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Partial purification and preparation of bovine lactoperoxidase and characterization of kinetic properties of its immobilized form incorporated into cross-linked alginate films

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Abstract

Lactoperoxidase (LPS), purified directly from bovine rennet whey by Toyopearl-SP cation-exchange chromatography and lyophilized by using dextran as supporting material, maintained almost 70 and 60% of its activity after almost 2 and 5 months storage at −18 °C, respectively. Incorporation of the prepared LPS into alginate films between 0.08 and 0.69 mg/cm^2 (516–4325 U/cm^2) caused the immobilization of most of the enzyme and gave films with LPS activity between 0.05 and 2.8 U/cm^2, determined in the presence of 8 μM H_2O_2. Between 2 and 24 μM H_2O_2 concentrations, a two-fold increase in H_2O_2 concentration caused 1.5–2.5-fold increase in LPS activity of films incorporated with 0.24–0.28 mg/cm^2 (1200 U/cm^2) LPS. The Q_{10} and E_a of immobilized enzyme activity between 4 and 16 °C were 1.69 and 34.6 kJ/mol, respectively. However, in the 16–30 °C range, the temperature change had almost no effect on LPS activity of films. The optimal activity of immobilized LPS was observed at pH 6.0, but the enzyme maintained 30–85% of its activity between pH 3.0 and 7.0. The immobilized LPS also had a high stability between pH 4.0 and 6.0. The results of this study showed the good potential of LPS-incorporated alginate films in forming a natural antimicrobial mechanism in different foods.

Keywords: Lactoperoxidase; Purification; Immobilization; Characterization; Alginate films; Antimicrobial packaging

1. Introduction

The increased demand for easily prepared minimally processed fresh produce and the related increase of food-borne microbial outbreaks (De Roever, 1998) have intensified the research on antimicrobial packaging technologies (Suppakul, Miltz, Sonneveld, & Bigger, 2003). Different chemicals, such as organic or inorganic acids, metals, alcohols, ammonium compounds or amines, can be incorporated into plastic or biodegradable packaging materials as antimicrobials (Appendini & Hotchkiss, 2002; Suppakul et al., 2003). However, due to the health concerns of consumers related to chemical preservatives and environmental problems associated with the use of plastic packaging materials, there is an increasing demand for the food industry to use natural biopreservatives with edible and/or biodegradable packaging materials (Appendini & Hotchkiss, 2002; Quintavalla & Vicini, 2002). Different materials employed to obtain edible packaging films include cellulose derivatives, zein, carrageenan, alginate, and whey proteins (Cha, Choi, Chinnan, & Park, 2002; Han, 2000; Padgett, Han, & Dawson, 1998; Quintavalla & Vicini, 2002; Suppakul et al., 2003). In most of the edible materials, the film formation occurs under mild conditions and this protects biopreservatives from denaturing effects, e.g. from classical thermal polymer processing methods, extrusion and injection molding (Appendini & Hotchkiss, 2002; Han, 2000; Suppakul et al., 2003). Thus, the incorporation of biopreservatives into biodegradable films is generally more suitable than their incorporation into plastic films.

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The most frequently used biopreservatives for antimicrobial packaging are lysozyme and nisin (Cha et al., 2002; Dawson, Hoffman, & Han, 2000; Hoffman, Han, & Dawson, 2001; Janes, Kooshesh, & Johnson, 2002; Padgett et al., 1998; Padgett, Han, & Dawson, 2000; Park, Daeschel, & Zhao, 2004; Teerakarn, Hirt, Acton, Rieck, & Dawson, 2002). These biopreservatives are effective mainly on Gr (+) bacteria. However, they become effective also on Gr (−) bacteria when combined with chelating agents such as EDTA (Branen & Davidson, 2004; Padgett et al., 2000). The other biopreservatives that may be used in antimicrobial packaging are bacteriocins, such as pediocin and lacticin, and antimicrobial enzymes, such as chitinase and glucose oxidase (Labuza & Breene, 1989; Suppakul et al., 2003). Recently, different studies have also been conducted on the use of a lactoperoxidase (LPS)-thiocyanate-H_2O_2 antimicrobial system in food preservation. The LPS system is part of the natural preservation system that exists in milk. Thus, addition of thiocyanate and/or H_2O_2 to milk, to activate naturally occurring LPS, is used to improve microbial quality of milk and cheese (Seifu, Buys, & Donkin, 2004; Seifu, Buys, & Donkin, 2005). The addition of LPS and other components of this antimicrobial system to thermally processed skim milk (Zapico, Medina, Gaya, & Nunez, 1998), meat and vegetable products (Elliot, McLay, Kennedy, & Simmonds, 2004; Kennedy, O'Rourke, McLay, & Simmonds, 2000; Touch, Hayakawa, Yamada, & Kaneko, 2004) and prevention of the development of pathogenic bacteria have also been studied. The studies related to use of LPS in antimicrobial packaging, on the other hand, are new and very limited. In their detailed review, Suppakul et al. (2003) suggested the use of LPS in antimicrobial packaging. Recently, the LPS was incorporated into edible whey protein films and tests of these films on different microorganisms and smoked salmon showed the good potential of this enzyme for use in antimicrobial packaging (Min & Krochta, 2005a; Min, Harris, & Krochta, 2005b; Min, Harris, & Krochta, 2005c). The mechanism of the antimicrobial action of LPS is based on conversion of thiocyanate (SCN^-) to antimicrobial products, such as hypothiocyanite ($OSCN^-$) ion, hypothiocyanous acid (HOSCN) and some other highly reactive and short-lived oxidation products in the presence of H_2O_2 (Pruitt, Tenovuo, Andrews, & McKane, 1982). This system generally shows a bactericidal effect on Gr (−) bacteria and a bacteriostatic effect on Gr (+) bacteria (Seifu et al., 2005). Also, it has antifungal (Jacob, Antony, Sreekumar, & Haridas, 2000) and antiviral (Pakkanen & Aalto, 1997; Seifu et al., 2005) activities. The synergistic effect of LPS with nisin has also been demonstrated (Boussouel, Mathieu, Revol-Junelles, & Milliere, 2000; Dufour, Simmonds, & Bramer, 2003; Zapico et al., 1998).

In this work, LPS was partially purified, directly, from bovine whey by fast flow cation-exchange chromatography and prepared (with lyophilization) by using dextran as supporting material. The obtained enzyme was then tested for its storage stability, incorporated into alginate films and its affinity for alginate films; kinetic characteristics important for its potential use in antimicrobial food packaging were determined. The alginate films are frequently used for coating of meat, poultry and fish (Lindstrom, Morimoto, & Cante, 1992). Thus, the data obtained in this study help to create a natural antimicrobial system in these products.

2. Materials and methods

2.1. Materials

Whole milk samples used to produce LPS enzyme were obtained at 2–2.5 month intervals from the same Holstein cow of a local farm in Urla, Izmir (Turkey). Toyopearl sulphopropyl (SP) cation-exchanger (SP-550C, fast flow, size: 100 μm) was obtained from Supelco (Bellefonte, PA, USA). Dialysis tubes (cut off: 12000 MW), dextran (from *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 cpc) were obtained from Sigma Chem. Co. (St. Louis, Mo, USA). Rennet was obtained from ICN Biomedicals Inc. (Aurora, Ohio, USA).

2.2. Partial purification of LPS

For purification of LPS from bovine whey the method of Ye, Yoshida, and Ng (2000) was used with slight modifications. The skim milk was obtained by centrifugation of 1 L of whole milk at 5000*g* for 20 min at 30 °C. The skim milk was then filtered from cheese-cloth, warmed up to 37 °C in an incubator and 90 mg rennet were added to 900 ml of the skim milk. After 1 h at 37 °C, the precipitates in whey were separated by filtration through a cheese-cloth and centrifugation at 10,000*g* for 25 min at 4 °C. 400 ml of the clarified whey were then loaded on to a 11.5 × 2.8 cm Toyopearl-SP column previously equilibrated with 0.05 M Na phosphate buffer at pH 6.5. The column was first washed with 500 ml of the equilibration buffer and then eluted with a linear gradient of 600 ml of 0–0.55 M NaCl prepared in the same buffer. 10 ml fractions collected from the column were assayed for their absorbance at 280 nm and LPS activity. This study was conducted by using LPS from three different purification studies. In the first purification study, the activity profile of LPS eluted was monitored quantitatively, as described in Section 2.4., whereas, in the other studies, it was monitored qualitatively by using the same reaction mixture.

2.3. Preparation of LPS and test of its stability

For the preparation of LPS, the enzyme containing fractions collected from the column were pooled and dialyzed for 24 h at 4 °C. The dialyzed enzyme was then lyophilized in a Labconco freeze-dryer (FreeZone 6 l, Kansas City, MO, USA), working approximately at −47 °C collector

temperature and 50–100 × 10^{-3} mBar vacuum, after dissolving 250–300 mg of dextran in the dialyzed enzyme extract as a supporting material. The sample container volume was two to three times the sample volume. The stability of the LPS produced by this method was determined by monitoring of enzyme activity during frozen storage at −18 °C. The preparation was dissolved in distilled water and its activity was determined as described in Section 2.4.

2.4. Determination of soluble LPS activity

The activity of soluble LPS was determined spectrophotometrically by using a Shimadzu (Model 2450, Tokyo, Japan) spectrophotometer equipped with a constant temperature cell holder working at 30 °C. The reaction mixture was formed by mixing 2.3 ml of 0.65 mM ABTS prepared in 0.1 M Na phosphate buffer at pH 6.0, 0.1 ml of enzyme solution and 0.1 ml of 0.2 mM H_2O_2 solution. All components of reaction mixture were brought to 30 °C before mixing. The increase in absorbance was monitored at 412 nm for 5 min and enzyme activity, calculated from the slope of the initial linear portion of absorbance vs. time curve, was expressed as units (0.001 absorbance change in 1 min). The average of three measurements was used to calculate soluble enzyme activity.

2.5. Preparation of films

To prepare films, 0.62–5.2 mg of LPS preparation was dissolved per gram of 2% (w/v) alginic acid solution by mixing slowly with a magnetic stirrer. 10 g portions of this solution were then spread onto glass Petri dishes (9.8 cm in diameter). The Petri dishes were dried at room temperature for three days and 0.8 ml of 0.3 M $CaCl_2$ was pipetted into them to cross-link the dried films. Before being assayed for LPS activity, the films obtained were peeled from the Petri dishes and immersed in a beaker containing 50 ml distilled water for 30 s, to remove excessive $CaCl_2$ that causes precipitations during LPS activity measurement. The average thickness of a cross-linked and dried control film (LPS-free) prepared by this method was determined by a scanning electron microscope (Philips XL 30S FEG, FEI Company, Eindhoven, Netherlands) as 12.8 ± 1.6 μm.

2.6. Determination of immobilized LPS activity in films

To determine the immobilized LPS activity in films, the obtained cross-linked and washed alginate films were carefully halved with a clean razor. A film half (area: 37.7 cm^2) was placed into a glass Petri dish containing 23 ml of 0.65 mM ABTS solution prepared in 0.1 M Na phosphate buffer at pH 6.0 and 2 ml of 0.1 mM H_2O_2 solution, brought to 30 °C before placing films (final H_2O_2 concentration in reaction mixtures was 8 μM). The Petri dishes were kept in an incubator at 30 °C and stirred 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 vs. time curve. The measurements were repeated for the remaining half of the film and the average of two measurements was considered in all film activity measurements. The activity was expressed as U (0.001 absorbance change in one minute) per cm^2 of the films.

2.7. Determination of LPS affinity for films

The affinity of LPS for alginate films was determined by modifying the method used by Appendini and Hotchkiss (1997). This method is based on considering the amount of enzyme activity retained in films after repeated activity measurements. In this study, after each activity measurement the films were additionally washed with cold distilled water. The films used in this study contained 0.52 mg/cm^2 (3252 U/cm^2) of LPS. The activity determination was conducted seven times and, after each activity determination, to remove the free enzyme and oxidized substrate absorbed, the films were washed by first placing for 3 min in a beaker containing 100 ml of cold distilled water and second placing for 15 min in another beaker containing 250 ml of cold distilled water, stirred magnetically. In these tests only, the immobilized LPS activity, measured at 412 nm, was calculated by a two point assay, by reading standard reaction mixture absorbance at 0 min and 30 min of reaction (Activity = $Abs_{30\ min} - Abs_{0\ min}$). This time period corresponds to the initial linear portion of absorbance vs. time curves. All measurements were applied for two films and averages were used to determine the effect of repeated activity measurements and washing on LPS activity retained in films.

2.8. Characterization of LPS activity in films

In all characterization studies, the characteristics were determined for immobilized LPS activity, in films at 30 °C, assayed by the standard method given in Section 2.6. All measurements were applied for two films and averages were used in determination of an effect on LPS activity expressed as U/cm^2 or percent initial activity.

The effect of prepared enzyme concentration on LPS activity of films was determined by measuring activity of films incorporated with LPS between 0.08 and 0.69 mg/cm^2 (516 and 4325 U/cm^2).

The effect of H_2O_2 concentration on LPS activity of films was determined by measuring activity of films incorporated with 0.24–0.28 mg/cm^2 (1200 U/cm^2) of LPS at different H_2O_2 concentrations (final concentrations in reaction mixture were between 2 and 24 μM).

The effect of temperature on LPS activity of films was determined by measuring activity of films incorporated with 0.23 mg/cm^2 (998 U/cm^2) LPS between 4 and 30 °C. The activation energy (E_a) for the activity was calculated by using an Arrhenius plot, formed by plotting natural logarithm of activity (U/cm^2) vs. reciprocal of temperature

($1/T$ (°K)). The temperature quotient (Q_{10}) of the enzyme activity was calculated from the equation $k_2/k_1 = (Q_{10})^{(T_2-T_1)/10}$, where k is reaction rate (activity) and T is temperature (°C).

The effect of pH on LPS activity of films was determined by measuring enzyme activity of films incorporated with 0.19 mg/cm^2 (1274 U/cm^2) of LPS in a reaction mixture containing 2 ml of 0.3 mM H_2O_2 and 23 ml of 0.65 mM ABTS solution prepared in 0.1 M acetate (at pH 3.0, 4.0 and 5.0) or Na phosphate (at pH 6.0 and 7.0) buffers.

The pH stabilities of LPS activity of films were determined by incubating films incorporated with 0.19 mg/cm^2 (1114 U/cm^2) LPS in 25 ml of 0.1 M acetate (at pH 3.0, 4.0 and 5.0) or 0.1 M Na phosphate (at pH 6.0) buffers at 4 °C for 24 h. The remaining activities of the films were determined, at pH 6.0, by using the following reaction mixture: 23 ml of 0.65 mM ABTS prepared in 0.1 M Na phosphate buffer at pH 6.0 and 2 ml of 0.3 mM H_2O_2. The remaining LPS activities of films were given as percent of initial activity determined at pH 6.0 by using films not incubated in buffer for 24 h.

2.9. Protein content

Protein content was determined by the Lowry procedure, using bovine serum albumin as standard (Harris, 1987). An average of five measurements was used to calculate the protein content.

3. Results and discussion

3.1. Partial purification of LPS

In different partial purification studies, the LPS elution from Toyopearl-SP column was initiated when salt concentration was between 0.21 and 0.26 M, and ended when salt concentration reached between 0.42 and 0.51 M. In all elutions, the most active LPS fractions came between 0.3 and 0.4 M salt concentrations. In batch 1, a large protein peak, containing two fractions but lacking LPS activity, was detected before LPS elution (Fig. 1). The elution profile of LPS activity in this batch suggested the presence of two major LPS enzyme forms in bovine whey (F_1 and F_2). In batch 2, conducted with another milk sample after several months, the LPS was not well separated from the major protein peak eluted before LPS-active fractions in batch 1, and several protein peaks were observed in the enzyme active region (Fig. 2). This increased the protein content of the collected LPS-active fractions. However, because of the higher LPS activity of whey sample used in batch 2, the specific activity of enzyme and purification fold of separation were not significantly different from those of the same parameters in batch 1 (Table 1). In batch 3, as occurred in batch 1, the major portion of LPS-active fractions was separated from the large protein peak, coming before enzyme. The bovine whey used in batch 3 contained the highest LPS activity, and its specific activity and recovery after column chromatography were also highest. The results of these chromatographic separations are quite different from those of Ye et al. (2000), that used

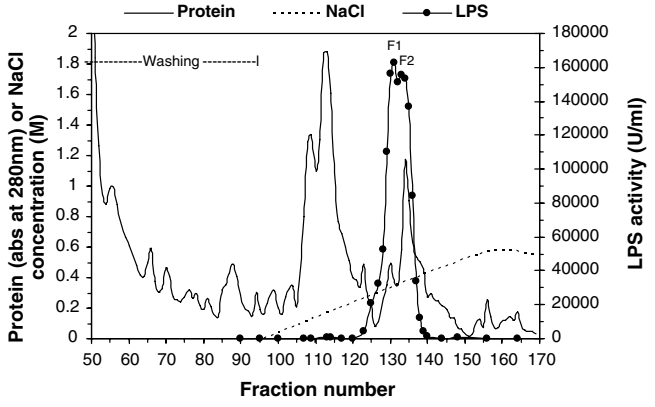

Fig. 1. LPS activity and protein profiles during Toyopearl-SP cation-exchange chromatography of bovine rennet whey (batch no: 1).

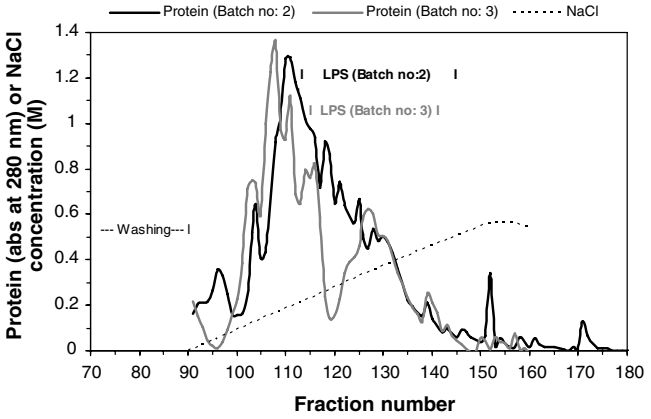

Fig. 2. Protein profiles and LPS-active regions during Toyopearl-SP cation-exchange chromatography of bovine rennet whey (batches no: 2 and 3).

Table 1
Summary of partial purification studies of LPS from bovine rennet whey

Batch no.	Volume (ml)	T activity (U)	T protein (mg)	S activity (U/mg)	Purity (fold)	Recovery (%)
1. Treatment of skim milk with rennet and centrifugation						
1	400	13,708,000	4374	3134	1	100
2	400	18,824,000	5032	3741	1	100
3	400	26,614,000	3632	7328	1	100
2. Toyopearl-SP cation-exchange chromatography						
1	150	3,651,750	76	48,049	15	27
2	330	9,698,700	182	53,290	14	52
3	248	21,588,400	95	227,246	31	81
3. 24 h Dialysis at 4 °C						
1	Nd[a]	Nd	Nd	Nd	Nd	Nd
2	300	9,447,000	153	61,745	17	50
3	267	16,950,495	74	229,061	31	64

[a] Not determined.

the same chromatographic procedure for the partial purification of LPS from bovine whey. These workers eluted LPS from the column between 0.16 and 0.19 M salt concentrations as a single peak. Also, the LPS-free large protein fraction containing two protein peaks, reported as lactoferrin-a and lactoferrin-b by these workers, were eluted from the column between 0.42 and 0.55 M salt concentrations. Ozdemir, Aygul, and Küfrevioglu (2001), on the other hand, determined two LPS fractions after cation-exchange chromatography on CM-Sephadex C-50. However, the LPS gave a single band in SDS-PAGE electrophoresis. Watanabe et al. (2000) explained the heterogenic profiles of LPS in disk-electrophoresis and ion-exhange chromatography by differences in the N-terminal amino acid residues of different LPS forms. However, as LPS is a glycoprotein that contains almost 10% carbohydrate, such an heterogeneity may also be related to the type of carbohydrate attached to the enzyme by glycosylation. In fact, Wolf, Ferrari, Traversa, and Biemann (2000) reported that the glycosylation sites of LPS are fully occupied with different high mannose and complex structures and this may vary molecular weight of LPS between 74,850 and 79,188 Da.

3.2. Stability of the prepared LPS during storage

In the literature, the general stabilizing effect of dextran on enzymes was reported by different workers (de la Casa, Guisan, Sanchez-Montero, & Sinisterra, 2002; Sasahara, McPhie, & Minton, 2003; Wasserman, 1984). The dextran produced by bacterial fermentation with *Leuconostoc mesenteroides* presents no toxicological concerns and it has been approved for food applications (Anonymous, 2000). As shown in Table 2, different batches of enzymes, lyophilized by using dextran, maintained almost 70–75% of their initial activity after 1–2 months at −18 °C. Further storage of one of the enzymes for almost 5 months increased the loss of enzyme activity only slightly. Thus, it appears that most of the activity in lyophilized LPS used in film making showed sufficient stability during several months of frozen storage.

3.3. Affinity of LPS for alginate films

As seen in Fig. 3, after the first activity measurement, conducted with 30 min of reaction at 30 °C and washing, the films incorporated with 0.52 mg/cm^2 of LPS maintained almost 80% of their activity. Thus, it is clear that most of the LPS-incorporated into alginate films was immobilized. In characterization studies, the LPS content of films was equal to almost half of the LPS content of films used in repeated activity measurements. Thus, little LPS leakage from the films is expected during characterization studies. In fact, in preliminary release tests conducted at 4 °C in distilled water (volume: 50 ml, stirring rate: 200 rpm) for 1800 min, no LPS activity release was determined from alginate films (area: 75.4 cm^2) incorporated with low amounts of LPS preparation (0.15 mg/cm^2 or 941 U/cm^2). The alginate films are composed of linear copolymers of D-mannuronic acid and L-guluronic acid that are cross-linked by the $CaCl_2$ (Lindstrom et al., 1992). Because of its high isoelectric point (pI 9.6), the LPS is mainly positively charged in the pH values studied. Thus, the enzyme may bind to films by the negatively charged carboxylic acid groups on polymeric chains of alginate. In this study, the LPS was prepared with dextran. Thus, the H-bonding of dextran to enzyme and alginate can also make a contribution to the immobilization of enzyme. After the third washing, on the other hand, a moderate drop (almost 40%) of enzyme activity was observed, whereas significant drops in activity occurred in the following washings. The reductions of enzyme activity at the latter stages might be related to the inhibitory effect of the substrate's blue coloured oxidation products absorbed by the films and could not be effectively removed by washing.

Table 2
Stability of partially purified LPS lyophilized by using dextran as supporting material and stored at −18 °C

Batch no.	Storage time (days)	Remaining activity	
		U/mg powder	%
1	0	6271	100
	28	4542	72
	48	4767	76
	161	3768	60
2	0	4363	100
	32	4424	101
	70	3005	69
3	0	6576	100
	20	5750	87
	33	4621	70

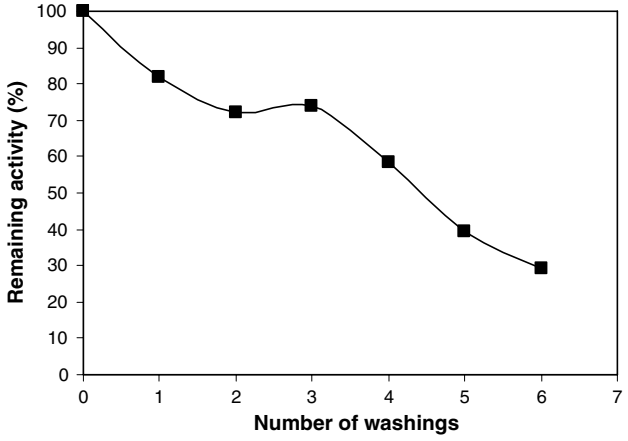

Fig. 3. Stability of LPS activity of alginate films during repeated activity measurements and washings.

3.4. Effect of enzyme preparation concentration on LPS activity of films

Incorporation of LPS (up to 0.16 mg/cm^2) did not notably change the LPS activity of alginate films in the presence of 8 μM H_2O_2 (Fig. 4). The initial lag period suggests the necessity of reaching a critical LPS concentration at the film surface where catalytic activity of films is controlled. Between 0.16 and 0.52 mg/cm^2 LPS concentrations, the increase of incorporated enzyme concentration increased the LPS activity of films linearly. However, at 0.69 mg/cm^2 LPS concentration, the slope of the curve increased further. The increase of activity at the highest LPS concentration may be due to the leakage of part of the enzyme in films to the reaction mixture.

3.5. Effect of H_2O_2 concentration on LPS activity of films

The effect of H_2O_2 on enzyme activity of alginate films incorporated with 0.24–0.28 mg/cm^2 (1200 U/cm^2) LPS was determined between 2 and 24 μM H_2O_2concentrations. As seen in Fig. 5, increase of H_2O_2 concentration increased the LPS activity of alginate films. In the H_2O_2 concentration range studied, the activities of films changed between 0.04 and 1.6 U/cm^2, and 1.5–2.5-fold increase in LPS activity was observed when H_2O_2 concentration was increased two-fold. The H_2O_2, an important component of the LPS antimicrobial mechanism, is a toxic compound, but it was reported that, at low concentrations (less than 100 μM) and in the presence of LPS and thiocyanate, mammalian cells are protected from this toxicity (Seifu et al., 2005). Thus, it seems that the obtained films showed good LPS activity at H_2O_2 concentrations not toxic to mammalian cells. Also, it appears that, by regulating the concentration of H_2O_2, it is possible to form and control the rate of an antimicrobial mechanism in alginate films incorporated with small amounts of LPS.

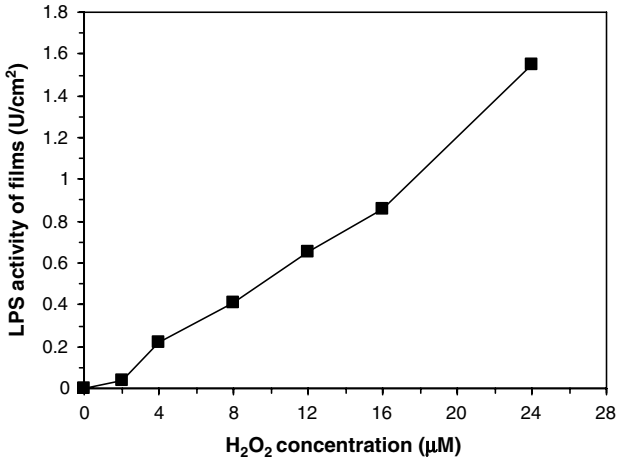

Fig. 5. Effect of H_2O_2 concentration on LPS activity of alginate films.

3.6. Effect of temperature on LPS activity of films

The effect of temperature on LPS activity immobilized in alginate films was determined between 4 and 30 °C, a temperature range to which packaged food is generally exposed. As seen in Fig. 6, between 4 and 16 °C, an increase of temperature increased the enzyme activity of films. In this temperature range, a 10 °C change in temperature caused a 1.69-fold (Q_{10}) change in LPS activity. The activation energy of enzyme between 4 and 16 °C was also calculated to be 34.6 kJ/mol. These E_a and Q_{10} values indicate the low dependency of LPS activity in alginate films on temperature change. Between 16 and 30 °C, on the other hand, the temperature had almost no effect on LPS activity of films. The low temperature dependency of the immobilized LPS in alginate films is an advantage for food applications of the enzyme when a certain amount of enzyme activity is desired in the product under variable processing and/or storage temperatures.

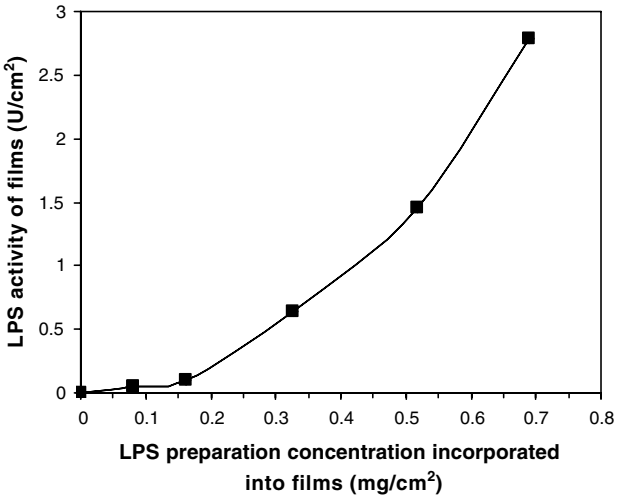

Fig. 4. Effect of enzyme preparation concentration on LPS activity of alginate films (H_2O_2 concentration : 8 μM).

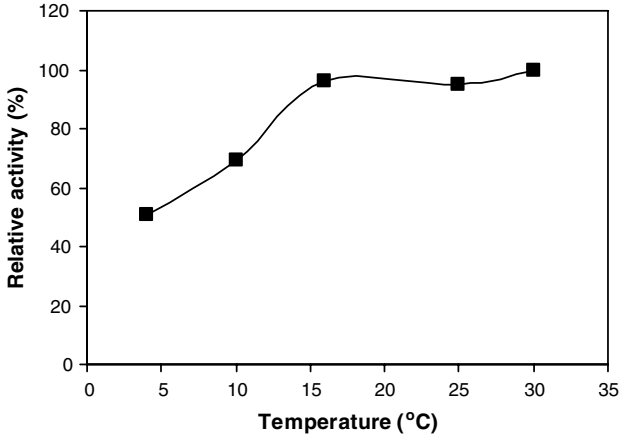

Fig. 6. Effect of temperature on LPS activity of alginate films.

3.7. Effect of pH on LPS activity of films

The effect of pH on LPS activity was determined between pH 3.0 and 7.0. At pH 4.0, the activity of enzyme was minimum (Fig. 7). However, the enzyme showed almost 30% of its activity at this pH value. The optimum activity of enzyme was at pH 6.0, but the enzyme is also very active at pH 7.0. However, at pH 7.0 the alginate films were decomposed during activity measurements. It is likely that the decomposition of films occurred because of the loosening of cross-linking interactions at this pH by the effect of oxidative changes catalyzed by LPS in the reaction mixture. The enzyme, on the other hand, maintained minimum 50% of its activity at pH 3.0 and 5.0. The broad pH spectrum of LPS suggests the suitability of using alginate films incorporated with LPS as a natural antimicrobial system for different foods. The pH optimum and profile of immobilized LPS in alginate films were almost same or not significantly different from that reported for soluble LPS in bovine milk. For example, Ozdemir et al. (2001) reported the optimum pH of soluble LPS to be 6.0 for ABTS substrate. Blel, Guingamp, Gaillard, and Humbert (2001) showed that the LPS in milk maintained a minimum 50% of its activity between pH 6.0 and 8.0 and it exhibited optimum activity at pH 6.7 against ABTS substrate. In their detailed review, Seifu et al. (2005) also reported that the optimum pH of soluble bovine LPS was between 5.0 and 6.0, depending on the concentrations of the substrates ABTS and H_2O_2 used in the assay.

3.8. pH stability of LPS activity of films

The stability tests showed that the LPS lost almost 90% of its activity in 24 h at pH 3.0 (Fig. 8). However, the enzyme activity is very stable between pH 4.0 and 6.0, a range where most foods fall in this category. The retention of most of the enzyme activity in alginate films in this pH range after 24 h once more showed the strong binding of LPS by the alginate films.

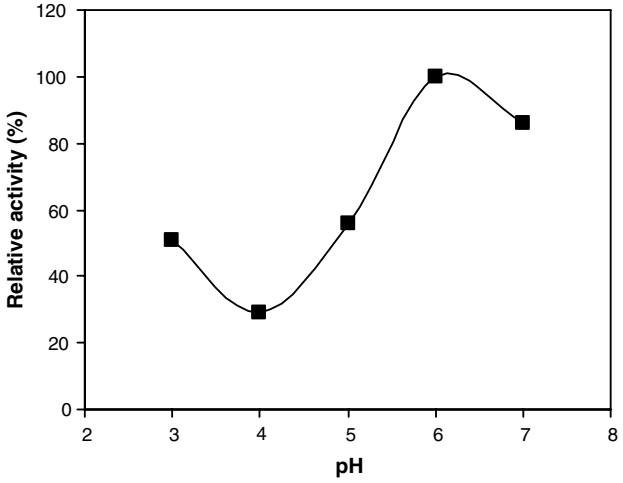

Fig. 7. Effect of pH on LPS activity of alginate films.

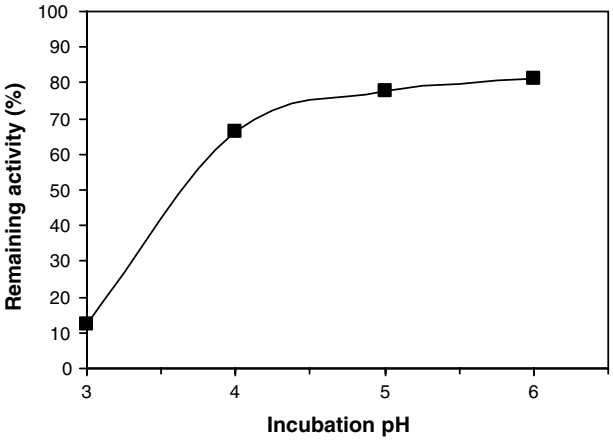

Fig. 8. pH stability of LPS activity of alginate films (Incubation period 24 h at 4 °C).

4. Conclusions

The preparations obtained by lyophilization of purified bovine LPS, by using dextran as supporting material, showed sufficient stability for several months at −18 °C. The incorporation of prepared LPS into alginate films caused the immobilization of most of the enzyme. Thus, the antimicrobial effect of LPS-incorporated alginate films might be controlled by the enzymatic transformation of antimicrobial compounds from the thiocyanate incorporated into films, added to food or naturally occurring in food. The high LPS activity of films at low H_2O_2 concentrations, very small effect of temperature changes on immobilized enzyme activity, broad pH range and pH stability of enzyme activity, all show the good potential of LPS-incorporated alginate films in forming a natural antimicrobial mechanism in different foods. Further studies are continuing in our laboratory to test the antimicrobial effect of LPS-incorporated alginate films on different microorganisms.

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