

DEVELOPMENT OF AN ENCAPSULATION SYSTEM IN FOOD LIPOSOMES FOR THE PROTECTION AND CONTROLLED RELEASE OF NISIN IN COOKED MEAT PRODUCTS

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Abstract – The effective use of the antimicrobial peptide nisin in meat products is restricted, notably, by its reaction with glutathione and proteases in raw meat. The purpose of this study is to prepare liposomes for meat/food applications in order to maximize the activity of nisin during food processing and storage. To achieve this goal, an encapsulation procedure in dipalmitoyl-phosphatidylcholine (DPPC) liposomes was developed without the formation of a hydrated film in solvents. Nisin was dissolved in acidic solutions (HCl 20 mM at pH2) prior to liposome formation and analysis in ground beef. The encapsulation efficiency of nisin in DPPC liposomes formed with hydrated film was greater than $46 \pm 2\%$. For nisin in liposomes made without a hydrated film, $30 \pm 2\%$ encapsulation efficacy was obtained. Liposomes were stable for up to 7 days at 4 °C and a slow release of active nisin was observed after that. Nisin is protected and release upon cooking when encapsulated in liposomes prepared from DPPC. For liposomes formed with nisin dissolved in HCl at pH 2, zones of inhibition were bigger than their counterparts in PBS buffer (0.017 M KH_2PO_4 , Na_2HPO_4 0.05 M and 1.5 M NaCl at pH 6.4). The use of organic acid to dissolve nisin provides also an increased inhibitory activity.

Key Words – Antimicrobial; Encapsulation; Liposome; Meat; Nisin

I. INTRODUCTION

Nisin is a polypeptide produced by *Lactococcus lactis* subsp. *lactis* [1]. It is used as a food preservative because of its antimicrobial activity against several Gram-positive bacteria that can grow in food, such as *Listeria monocytogenes* [2].

However, addition of nisin to meat products has been largely unsuccessful, unless used at high concentrations [3]. Previous reports indicated that the loss of free nisin activity in meat could be due

to the composition of the meat, such as the presence of proteases [4], and its reaction with glutathione [5].

In this study, we investigated the possibility to protect nisin by encapsulation in liposomes to provide a temperature control release system that enhances its efficacy and stability in meat. The most commonly used phospholipid for liposome formation is lecithin [6] but other phospholipids, like dipalmitoylphosphatidylcholine (DPPC), have more appropriate melting temperature for meat cooking applications. To prepare liposomes for food applications, it is necessary to employ techniques that meet the regulatory requirements for additives. The standard methods for liposomes preparation generally involve the use of solvents for the solubilisation of phospholipids (e.g., chloroform). Such solvents are not appropriate for a food application. Hence, nisin encapsulation procedures without the use of solvents were successfully developed for the controlled release of nisin in its active form.

II. MATERIALS AND METHODS

Culture and antimicrobial activity

Pediococcus acidilactici UL5 and *Listeria innocua* HPB 13 were kindly obtained from the Department of Food Science and Nutrition Science at University Laval and were used as indicator organisms to determine nisin activity.

Liposome preparation

The first liposomes were prepared using the method of Taylor et al. [7] which involve the formation of a hydrated film with chloroform. We prepared food grade liposomes by hydrating phospholipids directly into PBS buffer (0.017 M KH_2PO_4 , Na_2HPO_4 0.05 M and 1.5 M NaCl at pH

6.4) or HCl (20 mM, pH 2) by vortexing, avoiding the formation of hydrated film with chloroform.

Liposome samples were prepared from DPPC because its transition temperature (42°C) is higher than the temperature necessary to inactivate the enzymatic reaction of nisin with glutathione (30-32°C; [8]). Nisin was dissolved in HCl (20 mM, pH 2) to obtain a stock solution of 250 µg/mL and then further diluted with HCl or PBS at a concentration of 83.25 µg/mL of nisin. Antimicrobial activity of nisin was detected by the inactivation of the indicator organism (1%) on the spot-on-lawn assay as described by Gratia (1946, cited by Tagg et al. [10]). The size of the inhibition zone obtained was measured with a caliper.

Liposome stability

The liposomes were stored at 4°C and their stability was followed for 7 days by measuring the zone of inhibition produced on a lawn of the indicator organism as described above. Different temperatures (4, 25, 42, 50, 63 and 71°C) were tested for the liberation of active nisin. Liposomes were heated for 30 min. Liberation of nisin was detected by the inactivation of the indicator organism as indicated above. Activity of encapsulated nisin was determined for liposomes formed with or without a hydrated film for nisin dissolved in HCl (pH 2) or PBS (pH 6.4). Liposomes were purified by centrifugation (85,000 x g, for 1 h at 25°C). The pelleted liposomes were resuspended in either HCl or PBS. To determine the encapsulation efficacy, the liposome was heated above the transition temperature of the lipid (60°C) to release nisin. The preparation was again centrifuged to keep only the released nisin. Total protein was determined using the BCA Protein Assay Kit-Reducing Agent Compatible (Thermo scientific, Rockford, Illinois, USA).

Controlled release in meat system.

To determine the efficacy of nisin in extra lean beef ($\leq 10\%$ fat), encapsulated nisin was added to the meat samples (83.25 µg/g of pure nisin) and hand massaged to evenly distribute the encapsulated nisin in the meat matrix. Raw meat was stored at 4°C for various times (0, 15, 30 and 60 min) until it was tested directly in raw meat or cooked to a core temperature of 71°C. Briefly,

samples of raw meat (7 g) containing the encapsulated nisin were placed in a glass tubes of 1 cm in diameter and heated in a high precision ($\pm 0.001^\circ\text{C}$) circulating programmable water bath (Cole-Palmer Polystat Heated Circulating Bath, Cole-Parmer Canada Inc., Anjou, Qc, Canada) at 80°C. The temperature of the meat was followed using a data logger equipped with a type T thermocouple (Food tracker MultiPac21, Datapaq Inc., Wilmington, MA, USA). After treatment, samples were cooled in an iced water bath (4°C). To determine the release of nisin, 50 mL of MRS agar (15% agar) was poured into a small 100 mL beaker. Once the agar was solidified, a hole was punctured in the middle using a sterile glass tube to make a well of 1.5 cm in diameter and 4 cm in height. The raw or cooked meat sample was placed in the well and covered with 8 mL of soft MRS agar (7.5% agar) inoculated with the indicator organism (1%). The beaker was incubated for 24 h at $30 \pm 1^\circ\text{C}$. The size of the inhibition zone obtained was measured with a caliper.

Liposome preparation with naturel organic acid

Stock solution of nisin dissolved in HCl (20 mM, pH 2) to obtain 250 µg/mL was diluted with 20 mM lactic acid (pH 2) or 20 mM acetic acid (pH 2) to a final concentration of 83.25 µg/mL of nisin and then used in liposomes prepared from DPPC without a hydrated film as described above. Liposome stability at 4°C and nisin release at different temperatures (4, 25, 42, 50, 63 and 71°C) were tested as previously described.

III. RESULTS AND DISCUSSION

The encapsulation efficiency of nisin in DPPC liposomes formed with hydrated film was greater than $46 \pm 2\%$. The encapsulation efficiency of nisin in liposomes (nisin dissolved in HCl at pH 2 or PBS at pH 6.4) prepared without the formation of a hydrated film was $30 \pm 2\%$. In contrast, the encapsulation efficiency of nisin in commercial proliposome H was 34.6% [11]. Liposomes (500 µl) were heated (4, 25, 37, 42, 50, 63 and 71°C) for 30 min and 20 µl was directly spotted onto an inoculated soft agar plates. At 4 and 25°C, nisin is completely retained in the liposome and no inhibition zone was observed (data not shown). By increasing the temperature to 37°C and above, the

release of nisin occurred and zones of inhibition were formed (data not shown). These results indicate that temperatures $\geq 37^{\circ}\text{C}$ sufficiently affect the surface properties of all liposomes tested for nisin to be effectively released.

Encapsulated nisin was mixed into ground beef and stored at 4°C for various length of time before heating at 71°C . The activity of nisin was detected by the presence of inhibition zone of the indicator organism. Encapsulated nisin assays did not result in the formation of inhibition zones in raw meat no matter the exposure time (Fig. 1A and C) indicating that nisin remained in the capsule until heat treatment was applied and melt the liposome (Fig. 1B and D). The size of inhibition zones varied from 25 to 30 mm when nisin was dissolved in HCl (pH 2) but were less than 25 mm when nisin was dissolved in PBS (pH 6.4). From these results, we can conclude that nisin is protected inside the liposomes and that cooking temperature allows its release upon liposome melting. The observed activity after cooking support the results of Rose et al. [5] who demonstrated that nisin remained active when added to cooked meat. Indeed, the increasing temperature during cooking most probably led to the timely denaturation of glutathione [10] with the release of nisin from the melted liposome (fusion temperature of DPPC is 63°C).

The controlled release of nisin from liposomes made with nisin dissolved in 20 mM lactic acid (pH 2) or 20 mM acetic acid (pH 2) is currently under investigation. Preliminary results are promising and indicate that inhibition was as good as with HCl (data not shown). These acids are inhibitory against *L. innocua* (Table 1) and in the presence of nisin, the size of the inhibition zone is increased (Figure 2). Further experiments in ground beef are required to test those liposomes where nisin is dissolved in lactic or acetic acid.

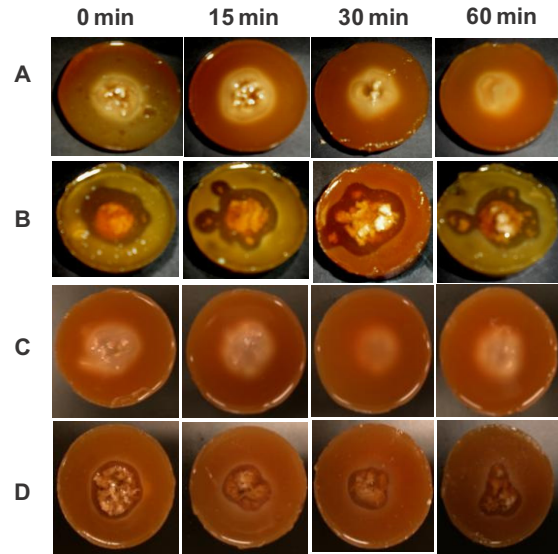


Figure 1. Efficacy of DPPC encapsulated nisin as a function of pH (HCl, pH 2 A, B and PBS buffer, pH 6.4 C, D) in ground beef. Nisin was tested at $83.25 \mu\text{g/g}$ meat. Encapsulated nisin was in contact with raw meat for 0, 15, 30 and 60 min (A, C) prior to heating to a core temperature of 71°C (B, D). The experiments were repeated three times.

Table 1. Antimicrobial activity (mm) of nisin in various acid solutions at pH 2 against *L. innocua*.

| | HCl | Lactic acid | Acetic acid |
|--------------|---------------|----------------|-----------------|
| Nisin + acid | $17,05 \pm 2$ | $24,2 \pm 2,5$ | 28 ± 2 |
| Acid | $1 \pm 0,5$ | 22 ± 1 | $24,05 \pm 1.5$ |

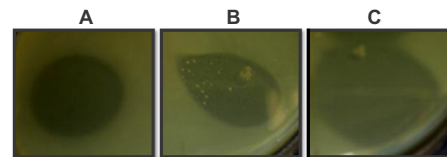


Figure 2. Antimicrobial activity (mm) of nisin in various acid solutions at pH 2 against *L. innocua*: HCl (A), lactic acid (B) and acetic acid (C). Nisin was tested at $83.25 \mu\text{g/g}$.

IV. CONCLUSION

In conclusion, encapsulation of nisin in liposomes formed with or without a hydrated film was effective in protecting nisin in raw meat and to permit its controlled release upon liposome melting during cooking. The additional inhibitory activity provided when nisin is dissolved at pH 2 (HCl, lactic and acetic acid), compared to pH 6.4 in PBS, largely compensated for the lower encapsulation efficacy observed in liposomes made without a hydrated film (data not shown).

The development of a food grade encapsulation procedure without solvent and HCl is promising. In this study, we encapsulated nisin but other bioactive molecules sensitive to cooking temperatures could possibly be protected as well provided that they are compatible with the chemical structure of the liposome.

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