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Development of a bioactive packaging cellophane using Nisaplin® as biopreservative agent

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ABSTRACT

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Aims: Production of a nisin-containing cellophane-based coating to be used in the packaging of chopped meat.

Methods and Results: The adsorption of nisin to cellophane 'P' type surface was studied at 8, 25, 40 and 60°C using different concentrations of nisin. Then, the antimicrobial activity of adsorbed nisin to cellophane surface was determined in fresh veal meat for effectiveness in reducing the total aerobic bacteria. The adsorption of nisin to cellophane was higher at 8°C. The developed bioactive cellophane reduced significantly the growth of the total aerobic bacteria (by *ca* 1·5 log units) through 12 days of storage at 4°C.

Conclusions: Bioactive cellophane packaging could be used for controlling the microbial growth in chopped meat.

Significance and Impact of the Study: Nisin-adsorbed bioactive cellophane would result in an extension of the shelf life of chopped meat under refrigeration temperatures.

Keywords: adsorption, bioactive cellophane, meat, nisin, packaging.

INTRODUCTION

The ability of food-borne pathogens and spoilage-causing bacteria to adhere to solid surfaces is a serious problem in food industries. These micro-organisms once attached to surfaces may be much more resistant to the antimicrobial effect of sanitizers commonly used in the food industry, thus being a source of contamination for food products (Chmielewski and Frank 2003).

In recent years, the attention has been focused on the prevention of initial adhesion of microbial contaminants by application of an antimicrobial substance to the surface rather than trying to remove the undesirable bacteria once they are adhered. Thus, various kinds of preservatives including enzymes, organic acids, metals, fungicides and bacteriocins have been proposed or tested for food packaging applications (Cagri *et al.* 2004; Mauriello *et al.* 2004).

The use of bacteriocins in packaging materials has received a great attention in the last years. Although many bacteriocins (such as pediocins, lacticin and plantaricin) have potential application in food products, the lantibiotic nisin is currently the only bacteriocin approved as Gras (Generally recognized as safe) food additive by both the Food and Drug Administration and WHO. This compound, produced by *Lactococcus lactis*, has a broad spectrum of antibacterial activity, which includes other lactic acid bacteria as well as spoilage bacteria and food-borne pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus* (Lee *et al.* 2003). In addition, nisin is heat-stable, non-toxic and sensitive to digestive proteases (Guerra and Pastrana 2002). In batch cultures, nisin production takes place during the active growth phase and completely stops when cells reach the stationary phase (Guerra and Pastrana 2002). The main variables affecting the production of nisin are temperature, pH, composition of the culture media and inducers (Parente and Ricciardi 1999).

The generally accepted mode of action of nisin on vegetative cells involves the prevention of peptidoglycan synthesis and the formation of pores in the cytoplasmic membrane of target cells. This leads to the cell death due to the rapid efflux of essential small cytoplasmic substances,

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such as amino acids, potassium ions, and nucleotides from the cytoplasm of a number of Gram-positive bacteria, such as *S. cohnii* and *Bacillus subtilis* (Gao *et al.* 1991). However, nisin has proved to adsorb successfully to various food packaging materials and still retain sufficient activity to inhibit pathogenic bacteria (Lee *et al.* 2003). Thus, the production of antimicrobial films using nisin could be an exciting development, which allows industry to simplify processing conditions and reduce the amount of added chemicals.

Studies dealing with the development of different bioactive food packaging materials (such as plastic and paper) using bacteriocins for use in the food packaging industry have been reported in the last years (Lee *et al.* 2003, 2004; Mauriello *et al.* 2004; Vartiainen *et al.* 2004). However, reports dealing with the development of antimicrobial packaging materials using cellophane are lacking. In addition, little is known about the influence of temperature on the adsorption of nisin to food contact surfaces such as cellophane.

In this paper, the nisin adsorption kinetic was studied to determine the optimal time for high nisin adsorption. Subsequently, the nisin adsorption isotherms were built at 8, 25, 40 and 60°C. Then, nisin was adsorbed to a cellophane surface of the type used to package hamburgers, in the optimal conditions of time and temperature. The stability of the bioactive cellophane was ascertained using an agar diffusion assay and its functionality in food systems was determined in fresh veal meat for effectiveness in reducing the initial population of total aerobic bacteria.

MATERIALS AND METHODS

Bacterial strain

In a previous work (Guerra and Pastrana 2002), the sensitivities of 19 bacterial strains (including one *Carnobacterium*, one *Leuconostoc*, two *Listeria*, three enterococci, two lactococci and 10 lactobacilli strains) to a solution of 1 g l^{-1} of Nisaplin® (Aplin & Barrett, Danisco Cultor, UK) were tested. In this study, *Enterococcus hirae* CECT 279 was found to be the most sensitive of the strains tested, and it was therefore chosen as the indicator strain for bioassays. This strain was obtained from the Spanish Type Culture Collection (CECT) and maintained as slants on Rothe agar (Panreac, Barcelona, Spain) at 4°C.

Bacteriocin preparation

Nisin was used in the form of the commercial preparation Nisaplin® (Aplin & Barrett) that contained (in %, w/w): pure nisin A, 2·5; proteins, 12·0; carbohydrates 6·0. Sodium phosphate buffer solutions were prepared using chemicals of analytical grade and distilled, deionized water. Stock solution of nisin was prepared by dissolving Nisaplin® in 0·01 M monobasic sodium monophosphate (pH 4·5) to assure complete solubilization of nisin. This suspension was then centrifuged (5000 *g* for 10 min) to remove insoluble whey proteins from the preparation. Subsequently, 0·01 M dibasic sodium monophosphate (pH 9·0) was added until a pH of 6·0 and a final concentration of 12 g l^{-1} of Nisaplin® (0·3 mg of nisin per ml) were obtained. Before and after centrifugation, the antibacterial activity of the suspension of Nisaplin® was determined against *E. hirae* CECT 279 using a turbidimetric method (Guerra and Pastrana 2002), and no loss of activity was detected. Different working solutions of nisin were prepared by dilution of the stock nisin solution with 0·01 M sodium phosphate buffer (pH 6·0).

Cleaning and preparation of the cellophane surface

The cellophane 'P' type surfaces used in this study were provided by 'La cellophane Española, SA' (Burgos, Spain). The material was washed with 1% (w/v) sodium dodecyl sulphate (Sigma, Madrid, Spain), rinsed with distilled, deionized water and washed again with ethanol (Panreac) during 10 min under shaking conditions each time. Finally, they were rinsed with distilled, deionized water and air-dried.

Construction of adsorption isotherms

As a previous step to the construction of the adsorption isotherms, the time necessary to reach the adsorption equilibrium was determined using two Nisaplin® solutions (1·2 and 12 g l^{-1}) prepared as described above. First, the cleaned cellophane 'P' type was cut in surface areas of 53·8 cm^2. The surfaces were contacted with 20 ml of the two Nisaplin solutions (in sodium phosphate buffer, pH 6·0) at 25°C (Daeschel *et al.* 1992). Triplicate samples were run simultaneously. At pre-established times (6, 9, 12, 24 and 36 h) the surfaces were removed from the nisin solutions and rinsed three times with distilled, deionized water to remove protein not tightly bound to the surface, and stored in a dessicator for about 12 h.

To obtain the adsorption isotherms at 8, 25, 40 and 60°C, the cleaned cellophane surfaces were contacted with the different nisin solutions under static conditions by contacting one surface with one volume (20 ml) of protein solution for 12 h. Triplicate samples of each material were run simultaneously. Before and after removing the surfaces, the antibacterial activities of the nisin solutions were quantified using the turbidimetric method described in Guerra and Pastrana (2002).

Because there was no decrease in activity of Nisaplin solutions when not in direct contact with cellophane, the activity of nisin adsorbed to cellophane was taken to be equal to the amount of activity lost in the two bacteriocin solutions. The individual concentrations of nisin (g l^{-1}) were then determined from a standard curve (nisin concentration vs antibacterial activity). The amounts of adsorbed nisin were determined from the difference between nisin concentrations in solution before and after adsorption (Norde and Zoungrana 1998).

Stability of the bioactive cellophane

The activity of adsorbed nisin to the cellophane surfaces was determined using the agar diffusion assay (Guerra and Pastrana 2002). *Enterococcus hirae* CECT 279 was grown overnight in Rothe broth (Panreac), pelleted by centrifugation (10 000 *g* for 5 min at 4°C) and washed three times in saline (0·8% NaCl). The pellet was resuspended in 10 ml of 0·8% saline and the suspension was spectrophotometrically adjusted (700 nm) with 0·8% saline to 10^5 CFU ml^{-1}. Rothe agar was cooled to 45°C and seeded with 10% (v/v) of the adjusted *E. hirae* suspension to provide a concentration of 10^4 CFU ml^{-1} into Rothe agar plates. Inoculated medium was poured into sterile Petri dishes to a volume of 25 ml. To prepare a surface with adsorbed nisin, dried rectangular pieces of cellophane 'P' type surface (2·6 × 1·8 cm) were immersed in 25 ml of a 12 g l^{-1} Nisaplin® buffered solution (0·01 M NaH_2PO_4, 0·01 M Na_2HPO_4, pH 6·0) for 12 h at 8°C under static conditions. Surfaces were then rinsed in 20 ml of 0·01 M phosphate buffer to remove non-adsorbed nisin. Controls consisted in cellophane surfaces immersed in 25 ml of 0·01 M sodium phosphate buffer (pH 6·0) for 12 h at 8°C. Then both surfaces were placed face down directly onto the surface of the seeded agar dish. The plate was kept for 4 h at 4°C to allow the diffusion of the nisin adsorbed to the surface. Finally, it was incubated at 30°C for 48 h to determine the widths of the clear inhibition zones around the surface.

Preparation of packaging cellophane and food sample

Disks of cellophane 'P' type (9 cm in diameter) were used for the preparation of bioactive packaging. These surfaces were previously prepared as described above and then contacted with a 12 g l^{-1} Nisaplin® buffered solution (0·01 M NaH_2PO_4, 0·01 M Na_2HPO_4, pH 6·0) for 12 h at 8°C.

The fresh veal meat, which was obtained from a local supermarket 24 h after slaughter, was ground in a sterilized household blender (Moulinex Coupe-Coupe, Ecully, France). Aliquots of 50 g of chopped meat were extended with a sterile spatula on the dried cellophane surfaces treated with nisin, resulting in thick layers of about 5 mm. They were then covered with other treated cellophane surface such that the entire area was covered. The controls consisted on 50 g samples of chopped meat extended on both cellophane surfaces without nisin and on cellophane surfaces previously treated with 0·01 M sodium phosphate buffer (pH 6·0). All samples were incubated at 4°C.

At selected intervals (1 day) up to 12 days, samples of chopped meat were withdrawn for bacteria enumeration. From each sample of 50 g of chopped meat, a 10-g sample was mixed with 90 ml of buffered peptone water using a Stomacher 400 for 1 min, and then serially diluted with sterile buffered peptone water. An aliquot (0·1 ml of each serial dilution) was inoculated on prepoured plates contained plate count agar (Merck, Darmstadt, Germany). Agar plates were incubated aerobically at 30°C for 48 h. Colonies were counted and results expressed as CFU per g of sample. All calculations were based on six repetitions: all counts were performed three times using two samples in each trial. Data were then reported as mean values and standard deviations.

Data sets were analysed by two-sample *t*-test (Pagano and Gauvreau 2001) to determine significant differences in the proliferation of the total aerobic bacteria between controls and treated food samples. Statistical significance occurs for a standard level of significance ($P < 0·05$).

RESULTS AND DISCUSSION

Nisin adsorption isotherms

The adsorption kinetic results of nisin (contained in both 1·2 and 12 g l^{-1} Nisaplin® solutions) to the cellophane surfaces at 25°C are showed in Fig. 1a. As can be seen, nisin adsorption exhibited a steep initial slope (in the first 12 h of incubation) followed by immediate attainment of a plateau. This showed that a time of 12 h was needed to reach equilibrium. Therefore, this incubation time was chosen to obtain the nisin adsorption isotherms to cellophane at 8, 25, 40 and 60°C (Fig. 1b). As can be seen in Fig. 1(b,c), the increase in temperature resulted in a decrease in the amounts of adsorbed nisin. Thus, a maximum load of 0·62 μg of nisin per cm^2 was obtained at 8°C. This decrease in protein adsorption with the increase in temperature has been observed before and it was related to the higher excitation state of protein molecules at higher temperature decreasing the attractive forces between them and the solid surfaces. However, the increase in the amounts of protein adsorbed at lower temperatures may be related to a reduction in translational energy of protein, which could enhance its positioning for adsorption or to a reduction in energy available for desorption (Kim and Hong 2000).

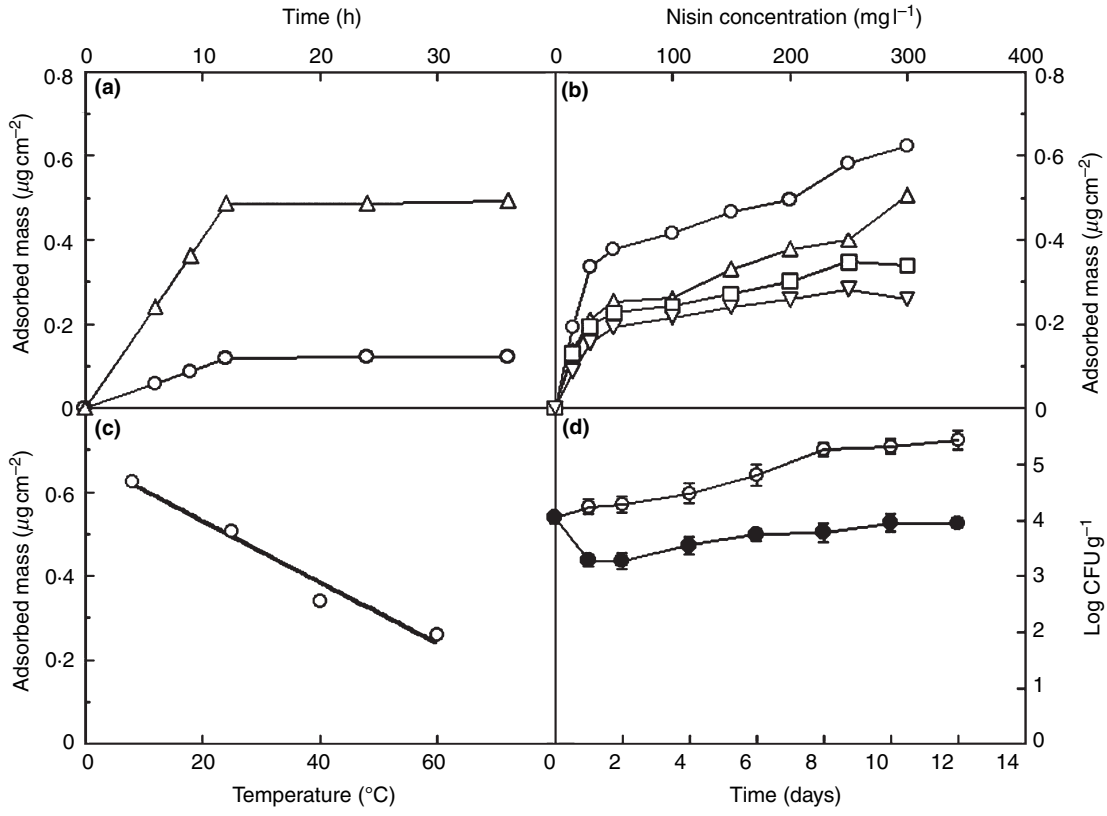

Fig. 1 (a) Kinetics of adsorption of nisin to cellophane for 1·2 (○) and 12 g l^{-1} (△) of Nisaplin® solutions at 25°C. (b) Adsorption isotherms of nisin to cellophane 'P' type at 8 (○), 25 (△), 40 (□) and 60°C (▽). (c) Effect of temperature in nisin adsorption. (d) Effect of bioactive cellophane surfaces impregnated with Nisaplin® (black symbols) and untreated cellophane surfaces (open symbols) against the total endogenic bacteria in fresh veal meat packaged with both cellophane surfaces. Error bars represent the standard deviation of two experiments carried out in triplicate. Mean values of control and treated samples were significantly different ($P < 0{\cdot}05$) after storage at 4°C for 1 day and longer

In all isotherms, adsorption of nisin to cellophane increased displaying two phases. The first exhibited a steep initial slope in the nisin concentration range of 0–50 mg l^{-1}, followed by a second in which the slope fell with rise in concentration. Thus, in the first phase, in which nisin showed a higher surface affinity, the rapid increase in adsorption could be related to the existence of sites capable of adsorbing the nisin molecules. The decrease in adsorption observed in the second phase probably occurred because vacant sites became more difficult to find with the progressive covering of the cellophane surface. However, as a plateau value did not attain in any case, it could be suggested that nisin adsorbed to cellophane forming at least two layers. Thus, the first nisin monolayer was probably completed when the nisin concentration in solution was 50 mg l^{-1}. The subsequent slow rise could represent the development of a second monolayer, in which, the nisin molecules are attracted by the own nisin molecules forming the first layer. Thus, the slope in the first phase was higher than that of the second phase because the forces generating the second and subsequent layers are weaker than those generating the first (Giles and Smith 1974).

Antimicrobial activity of the impregnated cellophane

The nisin antibacterial activity in the adsorbed state was tested using an agar plate bioassay, which was seeded with *E. hirae* CECT 279. The zones of activity (0·6 mm in thickness) measured around the periphery of the impregnated cellophane surface raised the concerns that nisin might be diffusing through the medium (Daeschel *et al.* 1992; Dawson *et al.* 2003) or that nisin retained its antimicrobial activity in the adsorbed state (Bower *et al.* 1998). The structure of nisin is stabilized by five thioether rings, making it a relatively rigid molecule that might desorb from the surfaces over the time (Bower *et al.* 1998), mainly the molecules placed in the external zone of multilayer film.

Effectiveness of bioactive inserts on fresh veal meat

To assess the shelf life implications of incorporating bioactive inserts in the packaging of fresh veal meat, total aerobic bacteria counts were determined. The total bacteria counts in the control inserts (non-nisin-containing cellophane) appeared to be significantly higher ($P < 0{\cdot}05$) than those in the samples treated with nisin after storage at 4°C for 1 day and longer.

The initial total population of these bacteria after chopping was in the region of 4·0 log units. Fresh veal meat packaged in the control inserts showed only a small increase in the total bacteria count after the first 4 days of incubation. However, from this time, the rate of proliferation of these bacteria increased and by the end of the experiment the initial total population had increased by approx. 1 log unit (Fig. 1d). Contrarily, in cellophane samples containing adsorbed nisin, there was an initial drop of 0·7 log units, then, the cells surviving nisin treatment resumed growth from the second day of incubation. However, the final total bacteria count level reached after 12 days of incubation (approx. 3·9 log units) was significantly lower ($P < 0{\cdot}05$) than the initial total bacteria count level. These results showed that bioactive cellophane to which nisin was adsorbed, gave a good protection against bacterial growth.

Other researchers have also observed a similar reduction in the total populations of bacteria, when meat samples were packaged with food packaging materials containing bacteriocins (Ming *et al.* 1997; Scannell *et al.* 2000; Dawson *et al.* 2002; Lee *et al.* 2003; Mauriello *et al.* 2004).

In conclusion, from a practical point of view, it is reasonable to suppose that the use of bioactive inserts would result in an extension of the shelf life of chopped meat. Additionally, the combination of this approach with refrigeration of fresh meat and storage in a vacuum or modified atmosphere would provide a food with enhanced microbial stability.

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