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Effect of high pressure on the antimicrobial activity and secondary structure of the bacteriocin nisin

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ABSTRACT

Effect of high pressure (HP) treatment on the antimicrobial properties and the structure of nisin was evaluated. Nisin solutions at pH 2.8 or 6.1 were treated by HP at 500 MPa – 10 min – 20 °C and their antimicrobial potency was determined. It appeared that HP clearly impacted the antimicrobial activity of nisin, with respective activity loss of 22.5% and 49.9% at pH 2.8 and 6.1. Structural analysis of nisin by circular dichroism and Fourier transform-infrared spectroscopies revealed that the decrease of nisin antimicrobial activity was likely due to the unfolding of the protein induced by HP. A loss of nisin β-turns structure, particularly significant at neutral pH, was linked to the drastic drop in antimicrobial activity, as these structures are implicated in the nisin interaction with the bacterial membrane.

Industrial relevance: The combination of nisin and high pressure (HP) can be use at an industrial scale to inactivate bacteria. Nisin is allowed as a food additive (E234) and can be added at a final concentration ranging from 120 to 500 IU/g, depending on the product. In this work, we showed that HP can induce a significant reduction of nisin activity (-22.5% at pH 2.8 and -49.9% at pH 6.1). Therefore, this activity loss could be taken into account to manage the final nisin concentration in HP-treated food products.

1. Introduction

Nisin is a bacteriocin produced by certain strains of *Lactococcus lactis* subsp. *lactis*, exhibiting a broad spectrum of inhibitory activity against Gram-positive bacteria (Delves-Broughton, Blackburn, Evans, & Hugenholtz, 1996). Due to its natural origin and its nontoxicity toward humans, this antimicrobial peptide is a matter of interest for the food industry. Nisin is composed of 34 amino acids of which 13 are involved in post-translational modifications, inducing the formation of five ring-like structures (Hasper, De Kruijff, & Breukink, 2004; Kupke & Gotz, 1996). By targeting the lipid II of vegetative forms of Gram-positive bacteria, nisin disrupts the transglycosylation leading to pore formation in the cell membrane and inhibition of the cell wall biosynthesis. Nisin also inhibits the outgrowth of germinated bacterial spores by preventing the establishment of a membrane potential and oxidative metabolism (Gut, Prouty, Ballard, van der Donk, & Blanke, 2008). Although nisin can act on the cell membrane of Gram-positive bacteria, Gram-negative bacteria are generally resistant since the outer membrane blocks the access of nisin to the cytoplasmic membrane. Similarly, the thick condensed membrane of dormant spores seems to protect them from nisin action.

Because high pressure treatment (HP) is known for inducing sub-lethal damages to the cell membrane, this non-thermal process has been combined many times with nisin. For example, Black, Kelly, and Fitzgerald (2005) showed that a reduction of 8 log of *Escherichia coli* in milk was achieved by combining a HP treatment at 400 MPa for 5 min at 20 °C with 500 IU/mL of nisin (Black et al., 2005). Under similar conditions, the independent effects of HP and nisin induced 5.19 ± 0.86 and 0 log-cycle reduction, respectively. However, Lee, Heinz, and Knorr (2003), did not find any synergy between HP (300 MPa; 3.3 min; 5 °C) and nisin (20 mg, equivalent to 640 IU/mL), as such combination tested on *E. coli* showed the same degree of inactivation as HP alone (Lee et al., 2003). Numerous studies also reported a high synergy between HP and nisin on the inactivation of bacterial spores (Aouadhi et al., 2013; Aouadhi, Mejri, & Maaroufi, 2015; Black et al., 2008). Nevertheless, strong variations of spore inactivation are observed, depending on the pH and the food matrices treated. Moreover, authors found that the addition of nisin after HP induces higher *Bacillus coagulans* spores inactivation than nisin addition only during HP (Roberts & Hoover, 1996; Stewart, Dunne, Sikes, &

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Hoover, 2000). Therefore, if the combination of nisin with HP is efficient to improve the inactivation of Gram-positive bacteria, it also permits the inactivation of nisin-resistant Gram-negative bacteria and spores.

Because nisin was shown to be stable at high temperature, it is generally assumed that HP have also no effect on the antimicrobial activity of nisin (Davies et al., 1998). However, until now, no study can be found on this subject. The structure of nisin is responsible for its antimicrobial activity, as a lanthionine ring on its N-terminus directly interacts with the bacterial cell membrane (Hsu et al., 2004). It is well known that pressure above 300 MPa can affect the secondary structure of proteins and peptides. Therefore, HP treatment could potentially affect the antimicrobial properties of nisin (Yang & Powers, 2016).

Nisin is the only bacteriocin allowed as a food additive (E234) and can be added at a concentration ranging from 120 to 500 IU/g, depending on the product (EFSA, 2006). Since the HP treatment is increasingly used at an industrial scale, it is important to examine the effect of HP on nisin activity.

In this study, the effect of HP on the antimicrobial activity and the structure of nisin were investigated at two different pH. Nisin was treated by HP at 500 MPa for 10 min at 20 °C in aqueous solutions at pH 2.8 and 6.1. Change in the antimicrobial activity was evaluated using a quantitative agar diffusion method and possible induction of secondary structure changes were investigated by infrared and circular dichroism (CD) spectroscopies.

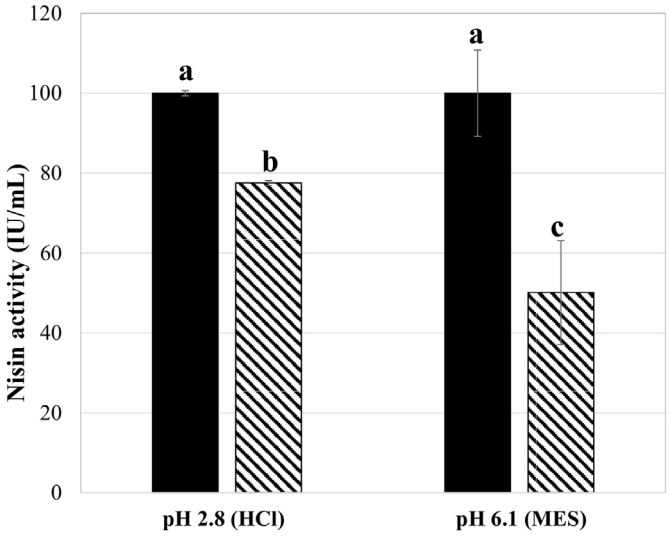

Fig. 1. Effect of high pressure on the antimicrobial activity of nisin. Nisin in solution in 0.02 M HCl (pH 2.8) or 0.1 M MES (pH 6.1) was treated by HP at 500 MPa – 20 °C – 10 min and its antimicrobial activity was determined by quantitative agar diffusion method using *Micrococcus flavus* DSM 1790. Black bars: nisin activity before HP. Stripped bars: nisin activity after HP. Error bars represent SD calculated from independent triplicates. The letters represent a significant difference ($p < 0.05$) obtained with Tukey's HSD (Honest Significant Difference) test.

2. Material and methods

2.1. Effect of HP on the antimicrobial activity of nisin

2.1.1. Preparation of nisin solutions

Nisin stock solutions of 1000 IU/mL were prepared by diluting 0.1 g of nisin powder (nisin at 2.5% in balance sodium chloride and milk sugars, Sigma Aldrich, France) into 80 mL of 0.02 M HCl at pH 2.8 (Sigma-Aldrich, France) or into 80 mL of 0.1 M 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer at pH 6.1(Sigma-Aldrich, France). The stock solutions in MES buffer and HCl were centrifuged 5 min at 11,200*g* - 25 °C (Eppendorf 5810 R, Montesson, France) and the supernatants were 10-fold diluted in 0.1 M MES or 0.02 M HCl, respectively. Nisin solutions of 100 IU/mL in MES or HCL were thus obtained.

A volume of 0.5 mL of each nisin solution was heat-sealed into polyethylene pouches (polyethylene transfer pipet, Dominique Dutscher) for the HP treatment.

The protein purity of the nisin powder used in this study was previously checked by denaturing gel electrophoresis on 8 to 16% acrylamide gel (Novex™ 8–16% Tris-Glycine Mini Gels, WedgeWell™ format, 12-wells) performed in Tris-SDS buffer (Sigma Aldrich, France).

2.1.2. HP treatment

HP tests were performed in a Top Industrie 700 MPa vessel (France) with a double-walled metal pressure chamber of 20 cm^3. Water was used as the pressure-transmission fluid. The internal temperature of the pressure chamber was maintained by a water bath (Minisat 240, Huber, Germany) connected to the double wall of the pressure chamber and monitored by a Pt100 thermocouple (Omega, USA). The temperature of the water bath was set in order to limit the adiabatic heating to 5 °C above the desired temperature set point. The pressure and the temperature during the HP treatment were recorded using instruNet World (iW) software. The compression rate was 3 MPa/s and the decompression was nearly instantaneous (< 3 s).

Nisin pouches were placed in HP vessel 5 min before treatment in order to let the temperature equilibrate and treated at 500 MPa for 10 min at 20 °C.

2.1.3. Estimation of the antimicrobial activity of a nisin solution

The activity of HP treated samples was determined by a quantitative agar diffusion method using *Micrococcus flavus* DSM 1790 as a nisin-sensitive strain, as described by Tramer and Fowler (1964). Briefly, standard curves of nisin (0–100 IU/mL) were plotted using stock solutions of 1000 IU nisin/mL prepared by diluting nisin powder into 0.1 M MES or 0.02 M HCl as described in the Section 2.1.1. The stock solutions in HCl and MES were filtered at 0.2 µm (filter: cellulose acetate, Sartorius, France) and serially diluted in sterile HCl or MES, respectively. The diluted solutions were randomized into 9 mm holes bored into an assay medium previously inoculated with *M. flavus* DSM 1790. After 48 h of incubation at 30 °C, the diameters of the inhibition-zones were measured. The nisin concentrations were then plotted against the inhibition-zone diameters. The following standard curves were obtained (with D corresponding to diameter of the inhibition-zone (mm) and [Nisin] to the nisin activity in IU/mL):

Standard curve for nisin in solution in 0.1 M MES:

$[Nisin]_{MES} = 18.02\ (D - 9)$; $R^2 = 0{,}97$.

Standard curve for nisin in solution in 0.02 N HCl:

$[Nisin]_{HCl} = 21.79\ (D - 9)$; $R^2 = 0{,}97$.

These equations are only valid for the concentration range 0–100 IU/mL, where the relation between the inhibition-zone diameter and the nisin concentration is linear.

The activity of HP-treated nisin samples was determined in the same conditions as the standard solutions. The average of three inhibition-zone diameters for each sample was used to calculate nisin concentrations using calibration curves.

2.2. Effect of HP on the secondary structure of nisin

2.2.1. Circular dichroism spectroscopy (CD)

Nisin solutions at 1.2 g/L were prepared by diluting nisin powder (Sigma Aldrich, France) in 0.02 M HCl or 0.1 M MES. Solutions were dialyzed against the same diluents (MES or HCl) with Pur-A-Lyzer™ Mega 1000 dialysis kit (1 kDa molecular weight cutoff) (Sigma-Aldrich, France). A volume of 0.5 mL of the dialyzed nisin solutions was heat-sealed into polyethylene pouches and treated by HP as described in Section 2.1.2. After treatment, solutions were centrifuged 5 min at 11,200*g* - 25 °C (Eppendorf 5810 R, Montesson, France) and the supernatants were analyzed by CD.

CD spectra were recorded between 185 and 260 nm for each of the