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Note

Antimicrobial Potential of Polylysine in Edible Films

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The antimicrobial activity of edible films from whey proteins, alginate, zein and chitosan incorporated with polylysine (PL) and PL-ethylenediaminetetraacetic acid (Na_2EDTA) combination have been tested on different bacteria including *Escherichia coli*, *Listeria innocua*, *Salmonella* Typhimurium and *Stapylococcus aureus*. The PL-containing films of whey proteins, alginate and chitosan were effective on *L. innocua*, but had limited effect on *E. coli*. On the other hand, the PL-containing zein films showed good antimicrobial activity on both *E. coli* and *L. innocua* as well as on *S. aureus*. PL-Na_2EDTA combination also gave zein films effective on *S.* Typhimurium. The incorporation of PL alone or PL-Na_2EDTA combination did not cause any significant change in mechanical properties of zein films. Zein has a good potential to develop novel antimicrobial packaging materials incorporated with PL.

Keywords: alginate, antimicrobial packaging, chitosan, polylysine, whey protein, zein

Introduction

The increased demand on easily prepared minimally processed fresh produce and the related increase in food-borne microbial outbreaks (De Roever, 1998) have intensified the research on antimicrobial packaging technologies (Suppakul *et al.*, 2003). Due to the health concerns of the consumers, producers show a particular interest in use of natural biopreservatives in antimicrobial packaging. Due to the environmental concerns and technological problems such as denaturing effects of thermal polymer processing methods, extrusion and injection molding, the incorporation of biopreservatives into biodegradable films is more suitable than their incorporation into plastic films (Han, 2000; Appendini and Hotchkiss, 2002; Suppakul *et al.*, 2003).

ε-Polylysine is a natural antimicrobial polypeptide which is formed by 25 to 35 L-lysine residues (Ting *et al.*, 1999; Geornaras and Sofos, 2005). It is produced from aerobic fermentation by *Streptomyces albulus* which is a non-pathogenic microorganism and approved by use in Japan as an antimicrobial preservative in foods (Hiraki *et al.*, 2003). Different Japanese foods that contain PL include sliced fish and fish surimi, boiled rice, noodle soup stocks, noodles and cooked vegetables (Hiraki *et al.*, 2003). Recently, U.S Food and Drug Administration also approved the PL as generally recognized as safe (GRAS) for use in cooked or sushi rice (FDA, 2004). The antimicrobial action of PL is attributed to its polycationic and surface active nature which enables its interaction with bacterial membranes (Ho *et al.*, 2000). The antimicrobial is effective on major G(+) and G(-) food pathogenic bacteria including *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella* Typhimurium (Geornaras and Sofos, 2005; Geornaras *et al.*, 2007). However, studies in the literature related to use of PL in antimicrobial packaging are scarce. In fact, it was only Zinoviadou *et al.* (2010) who tested PL in whey protein films and successfully applied the developed films to control spoilage flora of fresh beef. In this study, besides whey protein films, alginate, chitosan and zein films were incorporated with the PL and PL-Na_2EDTA combination, and the obtained films were tested for their antimicrobial effects on different bacteria and mechanical properties. The Na_2EDTA used frequently in combination with biopreservatives such as lysozyme and nisin to destabilize cell walls of G(-) bacteria (Padgett *et al.*, 1998; Branen and Davidson, 2004) was also first time combined with PL in an edible film to increase the effectiveness of antimicrobial packaging.

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Materials and Methods

Materials Whey protein isolate (BiPRO®, 97.8% pro-

tein) was obtained from Davisco Foods International, Inc. (MN, USA). Sodium alginate and maize zein were obtained from Sigma Chem. Co. (St. Louis, Mo., USA). Medium molecular weight chitosan was purchased from Aldrich (Sigma-Aldrich Co., St. Louis, Mo., USA). Polylysine (1 : 1 mixture of ε-polylysine and dextran) was kindly donated by Chisso America, Inc. (New York, USA).

Preparation of different edible films Different amounts of PL were added to film forming solutions to achieve final concentrations of 175, 350 or 700 μg PL/cm^2 in dried films. Na_2EDTA was only used in zein and chitosan films since it caused aggregation of film components within whey protein and alginate films. The final concentration of Na_2EDTA within dry films was 200 μg/cm^2. The average thickness of films produced in this study was determined by a scanning electron microscope (SEM) (Philips XL 30S FEG, FEI Company, Eindhoven, The Netherlands) by measuring each kind of film at 15 different points.

Whey protein films were prepared as described by Min *et al.* (2005). The film forming solution formed by this method was filtered through cheesecloth to remove air bubbles and different amounts of PL were added to the medium. The mixture was stirred at 300 rpm for 20 min to dissolve the ingredients and 4.3 g of it was spread evenly onto a 8.5 × 8.5 cm glass plate previously cleaned with ethanol. The films were dried at room temperature for 24 h (the average thickness of these films was 117 ± 10 μm).

The alginate films were prepared as described by Mecitoglu and Yemenicioglu (2007). Different amounts of PL were added into film forming solutions. The mixture was stirred for 20 min at 300 rpm to dissolve the ingredients and 10 g portion of it was cast into glass Petri dishes (9.5 cm in diameter). The films were dried at room temperature for 48 h and used after cross-linking with 0.8 mL of 0.3 M $CaCl_2$ and incubating for 30 min (the average thickness of these films was 27 ± 4.30 μm).

The chitosan films were prepared by slight modification of method given by Srinivasa *et al.* (2007). The chitosan solution was prepared by dissolving 1% (w/w) chitosan in a 0.5% (w/w) acetic acid solution by continuous stirring for 12 h. The solution was filtered through cheesecloth, degassed in a vacuum oven at ambient temperature and 2.2% glycerol (w/w) was added into it as a plasticizer. Different amounts of PL and/or Na_2EDTA were added to the medium and the mixture was homogenized with a homogenizer (Silent Crusher M, Heidolph, Germany) at 10000 rpm for 2 min. After homogenization, 20 g of solution was poured into sterile disposable plastic Petri dishes and then films were dried at 45°C for 22 h in an incubator aerated with an internal circulator (the average thickness of these films was 50 ± 6.11 μm).

Zein films were prepared as described in Padgett *et al.* (1998). After heating and cooling film forming solutions prepared by this method, different amounts of PL and/or Na_2EDTA were added to the medium. The mixture was further stirred for 25 min and 4.3 g of it was spread evenly onto a 8.5 × 8.5 cm glass plate previously cleaned with ethanol. The plates were dried at room temperature for 24 h (the average thickness of these films was 205 ± 16 μm).

Test of film antimicrobial activity *Escherichia coli* (NRRL B-3008) and *Listeria innocua* (NRRL B-33314) were supplied from Agricultural Research Service Culture Collection (Peoria, IL, USA). *Salmonella* Typhimurium (CCM 5445) and *Staphylococcus aureus* (RSKK 95047) were kindly provided by Dr. Handan Baysal (Izmir Institute of Technology, Izmir, TURKEY), and Dr. Gülsün Evrendilek (Abant Izzet Baysal University, Bolu, TURKEY), respectively. The overnight cultures were prepared in nutrient broth by conducting incubations at 37°C. For antimicrobial tests, 12 discs (1.3 cm in diameter) were prepared from films by a cork borer under aseptic conditions. During tests, 3 discs were placed carefully onto each Petri dish containing nutrient agar on which 0.1 mL culture was spread (average numbers of cells were kept between 10^8 and 10^9 cfu/mL). All Petri dishes were incubated at 37°C for 48 h and the area of the clear fully formed zones (f) observed was determined by measuring the zone diameter with a digital caliper. The areas of partially formed zones (p) occurred on one side of the discs were not measured. However, the numbers of p and negative discs (n) were noted.

Mechanical properties of films Mechanical properties of the films were determined by using a Texture Analyser TA-XT2 (Stable Microsystems, Godalming, UK) as described in Arcan and Yemenicioğlu (2011). At least 5 replicates of each film were tested.

Statistical analysis Analysis of variance (ANOVA) was applied using Minitab 14 (Minitab Inc., State College, PA) to determine the effects of PL and/or Na_2EDTA on the mechanical properties of films. Multiple comparisons of means were performed using Tukey's HSD (Honestly Significant Differences) test with a level of 95% confidence interval.

Results and Discussion

Antimicrobial activity of PL in whey protein films The whey protein films incorporated with 175 or 350 μg/cm^2 of PL did not show antimicrobial activity on *E. coli* (Table 1). However, an antimicrobial effect was observed on this bacterium when concentration of PL was increased to 700 μg/cm^2. To increase antimicrobial activity against *E. coli*, PL could not be combined with Na_2EDTA, since addition of this chemical caused extensive insoluble protein aggregate for-

Table 1. Antimicrobial activity of different edible films on *E. coli* and *L. innocua*.

Film type	Concentration (μg/cm^2)		Number of fully formed (f), partially formed (p) or negative zones (n)		Average area of clear fully formed zones (cm^2)	
	PL	Na_2EDTA	*E. coli*	*L. innocua*	*E.coli*	*L. innocua*
Whey Protein[a]	–	–	12n	12f	0	0.70 ± 0.09
Whey Protein	175	–	12n	12f	0	0.61 ± 0.09
Whey Protein	350	–	12n	12f	0	1.36 ± 0.13
Whey Protein	700	–	12f	12f	0.58 ± 0.09	2.04 ± 0.17
Alginate[a]	–	–	12n	12n	0	0
Alginate	175	–	5f / 3p / 4n	1f / 11n	0.36 ± 0.10	1.12
Alginate	350	–	5f / 3p / 4n	9f / 3p	0.29 ± 0.09	1.15 ± 0.28
Alginate	700	–	2f / 4p / 6n	12f	0.55 ± 0.05	1.60 ± 0.51
Chitosan	–	–	7p / 5n	12n	0	0
Chitosan	175	–	2f / 4p / 6n	11f / 1p	0.49 ± 0.01	0.65 ± 0.15
Chitosan	350	–	3f / 3p / 6n	12f	0.48 ± 0.11	1.00 ± 0.17
Chitosan	700	–	10f / 2p	12f	0.64 ± 0.20	1.62 ± 0.33
Chitosan	175	200	1f / 5p / 6n	nt[b]	0.55 ± 0.00	nt
Chitosan	350	200	6f / 4p / 2n	nt	0.82 ± 0.25	nt
Chitosan	700	200	9f / 3p	nt	0.79 ± 0.32	nt
Zein	–	–	12n	12n	0	0
Zein	175	–	12f	12f	0.34 ± 0.04	0.90 ± 0.16
Zein	350	–	12f	12f	0.39 ± 0.05	1.56 ± 0.26
Zein	700	–	12f	12f	0.70 ± 0.07	1.78 ± 0.24
Zein	175	200	12f	nt	0.68 ± 0.11	nt
Zein	350	200	12f	nt	0.93 ± 0,14	nt
Zein	700	200	12f	nt	1.14 ± 0,19	nt

[a]: use of Na_2EDTA with PL caused aggregation problems in whey protein and alginate films.
[b]: not tested on this bacteria.

mation in film forming solutions. The PL-containing whey protein films were also tested on *L. innocua* (Table 1). The control films and 175 μg/cm^2 PL-containing films showed a limited antimicrobial activity on *L. innocua*. It is likely that the limited antimicrobial activity of control films was due to some natural antimicrobial milk proteins in the whey such as lactoferrin (Recio and Visser, 2000). The increase of PL concentration from 175 to 350 μg/cm^2 and then 350 to 700 μg/cm^2 increased the antimicrobial activity of whey protein films against *L. innocua* almost 2.2 and 1.5 fold, respectively. This result clearly showed the significantly higher antimicrobial activity of PL in whey protein films on *L. innocua* than *E. coli*.

Antimicrobial activity of PL in alginate films The control alginate films are transparent and clear (Fig. 1A), but they turned semi-transparent and milky (Fig. 1B) by the addition of PL. This could be related with interaction of PL with the film matrix and its reduced solubility in dried and cross-linked films. The ineffectiveness of PL-containing alginate films on *E. coli* supported this hypothesis. The number of clear fully formed zones on *E. coli* reduced as PL concentration in the films was increased. This result indicated increased neutralizing interactions of PL in the films against *E. coli* at high PL concentrations. The alginate is composed of linear copolymers of D-mannuronic acid and L-guluronic

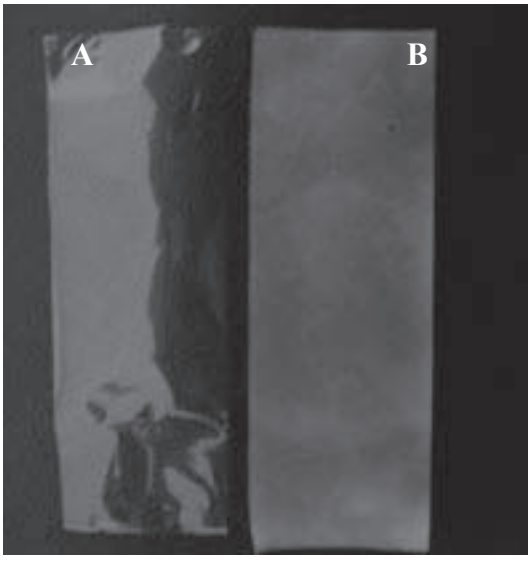

Fig. 1. Photographs of alginate films (A: control; B: 700 μg/cm^2 PL-containing films).

acid (Lindstrom *et al.*, 1992). Thus, because of its cationic nature, the binding of PL to negatively charged carboxylic acid groups on polymeric chains of alginate and engagement of its free positive charges are possible. The positive charges of PL are particularly effective on its action against G(-) bacteria which bear negative charges on their membrane surface (Ting *et al.*, 1999). On the other hand, the PL could not be supported with Na_2EDTA since use of this chelating agent also led to aggregation and insolubilization of film components. The films incorporated with 175 μg/cm^2 of PL also showed almost no antimicrobial activity on *L. innocua*. However, the increase of PL concentration to 350 or 700 μg/cm^2 increased the number and average area of clear fully formed zones on *L. innocua* considerably. The completely different responses of *E. coli* and *L. innocua* on the change of PL concentration suggested the different modes of inhibition of these bacteria by the PL.

Antimicrobial activity of PL in chitosan films Due to its cationic nature, the chitosan has been reported to have an inherent antimicrobial activity (Appendini and Hotchkiss, 2002). However, the control chitosan films lacking PL were ineffective against *E. coli*. The incorporation of 175 or 350 μg/cm^2 of PL into chitosan films gave only several clear fully formed zones on *E. coli*, but a limited antimicrobial activity was obtained against this bacterium at PL concentration of 700 μg/cm^2. The PL-Na_2EDTA combination was more effective than PL alone and it increased the number and area of fully formed zones against *E. coli* at 350 and 700 μg/cm^2 PL concentrations. However, there were still some partially formed or negative zones at these concentrations which indicated hardly reaching of the desired critical inhibitory concentration for *E. coli* at different portions of the films. The chitosan films tested at the same PL concentration range against *L. innocua* were found much more effective than those tested against *E. coli*. Almost all of the films tested gave fully formed zones against *L. innocua* with increased zone area by increasing the PL concentration.

Antimicrobial activity of PL in zein films Due to its hydrophobic character, zein had to be dissolved in ethanol. This prevented complete dissolution of hydrophilic PL in zein film forming solutions and caused formation of tiny aggregates in the obtained films. Effective stirring enabled homogenous distribution of aggregates within the films, but the sizes of these aggregates increased as PL concentration increased from 175 to 700 μg/cm^2 (Fig. 2).

Zein films incorporated with 175 μg/cm^2 of PL were effective on *L. innocua*. The 2 fold increase of PL concentration from 175 to 350 μg/cm^2 increased the average area of clear fully formed zones on *L. innocua* almost 1.7 fold. Further increase in PL concentration from 350 to 700 μg/cm^2 caused only a slight increase in zone area. However, the PL-Na2EDTA combination increased the effectiveness of zein films on *E. coli* considerably. For example, 175, 350 or 700 μg/cm^2 PL-containing zein films with 200 μg/cm^2 Na_2EDTA gave 2.0, 2.4 and 1.6 fold greater zones against *E. coli* than PL-containing films lacking Na_2EDTA, respectively.

The positive results of antimicrobial tests with zein films showed the good potential of these films for edible film applications of PL. Thus, to better evaluate their capacity, these films were further tested on *S. aureus* and *S.* Typhimurium (Table 2). The PL-containing zein films did not show any antimicrobial effect on *S.* Typhimurium. It is clear that this pathogenic G(-) bacterium is much more resistant than *E. coli* against action of PL. However, the use of PL-Na_2EDTA combination made a significant contribution to overcome this resistance and gave the largest clear zones observed in this study. The Na_2EDTA-containing zein films alone were not effective on *S.* Typhimurium, since these films formed turbid zones (results were not given) (similar effect was also observed for *E. coli*). The PL-containing zein films were also effective on *S. aureus*. The films caused clear fully formed zones against this bacterium for all tested discs which average area increased by the increase of PL concentration.

Mechanical properties of films The tensile strength (TS), elongation at break (E) and Young's modulus (YM) values of different films were given in Table 3. Except the increased TS of alginate films, the incorporation of PL at the highest concentration used in microbial tests caused no statistically significant change in mechanical properties of films ($P > 0.05$). PL-Na_2EDTA combination did not have a significant effect on mechanical properties of zein films ($P > 0.05$), but this combination caused a significant reduction in E of chitosan films ($P < 0.05$). The alginate films gave the highest TS and YM values, but they showed the lowest E values. On the

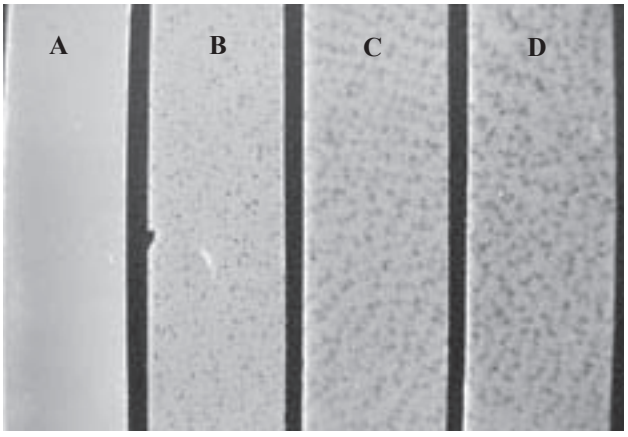

Fig. 2. Photographs of different zein films (A: control; PL concentrations: 175, 350, 700 μg/cm^2 in B, C and D, respectively).

Table 2. Antimicrobial activity of zein films on *S.* Typhimurium and *S. aureus.*

Concentrations (μg/cm^2)		Number of fully formed (f), partially formed (p) or negative zones (n)		Average area of clear fully formed zones (cm^2)	
PL	Na_2EDTA	*S.* Typhimurium	*S. aureus*	*S.* Typhimurium	*S. aureus*
–	–	12n	12n	0	0
175	–	12n	12f	0	0.91 ± 0.10
350	–	12n	12f	0	1.17 ± 0.17
700	–	12n	12f	0	1.45 ± 0.27
175	200	10f / 2n	nt[a]	1.52 ± 0.90	nt
350	200	12f	nt	2.10 ± 0.64	nt
700	200	12f	nt	2.97 ± 0.87	nt

[a]: not tested on this bacteria.

Table 3. Mechanical properties of different edible films.

Film Type	Concentrations (μg/cm²)		Mechanical properties		
	PL	Na_2EDTA	TS[f] (MPa)	E (%)	YM (MPa)
Whey Protein	–	–	1.34 ± 0.26[a]	116.92 ± 17.55[a]	0.20 ± 0.04[a]
Whey Protein	700	–	1.63 ± 0.26[a]	122.85 ± 30.32[a]	0.13 ± 0.02[a]
Alginate	–	–	61.90 ± 12.00[b]	1.95 ± 0.58[b]	48.45 ± 5.50[b]
Alginate	700	–	76.21 ± 22.50[c]	1.96 ± 0.57[b]	49.31 ± 4.55[b]
Chitosan	–	–	18.14 ± 3.89[de]	134.20 ± 13.08[a]	0.07 ± 0.01[a]
Chitosan	700	–	15.91 ± 4.73[de]	106.54 ± 19.38[a]	0.12 ± 0.02[a]
Chitosan	700	200	10.87 ± 2.69[ad]	81.30 ± 13.00[c]	0.11 ± 0.01[a]
Zein	–	–	7.93. ± 1.41[ae]	2.90 ± 0.77[b]	4.01 ± 0.51[c]
Zein	700	–	8.55 ± 0.68[ae]	2.75 ± 0.41[b]	4.17 ± 0.15[c]
Zein	700	200	7.50 ± 0.69[ae]	2.49 ± 0.38[b]	3.78 ± 0.14[c]

[a-e]: Different letters within the same column indicate significant differences ($P < 0.05$).
[f]TS: Tensile strenght; E: elongation; YM: Young's modulus

other hand, although whey protein and chitosan films gave lower TS values than alginate films, they showed the highest E values. The zein films were the only films showing both low TS and E values and this was in line with the literature reporting the classical brittleness problems of these films (Arcan and Yemenicioğlu, 2011).

In conclusion, the results obtained in this study showed that the PL showed better antimicrobial performance in zein films than in whey protein, alginate and chitosan films. Due to solubility problems within the films, the Na_2EDTA used to support antimicrobial activity of PL could not be used in alginate and whey protein films. The PL-Na_2EDTA combination could be used in zein and chitosan films, but zein films gave better antimicrobial activity than chitosan films. Zein showed minimum interaction with PL due to its hydrophobic character. Therefore, PL in zein was preserved and showed higher antimicrobial activity than PL in other edible film materials interacted with this agent and reduce its antimicrobial potential. The zein is a promising candidate for use with PL to obtain antimicrobial edible food packaging materials, since it is one of the abundant and cheap sources of biodegradable materials obtained as a major co-product of the rapidly growing bioethanol industry. In food industry, zein is currently applied mainly as food coating material for candies, fruits and nuts. A particular interest has also been focused on use of pre-cast zein films or coatings on antimicrobial and antioxidant packaging of ready-to-eat poultry and meat products (Herald *et al.*, 1996; Janes *et al.*, 2002; Marcos *et al.*, 2007). Further studies are needed to test the efficacy of PL-containing zein films in food applications.

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