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Development of flexible zein–wax composite and zein–fatty acid blend films for controlled release of lysozyme

Iskender Arcan, Ahmet Yemenicioğlu *

Department of Food Engineering, Faculty of Engineering, Izmir Institute of Technology, 35430, Gülbahçe Köyü, Urla, Izmir, Turkey

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ABSTRACT

The aim of this study was controlled release of lysozyme by modification of hydrophobicity and morphology of zein films using composite and blend film making methods. The incorporation of beeswax, carnauba or candelilla wax into films at 5% (w/w) of zein gave composite films containing amorphous wax particles, while incorporation of oleic acid into film at 5% of zein caused formation of blend films containing many spherical zein capsules within their matrix. The lysozyme release rates of composites reduced as the melting point of waxes increased. The composites and blends showed 2.5 to 17 fold lower lysozyme release rates than controls. The films were effectively plasticized by using catechin. The catechin also provided antioxidant activity of films (up to 69 μmol Trolox/cm^2) and contributed to their controlled release properties by reducing film porosity. The films showed antimicrobial activity against *Listeria innocua*. This work showed the possibility of obtaining advanced edible films having flexibility, antimicrobial and antioxidant activity and controlled release properties.

1. Introduction

There has been a growing interest to develop the functional properties of packaging materials and to obtain packed foods with better shelf-life and quality. Active packaging incorporating antimicrobials is one of the most promising areas since application of this method can improve safety of foods by inhibiting pathogenic bacteria or controlling spoilage flora using minimum amounts of active compounds (Appendini & Hotchkiss, 2002). Although different natural and chemical preservatives have successfully been incorporated into plastic, biodegradable and/or edible packaging materials, health concerns of the consumers and environmental problems caused a particular interest in using natural antimicrobial compounds in edible packaging materials (Appendini & Hotchkiss, 2002; Han, 2005).

The natural antimicrobial agents frequently employed in active packaging include antimicrobial enzymes, bacteriocins, essential oils and phenolic compounds (Appendini & Hotchkiss, 2002; Joerger, 2007; Mastromatteo, Mastromatteo, Conte, & Del Nobile, 2010). Lysozyme obtained from hen egg white is one of the most potential candidates for antimicrobial packaging since it has a GRAS status and it shows good stability and activity in different films and food systems under refrigerated storage temperatures (Mecitoglu et al., 2006; Ünalan, Korel, & Yemenicioğlu, 2011). Thus, lysozyme has recently been tested extensively in different plastic materials such as cellulose acetate, nylon, and PVOH (Gemili, Yemenicioglu, & Altinkaya, 2009; Joerger, 2007) and biopolymeric materials from zein, soy protein, carrageenan, whey protein, chitosan, alginate and pullulan (Joerger, 2007; Mendes de Souza, Fernández, López-Carballo, Gavara, & Hernández-Muñoz, 2010). This enzyme shows antimicrobial activity mainly on Gram-positive bacteria by splitting the bonds between N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycan in their cell walls. However, due to their protective outer membrane surrounding the peptidoglycan layer, it does not show antibacterial activity against Gram-negative bacteria. Thus, the application of lysozyme in active packaging targets mainly inhibition of the critical Gram-positive pathogenic bacteria such as *Listeria monocytogenes* (Duan, Park, Daeschel, & Zhao, 2007; Min, Harris, Han, & Krochta, 2005). However, when lysozyme is combined with ethylenediaminetetraacetic acid (EDTA), the outer membranes of Gram-negative bacteria are destabilized by EDTA and the obtained films become effective on the other critical pathogenic bacteria including *Escherichia coli O157:H7* and *Salmonella typhimurium* (Gucbilmez, Yemenicioglu, & Arslanoglu, 2007; Mecitoglu et al., 2006; Padgett, Han, & Dawson, 1998; Ünalan et al., 2011).

Antimicrobial packaging targets mainly the food surface on which microbiological changes occur most intensively (Appendini & Hotchkiss, 2002). However, a sufficient antimicrobial effect could not be achieved unless the release rate of antimicrobial compounds from the packaging materials to food surface could be adjusted considering the physical and chemical properties of food, growth kinetics of target pathogenic or spoilage microorganisms and the expected food shelf life (Han, 2005). In the literature, there are different studies which aim to design plastic, biodegradable or edible films particularly for controlled release of lysozyme. Buonocore, Conte, Corbo, Sinigaglia, and Del Nobile (2005)

* Corresponding author. Tel.: +90 232 7506292; fax: +90 232 7506196.
E-mail address: ahmetyemenicioglu@iyte.edu.tr (A. Yemenicioğlu).

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produced multilayered PVOH films or changed the degree of crosslinking for PVOH films to achieve controlled release of lysozyme. To achieve controlled release of lysozyme, Gemili et al. (2009) developed asymmetric cellulose acetate films with varying porosities while Bezemer et al. (2000) changed composition and molecular weight of copolymers in biodegradable polyethylene glycerol/poly butylene trephthalate films. Mendes de Souza et al. (2010) developed sodium caseinate films of which lysozyme release profiles could be changed by modifying pH and the amount of crosslinking agents such as $CaCl_2$, transglutaminase and glyoxal used in film making. Park, Daeschel, and Zhao (2004) formed chitosan–lysozyme composites and achieved different release rates for lysozyme by changing amount of enzyme within the composite structure. Gucbilmez et al. (2007) achieved a sustained lysozyme release from zein films by forming their composites with chickpea proteins.

Zein, a water insoluble hydrophobic storage protein found in corn and maize, attracts a particular interest as a biopolymer since it has excellent film forming and gas barrier properties; it is one of the rare proteins soluble in various organic solvents including ethanol and it is the major co-product of the oil and rapidly growing bioethanol industries (Manley & Evans, 1943; Selling, Woods, Sessa, & Biswas, 2008; Shukla & Cheryan, 2001; Wang et al., 2007; Zhang, Luo, & Wang, 2011). Thus, a particular interest has been focused on use of zein in active food packaging by incorporation of different natural antimicrobials including lysozyme (Gucbilmez et al., 2007; Mecitoglu et al., 2006; Padgett et al., 1998). However, the classical brittleness, and flexibility problems of zein films is a great limitation for their use as a free standing film and more widespread application as an active coating material. Moreover, to increase the potential application of active zein based films in food industry further studies are needed to develop smart controlled release mechanisms for different natural antimicrobial agents used in active packaging. Recently, Arcan and Yemenicioglu (2011) improved the flexibility of zein films effectively by using natural phenolic compounds like catechin, but studies to develop smart controlled release mechanisms for lysozyme containing zein films are scarce. In the present study, controlled release of lysozyme was achieved by exploiting the different morphologies of zein–wax composites and zein–oleic acid blends prepared by means of homogenization in presence of emulsifier lecithin. The classical brittleness problem of the obtained zein-based films was also eliminated by using the recently discovered natural zein plasticizer catechin (Arcan & Yemenicioglu, 2011). This study brings a novel approach by showing possibility of creating flexible active packaging with controlled release properties using an edible biopolymer.

2. Materials and methods

2.1. Materials

Maize zein (batch number: 058 K0093), (+)-catechin hydrate (≥98%), *Micrococcus lysodeikticus*, carnauba wax (No.1, refined), beeswax, candelilla wax, and oleic acid (90 %) were from Sigma Chem. Co. (St. Louise, Mo, USA). Soybean L-α-lecithin and glycerol were from Merck (Darmsdadt, Germany). All other chemicals were reagent grade. Fresh hen eggs used in production of lysozyme were obtained from a supermarket in Izmir (Turkey).

2.2. Preparation of films

Zein films were produced as described in Padgett et al. (1998). Briefly, 1.4 g zein was dissolved with 8.1 mL of ethanol (96 %) by mixing slowly with a magnetic stirrer for 25 min. Glycerol (0.4 mL) was then added to the medium as a plasticizer. The temperature of the mixture was then increased until it started to boil. The mixing was ceased and the solution was cooled to the room temperature after it had been boiled for 5 min. After that, the lysozyme (11.7 mg/g film forming solution) used as antimicrobial agent, catechin (50–100 mg/g film forming solution) used as an additional plasticizer for the zein (Arcan & Yemenicioglu, 2011) and lecithin (5% (w/w) of zein) used as emulsifier (for wax and oleic acid containing films only) were added into film forming solutions and the mixtures were homogenized (Heidolph, Germany, rotor Φ = 6.6 mm tip) at 10,000 rpm for 4 min. Then, 4.3 g portions of the homogenized film forming solutions were poured into the glass templates (W × L × H: 8.5 × 8.5 × 0.4 cm) and dried for 19 ± 2 h at 25 °C in an incubator unless otherwise was stated (see Section 2.6). This procedure was also used to obtain zein–wax composite and zein–fatty acid blend films by adding waxes (carnauba wax, candelilla wax and beeswax) or fatty acid (oleic acid) into film forming solutions at 5% (w/w) of zein. The waxes were added just before initiation of heating to melt them during boiling and ease their homogenization, while oleic acid was added after the boiling step following cooling to room temperature.

2.3. Production of lysozyme

Lysozyme was produced according to the method previously applied by Mecitoglu et al. (2006). For this purpose, carefully separated egg whites were diluted 3-fold with 0.05 M NaCl solution. To precipitate the egg white proteins other than the lysozyme, the pH of this mixture was set to 4.0 by adding several drops of 1 N acetic acid, and the solution was diluted with equal volume of 60 % (v/v) ethanol. After 6 h incubation at room temperature in the presence of 30 % ethanol, the mixture was centrifuged at 15.000× *g* for 15 min at 4 °C and the precipitate was discarded. The supernatant containing lysozyme was first dialyzed for 21 h at 4 °C by three changes of 2000 mL distilled water and then lyophilized by using a freeze drier (Labconco, FreeZone, 6 liter, Kansas City, MO, USA). The lyophilized enzyme was stored at −18 °C until it was used in film making.

2.4. Release tests and lysozyme activity

The release tests of films were conducted in water at 4 °C by applying shaking during the incubation period. Briefly, 4 × 4 cm pieces of films were placed into glass Petri dishes containing 50 mL of deionized water. The dishes were kept at 4 °C in an incubator and shaken with an orbital shaker working at 80 rpm. The release tests were conducted until equilibrium was reached for release of lysozyme or an insignificant increase was observed in lysozyme release. The lysozyme activity was monitored by taking 0.1 mL (×3) aliquots from the release test solution at different time intervals. The enzyme activities in collected aliquots were determined spectrophotometrically at 660 nm by using a Shimadzu (Model 2450, Japan) spectrophotometer equipped with a constant temperature cell holder working at 30 °C. The enzyme activities were calculated from the slopes of initial linear portions of absorbance *vs*. time curves and expressed as unit (U) which was defined as 0.001 change in absorbance in 1 min. All calculations were corrected by considering the activity removed by collected aliquots during sampling. The total lysozyme activity released from each film corresponded to maximum units released per cm^2 of the films (U/cm^2) at the equilibrium. All activity measurements were conducted for three times. The release curves were formed by plotting calculated released activities (U/cm^2) vs. time (h). The initial release rates of lysozyme were determined from the slope of the initial linear portion of release curve. The release rates were expressed as $U/cm^2/h$.

2.5. Scanning electron microscopy (SEM)of films

The cross-sectional morphology of selected films was determined by using SEM (Philips XL 30S FEG, FEI Company, Netherlands) under high vacuum mode at an operating voltage varying between 2 and 6 kV. Films were prepared for SEM by crashing, following freezing