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Original article

Active packaging of ground beef patties by edible zein films incorporated with partially purified lysozyme and Na_2EDTA

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Summary In this study, antimicrobial activity of zein films incorporated with partially purified lysozyme and disodium ethylenediaminetetraacetic acid (Na_2EDTA) has been tested on selected pathogenic bacteria and refrigerated ground beef patties. The developed films containing 700 μg cm^{-2} lysozyme and 300 μg cm^{-2} Na_2EDTA showed antimicrobial activity on *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella typhimurium*. The application of lysozyme and Na_2EDTA incorporated zein films on beef patties significantly decreased total viable counts (TVC) and total coliform counts after 5 days of storage compared to those of control patties ($P < 0.05$). Zein films incorporated with lysozyme and Na_2EDTA or Na_2EDTA alone significantly slowed down the oxidative changes in patties during storage ($P < 0.05$). Redness indices of patties coated with zein films were significantly lower than those of uncoated control patties during storage ($P < 0.05$). This study demonstrated the potential usage of zein films containing lysozyme and Na_2EDTA for active packaging of refrigerated meat products.

Keywords Antimicrobial packaging, ground beef patties, lysozyme, oxidation, zein films.

Introduction

Due to concerns over the risk of food-borne microbial outbreaks caused by minimally processed fresh and refrigerated products (De Roever, 1998; Devlieghere *et al*., 2004), extensive studies are conducted to develop and use new antimicrobial packaging technologies for food industry. A particular interest has been focused on using natural antimicrobial compounds in biodegradable edible packaging materials, since chemicals and plastics lost their attraction due to health and environmental concerns and problems (Appendini & Hotchkiss, 2002; Teerakarn *et al*., 2002; Lopez-Rubio *et al*., 2006; Rojas-Graü *et al*., 2009). The major natural antimicrobial agents tested for active packaging include antimicrobial enzymes, bacteriocins, plant phenolics and essential oils (Labuza & Breene, 1989; Appendini & Hotchkiss, 2002; Suppakul *et al*., 2003). These natural agents are incorporated into different biodegradable or edible packaging materials obtained from zein, cellulose derivatives, carrageenan, alginate, and whey protein isolates (Padgett *et al*., 1998; Han, 2000; Hoffman *et al*., 2001; Quintavalla & Vicini, 2002; Suppakul *et al*., 2003.

Lysozyme is one of the most commonly used natural antimicrobial agents incorporated into packaging materials (Appendini & Hotchkiss, 1997; Han, 2000; Quintavalla & Vicini, 2002; Mecitoğlu *et al*., 2006; Güçbilmez *et al*., 2007). This enzyme is effective mainly on Gram-positive bacteria and shows its antimicrobial activity by hydrolyzing β-1–4 glycosidic linkage between the *N*-acetylmuramic acid and *N*-acetylglucosamine of the peptidoglycan (PG) layer in bacterial cell wall (Masuda *et al*., 2001). The resistance of Gram-negative bacteria to lysozyme is due to lipopolysaccharide (LPS) layer surrounding their outer membrane and preventing the access of lysozyme to the PG layer (Davidson *et al*., 1993). To overcome the LPS layer of Gram-negative bacteria, lysozyme is generally combined with ethylenediaminetetraacetic acid (EDTA). Due to its metal chelating properties, EDTA destabilises the protective LPS layer of Gram-negative bacteria and increases their sensitivity to lysozyme (Branen & Davidson, 2004). This chelating agent is also very effective in preventing metal catalyzed oxidative changes in foods (Lindsay, 1996).

Recently, partially purified lysozyme obtained from hen egg white by a simple and economically feasible method based on ethanol precipitation of undesirable egg white proteins has been incorporated into zein films for antimicrobial packaging by Mecitoğlu *et al*. (2006) and Güçbilmez *et al*. (2007). The partially purified lysozyme was very stable and lost almost no activity in lyophilised form or in cast edible zein films stored at

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-18 and 4 °C for up to 8 and 4 months, respectively (Mecitoğlu *et al*., 2006). In this study, the developed zein films containing lysozyme were supported with Na_2EDTA and antimicrobial activity of films was tested on selected pathogenic bacteria and refrigerated ground beef patties. The potential benefits of the packaging treatment on oxidative stability and colour of ground beef patties were also evaluated. This study prepares a basis of using partially purified lysozyme containing zein films in active packaging of meat products.

Materials and methods

Materials

Fresh hen eggs used in preparation of partially purified lysozyme were obtained from a local supermarket in İzmir. Zein, *Micrococcus lysodeikticus*, dialysis tubes (12 000 MW), and 1,1,3,3-tetraethoxypropane (TEP, MW 220) were obtained from Sigma Chem. Co. (St Louis, MO, USA). $Na_2EDTA{\cdot}2H_2O$ was purchased form Riedel-de haën (Sigma-Aldrich Laborchemikalien, Seelze, Germany).

Preparation of partially purified lysozyme

Lysozyme was produced by the ethanol precipitation of non-enzyme protein impurities in the egg white as described in Mecitoğlu *et al*. (2006). The supernatant containing lysozyme was effectively dialyzed at 4 °C for 21 h by three changes of 2000 mL distilled water and then lyophilised by using a freeze drier (Labconco, FreeZone, 6 L, Kansas City, MO, USA) working under 50×10^{-3} and 100×10^{-3} mbar vacuum between −44 and −47 °C collector temperature. The lyophilised lysozymes (average activity: 15111 ± 935 units mg^{-1}) were stored at −18 °C until used in film preparation.

Determination of lysozyme activity

The activity of lysozyme was determined at 660 nm by a Shimadzu (Model 2450, Tokyo, Japan) spectrophotometer equipped with a constant temperature cell holder working at 30 ± 0.2 °C. The reaction mixture was prepared by mixing 2.3 mL *Micrococcus lysodeikticus* cell suspension (at 30 °C, prepared 0.26 mg mL^{-1} in 0.05 M Na–phosphate buffer at pH 7.0) and 0.2 mL enzyme solution (at 30 °C). The reduction in absorbance was monitored for 120 s and enzyme activity was calculated from the slope of the initial linear portion of absorbance vs. time curve. The activity of soluble enzyme in solutions was calculated by the following equation as unit (U) per mL. Unit was defined as 0.001 change in absorbance in 1 min. The average of three activity measurements was used in all tests:

$$\text{Activity (Unit mL}^{-1}) = (\text{Slope (abs min}^{-1})/0.001) \times (1/\text{enzyme volume (0.2mL)}) \times (\text{reaction volume (2.5mL)})$$

Preparation of films

Zein film forming solutions were prepared in ethanol as described by Mecitoğlu *et al*. (2006). The lysozyme and Na_2EDTA were added to film forming solutions to obtain final concentrations of 700 μg cm^{-2} (43 mg g^{-1}) and 300 μg cm^{-2} (19 mg g^{-1}) in dried films, respectively. The film forming solutions were then homogenised by a homogenizer-disperser (Silent Crusher M, Heidolph, Schwabach, Germany) at 8000 rpm (8F rotor with 6.6 mm diameter) for 2 min. Each solution was then cast onto a 8.5 × 8.5 cm glass plate placed on a balance and 4.3 g solution weight was spread evenly using a glass rod on its surface. The films were dried at room temperature (22 ± 1 °C maintained by an air conditioner) for 24 h. The films were cut into 3 × 3 cm pieces (four pieces from each film) used in packaging applications immediately. The average thickness of zein films determined with scanning electron microscope (SEM) (Philips XL 30S FEG; FEI Company, Eindhoven, the Netherlands) by taking ten measurements at different points of film were 128 ± 6, 131 ± 4, and 135 ± 6 μm for control, Na_2EDTA, and lysozyme and Na_2EDTA containing films, respectively. A representative cross-section photograph of the control film for measuring the film thickness is given in Figure S1, see Supporting Information online.

Determination of lysozyme activity of films

The soluble lysozyme activity of zein films was determined by release tests conducted in distilled water at 4 °C. The experiment was performed in a refrigerated incubator (VELP Scientifica, FOC 225I, Usmate, Italy). Briefly, the films (6 × 6 cm) were placed in glass Petri dishes (10 cm in diameter) containing 50 mL distilled water (4 °C). The dishes were stirred at 200 rpm using a magnetic stirrer (2 cm long teflon coated rod), until reaching of equilibrium for lysozyme release monitored by assaying enzyme activity at 0, 30, 60, 300, and 1400 min. The Petri dishes were closed tightly with their lids and wrapped with parafilm to prevent evaporation. The sampling was conducted by taking 0.6 mL aliquots from the release test solution and performing lysozyme activity measurements for three times (0.2 mL aliquot per measurement) as described in 'Determination of lysozyme activity' section. The enzyme activity was expressed as total units released per cm^2 of the films (U cm^{-2}). All calculations were corrected by considering the total activity removed during sampling.

The bound lysozyme activity of zein films was measured by using films exposed to release test. These films did not contain measurable soluble lysozyme activity. The obtained 6 × 6 cm films were cut into four equal-sized pieces and each piece (3 × 3 cm) was placed into separate Petri dishes containing 25 mL *Micrococcus lysodeikticus* solution (at 30 °C) used also in soluble activity determination. The Petri dishes were kept in an incubator at 30 °C and their contents' absorbance at 660 nm was monitored periodically in 5 min intervals for 90 min under continuous magnetic stirring at 200 rpm. The bound lysozyme activity was calculated by the following equation after determining the slopes of the initial linear portions of absorbance vs. time curves and it was given as U cm^{-2}.

$$\text{Activity (U cm}^{-2}) = (\text{Slope (abs min}^{-1})/0.001) \times (1/\text{film area (9 cm}^2)) \times (\text{reaction volume (25 mL)})$$

In vitro antimicrobial activity of films

Test of film antimicrobial activity was conducted by using *Listeria monocytogenes* (ATCC 7644), *Escherichia coli* O157:H7 (ATCC 700728), and *Salmonella typhimurium* (CCM 5445) as test microorganisms. These test microorganisms, *L. monocytogenes*, *E. coli* O157:H7, *S. typhimurium*, were kindly provided by Dr Özer Kınık (Ege University, Department of Dairy Technology, İzmir), Dr Ali Aydın (İstanbul University, Department of Food Hygiene and Technology, İstanbul), and Dr Handan Baysal (İzmir Institute of Technology, Department of Food Engineering, İzmir), respectively. The overnight cultures were prepared in nutrient broth by conducting incubations at 37 °C. For antimicrobial tests, sixteen discs (1.3 cm in diameter) from each film (6 × 6 cm) were prepared by a cork borer under aseptic conditions. Total of twelve discs were selected randomly and three out of twelve discs were placed into each Petri dish (total of four Petri dishes for each film type) containing nutrient agar, which had been previously inoculated with 0.1 mL of inocula containing from 10^6 to 10^7 CFU mL^{-1} of tested bacteria. The nutrient agar was used to determine the microbial loads of the inocula by incubating at 37 °C for 48 h. The Petri dishes were then incubated at 37 °C for 48 h and the areas of the fully formed zones (ffz) observed were determined by measuring the zone diameter with a digital caliper. The zones with diameters ≤1.1 cm and zones formed on only one side of the discs were designated as partially formed zones (pfz) and their numbers were reported. The numbers of negative zones (nz) were also counted and reported.

Packaging application

The performances of the developed films were evaluated by packaging of ground beef patties. The patties (containing 18% fat and 1% salt) lacking any antimicrobial and antioxidant agents were particularly produced for this study by the Pınar Meat Company, Inc., İzmir, Turkey. The meat used in patties was obtained from brisket (carcass refrigerated stored for 3–4 days after slaughtering and then brisket obtained left 2 days in shock freezing unit before used in pattie manufacturing). The three replicates used in this study were obtained at the same time from the same meat party. The patties were cut into 3 × 3 cm pieces (approximately 10 g) and divided into four groups randomly. One group was separated as control without applying any films. Film application was conducted for other three groups by placing 3 × 3 cm cut films at both sides of each piece of pattie. The three types of zein films applied to patties contained (i) no additives [ZF], (ii) 300 μg cm^{-2} Na_2EDTA [ZF(Na_2EDTA)], or (iii) 700 μg cm^{-2} lysozyme and 300 μg cm^{-2} Na_2EDTA [ZF(lysozyme-Na_2EDTA)]. After film application, all samples were wrapped first with stretch plastic film to create a good contact between zein film and pattie surface and then with aluminum foil to prevent unfolding or loosening of stretch film during storage. They were stored in a refrigerated incubator at 4 °C for 7 days and microbial load was determined at 0, 3, 5 and 7 days, while oxidation level and colour were determined at 0, 3 and 7 days. The films were peeled from sample surfaces before all analyses.

Microbiological analysis

For microbiological analysis, samples (10 g) were homogenised in sterile stomacher bags containing 0.1% sterile peptone water (90 mL) using a stomacher (Bag-Mixer® 400, Interscience, Bois Arpens, France) for 60 s. Serial dilutions were made and 1 ml aliquots of appropriate dilutions were pour plated with plate count agar (PCA, Fluka, Spain) for TVC and violet red bile agar (VRBA, Fluka, Spain) for TCC. After solidification, additional layer of VRBA was poured into the petri dishes. Incubations were conducted at 30 °C for 48 h for total viable count (TVC) and at 37 °C for 24 h for total coliform count (TCC). Experiments were performed in triplicate. The microbial counts were expressed as $\log_{10}$ of colony forming units (CFU) per g of sample.

Determination of oxidative stability

The oxidative stability of refrigerated stored patties was determined with the thiobarbituric acid (TBA) method as described by Bekhit *et al.* (2003). For analysis, 2.5 g sample was placed into a beaker containing 25 mL of

0.38% TBA and 15% trichloroacetic acid (TCA) prepared in 0.25 N HCl solution. The sample was homogenised at 10 000 rpm for 3 min using a homogenizer (Silent Crusher M, Heidolph, Schwabach, Germany) and three 5 mL aliquots obtained from homogenate were incubated in a 95 °C water bath for 15 min for colour development. Samples were cooled for 10 min in ice water and then centrifuged at $4500 \times g$ for 15 min. Their absorbance was measured at 532 nm by using a UV-VIS spectrophotometer (Shimadzu, Model 2450, Tokyo, Japan). A standard curve was formed using dilutions of 1 mM 1,1,3,3-tetraethoxypropane (TEP) and the results were expressed as mg malonaldehyde (MDA) per kg pattie. MDA extinction coefficient calculated from standard curve of TEP was 1.56×10^5 M^{-1} cm^{-1}. The average of three readings was used during tests of individual samples. The experiments were performed in duplicate, for two pieces of separately packed patties from each packaging treatment.

Determination of colour

The surface colour of patties was measured using a colour machine vision system (CMVS, ECS Inc., Gainesville, FL, USA). The samples were placed in a light box. An image of patties was taken with a charged coupled device (CCD) video camera located inside the light box. The 24-bit colour image was saved in the computer. The image was analysed to generate a discrete spectrum of the colours present in the sample, and the average CIE lightness (L^*), redness (a^*), and yellowness (b^*) values of all the pixels representing the sample. The a^*/b^* value was reported as redness index. Images were taken at 0, 3 and 7 days. The average of readings from surfaces of two different pieces of patties for each treatment was used for the calculation of L^* and a^*/b^* values.

Statistical analysis

Analysis of variance (ANOVA) was applied using Minitab 14 (Minitab Inc., State College, PA, USA) to determine the effects of antimicrobial activities of different films on *L. monocytogenes*, *E. coli* O157:H7, and *S. typhimurium* and to determine the effects of storage time and different packaging treatments on the microbiological, oxidative stability, and colour values of beef patties. Multiple comparisons of means were performed using Tukey's HSD (Honestly Significant Differences) test with a level of 95% confidence interval.

Results and Discussion

Lysozyme activity of zein films

The release tests conducted in distilled water at 4 °C until reaching of equilibrium showed the presence of 7550 ± 547 U cm^{-2} soluble lysozyme activity in films incorporated with both 11 025 U cm^{-2} lysozyme and Na_2EDTA. This corresponds almost 68% of the lysozyme activity incorporated into the films. The remaining lysozyme exists in bound form, possibly due to its trapping or affinity to film matrix. The bound lysozyme activity of films measured was almost 20.3 U cm^{-2} and this formed almost 0.3% of total (soluble + bound = 7570 U cm^{-2}) enzyme activity of films. Although it is considerably low, the bound activity may be beneficial since it can help maintaining sterility at film and food surface following diffusion of released soluble lysozyme through the depths of food. The homogenisation applied in this study during film making did not have a negative effect on enzyme activity since films obtained during preliminaries by applying stirring instead of homogenisation gave only 2% higher soluble enzyme activity (data not shown).

In vitro antimicrobial activity of zein films

The results of antimicrobial tests of partially purified lysozyme and/or Na_2EDTA incorporated zein films against different pathogenic bacteria, commonly associated with food poisoning are given in Table 1. As expected, the zein films containing lysozyme but lacking Na_2EDTA showed antimicrobial activity on *L. monocytogenes*, a Gram-positive bacterium. The films containing Na_2EDTA alone were significantly more effective on this pathogenic bacterium than films containing lysozyme alone ($P < 0.05$). But the most significantly effective films against *L. monocytogenes* were obtained by combining lysozyme with Na_2EDTA

Table 1 Antimicrobial activity of lysozyme and/or Na_2EDTA incorporated zein films on selected pathogenic bacteria

Type of film and bacteria tested	Zone types	Average area of ffz (cm^2)
Listeria monocytogenes		
ZF	12 nz	0[a]
ZF(Na_2EDTA)	12 ffz	1.94 ± 0.53[b]
ZF(lysozyme)	12 ffz	1.21 ± 0.12[c]
ZF(lysozyme-Na_2EDTA)	12 ffz	2.85 ± 0.85[d]
Escherichia coli O157:H7		
ZF	12 nz	0[a]
ZF(Na_2EDTA)	9 ffz/2 pfz/1 nz	2.28 ± 1.30[b]
ZF(lysozyme-Na_2EDTA)	10 ffz/1 pfz/1 nz	3.61 ± 1.49[b]
Salmonella typhimurium		
ZF	12 nz	0[a]
ZF(Na_2EDTA)	9 ffz/3 nz	2.58 ± 1.41[b]
ZF(lysozyme-Na_2EDTA)	12 ffz	3.27 ± 1.05[b]

ffz, fully formed zone; pfz, partially formed zone; nz, negative zone.
[a–d]Different letters within each type of film and bacteria tested denote significant difference at $P < 0.05$.
Data are mean values ± SD ($n = 12$).

($P < 0.05$). In fact, the zone area obtained by using zein films containing both lysozyme and Na_2EDTA gave 1.5 and 2.4 fold greater zones on *L. monocytogenes* than using films containing Na_2EDTA or lysozyme alone on this bacterium, respectively. The zein films containing combination of lysozyme and Na_2EDTA and Na_2EDTA alone were both effective on *E. coli* O157:H7 and *S. typhimurium*. Films containing combination of lysozyme and Na_2EDTA formed greater zones than Na_2EDTA containing films on indicated bacteria, but the areas of formed zones for these films were not significantly different from each other statistically ($P > 0.05$) (Table 1). The number of ffz on *E. coli* O157:H7 and *S. typhimurium* were also higher for films containing lysozyme and Na_2EDTA than those of films containing Na_2EDTA. The detection of pfz and nz in Na_2EDTA containing discs on *E. coli* O157:H7 (two pfz and one nz) and *S. typhimurium* (three nz) suggested some non-homogeneity in distribution of Na_2EDTA crystals within hydrophobic zein films. However, this problem was reduced considerably (one pfz and one nz on *E. coli* O157:H7 and no pfz and nz on *S. typhimurium*) for films containing lysozyme and Na_2EDTA combination.

Antimicrobial activity of zein films on refrigerated stored ground beef patties

The TVC of packed patties during refrigerated storage were given in Table 2. At the beginning of storage, at 0 and 3 days, no statistically significant effect of active packaging with lysozyme and Na_2EDTA or Na_2EDTA containing zein films was observed on TVC of ground beef patties. However, after 5 and 7 days of storage, the TVC of patties packed with lysozyme and Na_2EDTA containing films were significantly lower (0.75–1.9 decimals) than those of the other patties packed with or without control zein films and with zein films containing Na_2EDTA ($P < 0.05$).

The TCC of ground beef patties during refrigerated storage were also given in Table 3. The active packaging had no significant effect on initial TCC of patties ($P > 0.05$). However, after 5 days of storage, the TCC of patties packed with lysozyme and Na_2EDTA containing films were significantly lower than those of the others ($P < 0.05$). The zein films containing Na_2EDTA alone showed TCC lower than the controls only at the fifth day of storage. At the end of 7 days refrigerated storage, there were no statistically significant differences in TCC of ground beef patties. This result suggested reduction of available EDTA in the patties below inhibitory concentrations to coliform bacteria after fifth day and ineffectiveness of remained lysozyme whose activity on Gram-negative bacteria depends on EDTA.

In the literature, studies related to active packaging of meat products by using hen egg white lysozyme as bioactive agent were scarce. However, Datta *et al.* (2008) used lysozyme purified from oyster and commercial hen egg white lysozyme alone or in combination with nisin in

Table 2 TVC of refrigerated stored ground beef patties packed with different zein films

	TVC during storage at 4 °C (log_{10} CFU g^{-1})			
Type of film	**Day 0**	**Day 3**	**Day 5**	**Day 7**
Control	$5.20 \pm 0.01^{ab,D}$	$5.38 \pm 0.02^{b,C}$	$7.84 \pm 0.04^{a,A}$	$7.71 \pm 0.08^{a,B}$
ZF	$5.39 \pm 0.17^{a,B}$	$5.82 \pm 0.24^{a,B}$	$8.02 \pm 0.30^{a,A}$	$8.15 \pm 0.03^{a,A}$
ZF(Na_2EDTA)	$4.99 \pm 0.09^{b,B}$	$5.44 \pm 0.08^{ab,B}$	$6.94 \pm 0.49^{b,A}$	$7.65 \pm 0.42^{a,A}$
ZF(lysozyme-Na_2EDTA)	$5.09 \pm 0.02^{b,C}$	$5.10 \pm 0.16^{b,C}$	$6.14 \pm 0.14^{c,B}$	$6.90 \pm 0.34^{b,A}$

[a–c]Different letters within each storage time denote significant difference at $P < 0.05$.
[A–D]Different letters within each treatment denote significant difference at $P < 0.05$.
Data are mean values ± SD ($n = 3$).

Table 3 TCC of refrigerated stored ground beef patties packed with different zein films

	TCC during storage at 4 °C (log_{10} CFU g^{-1})			
Type of film	**Day 0**	**Day 3**	**Day 5**	**Day 7**
Control	$2.60 \pm 0.02^{a,D}$	$3.81 \pm 0.14^{ab,C}$	$5.52 \pm 0.04^{a,A}$	$5.06 \pm 0.12^{a,B}$
ZF	$2.90 \pm 0.34^{a,C}$	$4.35 \pm 0.09^{a,B}$	$5.62 \pm 0.10^{a,A}$	$5.48 \pm 0.07^{a,A}$
ZF(Na_2EDTA)	$2.68 \pm 0.04^{a,C}$	$3.96 \pm 0.41^{a,B}$	$5.02 \pm 0.12^{b,A}$	$5.32 \pm 0.36^{a,A}$
ZF(lysozyme -Na_2EDTA)	$2.60 \pm 0.10^{a,C}$	$3.25 \pm 0.06^{b,B}$	$4.34 \pm 0.02^{c,A}$	$4.76 \pm 0.48^{a,A}$

[a–c]Different letters within each storage time denote significant difference at $P < 0.05$.
[A–D]Different letters within each treatment denote significant difference at $P < 0.05$.
Data are mean values ± SD ($n = 3$).

calcium alginate coatings to decrease the growth of *L. monocytogenes* and *Salmonella anatum* in refrigerated smoked salmon. Duan *et al.* (2007) also employed commercial lysozyme in active packaging of Mozzarella cheese with chitosan films and enhanced their antimicrobial activity against *Pseudomonas fluorescens* and *L. monocytogenes*. On the other hand, several studies exist related to active packaging of ground beef or meat patties by employing different bioactive substances. For example, Emiroğlu *et al.* (2010) reported that soy protein films incorporated with oregano and thyme essential oils reduced the coliform and *Pseudomonas* spp. counts in fresh ground beef during refrigerated storage. These researchers, on the other hand, found no significant effects of active packaging on total viable count, lactic acid bacteria count, and *Staphylococcus* spp. Chidanandaiah *et al.* (2009) applied alginate coating containing mixed spice powder on buffalo meat patties and observed a decrease in total plate count, psychrophilic bacterial count and yeast and mold counts of samples during refrigerated storage.

Oxidative stability of refrigerated stored ground beef patties

The MDA contents of refrigerated stored ground beef patties determined by the TBA method were given in Table 4. The film application had no statistically significant effect on initial MDA contents of the patties ($P > 0.05$). Considerable increase occurred in the MDA contents of samples packed without zein film showed the high sensitivity of patties to oxidation during storage. However, active packaging of patties with zein films containing lysozyme and Na_2EDTA or Na_2EDTA alone slowed down the oxidative changes in these samples effectively. This result was in line with literature which reported effectiveness of metal chelating agent Na_2EDTA as an antioxidant to slow down the iron catalyzed oxidation in meat (Igene *et al.*, 1979; Shaila & Morrissey, 1987). At the end of 7 days refrigerated storage, the patties packed with control zein films also had significantly lower MDA contents than that of patties packed without any zein films. Thus, it seems that the antioxidant effect obtained by active packaging was related with both limitation of oxygen accessibility by the films and iron chelating properties of Na_2EDTA.

Table 4 TBA values of refrigerated stored ground beef patties packed with different zein films

	TBA values of patties during storage at 4 °C (mg MDA per kg pattie)		
Type of film	**Day 0**	**Day 3**	**Day 7**
Control	$4.60 \pm 0.14^{a,C}$	$9.59 \pm 0.83^{a,B}$	$24.39 \pm 1.20^{a,A}$
ZF	$4.56 \pm 0.39^{a,C}$	$7.04 \pm 0.45^{ab,B}$	$13.99 \pm 0.59^{b,A}$
ZF(Na_2EDTA)	$3.17 \pm 0.64^{a,A}$	$4.94 \pm 0.87^{b,A}$	$5.40 \pm 0.38^{c,A}$
ZF(lysozyme -Na_2EDTA)	$3.23 \pm 0.05^{a,B}$	$4.67 \pm 0.01^{b,A}$	$4.97 \pm 0.31^{c,A}$

[a–c]Different letters within each storage time denote significant difference at $P < 0.05$.
[A–C]Different letters within each treatment denote significant difference at $P < 0.05$.
Data are mean values ± SD ($n = 2$).

Colour changes of refrigerated stored ground beef patties

Lightness values (L^*) and redness indices (a^*/b^*) of refrigerated stored ground beef patties were given in Table 5. The changes in meat colour are very dynamic and related to the relative proportions of the three myoglobin forms, deoxymyoglobin, oxymyoglobin, and metmyoglobin (O'Sullivan *et al.*, 2003). The application of active packaging did not have a statistically significant effect on initial lightness values and redness indices of patties ($P > 0.05$). Lightness values showed a statistically insignificant change during 7 days of storage. A limited change in redness index was observed for control patties packed without any zein films. In contrast, redness indices of all patties packed with zein films decreased significantly during 7 days of storage ($P < 0.05$). This result suggested the limited contact of zein packed patties with molecular oxygen and lower amounts of oxymyoglobin formed in these samples.

Conclusions

The combinational use of partially purified lysozyme and Na_2EDTA gave films highly effective on major pathogenic bacteria such as *L. monocytogenes*, *E. coli* O157:H7, and *S. typhimurium*. The films also effectively delayed the multiplication of TVC and TCC numbers and the oxidative changes in beef patties during storage

Table 5 Effect of different zein films on colour of refrigerated stored ground beef patties

	Colour of patties during storage at 4 °C		
Type of film	**Day 0**	**Day 3**	**Day 7**
	Lightness (L^*) value		
Control	61.45 ± 3.46	58.53 ± 1.20	56.17 ± 1.49
ZF	59.72 ± 1.02	57.53 ± 2.32	57.91 ± 0.01
ZF(Na_2EDTA)	59.97 ± 0.22	58.50 ± 0.51	58.51 ± 0.94
ZF(lysozyme-Na_2EDTA)	59.49 ± 2.08	59.56 ± 0.69	59.14 ± 1.38
	Redness (a^*/b^*) index		
Control	$2.12 \pm 0.10^{a,A}$	$2.09 \pm 0.05^{a,AB}$	$1.58 \pm 0.19^{a,B}$
ZF	$2.18 \pm 0.02^{a,A}$	$1.77 \pm 0.09^{bc,B}$	$1.36 \pm 0.04^{a,C}$
ZF(Na_2EDTA)	$2.30 \pm 0.04^{a,A}$	$1.82 \pm 0.03^{b,B}$	$0.93 \pm 0.00^{b,C}$
ZF(lysozyme-Na_2EDTA)	$2.24 \pm 0.16^{a,A}$	$1.57 \pm 0.00^{c,B}$	$0.85 \pm 0.03^{b,C}$

[a–c]Different letters within each storage time denote significant difference at $P < 0.05$.
[A–C]Different letters within each treatment denote significant difference at $P < 0.05$.
Data are mean values ± SD ($n = 2$).

at 4 °C. The strong antioxidant activity of films was due mainly to their iron chelating capacity provided by Na_2EDTA. The results indicated that there was no beneficial effect of using lysozyme and Na_2EDTA combination in zein films on redness indices of beef patties. In fact, this study concluded that zein-partially purified lysozyme and Na_2EDTA based films may be used to enhance microbial safety and quality by controlling microbial load and delaying oxidative changes of active packed meat products.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. A representative cross-section photograph of the control film for measuring the film thickness by SEM (magnification ×150).

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