Antimicrobial Edible Packaging Based on Cellulosic Ethers, Fatty Acids, and Nisin Incorporation To Inhibit *Listeria innocua* and *Staphylococcus aureus*

V. COMA,* 1 I. SEBTL, 1–3 P. PARDON, 4 A. DESCHAMPS, 3 and F. H. PICHAVANT 4

1Laboratoire de Chimie des Substances Végétales, Institut du Pin, Université Bordeaux 1, 351 cours de la Libération, 33405 Talence, France; 2Laboratoire de Recherche en Génie Industriel Alimentaire, IUT A, Université Lyon I, Département Génie Biologique, Rue H. de Boissieu, 01000 Bourg-en-Bresse, France; 3Unité Sécurité Microbiologique des Aliments, ISTAB, Université Bordeaux 1, Avenue des Facultés, 33405 Talence, France; and 4Recherche Appliquée en Chimie Industrielle Organique, Institut du Pin, Université Bordeaux 1, 351 cours de la Libération, 33405 Talence, France

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ABSTRACT

Edible cellulosic films made with hydroxypropylmethylcellulose (HPMC) have proven to be inadequate moisture barriers. To improve its water vapor barrier properties, different hydrophobic compounds were incorporated into the HPMC matrix. Some fatty acids and derivatives were included into the film-forming solution prior to film formation. Stearic acid was chosen because of its high capacity to reduce significantly the water vapor transmission rate. Antimicrobial activity of edible HPMC film was obtained by the incorporation of nisin into the film-forming solution. Nisin is an antimicrobial peptide effective against gram-positive bacteria. The inhibitory activity of this bacteriocin was tested for inhibition of *Listeria innocua* and *Staphylococcus aureus*. The use of stearic acid was observed to reduce the inhibitory activity of active HPMC film against both selected strains. This phenomenon may be explained by electrostatic interactions between the cationic nisin and the anionic stearic acid. Further studies showed that antimicrobial activity of film varied with the nature of the hydrophobic compound incorporated, in decreasing order: film without lipid, methylstearate film, and stearic acid film. This corroborated the idea of electrostatic interactions.

In recent years, environmental pollution and safety of processed foods have become a major issue of concern. Research has focused on edible films and on use of several antimicrobial compounds incorporated into edible food packaging (12–14). As opposed to mixing antimicrobial compounds directly with food, their incorporation into film would localize functional effect at the food surface. This approach can be used to impart a strong localized functional effect, without excessively elevating the overall concentration of the additive in foods. A wide variety of edible compounds have drawn attention due to their film-forming ability, such as polysaccharides like hydroxypropylmethylcellulose (HPMC) investigated by Kamper and Fennema (10) and derivatives. Because of the hydrophilic nature of these polymers, only minimal moisture barrier properties can be expected. However, edible films composed of a hydrophilic polysaccharide matrix and a mixture of hydrophobic compounds such as fatty acids and derivatives have been developed to improve the moisture vapor barrier (8, 10, 11). *Listeria monocytogenes* and *Staphylococcus aureus* are unacceptable in foodstuffs, because of their pathogenicity. Existing methods of preservation may not be sufficient to preclude foodborne listeriosis (4, 5). Therefore, the development of complementary methods to inhibit the growth of both pathogenic bacteria such as packaging material-associated bacteriocins is an active area of research. Several reports have addressed the use of bacteriocins to suppress effectively the growth of gram-positive bacteria (6, 7, 15, 17). Of these, nisin is a bacteriocin produced by *Lactococcus lactis* subsp. *lactis*. It is a 34-residue-long lantibiotic that contains the unusual amino acid residues dehydrobutyrine, dehydroalanine, lanthionine, and β-methyl-lanthionine. It has antimicrobial activity against a broad spectrum of gram-positive bacteria such as *L. monocytogenes* and *S. aureus* (5, 16, 19) and gram-negative bacteria when the bacterial cell walls are weakened by lysozyme, NaCl, or EDTA (3, 18) before addition of nisin. Nisin has been shown to be nontoxic and recognized as safe by the American Food and Drug Administration in 1969. It has widely been used in the food industry as a safe and natural preservative (7). This publication reports the use of HPMC as a film-forming polymer. Fatty acids and derivatives were incorporated to improve the film moisture barrier. Film antimicrobial activity was due to nisin addition. Inhibitory activity of the edible film was tested on *L. innocua* and *S. aureus*. *L. innocua* was used instead of *L. monocytogenes* because of its nonpathogenicity to humans. However, its sensitivity to nisin is lower compared to *L. monocytogenes*. As a result, *L. monocytogenes* could be expected to behave similarly to *L. innocua*. The main objective of this study was to investigate the moisture barrier and antimicrobial properties of the HPMC-fatty acid film associated with nisin as preservative, in order to determine its efficacy as a...
potential active packaging material on \textit{L. innocua}, a model strain of \textit{L. monocytogenes}, and \textit{S. aureus} growth in food products.

**MATERIALS AND METHODS**

**Film formation.** Films were prepared using a modified procedure described by Kamper and Fennema (10), by dissolving 9 parts HPMC polymer (HPMC-Culmina 50; Aqualon, Alizay, France) in 200 parts distilled water, 100 parts absolute ethanol, and 1 part polyethylene glycol (PEG 400; Merck, Darmstadt, Germany). Nisin was presolubilized in phosphate buffer (pH 6.1) and the solution was added to water-alcohol mixture prior to HPMC incorporation. The nisin concentration was adjusted taking into account the dilution effect. Stearic acid (C18:0), palmitic acid (C16:0), methylpalmitate, methylstearate, and oleic acid (C18:1) were obtained from Stearinerie Dubois (Boulogne, France). These were added prior to film formation. The quantity of hydrophobic compounds was expressed in percentage of HPMC (wt/wt). The film-forming solution was heated at the melting points of the selected hydrophobic compound before fatty acid and derivatives addition. The solution was then homogenized for 15 min, degassed for 30 s, and plated onto glass at a thickness of 1 mm. The plates were heated at 90°C for 1 h. Films peeled from the plates had a 30 to 50 μm final thickness.

**Water vapor transmission rate (WVTR).** The WVTR of edible films was evaluated using NF ISO 2528 (1989). An aluminum cup containing anhydrous CaCl₂ desiccant (assay cup) or nothing (control cup) was sealed by the test film (50 cm² exchange film area) with paraffin wax at 50°C. It was placed in an environment of controlled humidity and temperature (90% relative humidity [RH] and 38°C or 50% RH and 23°C). The WVTR (g H₂O m⁻² over 24 h) was determined from the weight increase of the cup over time at the steady-state of transfer. All tests were conducted in triplicate.

**Organisms and maintenance.** \textit{S. aureus} 58156 and \textit{Micrococcus luteus} 270 were obtained from the Institut Pasteur collection (Institut Pasteur, Paris, France). \textit{Listeria innocua} 430 was selected from the USMA collection (ISTAB, University Bordeaux I, France). \textit{M. luteus} was grown at 30°C in nutritive broth (3178; Difco, Detroit, Mich.), whereas \textit{L. innocua} and \textit{S. aureus} were grown in tryptose broth (62176; Difco) at 37°C and agitated at 140 to 160 rpm for 18 to 24 h.

**Nisin.** Pure nisin (Aplin and Barrett Ltd., Dorset, UK) was dissolved in 0.05 M phosphate buffer, pH 6.1, to the desired concentration. For experiments with free nisin, the solution was heated at 90°C for 1 h to reproduce film preparation and stored at 4°C.

**Antimicrobial effectiveness.** An inhibition zone assay was conducted by inoculating the appropriate agar (12 g/liter) medium at 0.1% (vol/vol) with an overnight culture of the test strain. Seventy microliters of the nisin solution was then poured into wells (5 to 6 mm diameter) previously cut into the agar medium. Dishes were refrigerated at 4°C for 3 to 4 h to allow diffusion of the bacteriocin and then incubated at 30°C for 24 to 48 h. Zones of inhibition were measured to the nearest 0.5 mm. All experiments were performed in triplicate.

**RESULTS AND DISCUSSION**

In order to characterize \textit{L. innocua} and \textit{S. aureus} pathogenic strains in noninhibitory conditions, preliminary assays were conducted in triplicate. Cultures of bacteria, incubated in tryptose broth medium at 37°C, and stirred at 140 rpm determined a μₚₐₙₜ of 0.83 ± 0.15 min⁻¹ and 1.41 ± 0.14 min⁻¹ for \textit{L. innocua} and \textit{S. aureus}, respectively.

First, experiments were conducted to determine the ability of nisin to withstand temperature and solvent conditions used for edible film formation. The residual inhibitory activity was measured by the inhibition zone assay and compared to initial nisin activity. All experiments were replicated three times, and treatment means were separated using the Student’s t test at 95% probability. Results (data not shown) indicated that the film-forming conditions could be used without significantly \((P > 0.05)\) losing any nisin inhibitor properties.

In order to limit the dehydration phenomenon of food product, barrier moisture could be introduced by edible films. Because of the hydrophilic nature of HPMC films, a significant water vapor transfer was expected. To improve moisture barrier properties, edible hydrophobic compounds,
such as fatty acids and derivatives, were incorporated into film-forming solutions.

**WVTR determination.** In order to select a hydrophobic compound that contributed to a significant decrease in WVTR, the influence of either the length of the hydrocarbon chain, degree of unsaturation, and carboxyl function of fatty acid were investigated on moisture transfer. The WVTR was first determined at 23°C and 50% RH to reproduce an environment that is commonly encountered during food storage under commercial conditions (Table 1).

<table>
<thead>
<tr>
<th>Stearic acid (% wt/wt HPMC)</th>
<th>WVTR (g H₂O m⁻² per 24 h)ᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3,567 ± 218</td>
</tr>
<tr>
<td>5</td>
<td>784 ± 23</td>
</tr>
<tr>
<td>10</td>
<td>727 ± 107</td>
</tr>
<tr>
<td>15</td>
<td>455 ± 4</td>
</tr>
</tbody>
</table>

ᵃ Values are means of three experiments followed by their standard deviations.

Cellulosic films without hydrophobic compounds were able to act as minimal moisture barriers, due to the interactions of water molecules with hydrophilic polysaccharide polymers. Moreover, stearic acid exhibited a higher capacity to reduce the WVTR significantly compared to fatty acid derivatives. The incorporation of 30% (wt/wt HPMC) stearic acid allowed, for instance, a decrease in WVTR of about 144 to 58 g H₂O m⁻² over 24 h, whereas no improvement was obtained with other tested compounds.

Differences in WVTR between methylstearate and methylpalmitate films, stearic acid (C₁₈:₀), and oleic acid (C₁₈:₁) films, respectively, were in accordance with previously reported results (10, 11), given that the rate of transmission of water through a lipid film increased as the length of the lipid hydrocarbon chain decreased and the degree of unsaturation increased. However, the higher WVTR of methylstearate film, compared to stearic acid film, was not expected, because of the higher hydrophobic nature of the methylstearate. Films were then analyzed by Fourier transform infrared spectroscopy (Nicolet 210 apparatus) to determine whether an ester bond might be created between the carboxylic group of stearic acid (-COOH) and the hydroxyl group (-OH) of the cellulose derivative. In this case, the film would be more homogeneous, which could explain the lower WVTR of stearic acid films. The infrared spectra showed that there were no ester bonds in either film (data not shown).

According to Wong et al. (20), the increase of WVTR in composite films, when replacing fatty acids by their esters, could be due to variations in the film microstructure, including a decrease in density and pore formation that might be correlated with the permeability properties of the film. Therefore, film analysis by electron microscopy was conducted using a scanning electron microscope (JEOL 840A). Figure 1 shows that methylstearate films were consistently very porous, with more air globules than stearic acid films. As a result, the higher WVTR of film with methylstearate would be explained by a higher heterogeneity of the film, due to pore formation.

To test the effectiveness of C₁₈:₀ to limit moisture transfer under more difficult conditions, the WVTRs were also measured at 38°C and 90% RH. Data are presented in Table 2. As expected, WVTR was significantly increased by increased RH and temperature (10, 11). The incorporation of stearic acid decreased the WVTR from about 3,500
to 780 455-g H₂O m⁻² over 24 h, depending on the amount of incorporated fatty acid, corresponding to decreases of about 78 and 87%, respectively.

Stearic acid was selected as the hydrophobic compound in the edible coating because of its high capacity to reduce moisture transfer. Taking into account that more than 15% (wt/wt HPMC) of C₁₈:₀ contributed to the opacity phenomenon or nonhomogeneous film structures, this latter rate of incorporation was selected for the following experiments.

**Antimicrobial effectiveness.** In order to determine the MIC that had to be used in edible films, the antimicrobial effectiveness of different contents of free nisin was studied. Preliminary experiments in liquid medium showed that sensitivity to nisin was detected at 50 IU/ml for both organisms with the exception of stationary-phase *S. aureus* (data not shown). The sensitivity of *L. innocua* and *S. aureus* to nisin was also evaluated on solid medium using the inhibition zone assay. Results presented in Table 3 show that the MICs that had to be incorporated into film-forming solutions were 500 and 1,000 IU/ml for *L. innocua* and *S. aureus*, respectively. The MICs obtained on solid medium were higher than those observed in liquid media, mainly due to the limited diffusion of nisin in solid medium (2).

Given that the use of edible packaging material would be especially for solid food products, 1,000 IU/ml was chosen as the MIC for the inhibition of both microbial strains by active HPMC films.

**TABLE 3. Nisin antimicrobial effectiveness evaluated by the inhibition zone assay**

<table>
<thead>
<tr>
<th>Nisin concentration (×10² IU/ml)</th>
<th><em>L. innocua</em></th>
<th><em>S. aureus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>4 ± 1</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>6 ± 1</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>50</td>
<td>8 ± 1</td>
<td>10 ± 2</td>
</tr>
</tbody>
</table>

aData correspond to inhibition zone diameters (mm). Values are means of three experiments followed by their standard deviations.

**TABLE 5. Antimicrobial effectiveness of edible films against *M. luteus***

<table>
<thead>
<tr>
<th>Stearic acid (%)</th>
<th>5 × 10⁵</th>
<th>10⁶</th>
<th>5 × 10⁷</th>
<th>10⁸</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10 ± 1</td>
<td>11 ± 1</td>
<td>14 ± 2</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>10</td>
<td>72 ± 25</td>
<td>79 ± 1</td>
<td>93 ± 5</td>
<td>97 ± 12</td>
</tr>
<tr>
<td>20</td>
<td>67 ± 14</td>
<td>74 ± 7</td>
<td>94 ± 10</td>
<td>96 ± 8</td>
</tr>
<tr>
<td>30</td>
<td>54 ± 20</td>
<td>68 ± 16</td>
<td>86 ± 13</td>
<td>89 ± 7</td>
</tr>
<tr>
<td>40</td>
<td>51 ± 9</td>
<td>68 ± 11</td>
<td>82 ± 8</td>
<td>84 ± 10</td>
</tr>
<tr>
<td>50</td>
<td>—</td>
<td>74 ± 7</td>
<td>57 ± 20</td>
<td>70 ± 27</td>
</tr>
</tbody>
</table>

aData at 0% of stearic acid correspond to inhibition zone diameters (mm). For the other stearic acid percentages, data represent relative percentage of inhibition. Values are means of three experiments followed by their standard deviations.

**FIGURE 2. Nisin antimicrobial effectiveness evaluated by inhibition zone diameter (mm) on *M. luteus*.** Values are means of three experiments, and standard deviations are vertical lines.

**Antimicrobial effectiveness of edible films.** According to previously results, the antimicrobial effectiveness of active films based on 15% of stearic acid (wt/wt HPMC) as the hydrophobic compound and 1,000 IU/ml of nisin as the antimicrobial molecule was measured by the inhibition zone assay. As was not expected, no inhibition zone was observed and film did not exhibit any antimicrobial activity against either of the selected strains. This phenomenon could be due to a limited nisin desorption by interactions with film compounds. Therefore, a large number of edible films, made with different percentages of stearic acid and different nisin concentrations, were tested. Their inhibitory activity on selected strains was studied. Data are presented in Tables 4 and 5. First, a nisin concentration of 10⁵ IU/ml had to be reached in order to detect inhibition zones. Second, increasing parts of stearic acid incorporated into HPMC film resulted in a decrease in antimicrobial activity of the film against tested bacteria. Interactions between nisin and fatty acid might be responsible for the decrease in the antimicrobial activity. *M. luteus*, commonly used as a reference strain to measure nisin activity (7), was chosen to test this hypothesis and to study the influence of the nisin concentration on HPMC-fatty acid films. Results presented in Figure 2 confirm the high sensitivity of *M. luteus* to the bacteriocin compared to selected strains. First, it was possible to test films with lower nisin concentrations and to show, in most cases, an improvement in film activity.
TABLE 6. Antimicrobial effectiveness of edible film incorporating $5 \times 10^4$ IU/ml of nisin

<table>
<thead>
<tr>
<th>Hydrophobic compounds (%)</th>
<th>Stearic acid</th>
<th>Methylstearate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.0 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>2.7 ± 0.4</td>
<td>9.0 ± 1.0</td>
</tr>
<tr>
<td>50</td>
<td>2.6 ± 0.6</td>
<td>7.9 ± 1.1</td>
</tr>
</tbody>
</table>

* Film was tested on *M. luteus*. Data, which are the means of three experiments, correspond to zone inhibition diameters (mm) followed by their 95% Student t test deviations.

with increasing nisin concentrations. Second, the more the stearic acid concentration increased, the less the residual inhibitory activity of films was, whatever the nisin concentration. Jung et al. (9) reported similar effects on the activity of nisin in systems containing high levels of fat, and specified in their conditions that nisin activity was decreased by about 88% in the presence of 13% fat. Dean and Zottola (5), using nisin to inhibit *L. monocytogenes* in ice cream, reported that nisin exhibited a greater effect in 3% fat ice cream than in 10% fat.

Nisin is a cationic bacteriocin. The major determinant for the sensitivity of bacteria to nisin was the relative amount of negatively charged lipids in the target membrane.

Given that interactions between the cationic nisin molecules and anionic phospholipids contribute to the binding of nisin to the membrane (1), the same phenomenon could be presumed between positively charged nisin and negatively charged stearic acid. Therefore, further experiments were conducted to compare the inhibitory activity of stearic acid and methylstearate films on *M. luteus*. The stearic acid carboxyl function can be negatively charged depending on pH; contrary to the ester function of methylstearate. Data are shown in Table 6. The incorporation of 20% (wt/wt HPMC) hydrophobic compounds significantly decreased ($P > 0.05$) the antimicrobial property of the active film.

The nisin interaction with 20 and 50% (wt/wt HPMC) hydrophobic compounds incorporated into films was not significant ($P > 0.05$). However, when stearic acid was replaced by methylstearate, nisin activity increased. The difference between nisin interaction with stearic acid and methylsterate was significant ($P > 0.05$). Nisin retention was lower for HPMC films containing methylstearate.

Therefore, electrostatic interactions between stearic acid and nisin were especially presumed to be responsible for the lower antimicrobial activity of HPMC films. The cationic nisin was fixed by the anionic stearic acid into the film, and this explained the lower desorption of nisin from the film. The lower antimicrobial activity of the methylstearate film compared with the activity of the free lipid film showed that other interactions occurred, such as hydrophobic ones. The nature of the nisin–lipid interactions is currently being studied. The microstructure rearrangement of active films after lipid incorporation is also being investigated.

First, in the present study, the water vapor barrier of the edible packaging material from HPMC could be improved by using stearic acid as the hydrophobic compound. Second, although the rate of release of preservative from the film could be influenced by the film composition, the stearic acid content, especially because of the interactions between the bioactive peptide and the fatty acid, and nisin can be use as antimicrobial compounds in a potential edible film for the inhibition of some bacterial contaminants in food products. In order to subsequently optimize the desorption phenomena of the active molecule, the study of the different interactions between the bacteriocin and other components of the edible film will be studied, and the diffusion mechanisms in food products will be established.

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