Study of the performance of nisin supported in edible films

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Abstract

The antimicrobial activity of nisin supported in edible films prepared with suspensions of tapioca starch containing glycerol, was studied. Films were prepared by casting the systems after gelatinization. The effect of the edible film as antimicrobial barrier to external hazard as well as the diffusional characteristics of the nisin and its release characteristics were studied in parallel to antimicrobial inactivation. Studies were performed with L. innocua, after equilibration of edible films at a relative humidity (RH) of 57.5% and at 25°C. Results obtained showed that nisin supported in starch-based films is active and that the film is a useful barrier to further product contamination. Gradual release of the antimicrobial from the edible film can also help to preclude microorganism proliferation better than nisin directly added because it seems to counterbalance, at least partially, the inactivation of nisin.

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1. Introduction

Edible films are not expected to totally replace synthetic packaging. They can control moisture, gases, lipid migration as well as be used as carriers of additives and nutrients (Baker, Baldwin, & Nisperos-Carriedo, 1994; García, Martino, & Zaritzky, 1998). Cellulose, starch, proteins and lipids can be used to formulate edible films which must be completely neutral with reference to color, taste and odor. One important component of edible films is the plasticizer which is required to overcome film brittleness and improve its flexibility and extensibility (McHugh & Krochta, 1994).

In the last years much research has been done concerning the use of edible films as a way of supporting antimicrobials in food products as well as to provide the slow release of the antimicrobial. They have been studied as carriers of natamycin and potassium sorbate for antimicrobial surface application (Franssen, Rumsey, & Krochta, 2002), for slow release of lysozyme and nisin (Buonocore et al., 2003a, Buonocore, Del Nobile, Panizza, Corbo, & Nicolais, 2003b; Dawson, Hirt, Rieck, Acton, & Sotthibandhu, 2003; Ko, Janes, Hettiarachchy, & Johnson, 2001; Padgett, Han, & Dawson, 2000; Sebti, Blanc, Carnet-Ripoche, Sauvel, & Coma, 2004), for developing sorbate or benzoate carrier films (Chen, Yeh, & Chiang, 1996), for slow release of propylparaben (Chung, Chikindas, & Yan, 2001). In general, lost of activity of the antimicrobial due to storage conditions, reaction with food matrix and/or degradation pathways as well as antimicrobial effect on film performance when applied to food products, has not been well established in the bibliography.

Starches are a renewable resource widely available and can be obtained from different by products of harvesting and industrialization. They are widely used in the food industry and can be modified to enhance their functional properties (Zobel, 1994). The Food and Agriculture Orga-
nization (FAO) highlighted recently that tapioca is a good commercial cash crop and a major source of food security, and that it needs a competitive edge to thrive in the global starch market. Due to shortage or high price of traditional starch sources, such as wheat and corn, the tapioca starch is viewed as an alternative by the food companies for use as an ingredient (Anonymous, 2004).

Nisin is an antibacterial peptide produced by Lactococcus lactis that effectively inhibits Gram-positive bacteria and also the outgrowth of spores of Bacilli and Clostridia (Cleveland, Montville, Nes, & Chikindas, 2001; Hurst, 1981; Hurst & Hoover, 1993). It is the first antimicrobial peptide with a “generally recognized as safe” status in the United States for use in processed cheese (Food & Drug Administration, 1998). In addition, its use in various food products is allowed in several countries (Delves-Broughton, 1990).

According to previously mentioned facts it is important to clarify nisin behavior when supported in edible films with the object of analyzing usefulness of films as a hurdle for food preservation.

The objective of this research was to study the effect on L. innocua growth of nisin supported in edible films prepared with suspensions of tapioca starch containing glycerol.

2. Materials and methods

2.1. Film preparation

Mixtures of starch, glycerol and water (5.0:2.5:92.5 in weight) or starch, glycerol, nisin and water were prepared. In the case of films containing nisin, 15 g of water were replaced by a solution of nisin of a concentration such that each milliliter of final system contained 2000, 3000 or 5000 IU/ml of the antimicrobial. The pH of the systems was adjusted to 4.0 with citric acid solution (50%, w/w).

Gelatinization was performed at a constant rate of 1.8 °C/min for 30 min. The temperature of gelatinization was 70–75 °C. Sample final temperature was 82 °C. After gelatinization, vacuum was applied to remove air from the systems before casting. The mixtures were casted over glass plates and dried at 50 °C during 2 h. Drying was finished at 25 °C over calcium chloride. In the case of systems containing nisin, the antimicrobial was added after gelatinization to preclude affecting nisin activity. Addition was accomplished under agitation to assure system homogeneity.

Once formed, films were peeled from glass plates and conditioned at 25 °C, over saturated solution of NaBr (water activity, aw = 0.575) during 7 days. Nisin content was calculated after film stabilization taking into account the change of weight of the systems. The final concentration of the antimicrobial in the films resulted to be 881, 1322 or 2204 IU/cm², for an initial content of 2000, 3000 or 5000 IU nisin/ml of each system, respectively.

2.2. Materials used

Starch was provided by Industrias del Maíz S.A. (Argentina). Glycerol and citric acid used were of analytical grade (Mallickrodt, Argentina).

A stock solution of nisin (1 × 10⁹ IU/ml) was prepared by dissolving Nisaplin™ (Aplin & Barret Ltd., Dotset, UK) in sterile distilled water. The pH was adjusted to 2.0 with 0.1 N HCl to ensure high bacteriocin solubility and solution was stored at −20 °C.

2.3. Strains and growth conditions

L. innocua (CIP 8011, CCMA 29) was grown in 150 ml Tryptic Soy broth enriched with 0.6% (w/v) yeast extract (TSBYE), in a continuously agitated temperature-controlled shaker at 28 °C overnight. Three to five milliliters were inoculated in 150 ml of fresh TSBYE and agitated for 1–2 h, as previously explained, to obtain the final desired concentration of cells.

2.4. Agar diffusion technique

The agar diffusion test was used for determining the antimicrobial effect of films on L. innocua. 25 ml portions of TSYE agar were poured in Petri dishes. An aliquot of the culture was then transferred to the agar (final count = 1.5 × 10¹⁰ CFU/ml) obtaining the assay plate. Films with (1322 IU/cm²) or without nisin were then cut in 7 mm diameter disks, brought in contact with agar, maintained at 7 °C during 48 h and incubated at 37 °C for 48 h.

The antimicrobial effect of the film was determined by observing the existence of inhibition zones at the contact area as well as around disks.

2.5. Barrier to microbial contamination

Petri dishes containing TSYE agar with water activity controlled to 0.94 with dextrose (Merck Química Argentina, Buenos Aires, Argentina) according to Chirife, Ferro Fontán, and Benmergui (1980) and pH 5.0 (citric acid, 50%, w/w) were used to resemble a food product. Disks of 1 cm diameter were cut from films with (881; 1322 or 2204 IU/cm²) or without nisin and brought in contact with the surface of the agar. Then, 10 µl of a culture of L. innocua containing 2 × 10⁹ CFU/ml, were dispensed on the disks. Samples were incubated at 28 °C during 4 h and periodically sampled, to test bacterial viability.

2.6. Release to liquid medium and nisin inactivation

Trypetic soy broths with different pHs (5.5, 6.5 or 7.2) were used for preservative release studies. Three disks of the films of 1.4 cm diameter, with 1322 IU/cm² or without nisin (control) were immersed in each glass tube containing 20 ml of broth with L. innocua culture in exponential phase to yield approximately 10⁹ CFU/ml. Systems were incu-
3. Results and discussion

3.1. Diffusivity in solid medium

The antimicrobial properties of the films were evaluated by noting whether there was inhibition of bacterial growth at the film/medium interface, along with the existence of clear inhibitory zones around the film after incubation. The results of the research revealed that the starch based film itself could not inhibit the microbial growth at the film/medium interface. On the contrary, the starch film containing nisin yielded a low density of growth at the film/medium interface and a clear but narrow inhibitory zone around the film disk (total diameter of inhibition: 9 mm for an initial disk diameter of 7 mm), showing antimicrobial diffusion to the solid medium. Similar results were observed by Padgett et al. (2000) when studying the effect of nisin contained in corn zein films. It must be stated that edible film swelling, which determined film diameter increase along experimental work might have affected results obtained.

The diffusion observed is useful for microbial inhibition but it might result in an increase in diffusion rate from the surface into the foodstuff due to the high concentration gradient, not allowing to maintain a high concentration of the antimicrobial in the surface (Chen et al., 1996).

3.2. Films as barriers to contamination

Recent studies have focused on the application of edible films on the food surface preventing the diffusion of preservative into the food and inhibiting surface microbial growth (Franssen et al., 2002; Ozdemir & Floros, 2001; Sebti et al., 2004; Vodjani & Torres, 1989a, 1989b). One of the major potentials of this hurdle lies in the storage of semi-moist foods (Chen et al., 1996). According to this, an experiment was designed with the object of testing the efficacy of studied films as microbial barriers.

Results obtained showed (Fig. 1) a rapid decrease of L. innocua viable counts, followed by an additional slow inac-

![Graph](image-url)
ivation during the period evaluated. The antimicrobial effect increased with film nisin content, showing a reduction of 4 log cycle during 240 min of contact with a film with 2204 IU/cm². On the other hand, films without the natural antimicrobial did not affect initial counts of studied bacteria.

3.3. Release to liquid medium

3.3.1. Effect of pH

Nisin molecule is cationic but its net charge depends on pH (Thomas, Clarkson, & Delves-Broughton, 2000). At neutral pH, the positive charge diminishes due to loss of histidine protons and at acidic pH the charge is retained due to the high pK of lysine (Rollema, Kuipers, Both, de Vos, & Siezen, 1995). Nisin is, in general, more effective at acidic pHs and has a maximum activity at pH 5.5 (Dykes, Hancock, & Hatings, 1998). According to Ganzle, Weber, and Hammes (1999), the pH mediated changes that occur in the secondary structure of nisin influence the interaction between nisin and microbial membrane affecting antimicrobial activity.

For the purpose of studying nisin performance when supported in the edible starch films, samples with 1322 IU/cm² of nisin were immersed in broth media of different pHs. After 6 h of immersion, films studied swelled as visually determined through the increase in their diameter. This behavior, probably affected starch network organization and nisin release as previously stated by Buonocore et al. (2003b).

As can be observed in Fig. 2, L. innocua growth was smaller when nisin was present in the films. Cell reduction produced by nisin release in the first 2 h of the assay was similar for pH 6.5 (b) and 7.2 (a) and higher when medium pH was 5.5 (c). From then on, cells contained in the higher pH broths continued their growth while that contained in broth with pH 5.5, only increased slightly their number maintaining this behavior until 6.5 h. After 23 h of assay, counts were similar for all pH assayed. The counts for control tubes containing films without nisin increased along the assay except for pH 5.5 tubes which showed a delay of 6 hours; the decrease of pH from 7.2 to 5.5 depressed L. innocua growth for times shorter than 6.5 h of assay when nisin was absent. Anyhow, it is clearly observed that films with nisin were more inhibitory for studied microorganism than films without the antimicrobial, being nisin active at all pH evaluated.

3.3.2. Effect of different nisin concentrations

The effect of different nisin contents on L. innocua growth at pH 5.5 was studied. As can be observed in Fig. 3, the increase in nisin content produced a decrease in microbial growth. In general, the greatest effect was observed at, approximately, 1–2 h of contact and then, cells continued their growth with different rates, being smallest the one observed in the media with films containing 2204 IU/cm² of nisin. This trend seem to indicate a high initial release of preservative. This release of preservative could inhibit the microbial growth at the early stage of storage. Chen et al. (1996) observed a rapid release when working with edible films constituted by methylcellulose and chitosan and containing sorbate or benzoate as antimicrobial and stated that such high diffusion rate might in turn reduce the antimicrobial effect of the film for long term storage.

3.3.3. Nisin liberation in the absence of microorganism

The release of nisin from edible films (2204 IU/cm²) at pH 5.5 was also studied in the absence of microorganisms and compared with nisin behavior described in Fig. 3. Through the study of a control system, it was observed that L. innocua showed adequate viability (Fig. 4). An hour after the beginning of the experience, films studied produced 2.6 log cycles reduction in microbial population. Samples exposed for longer times showed similar effect
on microbial population, a fact that can be attributed to liberation coupled with reduction in nisin activity or to no additional liberation of the antimicrobial. It is interesting to remark that a greater population reduction was observed when liberation was studied in the absence of \emph{L. innocua} (compare Figs. 3 and 4 for equal nisin concentration). This trend might be attributed to the increase in external resistance to nisin diffusion when microorganisms are present during antimicrobial release.

3.3.4. Nisin inactivation

To clarify nisin behavior in the conditions of the assay, the effect of direct addition of nisin on microbial growth as a function of time was evaluated. Results show (Fig. 5) a maximum activity \((\log N = 2.7)\), for a nisin concentration of 150 IU/ml for times shorter than 1 h. Afterwards, and for all concentrations assayed, it was observed that population tended to increase probably due to nisin inactivation by combination with TSYE broth.

As a consequence, we can conclude that nisin released from films can also suffer loss of activity and that the constance in microbial population observed in Fig. 4 when films with antimicrobial were present might be ascribed to gradual liberation of nisin that compensated inactivation of the antimicrobial.

According to Chung et al. (2001) slow release might not be as effective as direct addition of the antimicrobial when the initial concentration of the microorganism is rather high. However, the expected microbial population in a preserved food must be low. Anyhow, a major advantage of slow release over direct addition is:

- Continuous microbial inhibition by slow delivery to food during an extended period fact that can help to reduce cross contamination during food use and storage rather than during preservation.
- Reduced destruction of the antimicrobial due to protection provided by film matrix. Anyhow, it must be remembered that the matrix might form complexes that can inhibit antimicrobial from acting (Kurup, Wan, & Chan, 1995; Ofman, Campos, & Gerschenson, 2004).

As can be observed through comparison of Figs. 4 and 5, although film performance in the first 1–2 h of contact was comparable to the direct presence of 50 IU/ml, the effect of gradual delivery of nisin from film during more than 8 hours produced a more efficient effect over \emph{L. innocua} growth than direct addition. This fact can help to reduce contamination during storage.

4. Conclusions

Presence of nisin in edible films formulated with tapioca starch and glycerol reduced \emph{L. innocua} growth, producing count decrease and acting as a barrier to contamination after processing.

The study of nisin performance through the agar diffusion technique was not satisfactory due to the competence between rate of nisin diffusion, diameter increase due to swelling and microbial growth. However, studied bacteria showed a decreased population under the film with nisin which also produced clear inhibitory zones.

The initial release of nisin to the liquid medium produced an important inhibition of \emph{L. innocua}, specially when
liquid had a pH 5.5. This inhibition increased with nisin content of the films. Although the nisin released from films suffered loss of activity, the gradual delivery of nisin still contained in films controlled growth of *L. innocua*, helping to reduce contamination along storage better than direct addition.

It can be concluded that antimicrobial activity of the films starch-based and containing nisin describe this hurdle as adequate to act as a barrier to further product contamination. Gradual release can also help to preclude microbial proliferation better than nisin directly present in the media. Additional studies must be performed to determine if starch modification or additional changes in composition (i.e. mixture of starch and lipids) might modify antimicrobial activity and release producing changes in the possibilities brought about by this hurdle.

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References


