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Characterization of carvacrol beta-cyclodextrin inclusion complexes as delivery systems for antibacterial and antioxidant applications

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ABSTRACT

Carvacrol is a good natural antimicrobial and antioxidant agent; however, its poor aqueous solubility and high volatility limit its application in food systems. Beta-cyclodextrin (BCD) is able to encapsulate hydrophobic molecules improving its aqueous solubility and reducing its volatility. BCD-carvacrol inclusion complexes were prepared using kneading (KN) and freeze drying (FD) methods. Sizes of BCD-carvacrol complexes were 441 ± 12 nm and 899 ± 44 nm and entrapment efficiencies were 83.79 ± 2.89% and 91.31 ± 0.41% for KN and FD BCD-carvacrol complexes, respectively. Polydispersity index was higher ($P < 0.05$) than 0.1 for both methods, indicating a polydisperse system. Differential thermograms and phase solubility study indicated formation of 1:1 stoichiometry inclusion complex. Trolox Equivalent Antioxidant Capacity (TEAC) values ranged from 7491 to 6421 μmol TE/g among treatments, where KN BCD-carvacrol complex showed the lowest ($P < 0.05$) antioxidant activity. Storage stability of BCD-carvacrol complexes proved beneficial to carvacrol encapsulation. Antimicrobial activity against *Escherichia coli* K12 and *Salmonella enterica* serovar Typhimurium LT2 showed that all BCD-carvacrol complexes inhibited bacterial growth at lower ($P < 0.05$) carvacrol concentrations (values ranged from 300 to 350 μg/mL) compared to free carvacrol (≥1000 μg/mL). Results indicate that these BCD-carvacrol complexes could have important applications in food systems due to their storage stability and improved antimicrobial activity.

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1. Introduction

Foodborne pathogen infections and reactive oxygen species (ROS) are two threats which humans, animals and food are continuously exposed to. Foodborne diseases remain a global public health challenge, as some diseases are controlled, others emerge as new threats. The Centers for Disease Control and Prevention (CDC) estimates that each year 48 million people get sick, 128,000 are hospitalized, and 3000 die of foodborne diseases. *Salmonella* and *Escherichia coli* are two of the major pathogens that cause foodborne illnesses (CDC, 2012). There is an urgent need for new effective intervention strategies in the food industry to help prevent foodborne illness. On the other hand, ROS such as hydroxyl radicals, superoxide radicals, singlet oxygen, and hydrogen peroxide radical may lead to oxidative stress, which has been related to aging and many pathological disorders, including cancer, atherosclerosis, inflammation, and neurodegenerative disorders (Yin, Xu, & Porter, 2011). Antioxidants are compounds which slow down or prevent the oxidation of other molecules (Brewer, 2011). They interact with free radicals and prevent the damage by ROS. Thus, the treatment with antioxidants is potentially a way to overcome oxidative stress. Food products also deteriorate when exposed to ROS (Brewer, 2011). Therefore, compounds possessing antimicrobial and antioxidant activities present great potential in food systems applications. In particular, phenolic compounds are known for their antimicrobial and antioxidant properties (Beena & Rawat, 2013).

There has been a growing interest in the use of natural antimicrobials and antioxidants for application in food products. This is mainly due to a consumer preference for natural ingredients combined with concerns about toxic effects of synthetic compounds (Puertas-Mejia, Hillebrand, Stashenko, & Winterhalter, 2002). Carvacrol (5-isopropyl-2-methylphenol) is a phenolic monoterpene constituent of essential oils produced by numerous aromatic plants and spices such as black cumin (*Nigella sativa* L.), marjoram (*Origanum majorana* L.), oregano (*Origanum vulgare* L.),

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and thyme (*Thymus vulgaris* L.) (Silva et al., 2012). Many biological effects have been described for carvacrol, such as pronounced antioxidant effect *in vitro*, inhibitory action against 3-nitrotyrosin and malondialdehyde formation, free radical scavenger, and anti-lipidperoxidative agent (Beena & Rawat, 2013; Silva et al., 2012). When compared to its isomer thymol, carvacrol has shown higher ($P < 0.05$) antioxidant activity by radical scavenging assay (DPPH, 2,2-diphenyl-1-picrylhydrazyl) (Beena & Rawat, 2013). In another study, using an aldehyde-carboxylic acid assay Lee, Umano, Shibamoto, and Lee (2005) demonstrated that carvacrol and thymol (5 mg/L) could inhibit oxidation almost completely for 30 days. Moreover, carvacrol also exhibits antibacterial, antifungal, antiviral, antitumor and anti-inflammatory activities (Beena & Rawat, 2013; Silva et al., 2012).

Carvacrol is Generally Recognized as Safe (GRAS) for consumption, and it is approved by the US Food and Drug Administration for food use and has been included by the Council of Europe in the list of chemical flavorings that may be added to foodstuffs (De Vicenzi, Stammati, De Vicenzi, & Silano, 2004). Carvacrol and thymol have been used as antiseptic in medical practice, agriculture, cosmetics and food industry. However, there are challenges to use carvacrol as antimicrobial and antioxidant in food products: (i) it has an extremely low flavor threshold and can drastically change the sensory properties of the food, (ii) it is highly insoluble in water due to its lipophilic nature and may have limited contact with pathogens in high moisture content foods (Kalemba & Kunicka, 2003); and (iii) it is oxidized, decomposed, or evaporated when exposed to the air, light, or heat (Locci, Lai, Piras, Marongiu, & Lai, 2004). Inclusion of carvacrol in cyclodextrins (CD) is one method to overcome these problems because this technique greatly reduces volatility, oxidation, and heat decomposition (Cabral-Marques, 1994; Szente & Szejtli, 2004).

CDs have a rigid structure with a hydrophilic outer surface and a singular hydrophobic cavity due the absence of hydroxyl groups. Due to their distinctive structure, CDs are able to form inclusion complexes, often a 1:1 interaction, with essential oils and several compounds, enhancing their solubility, chemical stability, and bioavailability (Del Valle, 2004). Beta-cyclodextrin (BCD) is one of the most widely used due to its cavity size that is suitable for common drugs with molecular weights between 200 and 800 g/mol and has been on the GRAS list since 1998, as a flavor carrier and protectant (Szente & Szejtli, 2004; Waleczek, Marques, Hempel, & Schmidit, 2003).

Several methods have been used to prepare cyclodextrin inclusion complexes such as coprecipitation, neutralization, spray-drying, coevaporation, kneading, and freeze-drying (Liu, Lo, Tsai, & Cham, 2010). Among them, kneading method, also known as slurry complexation, is a method that requires small amount of solvent in the preparation and gives a very good yield of inclusion. Consequently, it is conduced to a more easily scaled-up process and lower production costs (Hedges, Shieh, & Sikorski, 1995). Freeze drying is another method that produces a powdered sample in a very good yield of inclusion formation. The low temperature minimizes the loss of extremely volatile guests, being especially useful for heat labile guests (Del Valle, 2004). Many studies have reported carvacrol antimicrobial and antioxidant activities; however, how different encapsulation processes affect these activities when using BCD to form inclusion complexes has not been studied, nor has its physico-chemical properties and storage stability once encapsulated.

Considering the requirements of effectiveness and convenience of the application of natural antimicrobial in food systems, this project aimed to: (1) to prepare carvacrol-BCD inclusion complexes using kneading and freeze-drying methods and characterize their physicochemical properties; (2) to determine the resulting antimicrobial activity against *E. coli* K12 and *S.* Typhimurium LT2, antioxidant activity, and storage stability.

2. Materials and methods

2.1. Materials

Carvacrol 98% was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). β-cyclodextrin (BCD, average MW = 1135.01) was purchased from Alfa Aesar (Heysham, England), and tryptic soy agar (TSA), tryptic soy broth (TSB), peptone water, for bacterial growth and enumeration were purchased from Becton, Dickinson and Co. (Franklin Lakes, NJ, USA), Tween 20 was obtained from VWR (West Chester, PA, USA). HPLC-grade acetonitrile was purchased from EMD Chemicals (Darmstadt, Germany). For the antioxidant activity determination, potassium peroxydisulfate 99% (Alfa Aesar), ABTS, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) and diammonium salt were obtained from AMRESCO (Solon, OH, USA), and Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) from Tokyo Chemical (Tokyo, Japan). All other reagents were of analytical grade.

2.2. Preparation of beta-cyclodextrin inclusion complexes

2.2.1. Freeze-drying

Carvacrol (1.2 g) was dispersed in 500 mL of BCD aqueous solution (16 mmol/L – 9.08 g) using a 1:1 molecular ratio and mixed in a laboratory stirrer for 48 h at room temperature (25 °C) to allow for complex formation and prevent loss of volatiles to the atmosphere (Karathanos, Mourtzinos, Yannakopoulou, & Andrikopoulos, 2007). The solution was frozen at −20 °C and lyophilized at −50 °C under 1.09 Pa for 48 h in a Labconco Freeze Dryer-5 (Kansas City, MO, USA). The lyophilized samples were stored in sealed containers inside a desiccator at −20 °C until further use.

2.2.2. Kneading

Carvacrol (1.2 g) and BCD (9.08 g) were initially mixed in a 1:1 molecular ratio in a mortar for 10 min. Then a small amount of ethanol was added to make a homogeneous paste. The paste was further kneaded manually for 45 min. The obtained mass was dried in a desiccator under vacuum for 48 h and stored in a desiccator at −20 °C until further analyses (Manolikar & Sawant, 2003).

2.3. Entrapment efficiency (EE)

The amount of carvacrol entrapped in the inclusion complexes was determined spectrophotometrically (spectrophometer model Genesys 10S UV-Vis, Thermo Scientific, Madison, WI, USA) at 275 nm. For both inclusion complexes, 5 mg of sample was dissolved in 5 mL of 95 g/100 mL acetonitrile and left for 48 h at room temperature after being well mixed to allow enough time for all entrapped carvacrol to be in solution, providing the total carvacrol from inclusion complexes (entrapped in the BCD cavity plus the surface-adsorbed). The carvacrol adsorbed on the surface of the inclusion complexes were determined according to Marreto et al. (2008) by washing 0.5 g of sample with 5 mL of acetonitrile for 20 min with intermittent shaking. Before measurement, the solutions were centrifuged at 3200 × g for 15 min (centrifuge model Clinical 200, VWR International, Darmstadt, Germany) to remove any BCD from the solution, leaving only the active compound. A standard curve of carvacrol was prepared with concentrations ranging from 2.5 to 30 μg/mL, under the same conditions. The EE was calculated according to equation (1) (Gomes, Moreira, & Castell-Perez, 2011):

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