**Title: Synergy between the phage endolysin LysH5 and nisin to kill *Staphylococcus aureus* in pasteurized milk**

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**Abstract**

Phage-encoded endolysins are recently considered as new biocontrol tools to inhibit pathogens in food. In this work, we have studied the ionic requirements for optimal lytic activity of LysH5, the endolysin encoded by the staphylococcal bacteriophage phi-SauS-IPLA88. LysH5 activity was inhibited by the presence of $Mn^{++}$ and $Zn^{++}$ and enhanced by $Ca^{++}$, $Mg^{++}$ and NaCl. When LysH5 was combined with nisin, a bacteriocin currently used as a biopreservative in food, a strong synergistic effect was observed. The Minimum Inhibitory Concentrations of nisin and LysH5 were reduced 64- and 16-fold, respectively, as determined in checkerboard microtitre tests. In addition, nisin enhanced 8-fold the lytic activity of LysH5 on cell suspensions. The synergy observed *in vitro* was confirmed in challenge assays in pasteurized milk contaminated with *S. aureus* Sa9. Clearance of the pathogen was only achieved by the combined activity of both antimicrobials. As far as we know, this is the first study that exploits the possibilities of hurdle technology combining a phage-encoded endolysin and the bacteriocin nisin for efficient *S. aureus* inhibition in milk.

**Keywords:** endolysin, natural antimicrobials, dairy products, biopreservation.

## 1. Introduction

*Staphylococcus aureus* is a major human pathogen that causes a wide range of diseases including food poisoning due to the production of enterotoxins (Le Loir et al., 2003). Milk and dairy products are often implicated in staphylococcal outbreaks (De Buyser et al., 2001; Delbes et al., 2006). During the elaboration of dairy products, contamination by *S. aureus* may come from several sources including raw milk (notably milk from mastitic cows), biofilms in the processing plant environment as well as healthy human carriers.

Nisin, a bacteriocin produced by some strains of *Lactococcus lactis*, is used in more than 40 countries as a natural preservative in foods including dairy products (Gálvez et al., 2007). The combination of this bacteriocin with heat and non-thermal treatments, such as high pressure, pulsed electric fields and other antimicrobials has been also approached (Sobrino-López and Martín-Belloso, 2008). However, many reports have suggested that the ultimate failure of bacteriocin-based preservation systems may be due to the eventual growth of nisin-resistant strains that could compromise its use to control food-borne pathogens such as *Listeria monocytogenes* (Gravesen et al., 2002), *Clostridium botulinum* (Mazzotta et al., 1997), and *S. aureus* (Peschel et al., 1999).

Bacteriophage lytic enzymes have also attracted considerable interest as novel antimicrobials, mostly against gram-positive bacteria, and have been used for controlling bacterial infections and preventing pathogen colonization of mucosal membranes (Fischetti, 2008). Mixtures of endolysins with other antimicrobial agents have been previously assayed. Synergy between lysostaphin and the lysK endolysin against *S. aureus* has been reported (Becker et al., 2008). Similarly, the phage lytic

enzyme Cpl-1 acted synergistically with gentamycin, penicillin and with the phage endolysin Pal against several penicillin-resistant and sensitive *Streptococcus pneumoniae* strains (Loeffler and Fischetti, 2003; Djurkovic et al., 2005). On the contrary, there are hardly any reports on the combined activity of phage endolysins with food preservatives or other preservation technologies. Very recently, it has been shown that the endolysins KZ144 and EL188 sensitize *Pseudomonas aeruginosa* to high hydrostatic pressure (Briers et al., 2008)

We have previously identified and characterized the endolysin LysH5 encoded by the *S. aureus* phage phi-SauS-IPLA88. Bioinformatic analysis of the LysH5 protein sequence revealed three putative domains, a cysteine, histidine-dependent amidohydrolase/peptidase (CHAP) domain, an amidase-2 domain, and a C-terminal SH3b cell wall-binding domain. LysH5 lysed a wide range of staphylococci including bovine and human *S. aureus* and *Staphylococcus epidermidis* and it also inhibited *S. aureus* growth in milk (Obeso et al., 2008). In this work, we have analysed the ionic requirements for LysH5 activity and evaluated possible synergistic effects with the bacteriocin nisin in order to explore new biopreservation strategies based on hurdle technology to effectively inhibit *S. aureus* in milk.

## 2. Materials and Methods

**2.1. Bacterial strains and growth conditions.** *S. aureus* Sa9 was used as the indicator strain for endolysin activity. The organism was grown in 2xYT broth (Sambrook et al., 1989) at 37 °C for 18 h with vigorous shaking. *E. coli* BL21(DE3)/pLys containing the pRSETB-lysH5 plasmid was used to overexproduced the endolysin LysH5 (Obeso et al., 2008).

**2.2. LysH5 purification.** Exponentially cultures ($OD_{600}$ nm of 0.6-0.8) growing in shaking flasks at 37 °C were induced with 1 mM IPTG (isopropyl-beta-D-thiogalactopyranoside). Three hours after induction, cells were pelleted, washed with 20 mM $NaH_2PO_4$, pH 7.4 and frozen at -20 °C. For protein purification, 500 ml culture cell pellets were resuspended in 40 ml of cell resuspension buffer (iFOLD Protein Refolding System 2, Novagen, Madison, USA) and sonicated (15×5 s pulses with 15 s recovery on ice) following the manufacturer's instructions. Inclusion bodies containing LysH5 were obtained and stored at -80 °C. They were further desnaturalized in iFold Guanidine desnaturalization buffer and folding of the protein was monitored in the iFold protein refolding matrix that includes 96 buffer conditions. The refolded protein was added to 0.5 ml Ni-NTA (nickel matrix) slurry and eluted according to the manufacturer's instructions (Qiagen, Valencia, CA). Fractions containing LysH5 were dialyzed against 20 mM $NaH_2PO_4$ buffer, pH 7.0, diluted in glycerol (50% final concentration), and stored at -80 °C. Purity of each preparation was determined in 15% (w/v) SDS-PAGE gels. Electrophoresis was conducted in Tris-Glycine buffer at 20 mA for 1 h in the BioRad Mini-Protean gel apparatus. Protein was quantified by the Quick Start Bradford Protein Assay (BioRad, Hercules, CA).

**2.3. Turbidity reduction assays.** The turbidity assay was performed in a Microplate Spectrophotometer Benchmark Plus (BioRad, Hercules, CA) as previously described (Obeso et al., 2008). The enzymatic activity of LysH5 was determined in salt buffers composed of 50 mM phosphate buffer, pH 7.0 with NaCl ranging from 0 to 500 mM. Activity was also assayed in the presence of several cations ($CaCl_2$, $MgCl_2$, $MnCl_2$ and $ZnCl_2$) at concentrations ranging from 0 to 1 mM.

**2.4. Checkerboard microtiter tests.** Susceptibilities to nisin and the endolysin, and the putative synergism between both antimicrobials, were determined by microdilution in microtiter plates as previously described (Martínez et al., 2008). Minimum inhibitory concentrations (MICs) of nisin and endolysin were defined as the lowest concentration at which growth of the indicator organism was totally inhibited after 18 h incubation at 37 °C. To calculate MICs, two-fold serial dilutions of nisin from 3 μg/ml to 0.002 μg/ml and endolysin from 50 U/ml to 0.78 U/ml were used. To determine the synergistic effect between nisin and endolysin a checkerboard test (White et al., 1996) was carried out. Wells containing nisin (0.75 μg/ml to 0.00075 μg/ml) were combined with LysH5 (50 U/ml to 0.78 U/ml). In this test, up to 45 different combinations of nisin and the endolysin were tested. Controls with each nisin or endolysin concentration were also included separately. The fractional inhibitory concentration (FIC) was calculated as the MIC of the antimicrobial in combination divided by the MIC of the antimicrobial acting alone. Strong synergy exists if the sum of the two FICs [$\sum FIC = FIC_A + FIC_B$] is <0.5 (Hall et al., 1983). All the experiments were performed in duplicate.

A similar checkerboard test was performed with *S. aureus* Sa9 suspensions in 50 mM phosphate buffer, pH 7.0, to a final $OD_{600\ nm}$ of 1.5 as described in section 2.3. Minimum lysis concentrations (MLCs) of nisin and endolysin individually and in combination were determined. MLCs were defined as the lowest concentration at which total cell lysis was obtained after 15 min of incubation at 37 °C. The fractional inhibitory concentration (FIC) was calculated as described above. In this case, wells containing nisin (6 μg/ml to 0.005 μg/ml) were combined with endolysin (5 U/ml to 0.078 U/ml).

**2.5. Challenge tests in pasteurized milk.** Commercial pasteurized milk was inoculated with $10^2$ cfu/ml and $10^5$ cfu/ml of *S. aureus* Sa9. Immediately after, nisin (0.37 μg/ml and 0.75 μg/ml), endolysin (7.5 U/ml and 15 U/ml) and a mixture of both, were also added. The milk was incubated at 37 °C without shaking and samples were taken at various times. Survival of *S. aureus* was determined by plating decimal dilutions on plates of Baird–Parker selective agar (Scharlab, Barcelona, Spain) which were incubated at 37 °C for 24 h.

## 3. Results and Discussion.

**3.1. Improved purification and lytic activity of the endolysin LysH5.** Previous attempts to purify the endolysin LysH5 after overexpression of *E. coli* BL21(DE3)/pLys cultures containing pRSETB-lysH5 (Obeso et al., 2008) gave low yields owing to the poor solubility of the protein. Therefore, we proceeded to improve the recovery of LysH5 from inclusion bodies and subsequent refolding. Using the iFOLD Protein Refolding System 2, the highest solubility was obtained by refolding in buffer A: CHES 50 mM, PEG 3350 0.06%, $CaCl_2$ 0.25 mM, $MnCl_2$ 0.25 mM, $ZnCl_2$ 0.25 mM, TCEP 1 mM, pH 9. In this way, the purification yield was 128 Units per ml of induced *E. coli* culture, which means an increase of 35 times compared to our previous reports (Obeso et al., 2008).

To assess the optimal conditions for LysH5 activity, turbidity reduction assays with *S. aureus* Sa9 cells were carried out using 4 μg of LysH5 (10 U/ml, final concentration). Several concentrations of NaCl and the presence of other cations ($CaCl_2$, $MgCl_2$, $MnCl_2$ and $ZnCl_2$) were tested (Fig. 1). LysH5 activity was clearly inhibited by the presence of $ZnCl_2$ and $MnCl_2$, even at low concentrations (Fig. 1A). On the contrary,

$MgCl_2$ and $CaCl_2$ clearly enhanced activity of LysH5. A similar requirement of $Ca^{2+}$ (2-3 mM) was described for the *S. aureus* bacteriophage phi11 endolysin (Donovan et al., 2006). It has also been suggested that divalent cations may be associated with the pure enzymes and be required for activity and/or structural stability of peptidoglycan hydrolases (Llull et al., 2006). These $Ca^{2+}$ values are in accordance with the calcium content in milk and, thereby, may promote the lytic activity of these proteins in this food matrix. The endolysin LysH5 showed higher activity in the presence of any NaCl concentration tested with maximal activity at concentrations close to 100 mM (Fig. 1B). According to these results, the optimal buffer conditions for the turbidity reduction assays were defined as: phosphate buffer 50 mM, $CaCl_2$ 1 mM, $MgCl_2$ 1 mM, NaCl 100 mM, pH 7.0.

**3.2. *In vitro* synergy between nisin and LysH5.** Initially, minimum inhibitory concentrations (MICs) of nisin and the endolysin were determined by using exponentially growing cultures of *S. aureus* Sa9. The MICs of nisin and LysH5 were 3 μg/ml and 50 U/ml, respectively (data not shown). When both nisin and the endolysin were combined in the checkerboard microtiter test, a synergistic effect was observed. In the presence of subinhibitory concentrations of nisin, a lower endolysin concentration was needed to fully inhibit *S. aureus* Sa9 growth (Fig. 2A). Thus, growth inhibition by the two antimicrobials in combination was greater than either alone. From the checkerboard test, it could be concluded that the most effective conditions to inhibit bacterial growth were 0.75 μg/ml and 3.1 U/ml, or 0.045 μg/ml and 12.5 U/ml for nisin and LysH5, respectively. These values implied up to a 64-fold and 16-fold reduction of the nisin and endolysin MICs, respectively, when used in combination. The average

∑FIC was 0.155±0.06 (*n*=3) highlighting the strong synergy between both antimicrobials.

Many approaches to enhancing the antagonistic activity of nisin and expand its range of application have been tried. Nisin has been found to act synergistically with various antimicrobials including chelators (Fang and Tsai, 2003), small molecular weight substances from plants (Ettayebi et al., 2000), reuterin (Arqués et al., 2004), proteins such as lysozyme and lactoferrin (Branen and Davidson, 2004) and milk-derived peptides (López-Expósito et al., 2008). The positive interaction between the endolysin LysH5 and nisin could be due to a better access to their respective cleavage and binding site promoted by the other compound, or based on an enhanced peptidoglycan hydrolase activity through the dissipation of the membrane proton motive force by nisin. It has been shown that nisin triggered activity of the endolysin Lys44 by mimicking the holin disruption of the cytoplasmic membrane electrical and chemical gradients (Nascimiento et al., 2008).

To get a deeper insight into the synergy between nisin and LysH5, a checkerboard test was performed using metabolic arrested cells in buffer. These are the conditions which are routinely assayed to measure peptidoglycan hydrolytic activities. Under these conditions, the minimum lysis concentration (MLC) of LysH5 was 2.5 U/ml while nisin (more than 6 μg/ml) did not lyse the cells (data not shown). In this assay, the effective LysH5 concentration was lower than that needed to inhibit *S. aureus* growth in the MIC assay, showing than cell suspensions are more sensitive to the endolysin action than exponentially growing cells. It is possible to speculate that the higher sensitivity of *S. aureus* cell suspensions might be due to a lower peptidoglycan turnover in these metabolic arrested cells which would be unable to counteract the peptidoglycan breaks generated by LysH5. Nevertheless, the endolysin MLC against *S.*

*aureus* suspensions could be even reduced in the presence of nisin (Fig. 2B). The most effective conditions to properly lyse *S. aureus* Sa9 bacterial suspensions identified in this assay were 1.5 μg/ml and 0.3 U/ml or 0.180 μg/ml and 1.25 U/ml of nisin and endolysin, respectively. The ∑FIC for *S. aureus* suspensions was 0.077± 0.01 indicating again a strong synergistic activity where, the presence of nisin enhanced LysH5 activity up to 8-fold. These results further support that LysH5 activity might be increased by the permeabilization of the cytoplasmic membrane by nisin as described for the endolysin Lys44 (Nascimiento et al., 2008). Also, partial activation of autolysins by nisin may occur and facilitate LysH5 activity (Bierbaum and Sahl, 1985)**.** Nonetheless, the basis of the synergy between nisin and LysH5 deserves further investigation.

**3.3. Enhanced inhibition of *S. aureus* growth in milk.** In view of the synergy between nisin and LysH5 *in vitro*, we proceeded to verify if a combination of both antimicrobials would be more effective to inhibit *S. aureus* growth in milk. Challenge assays in pasteurized milk were carried out using two levels of *S. aureus* Sa9 contamination ($10^2$ cfu/ml and $10^5$ cfu/ml). Nisin (0.37 μg/ml and 0.75 μg/ml), endolysin LysH5 (7.5 U/ml and 15 U/ml) and a mixture of both were tested. The addition of LysH5 had only a slight inhibitory effect on *S. aureus* growth regardless the contamination level (Fig. 3). On the contrary, the addition of nisin inhibited *S. aureus* multiplication in milk and kept the initial cell counts constant (Fig. 3). However, a complete clearance of the pathogen was only obtained in the presence of the mixture (LysH5 and nisin) after 6 h of incubation. The inhibitory effect of both antimicrobials was already noticeable after 4 h and the counts were 4 and 6 unit-log below the control culture at both low (Fig. 3A) and higher (Fig. 3B) contamination levels, respectively.

As anticipated by the *in vitro* results, the mixture was also more efficient in a food matrix than each antimicrobial alone. A similar synergistic effect between nisin and bacteriophages against *S. aureus* in milk was previously described in short-time challenge tests (Martínez et al., 2008). However, bacteriophage cross-resistance arose in nisin-adapted mutants. This seems not to be the case with the endolysin LysH5 as these nisin mutants were still sensitive to LysH5 (data not shown). Another advantage over bacteriophages is that the combination of LysH5 and nisin was also successful at low pathogen concentration, opposite to the bacteriophages that require a minimum host threshold to be effective (Cairns et al., 2009). In conclusion, our results support the use of phage endolysins as non-traditional food preservatives in combination with the commonly used preservative nisin to inhibit pathogens in food matrices more efficiently. Further studies to determine endolysin stability under food processing conditions, as well as oral toxicity studies are in progress.

## 4. Acknowledgments

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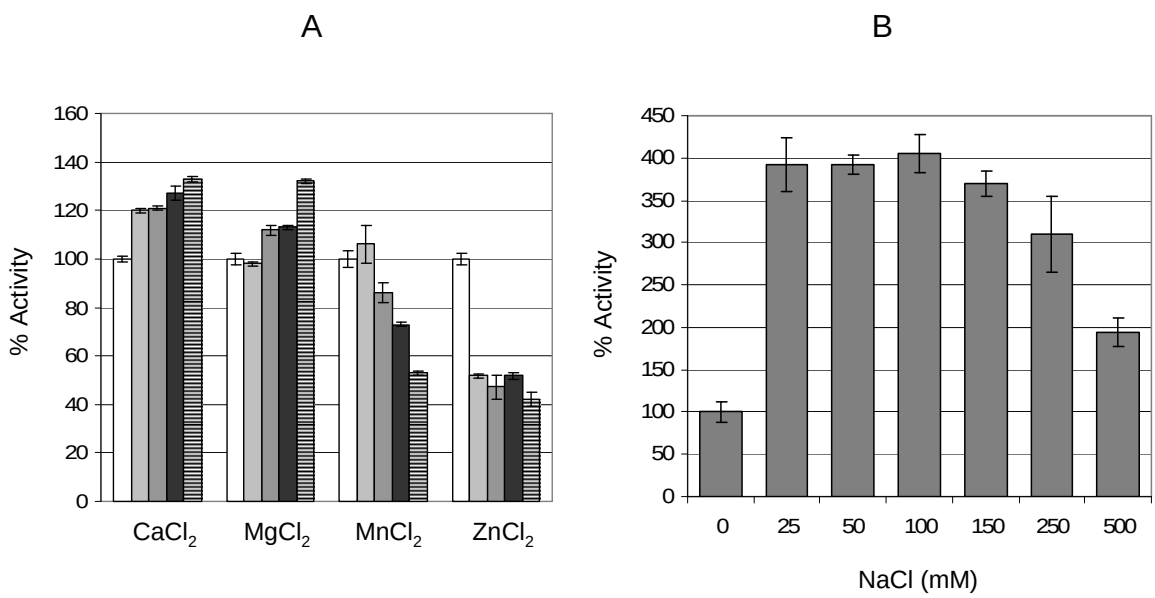
## 6. Figures

**Figure 1.** Optimization of turbidity reduction assay conditions for His-tagged LysH5 in the presence of several cations (A) and NaCl (B). (A) Cations were added to the reaction at 0.25 mM (light grey bars), 0.5 mM (dark grey bars), 0.75 mM (black bars), and 1 mM (striped bars). The lytic activity in phosphate buffer 50 mM, pH 7.0 without cations and NaCl was taken as 100% (white bars). Error bars represent standard deviation of three independent measurements.

**Figure 2**. Minimum Inhibitory Concentration (MIC) and Minimum Lysis Concentration (MLC) of the endolysin LysH5 in the presence of subinhibitory concentrations of nisin. A) *S. aureus* Sa9 culture. The MIC of nisin for *S. aureus* Sa9 was 3 μg/ml in these assay conditions. B) Turbidity reduction assay. The MLC of nisin for *S. aureus* Sa9 was > 6 μg/ml in these assay conditions.

**Figure 3.** Killing of *S. aureus* Sa9 with purified LysH5 and nisin in pasteurized whole milk. A) ♦, cell numbers of *S. aureus* Sa9; ■, LysH5 (7.5 U/ml); □, nisin (0.37 μg/ml); ▲, LysH5 (7.5 U/ml) and nisin (0.37 μg/ml). B) ♦, cell numbers of *S. aureus* Sa9; ■, LysH5 (15 U/ml); □, nisin (0.75 μg/ml); ▲, LysH5 (15 U/ml) and nisin (0.75 μg/ml). Values are the means of two independent experiments with standard deviation indicated by vertical bars.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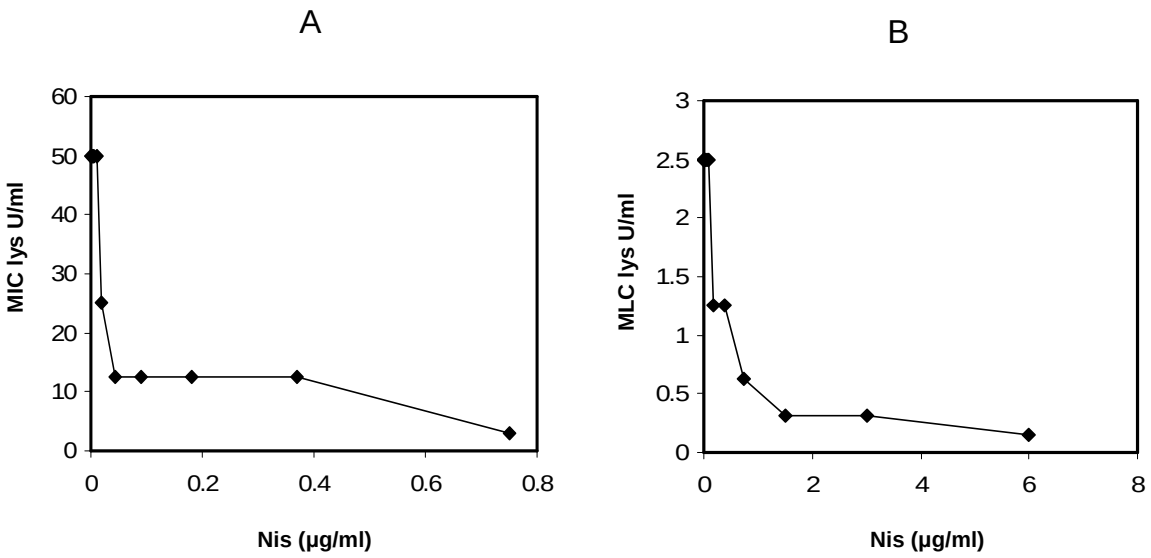
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