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ADSORPTION OF NISIN ON MONTMORILLONITE: A CONCENTRATION STRATEGY

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ABSTRACT

The adsorption of a commercial form of nisin, Nisaplin® (Npl) (Danisco), on montmorillonite (Mt), a natural inexpensive clay mineral accepted as food additive, was assayed. The intention was to develop food grade materials capable of releasing the peptide and avoiding negative interactions that affect nisin antimicrobial activity in food environments. A set of different Mt-Npl relation samples was prepared. The antimicrobial activity and the total organic carbon/total nitrogen (TOC/TN) content measured in nisin solutions after contact with the clay decreased as the amount of solid increased; thus, indicating the adsorption of the peptide on the support. Also, the Mt-Npl solids presented inhibitory activity against *Enterococcus faecium* C1. Additionally, Fourier Transform-Infrared Attenuated Total Reflectance spectroscopy (FT-IR-ATR) analysis of the Mt-Npl lyophilised systems showed that nisin was preferably adsorbed on Mt among Nisaplin® ingredients, suggesting a unique concentration and immobilization method for this antimicrobial peptide that could be applied in food preservation.

PRACTICAL APPLICATIONS

Montmorillonite (Mt) is an inexpensive abundant natural clay characterized by a moderate cation exchange capacity with high surface area. Nisaplin® is a commercial form of nisin, a polypeptide bacteriocin active against several Gram-positive food spoilage and pathogen microorganisms. The increasing demand for fresh and natural food favors the application of biopreservatives such as nisin. However, the interaction of these antimicrobials with other food components reduces their inhibitory effect when directly introduced into food systems. Thus, alternatives for the inclusion of active biomolecules in foods are necessary. Since Mt is a potential food additive, its use as a nisin immobilization agent provides a strategy to protect and optimize the gradual peptide liberation. This immobilization approach does not only offer a simple, rapid, and low cost method for the concentration of nisin, but also provides a feasible alternative procedure for the introduction of this antimicrobial peptide in food complex systems.

INTRODUCTION

In recent years, the demand for fresh, natural and minimally processed food has been in continuous increase (Deegan *et al.* 2006). Also, the use of food control strategies based on living organisms and/or their antimicrobial products, i.e. food biocontrol or biopreservation, has also become gradually more popular as it satisfies these requirements. Therefore, a natural alternative food biopreservative should have low effect on the nutritional and sensory properties, extend

the shelf life, require simple technological equipment and positively impact on the product final cost (Gálvez *et al.* 2010). For this, the use of bacteriocins synthesized by lactic bacteria as natural biopreservative alternatives has gained attention (Khan *et al.* 2010; Balciunas *et al.* 2013).

Nisin, a polypeptide bacteriocin produced by *Lactococus lactis* subsp. *lactis*, presents antimicrobial activity against Gram-positive food-associated pathogens such as *Clostridium botulinum*, *Bacillus cereus* and *Listeria monocytogenes* (Thomas and Delves-Broughton 2005). It is the most commercially important member of a large class of bacteriocins produced by lactic acid bacteria, approved as food additive by the Food and Agriculture Organization/World Health Organization (FAO/WHO) (Jones *et al.* 2005). Even so, the direct introduction of antimicrobials into food products is generally accompanied by an undesired dilution effect and a possible interaction with different food components, which might result in a decrease of the antagonistic action on the target cells. Therefore, the analysis of alternative methods to overcome these difficulties may improve the antimicrobial activity and stability of biopreservatives in complex systems (Were *et al.* 2004). The immobilization of nisin has been assayed by adsorption of the peptide on solid silica supports (Bower *et al.* 1995; Janes *et al.* 1998; Dawson *et al.* 2005) or encapsulation in inorganic and organic polymeric matrices (Daeschel *et al.* 1992; Lante *et al.* 1994), among others. Natural clay minerals, such as montmorillonite (Mt), have numerous advantages qualifying them as potential nisin carriers. First, they have low cost, and second, the American Food and Drug Administration (FDA) as well as the European Food Safety Authority (EFSA) consider natural phyllosilicates as safe food additives already been used successfully as vehicles for the controlled release of active ingredients of drugs (Joshi *et al.* 2009). Besides, the ingestion of clay minerals (geophagy) by humans has been well-documented for a variety of beneficial purposes, e.g. gastrointestinal protectors, mineral nutrients/supplements, etc. (Kikouama *et al.* 2009; Carretero *et al.* 2013).

In this work, the adsorption of a commercial form of nisin, Nisaplin® (Danisco, UK), on raw montmorillonite was assayed with the purpose of evaluating the application of clay as a simple, natural and low cost carrier. The objective was to develop food-grade materials with immobilized nisin capable of releasing effective amounts of the antimicrobial peptide, suitable for food preservation applications.

MATERIALS AND METHODS

Support and Peptide Sorbat

A commercial raw Mt obtained from a mine in Río Negro (Argentina), chemically and mineralogically characterized by Magnoli *et al.* (2008) (sample B), was used as inorganic support. The sodium fraction (≤ 2 μm) of this clay was obtained by saturation with NaCl, several rinsings with distilled water and centrifugation (10,000 × g, 15 min, 20C) (Sigma 4K10 Refrigerated Centrifuge, Germany). The Na-Mt obtained was dispersed in distilled water to a final concentration of 29.2 mg/mL and sterilized by autoclaving (121C, 15 min) before its use in adsorption assays. Nisaplin® powder (2.5% Nisin, 77.5% NaCl, 12% skim milk powder, 6% carbohydrates—Danisco, UK) was used as immobilized agent. Nisin solution was obtained by suspending 5 g of Nisaplin® powder in 50 mL of sterile distilled water to obtain a final nisin concentration of 2.5 mg/mL. The Nisaplin® suspension was centrifuged (3,000 × g, 10 min, 10C) (Eppendorf 5424 R Centrifuge, Germany) in order to eliminate insoluble ingredients of Nisaplin® and the supernatant recovered. The nisin solution obtained was stored at 4C until used.

Nisin Immobilized Systems

A series of antibacterial compounds was prepared by mixing different amounts of the Mt suspension with the nisin aqueous solution. After 2 h of stirring at 25C, the solids were recovered by centrifugation (10,000 × g, 15 min, 10C) (Eppendorf 5424 R Centrifuge, Germany), rinsed with distilled water and freeze-dried prior to further characterization. The supernatants recovered after nisin-Mt contact were also kept refrigerated for further analyses.

Each resulting nisin solid system was labeled according to its Nisin Loading Capacity (NL) as indicative of the fraction of the cation exchange capacity (CEC) of the clay that can be covered by the nisin present in solution for each system. This NL was determined as in Redding *et al.* (2002): $NL = g_{Nis}/(CEC_{Mt}.g_{Mt}.MW_{Nis}.X_{Nis})$ where NL (nisin loading) is the fraction of CEC satisfied by nisin molecules, CEC_{Mt} is the Mtcation exchange capacity (84.7 meq/100 g (Naranjo *et al.* 2013)), g_{Nis} is the nisin mass required to achieve the desired fraction of CEC, g_{Mt} is the clay mass, MW_{Nis} is the gram molecular weight of nisin, and X_{Nis} are the moles of charge per mole of nisin (determined as 5 mol/eq (Ibarguren *et al.* 2014)). The antibacterial compounds were designed in order to achieve nisin loadings (NL) equivalent to a CEC_{Mt} of 0.15, 0.30, 0.60, 1.50 and 3.00, and labeled Mt-Npl 0.15, Mt-Npl 0.30, Mt-Npl 0.60, Mt-Npl 1.50, and Mt-Npl 3.00, respectively.

Antimicrobial Activity Determination

The residual antimicrobial activity of nisin solutions recovered by centrifugation (10,000 × g, 15 min, 10C) (Eppendorf 5424 R Centrifuge, Germany), after contact with the solid, was determined by the serial 1:2 dilution method using *Enterococcus faecium* C1 (GenBank access code

EU428011) as indicator strain (Daba *et al.* 1991). Also, a modified agar diffusion technique was used to determine the activity of immobilized nisin against the same strain (Ibarguren *et al.* 2010). For this purpose, the agar wells were loaded with wet and lyophilised Mt-Npl systems and the inhibition halo zones were measured after 24 h incubation at 37C. Additionally, a pure nisin (Danisco, UK) aqueous solution (2.5 mg/mL) was used as antimicrobial control in this assay.

Total Organic Carbon and Total Nitrogen Determination

Total organic carbon and nitrogen (TOC/TN) content were measured in nisin solutions recovered after contact with the clay using a Shimadzu TOC-VCPN with TN Unit analyser (Shimadzu Co. Kyoto, Japan). Total carbon (TC), inorganic carbon (IC) and total organic carbon (TOC) were determined per oxidative combustion-infrared analysis, while total nitrogen (TN) was measured using the principles of oxidative combustion-chemiluminescence. All measurements were carried out in duplicate.

Fourier Transform-Infrared Attenuated Total Reflectance Spectroscopy Analysis

The peptide adsorption of the resulting lyophilised solid samples (Mt-Npl 0.15, 0.30, 0.60, 1.50, and 3.00) was analysed by Fourier Transform-Infrared Attenuated Total Reflectance spectroscopy (FT-IR-ATR). Also lyophilised Na-Mt, lyophilised Nisaplin® (Danisco, UK) and pure lyophilised nisin (Danisco, UK) were examined by this technique. Several scans were recorded with a Pt Single Reflection Diamond Attenuated Total Reflectance (ATR) Module (Bruker Alpha, USA) over the 375 to 4,000 cm^{-1} wavenumber range for each sample.

RESULTS AND DISCUSSION

The residual antimicrobial activity determined for nisin (Nisaplin®) solutions recovered after contact with clay, sequentially decreased with the increase of solid content in the reaction systems to the extent that the initial 3,200 UA/mL titre of the Nisaplin® solution (2.5 mg nisin/mL) lowered to 0 UA/mL after contact in Mt-Npl 0.15, Mt-Npl 0.30, and Mt-Npl 0.60 supernatants; and increased up to 75 UA/mL and 300 UA/mL in the supernatants of Mt-Npl1.50 and Mt-Npl3.00 systems, respectively. However, only Mt-Npl1.50 and 3.00 immobilized solid systems inhibited the growth of *E. faecium* C1, as observed by the halos detected by the agar diffusion technique (Fig. 1). The decrease of the residual antimicrobial activity of the supernatants recovered after contact with the clay, with increasing solid content, provided the first signal of the peptide adsorption. Nevertheless, although a complete loss of inhibition activity was detected in solutions after contact with Mt-Npl systems of lower NL value (i.e., more clay content), this did not mean that the resultant solid immobilized systems had the highest inhibition activity. Only Mt-Npl 1.50 and 3.00 inhibited the growth of *E. faecium* C1 by the agar diffusion technique, suggesting a possible NL threshold value over which adsorbed nisin is available for antimicrobial activity. Below this value, although the peptide is adsorbed to the clay, the inhibition activity could be blocked due to the interaction between the peptide and the solid support.

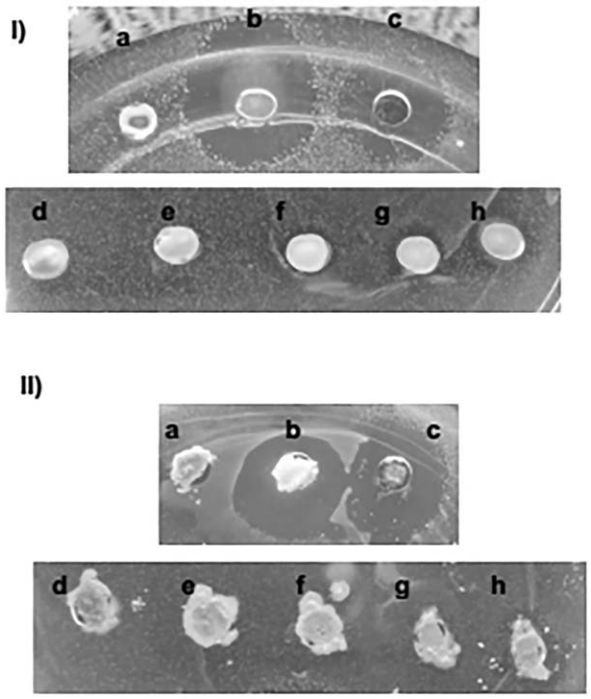

FIG. 1. INHIBITION HALOS OF MONTMORILLONITE-NISAPLIN® (Mt-Npl) **(I)** WET AND **(II)** FREEZE DRIED IMMOBILIZED SYSTEMS AGAINST *Enterococcus faecium* C1
(a) Mt (CONTROL), (b) PURE NISIN (2.5 mg/mL) (CONTROL), (c) NISAPLIN® (100 g/mL, 2.5 mg/mL NISIN) (CONTROL), (d) Mt-Npl 0.15, (e) Mt-Npl 0.30, (f) Mt-Npl 0.60, (g) Mt-Npl 1.50, (h) Mt-Npl 3.00.

The Mt-Npl adsorption isotherm was plotted with the data obtained from the Total Organic Carbon and Total Nitrogen (TOC/TN) content determined for Nisaplin® solutions recovered after contact with the solid (Fig. 2). As nisin is the major source of Nitrogen in the Nisaplin® solution, the amount of nisin adsorbed to the clay was obtained from the difference of TN in Nisaplin® solution prior and after contact with the different amounts of Mt. The Mt-Npl adsorption isotherm shape fits with the initial part of an "S" (solvent affinity-type) curve typical of "cooperative

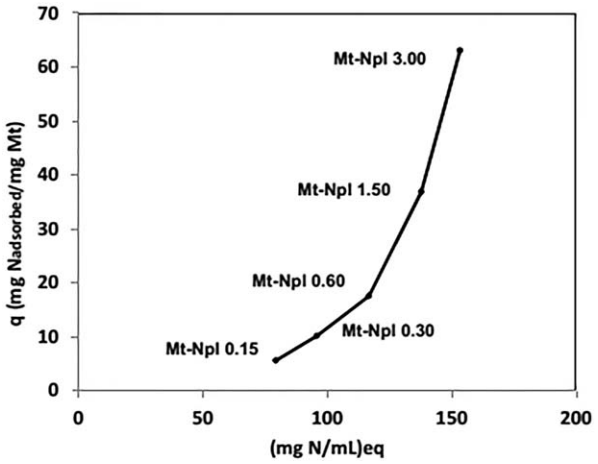

FIG. 2. ADSORPTION ISOTHERM OF NISIN (NISAPLIN®) ON MONTMORILLONITE (Mt) (25C) DETERMINED BY TOC/TN ANALYSES

adsorption" (Limousin *et al.* 2007). This model proposes that since sorbat-sorbat interaction is stronger than sorbat-sorbent interaction, the progressive adsorption of the solute facilitates the fixation of additional molecules to the sorbat (Bradl 2006). It can also be interpreted as a strong adsorption of the solvent, strong intermolecular attraction within the adsorbent layers, penetration of the solute in the adsorbent and monofunctional nature of the sorbate (Delle Site 2001). Thus, a side-by-side association between nisin adsorbed molecules occurs, facilitating the holding of the antimicrobial peptide to the clay surface as more solute is taken up, i.e. as nisin/clay ratio increases. This "cooperative adsorption" could explain the NL breakline value below which antimicrobial activity is not detectable in the Mt-Npl powders. At low NL, nisin molecules are mainly involved in the adsorption to the clay surface; as NL increases, the peptide side-by-side association leave more "outer" nisin molecules available for antimicrobial activity.

The FT-IR spectra of the clay, pure nisin, Nisaplin® and the different clay-nisin lyophilised powders are shown in Fig. 3. The Mt spectra shows a band at 995 cm^{-1}, assigned to Si-O and Si-O-Si stretching vibrations, as well as shoulders at 890 cm^{-1} which represent Al-OH bending vibrations (He *et al.* 2006). The band at 3,622 cm^{-1} is associated to the inner Si-OH signal which cannot be resolved from the broad band between 3,000 and 3,500 cm^{-1}, typical of adsorbed water. Bound interlayer water appears at 1,635 cm^{-1} (Acosta *et al.* 2003; Zhou *et al.* 2007). In the case of pure nisin, the more pronounced signals correspond to the amide I (1,638 cm^{-1}, stretching vibrations of the C═O bond) and amide II (1,520 cm^{-1} bending vibrations of the N-H bond) bands (Kong and Yu 2007). In addition, broad signals corresponding to adsorbed water (3,000–3,500 cm^{-1}) and C-H stretching (2,800–3,000 cm^{-1}) were observed. The peptide signals appear clearly weaker in the Nispalin® spectra, and some signals cannot even be resolved. However, the clay signals and both nisin amide peaks were detected in the Mt-Npl spectra, and both amide signals increase with increasing NL. The slight shift of the band at 995 cm^{-1} to higher wavelengths observed in the clay spectra reflects interactions between the peptide and the Mt Si-O tetrahedral layers upon intercalation (He *et al.* 2006). Normally, the analysis of the effect on bonded water provides a way to check if the intercalation of the sorbat in the solid clay matrix occurs (Acosta *et al.* 2003). In this case, the effect on bonded water in Mt-Npl powders could not be seen clearly since the pure nisin amide I band (1,638 cm^{-1}) and the adsorbed water band (~3,300 cm^{-1}) superimposed with the interlayer bonded water signals. However, it is important to note that the amide bands of the peptide adsorbed on Mt from Nisaplin® solution appear more intense than the signal of the non-adsorbed commercial nisin. Nisaplin® has different additives in its formulation, so this result suggested that nisin was preferably absorbed among Nisaplin® constituents, which conduced to the concentration of the peptide on the solid support.

Nisin has extensively been used as a food preservative and studied thoroughly in literature, but concentration strategies are still being considered. Thus, different techniques which avoid classic multi-step chromatographic methods (Garsa *et al.* 2014), like liquid-liquid extraction with organic solvents (Taylor *et al.* 2007) or aqueous two-phase micellar systems (Jozala *et al.* 2008), adsorption on hydrophobic and hydrophilic surfaces (Karam *et al.* 2013), among others, were proposed. The immobilization of nisin on hydrophilic supports results interesting due to the amphiphilic nature of the peptide, which allows the adsorption of the peptide and retention of the antibacterial activity on the adsorbed state

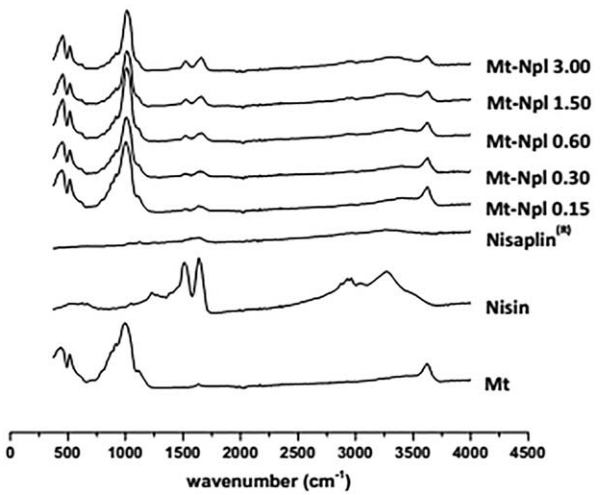

FIG. 3. FT-IR SPECTRA OF MONTMORILLONITE (Mt), PURE NISIN, NISAPLIN® AND MONTMORILLONITE-NISAPLIN® (Mt-Npl) SYSTEMS

(Daeschel *et al.* 1992; Karam *et al.* 2013). In this sense, the adsorption of nisin on montmorillonite is feasible since the protonation of amino residues in nisin molecule could favour electrostatic interactions with the negatively-charged surfaces of the clay mineral. In fact, we had previously verified the adsorption of pure nisin on montmorillonite, by means of a "frustrated intercalation" (Ibarguren *et al.* 2014). This intercalation model proposes a monolayer arrangement of nisin molecules between the layers located near the edges of the clay mineral. The results here obtained do not only confirm the positive interaction between the clay surface and the peptide in a more complex environment, but also show an option to enhance the use of commercial presentations of nisin with low content of the antimicrobial peptide (2.5% w/w), avoiding the addition of big amounts of the powder extract to obtain an antimicrobial effect on different food systems.

CONCLUSIONS

The adsorption of nisin from commercial Nisaplin® aqueous solution on Mt was verified and Mt-Npl systems with antimicrobial activity were obtained. This immobilization proposal offers a simple, rapid, and low cost method for the concentration of this peptide, and could be used as a feasible alternative method for the introduction of this antimicrobial peptide in food complex systems.

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