**PE8.06 Use of lysozyme-EDTA system for active packaging of cold stored beef burgers 38.00**

*Ahmet Yemenicioðlu (1) ahmetyemenicioglu@iyte.edu.tr, F Korel( 1), FYG Yener 1, Ý Arcan 1 Ý Uysal 1*
*(1)Izmir Institute of Technology, Turkey*

**Abstract—In this study, the effects of packaging with zein films incorporated with partially purified lysozyme (LYS) and Na2EDTA on microbial load, oxidative changes and color of cold stored beef burgers were investigated. Packaging of burgers with zein films incorporated with LYS and Na2EDTA was beneficial to reduce the initial total viable count (TVC) of burgers. However, these films have limited effect on suppression of TVC in burgers during cold storage. The modification of film structure by use of surface active legume proteins obtained from chickpeas (LPE) increased the distribution of hydrophilic LYS within zein films. This reduced the soluble enzyme in films, while increasing the bound and trapped enzyme and its immobilized activity in the films. Active packaging of burgers with zein films containing LYS, Na2EDTA and LPE did not cause a significant reduction in the initial TVC of burgers, but these films are more effective on suppression of TVC during cold storage. The Na2EDTA in films prevented the oxidative changed in cold stored burgers very effectively during 15 days of cold storage. However, no beneficial effects of zein films on burger color were determined. This study showed the good potential of LYS, Na2EDTA and LPE containing active zein films to improve microbial and oxidative quality of cold stored beef burgers.**

Authors are with Izmir Institute of Technology, Department of Food Engineering, Gulbahce Köyü, 35430 Urla, Izmir. (corresponding author to provide phone: +90-232-7506292; Fax: +90-232-7506196; e-mail: ahmetyemenicioglu@iyte.edu.tr)

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## I. INTRODUCTION

Due to increased food-borne microbial outbreaks caused by the easily prepared and minimally processed fresh produce [1], there is a great interest in antimicrobial packaging technologies. Although some chemical antimicrobials and antioxidants can effectively be used with plastic films for active packaging [2,3,4], there are significant health and environmental concerns related to these materials [5,6]. In contrast, the edible films and natural antimicrobials or antioxidants are readily accepted by the consumers and not considered as chemicals. Thus, extensive research has been conducted to adopt biopreservatives such as antimicrobial enzymes and bacteriocins and natural antioxidants into edible and biodegradable functional packaging materials [3,4,5,7,8,9].

Recently, we have produced partially purified lysozyme (LYS) from hen egg white by a simple and economically feasible method based on ethanol precipitation of undesirable egg white proteins and incorporated the lyophilized enzyme into zein films for antimicrobial packaging [10]. The LYS shows its antimicrobial activity mainly on G(+) bacteria by hydrolyzing their peptidoglycan (PG) layer at the cell wall. The enzyme shows also antimicrobial activity on G(-) bacteria when the protective layer abound their PG layer is destabilized by EDTA [10]. The main disadvantage of using partially purified LYS in zein films is the non-homogenous distribution of hydrophilic enzyme preparation in hydrophobic films. This causes formation of hydrophilic LYS aggregates within the films which releases rapidly form the films in water. High release rates of antimicrobials are not preferred for antimicrobial packaging since this left the most critical food surface unprotected after released antimicrobial diffused from food surface to interior parts of food. Thus, in our following studies, we have incorporated LYS into zein films in combination with surface active legume proteins (LPE) obtained from chickpeas [11]. The emulsifying activity of LPE improved the distribution of LYS in zein films and increased their bind enzyme activity, while also leaving a considerable soluble activity within their matrix. In this study, effects of different zein films developed by use of LYS, $Na_2EDTA$ and LPE were tested on quality of cold stored beef burgers.

## II. MATERIALS AND METHODS

*Materials.* The beef burgers (containing 18 % fat and 1 % salt) used in this study were produced by Pınar Et A.Ş. (İzmir, Turkey) without using any antimicrobial or antioxidant agents. Zein, *Micrococcus lysodeikticus* and dialysis tubes (12000 MW) were from Sigma Chem. Co. (St. Louis, Mo., USA). $Na_2EDTA.2H_2O$

was purchased form Riedel-de haën (Sigma-Aldrich Laborchemikalien, Seelze, Germany).

*Preparation of legume protein extract.* The legume protein extract (LPE) was obtained from acetone powder of thermally

processed chickpeas as described by Arcan and Yemenicioğlu

[12]. The water soluble protein content of lyophilized LPE was determined almost 51% by the Lowry method [13].

*Preparation of partially purified lysozyme.* Partially purified LYS was produced and lyophilized according to Mecitoğlu et al [10] from hen egg white by ethanol precipitation of protein impurities.

*Preparation of films.* Zein films were prepared by slight modification of the method described in Padgett et al. [14]. Briefly, 1.4 g zein was dissolved with 8.1 mL of ethanol (97 %) by mixing slowly with a magnetic stirrer for 25 min. 0.39 mL glycerol was then added to the medium and the temperature of the mixture was increased until it started to boil. The mixing was then ceased and the film solution was boiled for 5 min. After cooling to room temperature, LYS, $Na_2EDTA$ or LPE were added to the mixture and it was further stirred for 25 minutes. 4.3 g of the film forming solution was then spread evenly onto a 8.5 x 8.5 cm glass plate cleaned previously with ethanol and the plate was dried at room temperature for 24 h. The films were then cut into four 3.2 x 3.2 cm pieces (for packaging) or one 6.0 x 6.0 cm piece (for activity determination). The final concentrations of ingredients in dried films were 0.3 mg/cm$^2$ for each of LYS, $Na_2EDTA$ and LPE. The average thickness of control standard zein films was 0.18 ± 0.05 mm (determined by a micrometer by conducting 20 measurements in films).

*Soluble lysozyme activity of films.* The soluble LYS activity of films was determined by release tests conducted at 4 $^oC$. The films (6 x 6 cm) were placed in glass petri dishes (10 mm in diameter) containing 50 mL distilled water (4 $^oC$) and released LYS activity was monitored for 1400 min at different time intervals until reaching of equilibrium. During tests Petri dishes were covered with parafilms to prevent evaporation and stirred continuously at 200 rpm with a magnetic stirrer. The LYS activity was determined by taking 0.6 mL aliquots and conducting activity measurements for three times by using 0.2 mL of sample. The activity of LYS was determined spectrophotometrically at 660 nm by using a Shimadzu (Model 2450, Japan) spectrophotometer at 30 $^oC$. The reaction mixture was prepared by mixing 2.3 mL, 0.26 mg/mL *Micrococcus lysodeikticus* cell suspension (at 30 $^oC$, prepared in 0.05 M, pH 7.0 Na-phosphate buffer) and 0.2 mL enzyme containing sample (incubated at 30 $^oC$ for 5 min). The absorbance was monitored for 5 min and enzyme activity was calculated from the formula of Activity (U)= (slope/0.001) x reaction volume (2.5ml). The activity was then expressed as U released per cm$^2$ of the films (U/cm$^2$).

*Bound lysozyme activity of films.* In this study, the enzyme retained in zein films after release test was designated as bound enzyme. The films obtained from release tests were cut into 3 x 3 cm pieces. The films were stored in petri dishes at 4 $^oC$ and one piece was assayed for bound activity at 0$^{th}$, 5$^{th}$ and 7$^{th}$ days. For test of bound activity, the films were placed into glass Petri dishes containing 25 mL, 0.26 mg/mL *Micrococcus lysodeikticus* solution (at 30 $^oC$, prepared in 0.05 M, pH 7.0 Na-phosphate buffer). The petri dishes were kept in an incubator at 30 $^oC$ and their contents' absorbance at 660 nm was monitored periodically under continuous magnetic stirring at 200rpm. The lysozyme activity of the films were determined from the formula of Activity (U)= (slope/0.001) x reaction volume (25ml). The activity was then expressed as U per cm$^2$ of the films (U/cm$^2$).

*Packaging applications.* All packaging applications were conducted aseptically by the same trained persons. The burger pieces and films were handled by sterile glows and tweezers sterilized by 70 % ethanol before each set of applications. During applications 3.2 x 3.2 cm films were placed carefully at both sides of 3 x 3 cm burger pieces. The burgers were then packed firstly by wrapping with a stretch plastic film and then secondly by wrapping with an aluminum foil. All samples were stored in a refrigerated incubator at 4 $^oC$ for 26 days and their microbial loads, oxidation states and color were determined at different time intervals.

*Microbiological analysis.* For analysis, samples were placed aseptically in flasks containing 150 mL, 0.1 % peptone water, and the flasks were shaken for 5 min at 150 rpm in a shaker. 0.1 mL portions of isolates were then plated or used in serial dilutions prepared by 0.1

% peptone water. Triplicate plating were done from each dilution by the spread plate method on PCA plates for the determination of total viable count (TVC). All incubations were conducted at 37 $^{o}$C, and plates were counted at the end of 48 h. The microbial loads were expressed as $\log_{10}$ cfu/g.

*Determination of oxidative stability.* For this purpose, the TBA method was applied as described by Bekhit et al [15]. For analysis, 5g sample was placed into a Waring blender micro jar containing 50 mL of 0.38 % TBA and 15 % TCA prepared in 0.25 N HCl solution. The sample was homogenized for 1 min and two 10 mL aliquots obtained from homogenate were boiled for 10 min in a water bath. The boiled samples were then centrifuged at 4500 x g for 20 min and their absorbance was measured at 532 nm by using a UV-VIS spectrophotometer (Varian, Cary 100, Australia). The average of two measurements was used to determine the 532 nm value of each sample.

*Determination of color.* The surface color of cold stored burgers was determined by using a Chroma Meter (Minolta, CR 400, Japan) and measuring L*, a*, b* values (CIE). The L* value was reported as lightness value, whereas a*/b* value was reported as redness index. The average of minimum three readings from burger surfaces was used for the calculation of parameters.

*Statistical analysis.* In microbiological studies the effect of storage time or different packaging treatments on microbial load was analyzed by ANOVA using PROC GLM procedure of SAS. Means with a significant difference ($P \leq 0.001$) were compared using the Duncan test.

## III. RESULTS AND DISCUSSION

*Soluble and bound lysozyme activity of films.* The soluble enzyme activity of LYS containing zein films prepared with and without LPE were 1385 and 2291 U/cm$^2$, respectively. The use of LPE reduced the soluble enzyme activity of films almost 40%. However, films containing LPE showed significantly higher bound LYS activity than films lacking this agent (Table 1). As we reported previously [12], this occurred due to better distribution of hydrophilic LYS aggregates within the hydrophobic zein film by effect of surface active legume proteins and trapping of enzyme within the film matrix. The bind enzyme activity of zein films containing LPE reduced by 40-45% by cold storage, while a 2-3 fold increase occurred in bind enzyme activity of films lacking LPE. However, the films containing LPE still showed almost 10 fold higher bind LYS activity at the end of one week storage.

*Effect of zein films on total viable count (TVC).* The initial TVC of burgers showed that the use of standard zein films and zein films incorporated with $Na_2EDTA$-LPE, LYS-$Na_2EDTA$ and LYS-$Na_2EDTA$-LPE reduced the initial TVCs of beef burgers significantly ($P < 0.001$). However, the most significant reduction in initial TVC occurred in burgers packed by zein films incorporated with LYS-$Na_2EDTA$. At the end of 7 days cold storage, the TCVs of samples were not significantly different from their initial TVCs. However, between 7$^{th}$ and 12$^{th}$ days of cold storage, a significant increase in TVCs (2.1 to 2.7 decimal) occurred in packed burgers, except TVC of those burgers packed by using zein films incorporated with LYS-$Na_2EDTA$-LPE. At the 12$^{th}$ day of cold storage, the TVC of burgers packed by using zein films containing LYS-$Na_2EDTA$-LPE combination was 1.3 $\log_{10}$ CFU/g lower than 6 $\log_{10}$ CFU/g, considered as a limit in the shelf-life determination studies, whereas the TVCs of all other samples approached, reached or exceeded this limit. These results showed that the use of LYS-$Na_2EDTA$-LPE combination in zein films is more effective to suppress TVC in packed burgers during cold storage than the use of LYS-$Na_2EDTA$ which is effective mainly on reduction of initial TVC. These findings compared well with activity measurements which showed a more balanced distribution of soluble and bind activity in films containing LPE. It is clear that the free soluble LYS released rapidly at the beginning of cold storage from LYS-$Na_2EDTA$ containing films and showed high initial antimicrobial activity. But, the surface of burgers remained unprotected when the released enzyme at the surface diffused to interior parts of food. In contrast, the high bound LYS in LPE containing films maintained the antimicrobial activity at film contact surface during cold storage.

*Effect of zein films on oxidative stability.* As seen in Fig 2, considerable increases occurred in the absorbance values of control samples packed without any zein films and with control zein films. In contrast, during 14 days cold storage, the burgers packed by zein films containing $Na_2EDTA$-LPE or LYS-$Na_2EDTA$-LPE showed almost no increase in their absorbance at

532 nm. The LYS-$Na_2EDTA$ combination, on the other hand, was less effective and caused a slight increase in the absorbance of burgers. These results clearly showed the effectiveness of $Na_2EDTA$-LPE combination in prevention of oxidative changes in cold stored burgers. The increased oxidative stability of burgers is due mainly to iron chelating effect of $Na_2EDTA$. $Na_2EDTA$ acts synergetically with the free radical scavengers. Therefore, it is more effective with chickpea LPE which free radical scavenging activity was demonstrated recently by our research group [12].

*Effect of zein films on color.* The redness indexes of packed burgers cold stored for 7 days changed between 0.5 and 0.71 (Table 2). The initial redness index of burgers was 1.43. Thus, it is clear that the packaging and cold storage initially caused a reduction in the redness index of burgers. However, at the end of 14 days, the redness indexes of packed burgers increased and ranged between 0.76 and 1.05. The changes in meat color are very dynamic and depend on the interconversions of myoglobin (purplish red), metmyoglobin (brownish-red) and oxymyoglobin (bright red) [16], but the results showed that there is no beneficial effect of using LPE and $Na_2EDTA$ in zein films on maintenance of burger redness. The L* values of burgers also suggested no significant darkening or browning in burger color due to metmyoglobin formation.

## IV. CONCLUSION

This study clearly showed the importance of having a balanced soluble/bound LYS activity ratio in edible zein films used for active packaging of beef burgers. Films having high soluble/bound LYS activity ratio is effective on microbial load at initial periods of cold storage, while films with lower soluble/bound ratio suppress microbial growth mainly during cold storage period. The use $Na_2EDTA$ in films supported antimicrobial activity of LYS and effectively prevented the oxidative changes in burgers. The zein films containing lysozyme-$Na_2EDTA$-LPE are suitable to increase microbial and oxidative quality of burgers during cold storage.

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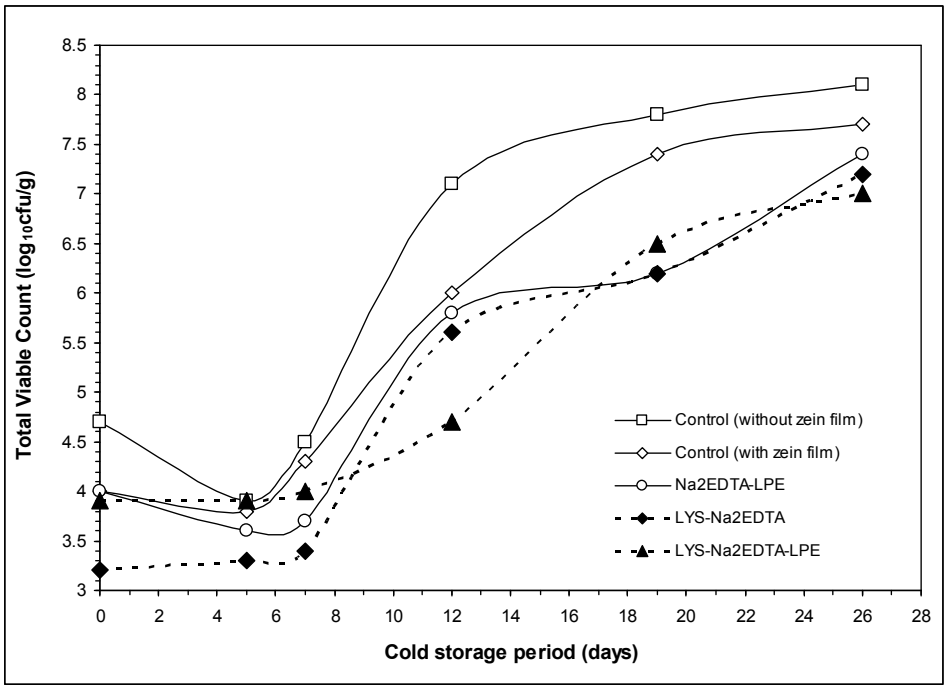


Fig. 1. Effect of active packaging on microbial load of cold stored beef burgers

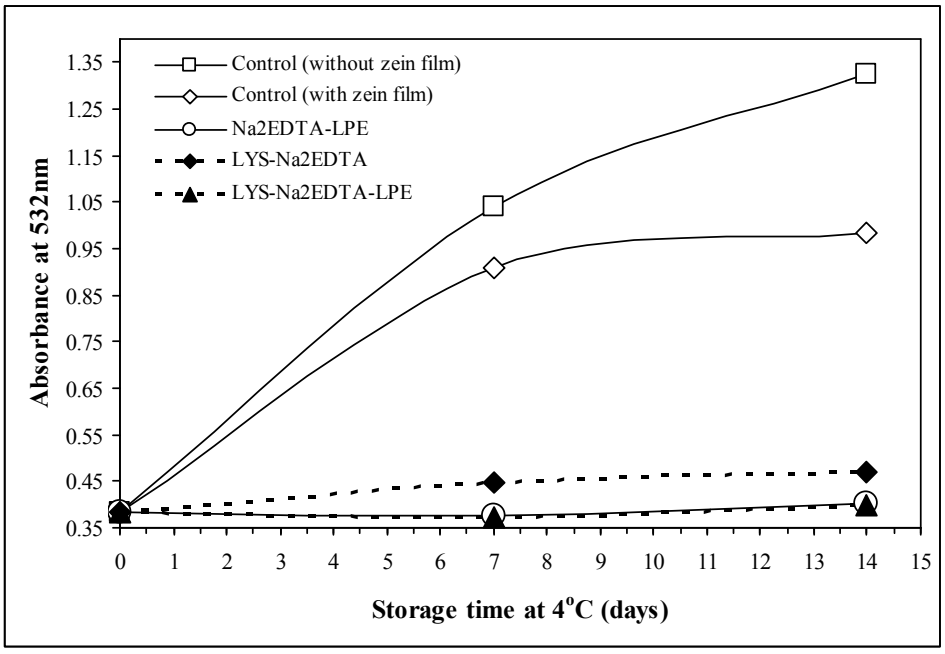


Fig. 2. Effect of active packaging on oxidative stability of cold stored beef burgers

Table 1. Bound lysozyme activities of different zein films

| Type of film | Change of activity (U/cm$^2$) during storage at 4 $^o$C (days) | | |
|---|---|---|---|
| | 0 | 5 | 7 |
| LYS | 6 | 18 | 13 |
| LYS-LPE | 210 | 116 | 128 |

Table 2. Effect of active packaging on color of beef burgers

| Type of film | Lightness (L*)[a] | | Redness index (a*/b*)[b] | |
|---|---|---|---|---|
| | Storage time at 4 $^o$C (days) | | | |
| | 7 | 14 | 7 | 14 |
| Control (without film) | 60.6 | 52.0 | 0.54 | 1.0 |
| Control (with film) | 53.9 | 55.6 | 0.71 | 0.83 |
| $Na_2$EDTA-LPE | 57.4 | 53.7 | 0.50 | 1.05 |
| LYS-$Na_2$EDTA | 55.4 | 57.3 | 0.58 | 1.03 |
| LYS-$Na_2$EDTA-LPE | 54.4 | 54.5 | 0.6 | 0.76 |

[a] Initial L* of burgers:52.2; [b]initial a*/b* value of burgers:1.43.