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Short communication

Antibacterial effectiveness of chitosan–propolis coated polypropylene films against foodborne pathogens

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ABSTRACT

Antibacterial properties of chitosan are well documented in the literature. However its antibacterial effectiveness in the film form is controversial due to the methodological differences in test methods used. In this study, antibacterial effectiveness of chitosan-coated polypropylene films alone and incorporating ethanolic extract of propolis (EEP) were evaluated against six foodborne pathogens (*Bacillus cereus*, *Cronobacter sakazakii*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella typhimurium* and *Staphylococcus aureus*) using the ISO 22196 method designed for the antibacterial treated plastic products. The results demonstrated that chitosan coated film exhibited the broad-spectrum antibacterial activity. Incorporation of EPP to coating at 10% (propolis resin/chitosan) enhanced antibacterial activity against all pathogens tested. Results of this study revealed that chitosan has antibacterial activity in the film form and that propolis is a promising antimicrobial for the food packaging applications.

1. Introduction

Foodborne pathogens continue to cause major public health problems worldwide and antimicrobial packaging systems can help reduce the food safety risks related to foodborne pathogens by controlling post-processing contamination on the food surface [1,2].

Chitosan, a linear polysaccharide, is a deacetylated derivative of chitin [3]. In addition to its inherent antimicrobial characteristic, dilute acid solutions of chitosan have good film-forming properties; therefore it is potentially useful in gels and coatings [4]. Nevertheless, chitosan films present a very high hygroscopicity and they may loss their physical integrity in the presence of moisture [5]. Due to this restriction, films made from chitosan cannot completely replace to synthetic polymer films in food packaging applications. Coating of synthetic films with chitosan provides an alternative way for overcoming this shortcoming by combining the advantages of chitosan and synthetic polymers [6]. Moreover, chitosan layer can be used as efficient carrier for bioactive compounds such as antimicrobial substances [7].

Propolis is a chemically very complex resinous bee product collected by bees from tree buds and its resin consists of flavonoids and phenolic acids and their esters, waxes, essential oils, pollen and various organic compounds [8]. The antibacterial activity of propolis, mainly attributed to the flavonoids and the phenolic acids, can be explained by the several mechanisms such as alteration of membrane permeability and inhibition of protein synthesis, due to complex composition of propolis and synergistic activity between phenolic and other compounds [9,10]. Recent studies have shown that propolis has the potential to be used in biopolymer-based antimicrobial food packaging systems as a natural alternative antimicrobial agent [11,12]. Nevertheless, no study has to date been published to handle the incorporation of propolis in chitosan films with the coatings for food packaging applications.

There are a great number of studies devoted to evaluate the antibacterial effectiveness of chitosan films and coating. However, due to lack of standardization in the antibacterial test methods used, several inconsistent results were reported in these studies [1,13–16]. Therefore, in this study antibacterial effectiveness of chitosan coated plastic films against six important foodborne pathogens was evaluated using current ISO standard method [17] for the antibacterial-treated plastic products. Additionally, the effect of addition of ethanolic extract of propolis (EEP) to the coating solution on the antibacterial activity was evaluated.

2. Materials and methods

2.1. Materials

Medium molecular weight chitosan was purchased from Sigma–Aldrich (Milwaukee, USA). A crude sample of bee (*Apis mellifera*) propolis was collected from beehives located in Konya region, Turkey. All chemicals used for preparing the EEP and film solutions

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were purchased from Merck (Darmstadt, Germany). Commercial one side corona-treated polypropylene (PP) film of 50 μm thickness (Polinas, Manisa, Turkey) was used as the base film since untreated PP does not provide sufficient sites for binding the biopolymer [6].

Three Gram-positive foodborne pathogenic bacteria (*Bacillus cereus* ATCC 11778, *Listeria monocytogenes* ATCC 7644 and *Staphylococcus aureus* ATCC 25923) and three Gram-negative foodborne pathogenic bacteria (*Cronobacter sakazakii* ATCC 51329, *Salmonella typhimurium* ATCC 14028 and *Escherichia coli* O157:H7 NCIMB 13861) were used in this study. Lyophilized cultures of microorganisms, except for *E. coli* O157:H7, were supplied from Microbiologics Inc. (Saint Cloud, USA). Culture of *E. coli* O157:H7 was kindly provided by STA Food Control Laboratory (Mersin, Turkey).

2.2. Ethanolic extract of propolis (EEP)

Grounded propolis was extracted with 80% ethanol in the dark for 7 days, with periodical strong hand shaking. The resulting aqueous-ethanol extract was filtered and concentrated at 50 °C, and the obtained resin was dissolved in 95% ethanol to a final concentration of 200 mg/ml. This final solution was incorporated to the coating solution.

2.3. Preparation of coating solutions

The chitosan solution was prepared by dissolving chitosan in an aqueous solution (1%, v/v) of acetic acid, to reach a final concentration of 2% (w/v). Glycerol was added to chitosan solution as plasticizer at the concentration of 2% (w/v). Tween 20 was added to chitosan solution at concentration of 0.05% (v/v) in order to improve its wettability and adhesion properties [18]. To obtain chitosan–propolis composite coating solution, EEP solution was incorporated into chitosan solution to reach final concentration of EEP resin/chitosan in the solution at 10% by weight.

2.4. Coating of polypropylene (PP) films

PP films were taped to a glass plate and coating solutions were cast onto the PP films with a thin-layer chromatography plate coater (Desega, Heidelberg, Germany) to obtain a 0.5 mm wet coating thickness. Then, coated PP films were dried at room temperature.

2.5. Preparation of inoculums

Stock cultures of microorganisms were stored in brain heart infusion broth (Merck) supplemented with 20% glycerol at −18 °C. Working cultures were grown on nutrient agar (Merck) slants and kept at 4 °C. Cell suspensions of microorganisms were prepared and adjusted to cell density of approximately 5×10^6 CFU/ml in 1/500 nutrient broth (Merck). These suspensions were used as inoculum in in vitro antibacterial activity assay.

2.6. Antibacterial activity

Antibacterial activities of coated PP films were determined by quantifying the survival of bacteria held in intimate contact for 1, 6 and 24 h at 35 °C with the coated sides according to ISO 22196 standard [17]. The plain PP film was used as control.

Film samples (50 mm × 50 mm) sterilized by UV treatment were placed into sterile petri dishes with the coated surface uppermost. An aliquot (200 μl) of test inoculums were pipetted onto film samples. Then inoculated film samples were covered with a piece of UV sterilized plain PP film (40 mm × 40 mm). Petri dishes containing the inoculated film samples were incubated at 35 °C under relative humidity of above 90%.

Microbiological counts were performed on the film samples immediately after inoculation and 1, 6, and 24 h of incubation. Microorganisms were recovered from film samples by D/E neutralizing broth (Acumedia, Lansing, USA) and inoculated onto tryptic soy agar plates (Lab M, Bury, UK). After incubation at 35 °C for 48 h, colonies grown on plates were counted and microorganism counts were calculated as log CFU/sample.

3. Results and discussion

Chitosan coated film showed a remarkable reduction in the counts of all microorganisms tested ranging from 2.07 to 3.26 log compared to control film during the 24 h exposure period (Fig. 1). These reduction values are satisfactory to demonstrate the antibacterial efficacy according to the criterion (≥2 log) defined in the Japanese standard JIS Z 2801 [19], which is identical to ISO 22196. Nevertheless, a pass/fail criterion for the antibacterial efficacy is not defined in the ISO 22196.

Results of present study revealed that chitosan coated film have a broad-spectrum of antibacterial activity. Results have also shown that inhibitory efficiency of chitosan coated film against Gram-positive bacteria (3.11–3.26 log) was higher compared to Gram-negative bacteria (2.07–2.23 log). Similarly, in previous studies [20,21], chitosan was found more effective on Gram-positive bacteria than Gram-negative. Strong antibacterial activity of chitosan against Gram-positive bacteria was explained by Fernandez-Saiz et al. [22], in terms of positively charged chitosan molecules interacting with the highly negatively charged teichoic acid backbone in their cell wall. This interaction influences the permeability of bacterial cell membranes and cause leakage of the cell contents [21,23].

In the present study, antibacterial activity of chitosan-coated film can be attributed to direct contact of bacterial cells with chitosan. As chitosan is in a solid form, only microorganisms in direct contact with the active sites of chitosan are inhibited [24]. Intimate contact principle of ISO 22196 based on low-volume of inoculum and relatively large test surface makes possible direct contact between bacterial cells and active sites of chitosan.

Another possible mechanism to explain antibacterial activity of chitosan-coated film is the soluble chitosan molecules that are released from the solid-state film upon liquid phase contact. Tang et al. [25] reported that the antibacterial effects of the cross-linked chitosan films decreased significantly with an increase in the cross-linking agent content. Fernandes-Saiz et al. [5] evaluated the dissolution of chitosan from chitosan films in liquid medium and reported a direct correlation between glucosamine release and antibacterial activity. In accordance with these findings, Ye et al. [26] reported that chitosan coated plastic film considerably inhibited the growth of *L. monocytogenes* in liquid medium in a chitosan concentration-depended manner.

The present study clearly contrasts with a recent study [16] dealing with the antibacterial effectiveness of chitosan film against *E. coli* and *S. aureus*, in which chitosan film did not exhibit any bactericidal properties by agar diffusion method. Similarly unsatisfactory results obtained by the agar diffusion method were reported in previous studies [1,13]. Author of these studies concluded that chitosan in film form is incapable of diffusing through the adjacent agar media and failed to exhibit any bactericidal properties in the film form due to lack of interactions between the biopolymer chains and microbial cell walls. Nevertheless, this conclusion contrasts with the highly hydrophilic character of chitosan films. Results of study of Lagaron et al. [15] in which the molecular structure of chitosan films was studied by ATR-FTIR spectroscopy upon direct contact with nutrient agar plates demonstrated that a considerable release of the protonated groups took place by capillarity