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Carbohydrate Polymers 49 (2002) 139–144

Carbohydrate Polymers

www.elsevier.com/locate/carbpol

# Active edible polysaccharide coating and interactions between solution coating compounds

I. Sebti[a,b,c], V. Coma[a,*]

[a]*Laboratoire de Chimie des Substances Végétales, Institut du Pin, Université Bordeaux 1, 351 cours de la Libération, 33405 Talence, France*
[b]*Laboratoire de Recherche en Génie Industriel Alimentaire, IUT A, Université Lyon 1, Département Génie Biologique, Rue H. de Boissieu, 01000 Bourg-en-Bresse, France*
[c]*Unité Sécurité Microbiologique des Aliments, ISTAB, Université Bordeaux 1, Avenue des Facultés, 33405, Talence, France*

Received 3 January 2001; revised 22 May 2001; accepted 18 July 2001

**Abstract**

To control pathogenic strain growth on food surface, anti-microbial edible coatings were obtained from hydroxy propyl methyl cellulose associated with nisin. The inhibitory activity of nisin was confirmed on *Listeria innocua* and *Staphylococcus aureus*. To improve packaging moisture barrier properties, stearic acid (SA) was used, but preliminary experiments showed a decrease in the inhibitory activity of coatings, probably from electrostatic interactions with the bacteriocin. The study of interactions between both the compounds was then conducted to subsequently optimize nisin desorption. First, the negatively charged carboxyl groups of SA were confirmed by trapping cationic ion method, allowing electrostatic interactions. Secondly, a comparative study of nisin fixation in SA or methyl stearate showed that SA–nisin interactions were mainly electrostatic. Finally, calcium ion addition to the nisin–SA mixture showed a potential application in order to improve packaging activity by increasing nisin desorption from coatings. 

*Keywords*: Carbohydrate edible packaging; Polypeptide nisin; Fatty acid; Active coatings; Electrostatic interactions

## 1. Introduction

Improvement in the safety and quality of processed foods by using anti-microbial edible films or coatings has received increasing interest (El Ghaouth, Arul, Ponnampalam & Boulet, 1991; Fang, Li & Shih, 1994; Kesler & Fennema, 1986, 1989a,b; Muzzarelli, Tarsi, Filippini, Giovanetti, Biagini & Varaldo, 1990; Rico-Pena & Torres, 1990; Siragusa, Cutter & Willet, 1999). Packaging containing bacteriocins to especially control the growth of pathogenic strains of *Listeria monocytogenes* and *Staphylococcus aureus* strains is a promising area of research. Several reports have addressed the use of nisin, a bacteriocin produced by a *Lactococcus* strain, to effectively suppress the growth of *L. monocytogenes* (Dean & Zottola, 1996; Delves-Broughton, Blackburn, Evans & Hugenholtz, 1996; Schillinger, Chung, Keppler & Holzapfel, 1998; Winkowski, Ludescher & Monville, 1996). Instead of applying nisin by spraying, it was incorporated into edible hydroxy propyl methyl cellulose (HPMC) films or coatings. Taking into account the hydrophilic nature of cellulose based films, stearic acid (SA) was used to improve the moisture barrier. Earlier studies showed that anti-bacterial effect of HPMC—SA film associated with nisin decreased with increasing SA content, indicating a possible interaction between SA and nisin (Coma, Sebti, Pardon, Deschamps & Pichavant, 2001).

The first purpose of this study was to assess the inhibitory activity against *Listeria innocua* and *S. aureus* of an active carbohydrate HPMC edible coating to check its efficiency as a potential 'active food packaging' material. *L. innocua* was used instead of *L. monocytogenes* because it is non-pathogenic to humans. In addition, *L. monocytogenes* behaved similar to *L. innocua* (Sebti, Deschamps & Coma, 2001). The second purpose was to study the interactions between both the components to understand why the ability of the coating to inhibit microbial growth is dependent on lipid content.

## 2. Materials and methods

### 2.1. Materials

*Coating compounds*: HPMC (Culminal 50, Aqualon, France), polyethyleneglycol 400 (PEG Merck, Germany),

* Corresponding author. Tel.: +33-5-57-962913, fax: +33-5-56-846422.
*E-mail address:* v.coma@ipin.u-bordeaux.fr (V. Coma).

0144-8617/02/$ - see front matter 
PII: S0144-8617(01)00315-0

stearic acid (SA) and methyl stearate (MS) (Lots 4751 and 5376 respectively, SIGMA Chemical, USA) were used.

*Organisms and maintenance*. *Micrococcus luteus* 270 (Institut Pasteur, France) was grown at 30°C in nutritive broth (DIFCO 3178, USA], whereas *L. innocua* 430 (USMA collection, University Bordeaux 1, France) and *S. aureus* 58156 (Institut Pasteur, France) were grown in tryptose broth (DIFCO 62176, USA) at 37°C and agitated at 140–160 rpm for 18–24 h.

*Nisin*. Pure nisin (Aplin and Barrett Ltd, UK) was dissolved in 0.05 M phosphate buffer pH 6.1 and stored at 4°C. Nisin concentration is determined in international unit per milliliter (IU $ml^{-1}$).

## 2.2. Methods

*Statistical treatment*. All experiments were replicated at least three times. Treatment means were separated using the Student's *t*-test at 95% probability ($p > 0.05$).

### 2.2.1. Anti-microbial properties of edible coating

*Coating forming solution*. Coating forming solutions were prepared using the procedure described by Coma et al. (2001), by dissolving 9 parts of HPMC in 200 parts of distilled water, 100 parts of absolute ethanol and 1 part of PEG 400. Nisin was pre-solubilized in phosphate buffer (pH 6.1) prior to incorporation. The coating forming solution was heated to the melting point of SA prior to fatty acid addition, homogenized for 15 min and then cooled to 45–50°C before casting in Petri dishes. SA ($C_{18:0}$) content was expressed as a percentage of HPMC part (w/w).

*Nisin stability*. An inhibition zone assay was conducted from a nutritive agar (12 g $l^{-1}$) medium inoculated with 0.1% (v/v) of an overnight culture of *M. luteus*. Fresh and treated nisin (nisin dissolved in water/ethanol [3/0, 2/1, 1/2] and heated at 80°C for 1 h) were then analyzed by the agar diffusion method (Coma et al., 2001). Zones of inhibition were measured to the nearest 0.5 mm. The experiment was performed in triplicate.

*Anti-microbial effectiveness of edible coatings*. About 20–30 and 100–200 UFC/Petri dish of *L. innocua* or *S. aureus* suspension were inoculated on surface tryptose agar medium. After drying, nisin solutions or coatings of about $5 \times 10^3$ and $10^4$ IU $ml^{-1}$, were poured into Petri dishes, dried and incubated at 37°C for 24–48 h prior to numeration. For control plates, nisin or coatings were replaced by phosphate buffer, pH 6.1. The percentage inhibition was calculated using the following equation

$$\frac{\text{UFC number in control plates} - \text{UFC number in test plates}}{\text{UFC number in control plates}} 100 \quad (1)$$

The experiment was replicated six times and data were treated statistically.

### 2.2.2. Study of interaction between hydrophobic compound and nisin

For the following assays, mixtures were stirred at 500 rpm for 10 min at 80°C, cooled and filtered through a 0.22 μm filter (Millex GV, Millipore) before being analyzed. Residual nisin or calcium ion $Ca^{2+}$, not trapped by SA or MS, were quantified using a BCA protein micro-assay kit (Pierce, Rockford) at 562 nm or atomic absorption (Perkin-Elmer instrument) at 422.5 nm. The hydrophobic compound content added to 40 ml nisin solutions was expressed as a percentage (w/w) of HPMC. The experiment was replicated three times and data were treated using the Student's *t*-test ($p > 0.05$).

*Nisin–SA interaction*. Different SA percentages were mixed with nisin solutions at $10^3$ and $1.2 \times 10^4$ IU $ml^{-1}$. The percentage of fixed nisin was calculated as follows

$$\text{Fixed nisin (\%)} = \frac{\text{initial nisin} - \text{residual nisin}}{\text{initial nisin}} 100 \quad (2)$$

*Calcium ion ($Ca^{2+}$)–SA interaction*. Solutions of different concentrations of $Ca^{2+}$ were mixed with 25 g $l^{-1}$ of SA. The fixed calcium was calculated as follows

$$\text{Fixed calcium (g } l^{-1}) = \text{initial calcium} - \text{residual calcium} \quad (3)$$

*Nisin–SA or MS interaction*. Different SA or MS percentages were mixed with $3 \times 10^3$ IU $ml^{-1}$ of nisin. The fixed nisin was calculated using Eq. (2).

*Nisin–calcium–SA interaction*. First, solutions containing $10^4$ IU $ml^{-1}$ of nisin were mixed with 3% of SA and treated as described earlier. After measuring residual nisin by BCA micro-assay, the $Ca^{2+}$ solution was added to a final $Ca^{2+}$ concentration about of 8 or 80 mg $l^{-1}$ and residual nisin was then quantified (case 1):

$$(AS)COO^- + Nis^+ \rightarrow [(AS)COO^{-/+}Nis]_{complex} + Ca^{2+}$$

$$\rightarrow [(AS)COO^{-/+}Nis]_{complex} + Ca^{2+}$$

$$\text{or} \quad [(AS)COO^{-/2+}Ca]_{complex} + Nis^+$$

Secondly, solutions containing 8 or 80 mg $l^{-1}$ $Ca^{2+}$ were mixed with 3% of SA, before adding nisin to a final concentration about of $10^4$ IU $ml^{-1}$. Residual nisin was then measured (case 2).

$$(AS)COO^- + Ca^{2+} \rightarrow [(AS)COO^{-/2+}Ca]_{complex} + Nis^+$$

$$\rightarrow [(AS)COO^{-/+}Nis]_{complex} + Ca^{2+}$$

$$\text{or} \quad [(AS)COO^{-/2+}Ca]_{complex} + Nis^+$$

Thirdly, a solution containing $10^4$ IU $ml^{-1}$ of nisin and 8 or 80 mg $l^{-1}$ $Ca^{2+}$ was added to 3% of SA. The resulting solution was treated as described earlier and then the

Table 1
Anti-microbial effectiveness on *M. luteus* of $5 \times 10^3$ IU ml$^{-1}$ of fresh and treated nisin (80°C, 1 h, water/ethanol: 3/0, 2/1 and 1/2) evaluated by the inhibition zone assay. Data correspond to inhibition zone diameters (mm). Values, followed by their standard deviations, are means of three experiments. Treatment means were separated using the Student's *t*-test ($p > 0.05$)

| | Inhibition zone diameter (mm) |
|---|---|
| 3 parts water | 14 ± 1 |
| Water/ethanol: 2/1 | 14 ± 1 |
| Water/ethanol: 1/2 | 13 ± 1 |

residual nisin was assessed (case 3).

$$(AS)COO^- + Ca^{2+} + Nis^+ \rightarrow [(AS)COO^{-/+}Nis]_{complex} + Ca^{2+}$$

$$\text{or} \qquad [(AS)COO^{-/2+}Ca]_{complex} + Nis^+$$

The only expected major complexes are indicated in the preceding theoretical representations. Experiments were replicated six times and treated using the Student's *t*-test.

## 3. Results and discussion

*Nisin stability*. To determine the ability of nisin to withstand temperature and solvent conditions used for edible coating formation and interaction experiments, the residual inhibitory activity of the treated nisin was compared to the initial fresh nisin activity. Results (Table 1) indicated that our conditions could be used without significant ($p > 0.05$) reduction in the nisin inhibition properties, confirming the fact that no chemical or structural changes occurred for nisin.

*Anti-microbial effectiveness of edible coating*. Table 2 lists the inhibition percentages of *L. innocua* and *S. aureus* strains obtained by active coating with different nisin and SA contents.

With a low initial level of *L. innocua* (about 19 UFC/Petri dish), the microbial growth was completely inhibited irrespective of the nisin and SA content level. With higher initial bacterial loading, anti-microbial activity increased weakly with increasing nisin concentration. However, a significant difference was obtained between inhibition from a free nisin solution and coating without SA. HPMC coating seemed to improve microbial inhibition probably by limiting gas transfer, especially oxygen, through the coating and/or allowing sufficient nisin on the surface of the model food, compared to the rapid diffusion of free nisin.

The inhibition percentage dramatically decreased when SA was incorporated, reaching an inhibition level lower than for free nisin solution. Presumably, interactions between nisin and SA limited the release of nisin from the coating.

For *S. aureus*, an initial level of 24 ± 7 bacteria/Petri dish was tested and results were comparative with a bacteria population of 184 ± 42 bacteria/Petri dish. However, there were significant improvements while using a higher concentration of nisin against both initial microbial levels, except with film without SA. For the latter, the observations and hypothesis were virtually identical with those of the earlier *L. innocua* study.

By and large, *L. innocua* appeared less sensitive to active coating than *S. aureus* due to its lower nisin sensitivity. Consequently, cellulosic coatings without SA associated with $5 \times 10^3$ IU ml$^{-1}$ of pure nisin offered a great advantage in preventing *L. innocua* and *S. aureus* surface growth. However, SA incorporation, in order to improve the water vapor barrier properties of the coating, decreased inhibition against both bacteria, raising the problem of the usefulness of the coating. Interactions between nisin and SA occurred. Similar effects on the activity of nisin in systems containing high levels of fat were reported by Jung, Bodyfelt and Daeschel (1992). These authors observed a decrease in nisin activity about 88% in the presence of 13% fat. Dean and Zotolla (1996), used nisin to inhibit *L. monocytogenes* in ice cream, and reported that nisin exhibited a greater effect in 3% fat ice cream than in 10% fat. To subsequently optimize the desorption phenomenon, experiments were carried out to determine the nature of these interactions.

*Nisin and SA interactions*. The basic character of nisin, due to its four positively charged sites supported by the lysine residues and by the $NH_2$ N terminal (Fig. 1), leads

Table 2
Inhibition effectiveness of either free nisin solution and nisin coating forming solution with 0 and 15% SA content. Data at 0 IU ml$^{-1}$ correspond to CFU number control plates. For the other nisin concentrations, values correspond to inhibition percentages. Data, followed by their standard deviations are means of six experiments. Treatment means were separated using the Student's *t*-test ($p > 0.05$)

| | | | | Coating forming solution | | | |
|---|---|---|---|---|---|---|---|
| | | Free nisin solution | | 0% SA | | 15% SA | |
| Nisin content (IU ml$^{-1}$) | 0 | $5 \times 10^3$ | $10^4$ | $5 \times 10^3$ | $10^4$ | $5 \times 10^3$ | $10^4$ |
| *L. innocua* | 19 ± 2 | | | Total inhibition | | | |
| | 138 ± 15 | 35 ± 4 | 40 ± 9 | 67 ± 12 | 67 ± 11 | 19 ± 4 | 33 ± 13 |
| *S. aureus* | 24 ± 7 | 56 ± 9 | 87 ± 12 | 100 ± 0 | 92 ± 16 | 79 ± 16 | 97 ± 4 |
| | 184 ± 42 | 58 ± 11 | 82 ± 5 | 100 ± 0 | 100 ± 0 | 78 ± 6 | 99 ± 1 |

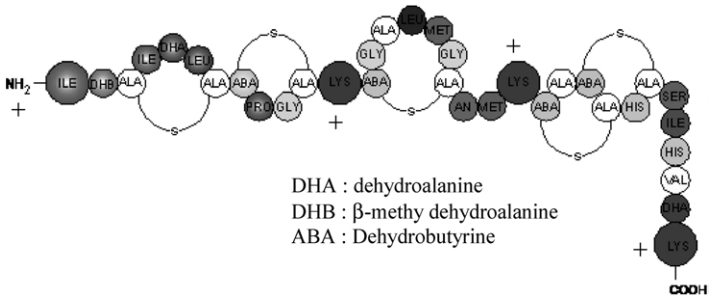


Fig. 1. Nisin structure (+) are net positive charge.

us to confirm that the peptide is positively charged and as our pH conditions, $6 < pH < 7$ (Breukink & Ben de Kruijff, 1999; Lins, Ducarme, Breukink & Brasseur, 1999).

SA is a weak acid. Because it is non water-soluble, the determination of the $pK_A$ is difficult. Taking into account the $pK_A$ of water soluble acids (Table 3) and, for instance, a $pK_A < 6$ for a $C_8$ hydrocarbon chain, and given that the hydrocarbon chain length from $C_3$ weakly influences $pK_A$, our conditions seemed sufficient to ensure that SA is negatively charged. Therefore for a pH between 6–7, nisin and SA were probably positively and negatively charged respectively, which suggests that lipid–nisin interactions could be electrostatic. This hypothesis is investigated as follows.

Fig. 2 represents the effects of initial nisin and SA contents on the percentage of fixed bacteriocin. First of all, the addition of SA to a solution of $10^3$ IU ml$^{-1}$ of nisin resulted in a total chelation of nisin with only 1% of SA. Obviously, it was not possible to study the kinetics of fixation, which could evaluate affinities between both molecules. The nisin concentration was then increased to $1.2 \times 10^4$ IU ml$^{-1}$. With less than 5% of SA, a pseudo-linear relationship was thus obtained between fixed nisin and SA content. When SA percentages exceeded 5%, no residual nisin could be detected. Therefore, SA strongly chelated with the nisin and the interactions involved could be attributed to various phenomena. For anti-microbial activity, several researchers have reported that nisin molecules initially bind to the target membrane of sensitive bacteria through electrostatic interactions between the positively charged nisin molecule and the anionic phospholipids of the lipid bilayer (Abee, 1995). In a second phase, the nisin monomer is inserted into the membrane and the inserted monomers aggregate laterally through hydrophobic interactions to form 'barrel-staves'. These pores completely dissipate $\Delta\psi$ and $\Delta$pH and induce bacterial death. Electrostatic and hydrophobic interactions may exist between nisin and SA (Monville & Chen, 1998). To test whether the electrostatic interactions are a reasonable explanation, calcium cationic ions were first mixed with SA in order to check the negative charge of the carboxylic function under our experimental conditions. Secondly, a study comparing SA and MS, was conducted.

Table 3
$pK_A$ of acids Chemistry Laboratory Course Chemistry Department, 1253 University of Oregon, Eugene, OR 97403-1253, 541-346-4601 (1999)

| Acids | $pK_A$ |
|---|---|
| Formic acid | 3.75 |
| Propanoic acid | 4.86 |
| Pentanoic acid | 4.84 |
| Hexanoic acid | 4.84 |
| Heptanoic acid | 4.89 |
| Octanoic acid | 4.89 |

*Calcium ion ($Ca^{2+}$)–SA interaction*. To test whether the theoretical negative charge of SA under our conditions is in agreement with experimental observations, the fixation of $Ca^{2+}$ on SA was studied with different initial contents of $Ca^{2+}$ (Table 4). The Cationic ion was chelated by SA and this fixation was dependent on the initial $Ca^{2+}$ concentration, indicating that the carboxylic function of SA was negatively charged and hence available for electrostatic interactions. SA chelated lower calcium contents than expected, on a stochiometric basis. No molecular relationship was established between fixed $Ca^{2+}$ and initial SA content. Moreover, $Ca^{2+}$ ion fixation increased with initial calcium content. Further experiments are being conducted in order to understand this phenomenon.

*Nisin–SA or MS interaction*. A comparative study of nisin with either SA or MS was conducted. Taking into account that SA and MS have similar hydrophobic alkyl chains, any difference in the evolution of the SA or MS–nisin interaction would be explained by electrostatic interactions involving the carboxylic functionality SA-$COO^-$. Residual free nisin was measured at different SA or methyl ester levels (Fig. 3). A significant lower fixed nisin from MS than SA were obtained, indicating that nisin–SA interaction were mainly electrostatic. Only about of 20% of initial nisin was fixed by high percentages of MS, leading to about 80% of electrostatic binds in the nisin– SA interaction system. The other interaction involved was certainly hydrophobic. SA replacement by MS improved inhibitory activity of edible coatings, but moisture barrier properties were not satisfactory.

*Nisin–$Ca^{2+}$–SA interaction*. Taking into account that the decrease in the anti-microbial activity of the coating is likely to be the result of electrostatic interactions between cationic nisin $Nis^+$ and anionic SA (SA)$COO^-$, experiments

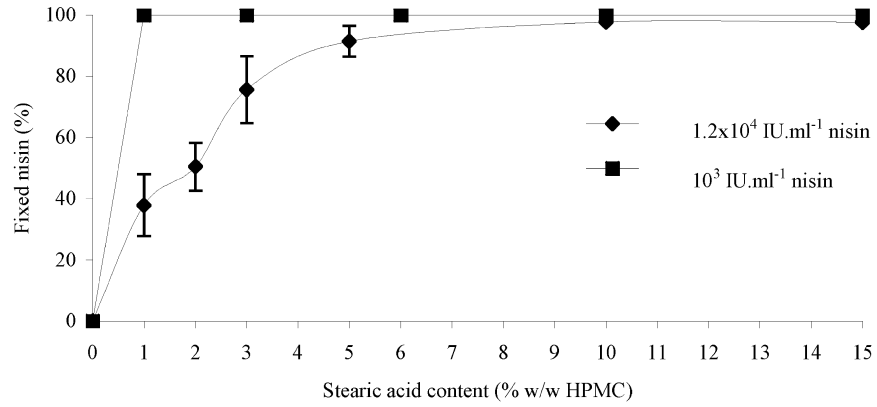


Fig. 2. Evolution of the percentage of fixed nisin from $10^3$ and $1.2 \times 10^4$ IU ml$^{-1}$ initial nisin versus SA content. Nisin was measured by BCA protein micro-assay. Each point of the graph is the mean of three experiments. Treatment means were separated using the Student's $t$-test ($p > 0.05$) and the standard deviations are vertical lines.

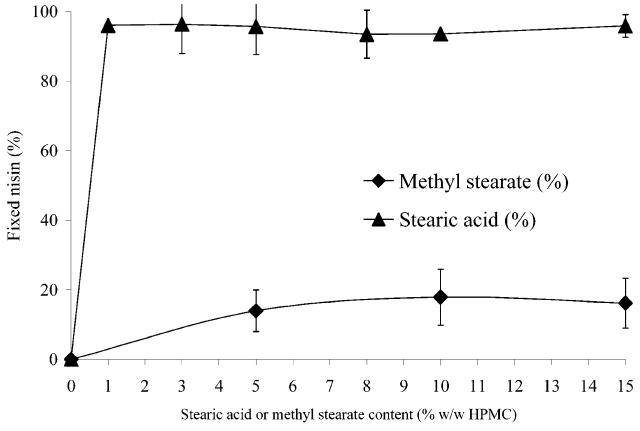


Fig. 3. Evolution of the percentage of fixed nisin versus SA or MS content. $3 \times 10^3$ IU ml$^{-1}$ initial nisin was used (see text for calculating fixed nisin). Nisin was measured by BCA protein micro-assay. Each point of the graph is the mean of three experiments. Treatment means were separated using the Student's $t$-test ($p > 0.05$) and the standard deviations are vertical lines.

were performed to study the influence of addition of positive ions in the packaging formulation, in order to improve nisin desorption by competition. $Ca^{2+}$ was selected and a level of about 8 mg l$^{-1}$ was chosen to theoretically saturate the available negative charges from SA (Table 5).

Nisin and SA were mixed prior to calcium incorporation (case 1). $Ca^{2+}$'s ability to replace nisin in $[(AS)COO^{-/+}Nis]_{complex}$ was investigated. SA fixed more than 97% of initial nisin, when 8 mg l$^{-1}$ of $Ca^{2+}$ concentration was used.

Table 4
Evolution of free calcium content after SA addition. Calcium cationic ions were measured by atomic absorption. Data, followed by their standard deviations, are means of three experiments. Treatment means were separated using the Student's $t$-test ($p > 0.05$)

| | $Ca^{2+}$ concentration (g l$^{-1}$) | | |
|---|---|---|---|
| Initial $Ca^{2+}$ content | 7.95 ± 0.27 | 3.78 ± 0.13 | 1.77 ± 0.13 |
| Fixed $Ca^{2+}$ after SA addition | 0.87 ± 0.03 | 0.32 ± 0.01 | 0.15 ± 0.06 |

Calcium addition did not reduce nisin fixation by SA. No significant difference was observed with regard to the case of nisin–SA mixture, in the absence of calcium.

$Ca^{2+}$ and SA were mixed prior nisin incorporation (case 2). The comparison of cases 1 and 2 showed that, calcium significantly reduced nisin fixation by SA ($p > 0.05$). However, 96% nisin fixation by SA were still obtained.

In case 3, nisin, $Ca^{2+}$ and SA were mixed simultaneously. The affinity of positively charged compounds for SA was studied. Data (Table 5) showed that SA addition to nisin fixed nearly 92% of the initial nisin. The simultaneous mixture of the three compounds seemed to significantly ($p > 0.05$) reduce nisin chelating, with regard to cases 1 and 2.

Finally, the three cases showed that, 8 mg l$^{-1}$ calcium addition weakly prevented nisin fixation by SA (92–97% nisin chelation). A calcium concentration of 8 mg l$^{-1}$ did not present a real potential to limit the SA–nisin interaction. Therefore, a higher calcium ion concentration of 80 mg l$^{-1}$ was used and tested under the same conditions (Table 5). At this high concentration, calcium prevented nisin fixation by SA in cases 2 and 3. In case 1, the $[(AS)COO^{-/+}Nis]_{complex}$ was disrupted and the cationic ion displaced nisin on SA.

In conclusion, nisin food coatings free of hydrophobic

Table 5
Nisin–SA interaction in 8 and 80 mg l$^{-1}$ calcium concentration solution. Initial nisin was 17680 ± 2000 IU ml$^{-1}$. Residual nisin was quantified by BCA protein micro-assay. All values are means of six experiments followed by their standard deviations. Treatment means were separated using the Student's $t$-test ($p > 0.05$)

| | | Free nisin (IU ml$^{-1}$) | |
|---|---|---|---|
| | | 8 mg l$^{-1}$ | 80 mg l$^{-1}$ |
| Case 1 | $[(AS)COO^{-/+}Nis]_{complex} + Ca^{2+}$ | 370 ± 40 | 15890 ± 1120 |
| Case 2 | $[(AS)COO^{-/2+}Ca]_{complex} + Nis^{+}$ | 590 ± 140 | |
| Case 3 | $(AS)COO^{-} + Ca^{2+} + Nis^{+}$ | 1120 ± 160 | |

compounds have a potential application in preventing surface microbial growth. SA incorporation to give a water barrier in active coating formulations with nisin decreased their inhibitory activity against the bacteria tested. This low activity was explained mainly by electrostatic interactions between the cationic nisin and the anionic SA, preventing nisin desorption from the coatings. Earier studies (Sebti, Pichavant, Pardon & Coma, 2000) indicated that SA replacement by MS improved inhibitory activity of edible films, but the moisture barrier properties were not satisfactory.

Because nisin and calcium instantaneously fixed SA, it was not possible to calculate the kinetics of fixation and affinity coefficients, which complicated the optimization of nisin desorption from the coatings and the improvement of edible coatings inhibitory activity.

The use of high $Ca^{2+}$ concentration decreased nisin fixation by SA. Calcium ion incorporation into the coating forming solution should be studied to improve coating and film inhibitory activity against *L. innocua* and *S. aureus*.

## Acknowledgements

The authors thank J. Delves-Broughton, of Aplin and Barrett Limited (15 North Street, Beaminster, Dorset, DT8 3TZ England) for providing pure nisin and for his technical assistance. Thanks are also extended to Rhônes–Alpes region (France) for technical support and financial subventions. Many thanks to A. Hadj Sassi (USMA researcher, University Bordeaux 1, France) for his scientific and technical support.

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