

Antimicrobial Activity of Lactoperoxidase System Incorporated into Cross-Linked Alginate Films

FATIH Y.G. YENER, FIGEN KOREL, AND AHMET YEMENICIOĞLU

ABSTRACT: In this study, the antimicrobial effect of lactoperoxidase (LPS) incorporated alginate films was investigated on *Escherichia coli* (NRRL B-3008), *Listeria innocua* (NRRL B-33314), and *Pseudomonas fluorescens* (NRRL B-253) in presence of different concentrations of H₂O₂ (0.2, 0.4, and 0.8 mM) and KSCN (1, 2, and 4 mM). The incorporation of 70 nmol ABTS/min/cm² LPS into alginate films gave 0.66 to 0.85 nmol ABTS/min/cm² enzyme activity at 0.2 to 0.8 mM H₂O₂ concentration range. The antimicrobial activity of LPS system on target bacteria changed according to the concentrations of KSCN and H₂O₂. The growth of all tested bacteria was prevented for a 6-h period by applying LPS system in presence of 0.4 or 0.8 mM H₂O₂ and 4 mM KSCN. At 0.8 mM H₂O₂ and 4 mM KSCN, the LPS system also inhibited growth of *L. innocua* and *P. fluorescens* for a 24-h incubation period, whereas *E. coli* growth could not be inhibited for 24 h under these conditions. At 0.2 mM H₂O₂ and 1 to 4 mM KSCN, a considerable inhibitory effect was obtained only on *P. fluorescens*. The decreasing order of the resistance of studied bacteria to LPS system is as follows: *E. coli*, *L. innocua*, and *P. fluorescens*. The developed antimicrobial system has a good potential for use in meat, poultry, and seafood since alginate coatings are already used in these products. Further studies are needed to test the LPS incorporated edible films in real food systems.

Keywords: alginate, antimicrobial packaging, edible films, lactoperoxidase

Introduction

Antimicrobial edible films and coatings have received attention since they have a good potential to delay microbial spoilage of food and to reduce the risk of surface contamination of food by pathogenic microorganisms (Quattara and others 2000; Cagri and others 2002; Eswaranandam and others 2004). Due to their greater acceptance by the growing "natural foods" market, incorporation of biopreservatives, especially bacteriocins and antimicrobial enzymes, and plant extracts into edible films have gained significant interest in the food industry (Hoover and Steenson 1993; Dean and Zottola 1996; Delves-Broughton and others 1998; Han 2000).

Lactoperoxidase (LPS) system is considered for use in food packaging since it has a broad antimicrobial spectrum. The enzyme shows bactericidal effect on Gram (–) bacteria and bacteriostatic effect on Gram (+) bacteria (Seifu and others 2005). Also, it has antifungal (Jacob and others 2000) and antiviral (Pakkanen and Aalto 1997; Seifu and others 2005) activities. LPS is an enzyme found in the milk, saliva, and tear secreted in mammary, salivary, and lachrymal glands of mammals, respectively (Wolfson and Sumner 1993). The LPS system consists of 3 components: LPS, thiocyanate, and hydrogen peroxide (H₂O₂). The enzyme catalyzes the oxidation of thiocyanate (SCN[–]) by use of H₂O₂ and generates intermediate antimicrobial products such as hypothiocyanite (OSCN[–]) and hypothiocyanous acid (HOSCN). These highly reactive products inhibit microorganisms by the oxidation of sulphhydryl (–SH) groups in their enzyme systems and proteins (Kussendrager and van Hooijdonk 2000). The structural damage of microbial cytoplasmic membranes by oxidation of –SH groups is reported as the principal

reason that causes the death of microbial cells (Reiter and Harnulv 1984; Kussendrager and van Hooijdonk 2000).

In the literature, there are different studies related to antimicrobial potential of LPS system against major food pathogenic bacteria. In these studies, antimicrobial activity of soluble LPS and its components has been tested against *Listeria monocytogenes*, *Staphylococcus aureus*, *E. coli*, *Brucella melitensis*, and *Salmonella enteritidis* (Kennedy and others 2000; Seifu and others 2004; Touch and others 2004). The soluble enzyme has also been tested in different food systems to improve microbial quality of milk, cheese (Seifu and others 2004, 2005), meat, and vegetable products (Kennedy and others 2000; Elliot and others 2004; Touch and others 2004). The concept of using LPS system in antimicrobial packaging is new. In fact, the LPS and its components have only been incorporated into edible whey protein films (Min and Krochta 2005; Min and others 2005a, 2005b). Recently, in our laboratories, the LPS has also been incorporated into alginate films. The enzyme incorporated into these edible films bound and immobilized effectively onto films following cross-linking and it shows appropriate stability and activity at a broad temperature and pH range (Mecitoğlu and Yemenicioğlu 2007). In this study, the antimicrobial activity of LPS incorporated into alginate films and its components has been tested on different bacteria including *E. coli*, *Listeria innocua*, and *Pseudomonas fluorescens*. The specific objectives of this research were to determine the effective concentrations of LPS components against the test bacteria and to test the resistance of different bacteria against the developed antimicrobial system supposed to be used in food coating applications.

Materials and Methods

Materials

Tyoppearl sulphopropyl (SP) cation-exchanger (SP-550C, fast flow, size: 100 μm) was purchased from Supelco (Bellefonte, Pa., U.S.A.). Dialysis tubes (cut off: 12000 MW), dextran (from

MS 20080157 Submitted 2/28/2008, Accepted 11/3/2008. Author Yener is with Biotechnology and Bioengineering Program, and authors Korel and Yemenicioğlu are with Food Engineering Dept., Faculty of Engineering, Izmir Inst. of Technology 35430, Urla, Izmir, Turkey. Direct inquiries to author Korel (E-mail: figenkorel@iyte.edu.tr).

Leuconostoc mesenteroides, 73.200 MW), ABTS (2,2-azino-bis-(3-ethylbenz-thiazoline-6-sulfonic acid)), and the sodium salt of alginic acid (from *Macrocystis pyrifera*, viscosity of 2% solution at 25 °C is 3500 cp) were obtained from Sigma Chem. Co. (St. Louis, Mo., U.S.A.). Rennet was purchased from ICN Biomedicals Inc. (Aurora, Ohio, U.S.A.). Nutrient agar and nutrient broth were obtained from Fluka (Spain). The microorganisms, *E. coli* (NRRL B-3008), *L. innocua* (NRRL B-33314), and *P. fluorescens* (NRRL B-253), were supplied from U.S. Department of Agriculture (USDA), Microbial Genomics and Bioprocessing Research Unit (Peoria, Ill., U.S.A.).

Partial purification and preparation of LPS

The partial purification of LPS from bovine whey was conducted with column chromatography by minor modification of the method of Ye and others (2000). In this method, the LPS was produced from rennet whey of skimmed milk using Toyopearl sulphopropyl (SP) cation-exchange column (11.5 × 2.8 cm). The column was equilibrated with 0.05 M sodium phosphate buffer at pH 6.5, washed with 500 mL of the same buffer, and then eluted with a linear gradient of 600 mL of 0 to 0.55 M NaCl (prepared in the sodium phosphate buffer). The LPS active fractions eluted between 0.3 and 0.4 M NaCl concentration were detected qualitatively by using the reaction mixture given in soluble enzyme activity determination.

The enzyme was prepared according to the method of Mecitoğlu and Yemenicioğlu (2007). For this purpose, LPS active fractions were pooled and dialyzed (cut off: 12.000 MW) for 24 h at 4 °C. The dialyzed extract was then lyophilized in a Labconco freeze-dryer (FreeZone 61, Kansas City, Mo., U.S.A.), after dissolving 250 to 300 mg dextran in it as a supporting agent. The enzyme prepared by this method and stored at -18 °C maintained almost 70% of its activity for at least 2 mo (Mecitoğlu and Yemenicioğlu 2007). The activity of lyophilized LPS was determined before each film preparation.

Preparation of alginate films

The alginate films were prepared according to the method given by Mecitoğlu and Yemenicioğlu (2007). Briefly, 0.2 to 0.6 mg of lyophilized LPS preparation were dissolved per gram of 2% (w/v) alginic acid solution by mixing slowly with a magnetic stirrer. The concentration of LPS preparation was selected carefully to set enzyme activity of all films at 70 nmol ABTS/min/cm². Ten-gram portions of this solution were then spread onto glass Petri dishes (9.5 cm in diameter). The Petri dishes were covered with a tent and dried at room temperature for 3 d. To cross-link the dried films, 0.8 mL of 0.3 M CaCl₂ was pipetted onto the Petri dishes. The films were peeled from the Petri dishes and washed with 10 mL sterile deionized water for 15 s to remove the excessive CaCl₂, which causes precipitations during LPS activity measurements. The average thickness of a cross-linked and dried control and LPS incorporated films prepared by this method was determined by a scanning electron microscope (Philips XL 30S FEG, FEI Co., Eindhoven, The Netherlands) as 13.05 and 18.87 μm, respectively.

Determination of soluble enzyme activity in LPS preparations

The soluble LPS activity of enzyme preparations was determined spectrophotometrically by using a Shimadzu (Model 2450, Tokyo, Japan) spectrophotometer equipped with a constant temperature cell holder working at 30 °C. Before activity determination, the lyophilized LPS was dissolved in distilled water. The reaction mixture consisted of 2.3 mL of 0.65 mM ABTS prepared in 0.1 M sodium phosphate buffer at pH 6, 0.1 mL of enzyme solution, and 0.1 mL

of 0.2 mM H₂O₂ solution. All components of the reaction mixture were brought to 30 °C before mixing. The absorbance was monitored at 412 nm for 5 min. The enzyme activity was calculated from the slope of the initial portion of absorbance against time curve and expressed as amount of ABTS oxidized per minute per milligram of LPS preparation. The molar extinction coefficient of ABTS at 412 nm was 32400 per M/cm (Touch and others 2004). The average of the 3 measurements was used to calculate enzyme activity.

Determination of soluble or bound LPS activity in films

To determine the soluble LPS activity in the films, cross-linked alginate films incorporated with LPS were placed into glass Petri dishes containing 50 mL of cold deionized water (4 °C). The Petri dishes, covered with parafilm to prevent evaporation, were incubated at 4 °C for 24 h and stirred at 200 rpm using a magnetic stirrer. The deionized water was then tested for residual enzyme activity. To increase the sensitivity of the enzyme activity test, the standard reaction mixture was changed to 2.2 mL of 0.65 mM ABTS prepared in 0.1 M sodium phosphate buffer at pH 6, 0.2 mL of deionized water obtained from the test medium, and 0.1 mL of 0.4 mM H₂O₂ solution. However, in this study, no soluble LPS activity was determined in the alginate films.

To determine the activity of bound LPS in the alginate films, the cross-linked and washed (15 s in 10 mL sterile deionized water) films were carefully halved with a clean razor. A film half was then placed into a glass Petri dish containing 23 mL of 0.65 mM ABTS solution prepared in 0.1 M sodium phosphate buffer at pH 6.0 and 2 mL of 0.2, 0.4, or 0.8 mM H₂O₂ solution. The solutions were brought to 30 °C before placing the film into the Petri dishes. The Petri dishes were incubated at 30 °C under continuous stirring at 200 rpm with a magnetic stirrer. The activity monitored by measuring the reaction mixture absorbance at 412 nm at different time intervals was determined from the slope of the initial linear portion of absorbance against time curve. The measurements were performed for the remaining half of the films and the average of 2 measurements was considered to calculate the activity. The enzyme activity was expressed as amount of ABTS oxidized per minute per square centimeter of the films.

Antimicrobial activity of LPS system

The bacterial strains, *E. coli* (NRRL B-3008), *L. innocua* (NRRL B-33314), and *P. fluorescens* (NRRL B-253), were maintained in nutrient broth containing 15% glycerol at -80 °C prior to the analyses. During activation of bacteria, *E. coli* and *L. innocua* were incubated using nutrient broth at 37 °C for 16 to 18 h, whereas *P. fluorescens* in nutrient broth was incubated at 26 °C for 16 to 18 h. All bacteria reached the stationary phase under these conditions (data not shown).

The cross-linked alginate films incorporated with LPS (or lacking LPS for controls) were prepared for the test by washing with 25-mL sterile deionized water for 15 s and cutting into 1.3-cm diameter discs using a sterilized cork borer under aseptic conditions. The discs were placed into test tubes containing nutrient broth (3 mL), 1 of the cultures (0.5 mL), 1, 2, or 4 mM KSCN (0.1 mL), and 0.2, 0.4, or 0.8 mM H₂O₂ (0.1 mL). Sterile distilled water (0.1 mL) was used instead of KSCN and/or H₂O₂ in some reaction mixtures lacking these reactants. The initial number of different bacteria in reaction mixtures changed between 3 and 4 log₁₀ CFU/mL. After preparation of reaction mixtures, tubes inoculated with *E. coli* or *L. innocua* were incubated at 37 °C for 24 h, whereas tubes inoculated with *P. fluorescens* were incubated at 26 °C for 24 h. The microbial growth in the tubes was enumerated on

nutrient agar by taking samples from reaction mixtures at 0, 6, and 24 h of incubation by using pour-plate method. The duplicate plates for each dilution were incubated at 37 °C for 24 h for *E. coli*, at 37 °C for 48 h for *L. innocua*, and at 26 °C for 48 h for *P. fluorescens*. The microbial counts were calculated as CFU per milliliter (N). The results of antimicrobial tests were expressed by plotting $\log N_t/N_0$ against incubation period of reaction mixtures (hours).

Data analysis

Statistical analysis was performed using MINITAB® release 13 (Minitab Inc., State College, Pa., U.S.A.). The data for LPS activity and the changes in microbial counts during 24 h of incubation were analyzed using analysis of variance (ANOVA) and Tukey's HSD significance test. Significance was accepted at $P < 0.05$.

Results and Discussion

LPS activity and concentrations of other components of antimicrobial system

In this study, the lyophilized LPS was incorporated into alginate films to exploit a naturally occurring antimicrobial mechanism. The results of our previous findings showed tight binding of lyophilized LPS in alginate films unless its incorporated concentration exceeded 700 $\mu\text{g}/\text{cm}^2$ (Mecitoğlu and Yemenicioğlu 2007). Thus, the level of LPS incorporated was maintained below 600 $\mu\text{g}/\text{cm}^2$ to prevent its solubilization from the films. The binding of LPS to alginate films and lack of its soluble form in films was also confirmed in this study by conducting soluble enzyme activity measurements following incubation of films in distilled water at 4 °C for 24 h. The polymeric chains of alginate can form negative charges by ionization of their carboxylic acid groups and LPS has a very high pI value (9.6). Thus, it is proposed that the positive charges of LPS formed under close to neutral conditions of film-making caused binding of the enzyme to the film matrix. The LPS was prepared with dextran. Thus, the H-binding of this polysaccharide to LPS and alginate could also make a contribution to the binding of enzyme (Mecitoğlu and Yemenicioğlu 2007).

The bound and retained activity in alginate films incorporated with 70 nmol ABTS/min/cm² LPS was determined at H₂O₂ concentrations of 0.2, 0.4, or 0.8 mM (Figure 1). The results revealed that the activity of LPS did not change significantly ($P \geq 0.05$) at the studied concentration range of H₂O₂. However, a slight reduction in average enzyme activity at 0.4 and 0.8 mM suggested a possible inhibitory effect of H₂O₂ on LPS. This result complies with that of Fonteh and others (2005) who reported substrate inhibitory activity of H₂O₂ on LPS above 0.2 mM concentration. In the literature, the H₂O₂ concentrations used to investigate antimicrobial activity of LPS changed between 0.25 and 0.5 mM (Zapico and others 1998; Jacob and others 2000; Garcia-Graells and others 2003). It seems

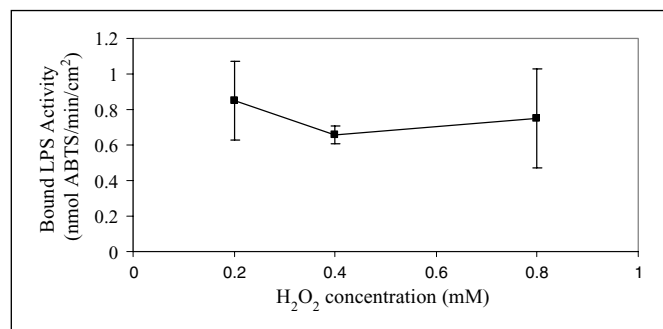


Figure 1 – Immobilized LPS activity of cross-linked alginate films at different H₂O₂ concentrations.

that the higher concentrations of H₂O₂ have been preferred due to the rapid degradation of H₂O₂ by the LPS used in tests and other H₂O₂ decomposing factors such as bacterial enzyme systems and reducing compounds in the test medium.

The range of KSCN concentrations applied in this study (1 and 4 mM) was selected from 8 different concentrations between 0.1 and 40 mM with a preliminary test by considering minimum amounts of this chemical necessary for consumption of > 90% of 0.4 mM H₂O₂ (H₂O₂ concentration was determined by semiquantitative test papers, Merck, Darmstadt, Germany) in a reaction mixture containing LPS incorporated discs and *E. coli* culture within 24 h of incubation at 37 °C.

Antimicrobial activity of LPS system

The antimicrobial activity of LPS system employed in presence of 0.2, 0.4, or 0.8 mM H₂O₂ and 4 mM KSCN was seen in Figure 2A. The LPS system caused low inhibitory effect on growth of *E. coli* in presence of 0.2 mM H₂O₂ and 4 mM KSCN. However, a significant inhibitory effect on this bacterium was observed at 6 h of incubation when H₂O₂ was increased to 0.4 or 0.8 mM in presence of 4 mM KSCN. In all reaction mixtures, the inhibitory effect of LPS system on *E. coli* exhausted when incubation periods were extended to 24 h. Figure 2B shows the antimicrobial effect of LPS system on *E. coli* in presence of 0.2 mM H₂O₂ and 1 or 2 mM KSCN. Under these conditions, the LPS system did not show a considerable antimicrobial activity on *E. coli*, and interestingly it became more effective with 1 mM KSCN than 2 mM KSCN at the end of 6-h incubation. It seems that the low concentration of H₂O₂ and KSCN controlled the rate of enzymatic transformation and prevented the rapid exhaustion of formed antimicrobial metabolites.

Figure 3A shows the antimicrobial activity of LPS system on *L. innocua* at 0.2 to 0.8 mM H₂O₂ and 4 mM KSCN concentrations. Similar to results obtained for *E. coli*, the LPS system was not very effective on *L. innocua* in presence of 0.2 mM H₂O₂ and 4 mM KSCN. The LPS system prevented the growth of *L. innocua* for a 6-h period at 0.4 mM H₂O₂ and 4 mM KSCN concentrations, but the growth of bacteria under these conditions could not be prevented at the end of 24 h of incubation. The elevation of H₂O₂ concentration to 0.8 mM at the same KSCN concentration became very effective on inhibition of *L. innocua* by reducing and keeping its counts below the initial counts for a 24 h period. This result clearly showed the greater inhibitory effect of LPS system on *L. innocua* than *E. coli* at highest H₂O₂ and KSCN concentrations. On the other hand, in presence of 0.2 mM H₂O₂ and 1 or 2 mM KSCN, the LPS system showed low inhibitory effect on growth of *L. innocua* (Figure 3B).

The effect of LPS system on *P. fluorescens* was given in Figure 4A. The growth of *P. fluorescens* was inhibited by the LPS system for a 6-h incubation period in presence of 0.2, 0.4, or 0.8 mM H₂O₂ and 4 mM KSCN. The LPS system employed in presence of 0.2 mM H₂O₂ and 4 mM KSCN could not prevent growth of *P. fluorescens* at the end of 24 h. However, LPS system employed in presence of 0.4 or 0.8 mM H₂O₂ at the same KSCN concentration delayed and prevented the growth of *P. fluorescens* at the end of 24 h, respectively. In presence of 0.2 mM H₂O₂ and 1 or 2 mM KSCN, the LPS system showed an inhibitory effect and maintained the *P. fluorescens* counts below initial count for a 6-h period (Figure 4B). However, at these concentrations, the effect of LPS system exhausted and it showed no antimicrobial activity at the end of 24 h. On the other hand, it is interesting to note that the counts of *P. fluorescens* in reaction mixtures lacking H₂O₂ but containing other components of reaction mixtures were higher than those of the control and reaction mixture containing only LPS. This occurred due to the activatory effect of KSCN on *P. fluorescens* and it was confirmed by repeated

tests with same reaction mixtures (data not given). Thus, it is clear that the use of LPS system against this bacterium needs application of high H₂O₂ concentrations to prevent excessive amounts of untransformed KSCN.

In the literature, there are very few reports related to use of LPS system in food antimicrobial packaging. The enzyme system was first incorporated into whey protein films by Min and others (2005a). These researchers supported the

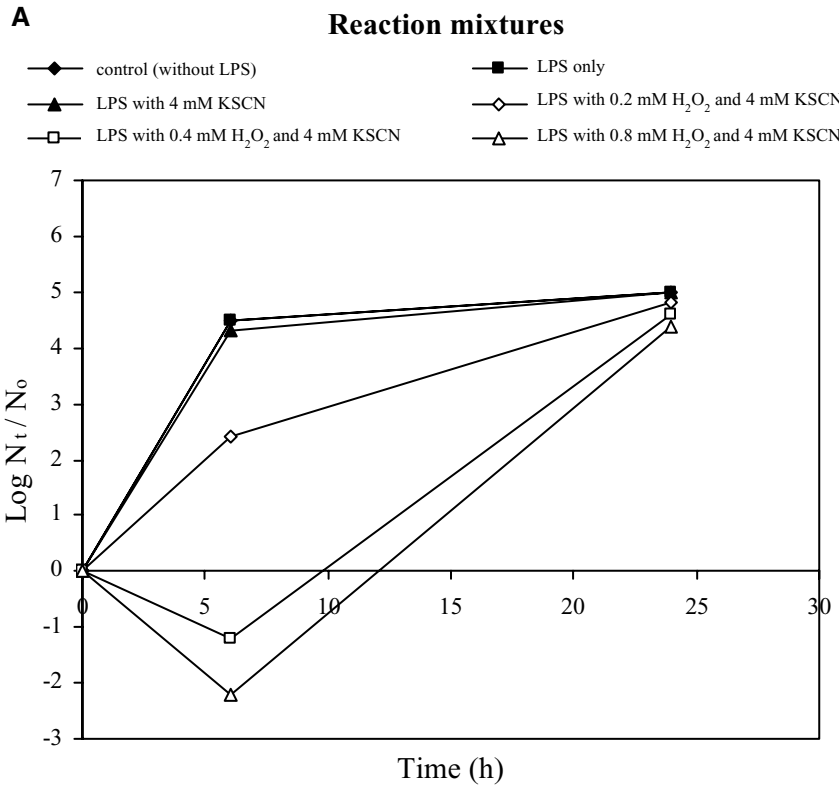
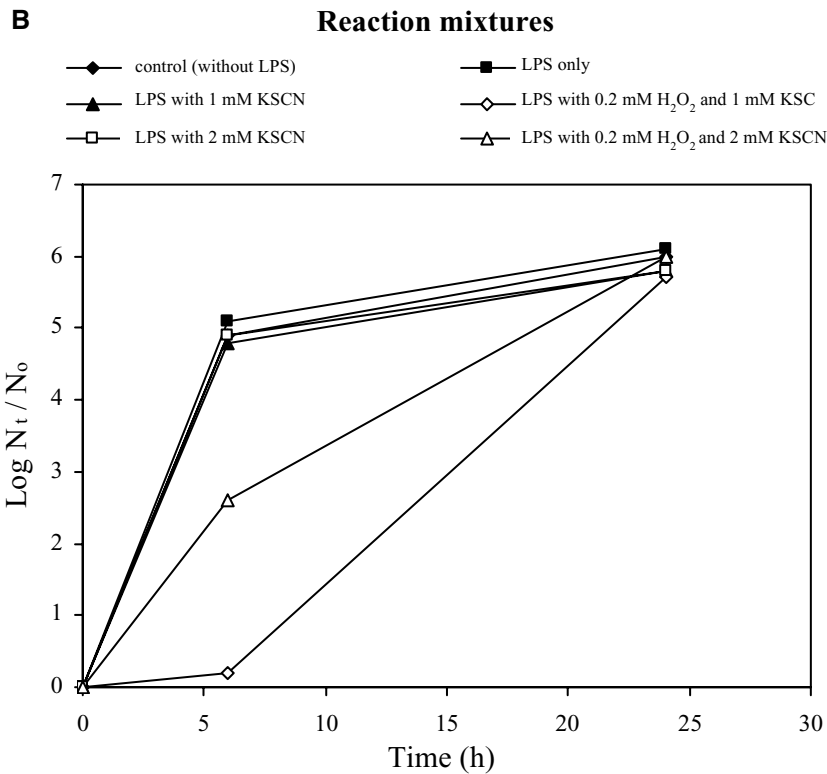


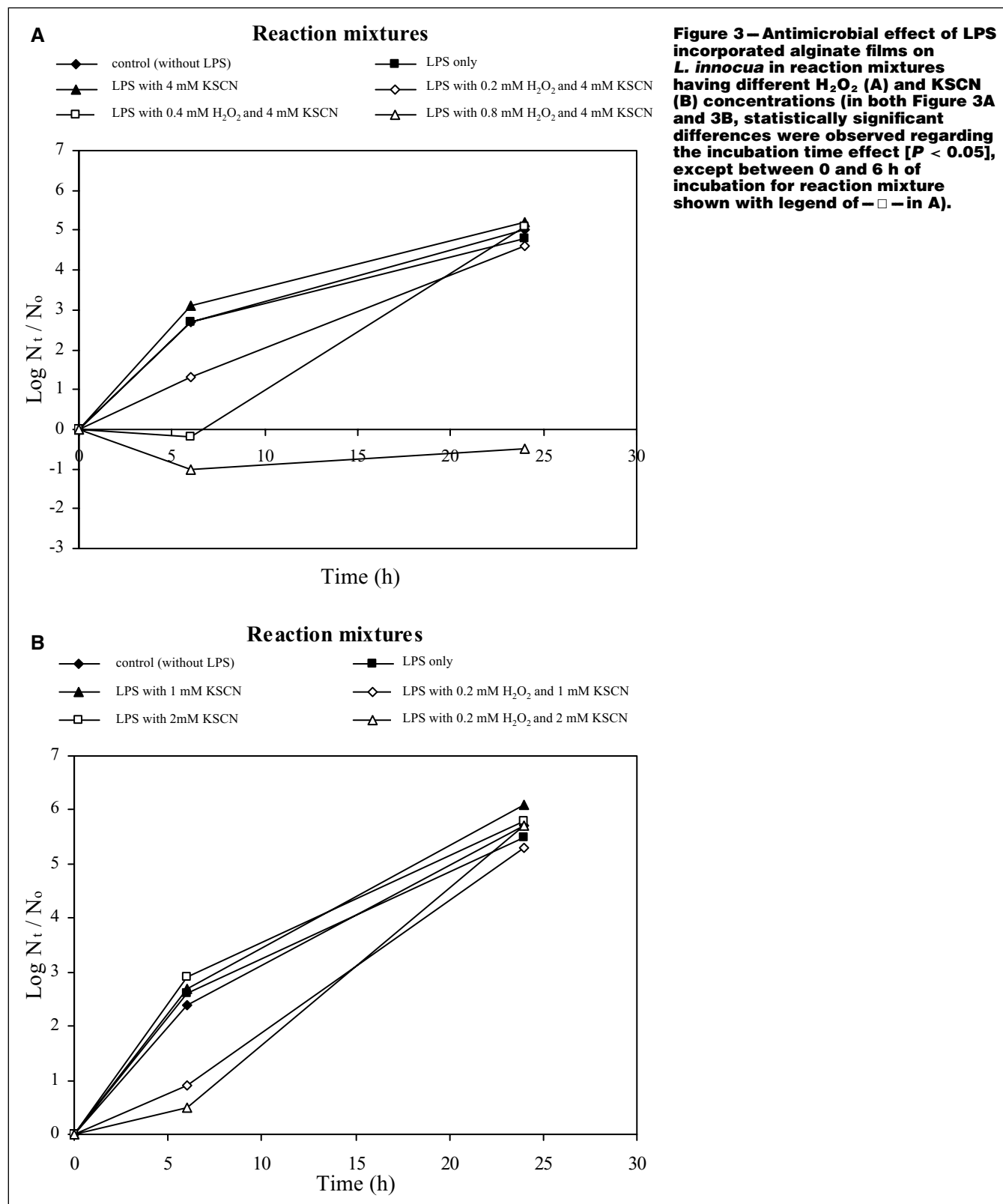
Figure 2—Antimicrobial effect of LPS incorporated alginate films on *E. coli* in reaction mixtures having different H₂O₂ (A) and KSCN (B) concentrations (in both Figure 2A and 2B, statistically significant differences were observed regarding the incubation time effect [*P* < 0.05], except between 0 and 6 h of incubation for reaction mixture shown with legend of —◇— in B).



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lactoperoxidase-KSCN-H₂O₂ system with glucose oxidase–glucose system to generate additional H₂O₂ for the LPS. The LPS system was then activated to form the antimicrobial metabolites and the reaction mixture was incorporated into whey protein isolate films. These films completely inhibited *S. enterica* and *E. coli* O157:H7, inoculated onto agar either before placing the

film disc or after placing the film discs. It was also reported in another study by the same research group that the whey protein isolate films incorporated with LPS metabolites were also effective on *L. monocytogenes* and the developed system successfully extended the shelf life of smoked refrigerated salmon (Min and others 2005b).



Conclusions

The antimicrobial system formed by LPS incorporated alginate films, KSCN and H₂O₂ showed antimicrobial activity on different tested bacteria. The decreasing order of the resistance of bacteria to LPS system at the studied conditions is as follows: *E. coli*, *L. innocua*, and *P. fluorescens*. The duration of antimicrobial effect of LPS system depends on activity of enzyme and initial concen-

trations of H₂O₂ and KSCN. During antimicrobial tests, the LPS has been employed at high incubation temperatures necessary for the bacterial growth, but this accelerated the enzyme activity and formation and degradation of reactive antimicrobial metabolites. Thus, the duration of antimicrobial effect of the developed system would be extended when the alginate films will be applied to refrigerated foods. Most of the patented applications of alginate

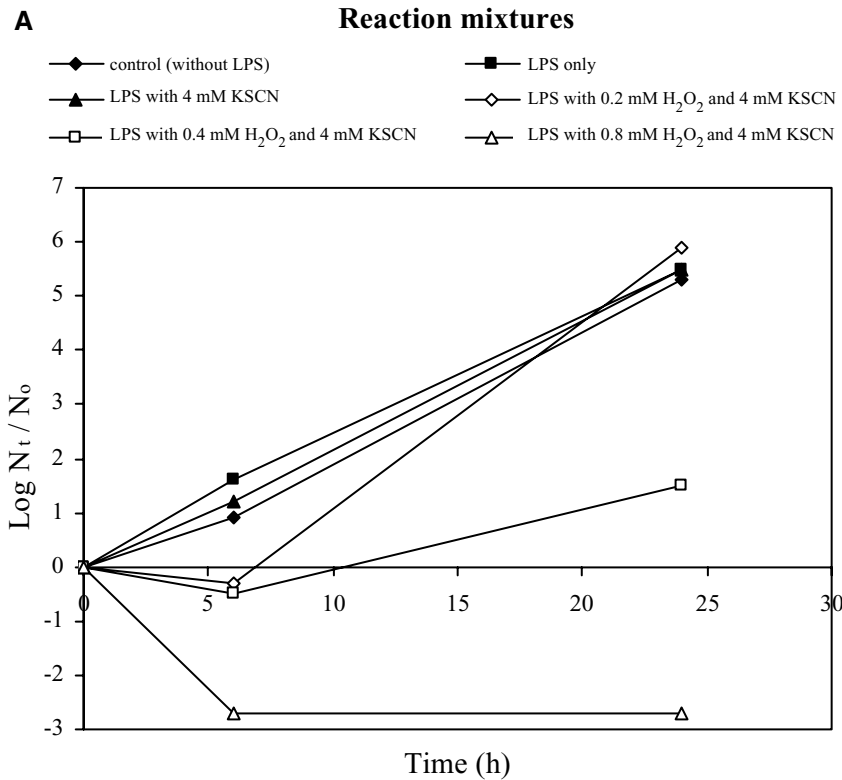
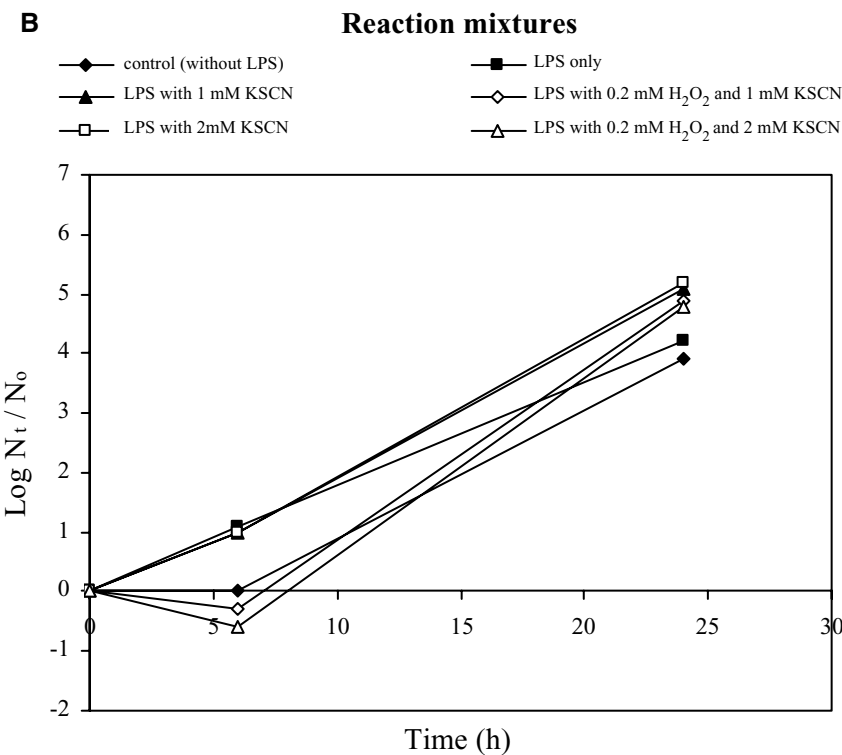


Figure 4 – Antimicrobial effect of LPS incorporated alginate films on *P. fluorescens* in reaction mixtures having different H₂O₂ (A) and KSCN (B) concentrations (in both Figure 4A and 4B, statistically significant differences were observed regarding the incubation time effect [*P* < 0.05], except between 0 and 6 h of incubation for reaction mixtures shown with legends of –◇– in A, –◆– and –◇– in B, and between 6 and 24 h of incubation for reaction mixture shown with legend of –△– in A).



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films have been developed for coating of meat, poultry, and seafood (Lindstrom and others 1992). Thus, the LPS incorporated antimicrobial films have a good potential to find food applications. Further studies are needed to test the LPS incorporated edible films in real food systems.

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