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Functionalization of chitosan by a free radical reaction: Characterization, antioxidant and antibacterial potential

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Ascorbic acid (PubChem CID: 54670067)
Acetic acid (PubChem CID: 176)
2,2-Diphenyl-1-picrylhydrazyl radical (DPPH) (PubChem CID: 74358)
2,2′-Azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) (PubChem CID: 5815211)
6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (PubChem CID: 40634)
Folin-Ciocalteu reagent (PubChem CID: 452323)
Sodium carbonate (PubChem CID: 10340)

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ABSTRACT

Chitosan was functionalized with epigallocatechin gallate (EGCG) by a free radical-induced grafting procedure, which was carried out by a redox pair (ascorbic acid/hydrogen peroxide) as the radical initiator. The successful preparation of EGCG grafted-chitosan was verified by spectroscopic (UV, FTIR and XPS) and thermal (DSC and TGA) analyses. The degree of grafting of phenolic compounds onto the chitosan was determined by the Folin-Ciocalteu procedure. Additionally, the biological activities (antioxidant and antibacterial) of pure EGCG, blank chitosan and EGCG grafted-chitosan were evaluated. The spectroscopic and thermal results indicate chitosan functionalization with EGCG; the EGCG content was 25.8 mg/g of EGCG grafted-chitosan. The antibacterial activity of the EGCG grafted-chitosan was increased compared to pure EGCG or blank chitosan against *S. aureus* and *Pseudomonas* sp. ($p < 0.05$). Additionally, EGCG grafted-chitosan showed higher antioxidant activity than blank chitosan. These results indicate that EGCG grafted-chitosan might be useful in active food packaging.

1. Introduction

The antioxidant/antimicrobial molecules covalently grafted onto polymers improve their stability and functional properties as well as enlarge the field of the potential applications of polymers (Akagawa, 2008; Lee, Woo, Ahn, & Je, 2014; Zhu & Zhang,

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2014). Different synthetic strategies such as free radical grafting by the hydrogen peroxide/ascorbic acid pair redox have been developed for this purpose. This strategy, rather than another grafting reaction, allows the chemical functionalization of polymers with high reaction yields without the generation of toxic compounds (Spizzirri et al., 2010; Yuan, Qiu, Su, Cao, & Jiang, 2016). These modified polymers could offer numerous advantages in active packaging technology such as decreasing the concentration of antimicrobial/antioxidant (Božič, Štrancar, & Kokol, 2013), homogeneous distribution of the active compound onto the polymeric matrix, and the increased thermal stability of the antimicrobial/antioxidant (Curcio et al., 2009). This increased thermal stability is the most important because extrusion is one of the main processes used in the preparation of packaging technology. Previous studies have reported degradation of active compounds at temperatures higher than 100 °C (Beigmohammadi et al., 2016).

Chitosan is a biopolymer that presents reactive functional groups susceptible to chemical modification and has been shown to be a functional polymer to covalently graft antioxidant/antimicrobial activity onto its backbone (Choi, Nam, & Nah, 2016; Wang et al., 2016). Several forms of bioactivity such as antifungal, antimicrobial, and antioxidant activity of chitosan have been reported (Xie, Hu, Wang, & Zeng, 2014). Therefore, a synergistic effect of the antimicrobial or antioxidant compound grafted onto chitosan could exist. Aljawish, Chevalot, Jasniewski, Paris et al. (2014) grafted ferulic acid (FA) onto chitosan by laccase-catalyzed oxidation and reported an increase in antioxidant activity (20%) of the modified chitosan compared to blank chitosan. However, the FA grafted onto chitosan showed lower scavenging activity than pure FA.

Many active compounds such as citral (Jin, Wang, & Bai, 2009), eugenol, carvacrol (Chen, Shi, Neoh, & Kang, 2009), gallic acid (Hu et al., 2015), fluoroquinolone (Cirillo et al., 2014), hydroxycinnamic acid (Lee et al., 2014; Liu et al., 2015), catechin (Curcio et al., 2009; Mi, 2013; Zhu and Zhang, 2014), and oxazolidine (Parisi et al., 2014) have been covalently grafted onto polymers. However, the covalently grafted polymers do not inhibit the growth of gram-negative bacteria, particularly *Pseudomonas* sp., which is one of the bacteria mainly responsible for the deterioration of refrigerated products containing meat and fish and is also the group of bacteria least sensitive to the action of essential oils and bioactive components of plant origin (Tajkarimi, Ibrahim, & Cliver, 2010). However, epigallocatechin gallate (EGCG), a flavonoid and the principal active compound of green tea (*Camellia sinensis*), has received considerable attention and is considered a potential alternative to synthetic additives. EGCG is categorized as Generally Recognized as Safe (GRAS) by the US Food and Drug Administration and can inhibit the growth of *Pseudomonas* sp. (also *Stenotrophomonas maltophilia*) (Gordon & Wareham, 2010), *Escherichia coli* (Nakayama et al., 2013), *Pseudomonas aeruginosa* (Zhang et al., 2014), *Streptococcus mutans* (Hu et al., 2013), *Staphylococcus aureus* (Novy, Rondevaldova, Kourimska, & Kokoska, 2013), *Vibrio cholera* (Friedman, 2007), *Campylobacter jejuni* (Yanagawa, Yamamoto, Hara, & Shimamura, 2003) and *Clostridium perfringens* (Lee, Kim, Kim, & Kim, 2009).

Additionally, antioxidant activity of EGCG has been reported in *in vitro* assays (Cvetkovic et al., 2015; Fernando & Soysa, 2015; Hu & Kitts, 2001; Potapovich & Kostyuk, 2003; Zorilla, Liang, Remondetto, & Subirade, 2011) and food systems such as pork muscle (Zhong & Shahidi, 2012), mackerel (*Scomber scombrus*) (Banerjee, 2006), and minced beef (Tang et al., 2006). Studies have reported that EGCG shows a higher antioxidant activity than natural compounds such as carvacrol, eugenol, and thymol as well as synthetic antioxidants such as THBQ, BHT, BHA and α-tocopherol, which are commonly employed by the food industry (Wanasundara & Shahidi, 1996).

No previous studies have attempted to evaluate the antioxidant and antibacterial activity that may be achieved simultaneously when EGCG is grafted onto a chitosan backbone. In the present work, EGCG was covalently grafted onto a chitosan backbone and characterized. Additionally, antioxidant and antimicrobial activity against two typical foodborne bacteria *Staphylococcus aureus* (gram-positive) and *Pseudomonas* sp. (gram-negative) were evaluated.

2. Materials and methods

2.1. Materials

Epigallocatechin gallate (EGCG) (94 wt%) was purchased from Teavigo®, DSM Nutritional Products (Kaiseraugst, Switzerland). Chitosan from crab shells (average MW = 121 kDa, 80% deacetylation) (Martínez-Camacho et al., 2010), hydrogen peroxide (H_2O_2), ascorbic acid, acetic acid, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), 2,2′-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Folin Ciocalteu reagent and sodium carbonate were purchased from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO).

2.2. Synthesis of EGCG grafted-chitosan

EGCG grafted-chitosan was prepared according to the method of Curcio et al. (2009), with a slight modification. Chitosan (0.5 g) was dissolved in 50 mL of acetic acid water solution (0.4% v/v). Then, 1 mL of 1.0 M hydrogen peroxide containing 0.054 g of ascorbic acid was added. After 30 min, 0.5 g of EGCG was introduced into the reaction. The grafting reaction was maintained at 25 °C for 24 h under atmospheric conditions. Finally, the reaction was purified into Amicon ultra centrifugal filter tubes (MWCO 100,000 Da) with washes of acetic acid water solution (0.4% v/v) by centrifugation for 35 min at 3500 rpm and 25 °C (Sorvall Legend XTR Centrifuge, Eppendorf, Hamburg, Germany). EGCG grafted-chitosan was verified to be free of unreacted EGCG and any other compounds by spectrophotometric analysis after the purification step. EGCG grafted-chitosan solution was lyophilized. Blank chitosan as a control was prepared under the same conditions but in the absence of EGCG.

2.3. Structural characterization of EGCG grafted-chitosan

To verify that the EGCG was covalently grafted onto chitosan, the EGCG, EGCG grafted-chitosan and blank chitosan as a control were characterized using UV–vis, FTIR, XPS, DSC and TGA techniques.

2.3.1. UV–vis analysis

To prove the existence of a chemical bond between EGCG and chitosan, pure EGCG (0.3 mg/mL), blank chitosan (10 mg/mL) and EGCG grafted-chitosan (10 mg/mL) solutions were analyzed using a UV–vis Spectrophotometer (Varian Co. Ltd., California, USA). Acetic acid water solution (0.4% v/v) was the blank solution.

2.3.2. FTIR analysis

The EGCG, blank chitosan and EGCG grafted-chitosan were dispersed in KBr, pelletized and analyzed using a Perkin-Elmer Spectrum 2000 spectrometer (Perkin-Elmer Co., Norwalk, CT). The absorbance measurements were carried out within the 4000–400 cm^{-1} range, with 16 scans and a resolution of 4 cm^{-1}.

2.3.3. X-ray photoelectron spectroscopy analysis

Surface composition of blank chitosan and EGCG grafted-chitosan was determined by X-ray photoelectron spectroscopy (XPS), using a photoelectron spectrometer PHI 5100 Perking-Elmer with an AlKα X-ray source (1486.6 eV photons) at a constant dwell