom flask. Then, the enzyme was added in a proportion of 1 mg of the protein per 500 mg of total reactants and the mixture was stirred for 1 min. Hydrogen peroxide (5% vol/vol) (0.05 mg of enzyme per mL of H_2O_2 5%) was added dropwise in 10 min. intervals for 1 h. After complete the reaction time, the mixture was filtered under vacuum in order to remove the non-soluble TBQ, oxidized form of TBHQ, generated as a byproduct of the reaction. The filtrate was adjusted to pH 7 to precipitate the product, and then centrifuged at 4000 rpm for 10 min. The obtained solid was intensively washed with ethanol to remove traces of non-reacted TBHQ or its oxidized form and then lyophilized to give chitosan-*g*-TBHQ as a white powder.

2.3. Crosslinking of chitosan and chitosan-g-TBHQ with agavin using CA

Samples of individual chitosan or chitosan-*g*-TBHQ, agavin, CA and sodium phosphate monobasic were added to a HCl solution (0.1 M) to final concentrations of 0.3% wt/vol, 0.6% wt/vol, 2.5% wt/vol and 2.5% wt/wt, respectively (sodium phosphate/CA) (Alonso et al., 2009). The reaction was conducted at 70 °C under magnetically stirring for 1 h. After cooling to room temperature, the mixture is adjusted to pH 7 in order to precipitate the product and then, centrifuged at 4000 rpm for 10 min. The obtained solid was washed with ethanol and lyophilized to give chitosan-co-agavin or chitosan-*g*-TBHQ-co-agavin as white powders; in all crosslinking reactions we obtain 80 ± 2% of mass yield.

2.4. Products characterization

NMR spectra were acquired in a Brucker AC 200 at 400 MHz spectrometer. Samples were dissolved in deuterated water for agavin, DMSO-d^6 for TBHQ and deuterated water with trifluoroacetic acid (10 vol%) for chitosan and products. Fructose:glucosamine ratio in the products was obtained by Eq. (1).

$$\text{Ratio}_{\text{fru:N-glc}} = \frac{6}{7}\left(\frac{\text{DA}\int \text{Mas}}{2\int \text{DAp}} - 1\right) \quad (1)$$

where DA is the degree of acetylation in the native chitosan expressed as molar fraction (0.193); $\int$Mas is the integration of the area assigned to the massive signals of agavin and chitosan, where 7 is the number of measurable hydrogens assigned to agavin and 6 is the number of measurable hydrogens assigned to chitosan; 2 is the stoichiometric relation between the measurable hydrogens of chitosan and those for the acetyl group; $\int$DAp is the integration of the area assigned acetyl signal.

The insertion of the TBHQ in the final product was calculated according to Eq. (2).

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