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# Hen Egg White Lysozyme Permeabilizes *Escherichia coli* Outer and Inner Membranes

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**ABSTRACT:** Natural preservatives answer the consumer demand for long shelf life foods, synthetic molecules being perceived as a health risk. Lysozyme is already used because of its muramidase activity against Gram-positive bacteria. It is also described as active against some Gram-negative bacteria; membrane disruption would be involved, but the mechanism remains unknown. In this study, a spectrophotometric method using the mutant *Escherichia coli* ML-35p has been adapted to investigate membrane disruption by lysozyme for long durations. Lysozyme rapidly increases the permeability of the outer membrane of *E. coli* due to large size pore formation. A direct delayed activity of lysozyme against the inner membrane is also demonstrated, but without evidence of perforations.

**KEYWORDS:** *lysozyme, antimicrobial activity, membrane permeability, Gram-negative bacteria*

## INTRODUCTION

Food additives, including preservatives, worry 66% of the European consumers, and synthetic additives are perceived as more dangerous than natural additives.[1,2] On the other hand, consumers demand safer food products, with a long shelf life. Research for novel, natural food preservatives is thus stimulated, and biological resources are therefore widely screened. Special attention is given to those molecules that have a wide antimicrobial spectrum. Peptides and proteins are considered as potential candidates. One of the natural antimicrobial proteins widely studied is hen egg white lysozyme (HEWL). HEWL is well-known for its muramidase activity against Gram-positive bacteria. It is therefore used as a food additive (E1105) to control Gram-positive spoilers in winemaking and cheese refining.[3−5] Several studies suggest that HEWL also acts against some Gram-negative bacteria; mechanisms such as perturbation of DNA or RNA synthesis and membrane permeabilization would be responsible for lysozyme activity against these micro-organisms.[3,6−8] The efficacy of lysozyme against Gram-negative bacteria can be increased by modifying the protein by proteolysis to obtain small active peptides,[9−11] by fusion of chemical moieties,[12−16] or by heat-denaturation.[17−20]

Membrane disruption is a major mechanism by which antibacterial peptides and proteins act on both Gram-negative and Gram-positive bacteria. The cationic and amphipathic character of most of these peptides and proteins suggests electrostatic and hydrophobic interactions with the bacterial cell wall. Such interactions could disturb the bacterial membrane, leading to bacterial cell death, or translocation of the peptide or protein into the cytoplasm, where it interacts with intracellular targets.[21,22]

Membrane permeabilization is an attractive hypothesis to explain lysozyme activity against Gram-negative bacteria, considering the recent discovery of lysozyme inhibitors in the periplasm of species such as *E. coli*.[23,24] Gram-negative bacteria are naturally protected against lysozyme by the outer membrane, a physical barrier preventing entrance into the cell of molecules bigger than 650 Da.[25] However, the presence of periplasmic lysozyme inhibitors in some Gram-negative bacteria suggests that lysozyme is able to cross their outer membrane. Yet, very little is known about the membrane activity of lysozyme. Especially, there is no experimental evidence of the capability of lysozyme to directly act, or not, on the inner membrane of Gram-negative bacteria.

Different techniques have been used to detect bacterial membrane permeabilization, such as detection of potassium leakage, LPS monitoring, NPN uptake, $DiSC_3$ uptake, or atomic force microscopy (AFM).[26−33] One of the most popular and simple assays is a spectrophotometric method using *Escherichia coli* ML-35p. This method was first described by Lehrer in 1988 and was later optimized for smaller sample volumes by Epand in 2010.[32,33] It has been used to detect membrane activity of antibacterial peptides such as cecropin A, melittin, ceragenins, Cg-BPI, and indolicidin and of antibacterial proteins such as human defensins and HEWL.[32−35] *E. coli* ML-35p is constitutive for $\beta$-galactosidase expressed in the cytoplasm and produces a $\beta$-lactamase in the periplasm, which is encoded on a plasmid (pBR322). This bacterial strain also lacks lactose permease.[32,33] The measurement of $\beta$-lactamase and $\beta$-galactosidase activities thus enables the detection of outer and inner membrane permeabilization, respectively (Figure 1).

In the literature, membrane permeability has been measured using the *E. coli* ML-35p mutant with a contact time never

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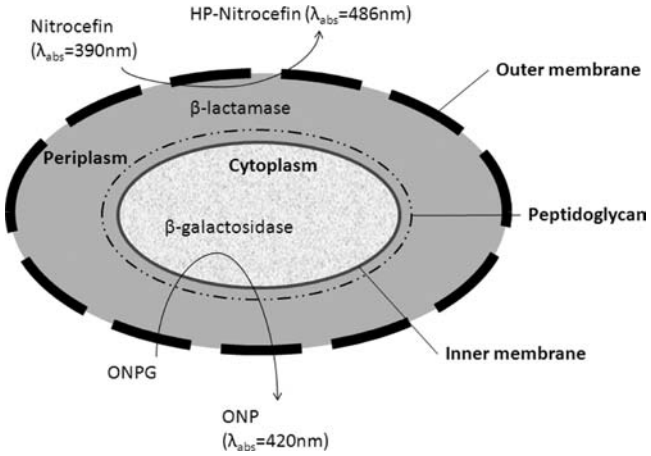


**Figure 1.** Schematic representation of *E. coli* ML-35p cell, with locating *β*-lactamase and *β*-galactosidase. Both enzymes become accessible to their substrates (nitrocefin and ONPG, respectively) when outer and inner membrane permeabilization occurs; the respective products HP-nitrocefin ($\lambda_{abs}$ = 486 nm) and ONP ($\lambda_{abs}$ = 420 nm) are then released.

exceeding 2 h between the studied antimicrobial agent and the bacterial cells. However, it is imaginable that membrane permeabilization could sometimes result from slow phenomena, especially when proteins are concerned. Indeed, the mechanisms described to explain the antimicrobial activity of peptides and binding of lysozyme upon phospolipid bilayers generally suppose conformational modifications.[21,36] Then, protein structure changes at the water/lipid interface could be slow events, because of the higher molecular mass and rigidity of proteins, compared to small and flexible antibacterial peptides. Especially, HEWL is known as a highly structured and stable protein consisting of 129 amino acids cross-linked by 4 disulfide bridges.[37] In the present study, Lehrer's protocol has thus been modified for long duration experiments to investigate membrane perturbation by lysozyme. Improvements were necessary to circumvent inconveniences such as evaporation, sedimentation, and signal instability.

## MATERIALS AND METHODS

If not stated otherwise, chemicals were obtained from Sigma-Aldrich (Saint-Quentin, France).

**Bacterial Strains.** The bacterial strain *E. coli* ML-35p, which is lactose permease deficient and expresses *β*-lactamase and *β*-galactosidase in the periplasm and cytoplasm, respectively, was kindly provided by Destoumieux-Garzon, initially supplied by Lehrer. *E. coli* ML-35p was grown overnight (18 h) in TSB (AES, Bruz, France) with 50 *μ*g/mL ampicillin at 37 °C under stirring (130 rpm). The bacterial culture was washed twice in Tris-HCl buffer (50 mM, pH 7.0). The absorbance of the final suspension was around 1.0 at 620 nm, corresponding to about $10^9$ CFU/mL. The $10^8$ and $10^7$ CFU/mL solutions were prepared by appropriate 10-fold dilutions of the previous culture.

**Signal Stability of the Substrates and Products of *β*-Lactamase and *β*-Galactosidase.** To evaluate the signal stability of ONPG, ONP, and nitrocefin (Merck Chemicals, Darmstadt, Germany), solutions of 1, 0.07, and 0.015 g/L of these three reagents, respectively, were prepared in 50 mM Tris-HCl buffer, pH 7.0. The hydrolysis product of nitrocefin (HP-nitrocefin) was produced by the enzymatic reaction (1 h, 25 °C) between nitrocefin (0.015 g/L) and penicillinase from *Bacillus cereus* (3.5 g/L) in 50 mM Tris-HCl buffer, pH 7.0. Three hundred microliter aliquots of each solution (ONPG, ONP, nitrocefin, HP-nitrocefin) were dispensed into microplate wells. The absorbance of the four substances was subsequently measured by a spectrophotometer Spectramax M2 (Molecular Devices, UK) for 10 h, after sealing or not the microplates with a Clear Seal film (4titude, Surrey, UK). The stability of ONPG and ONP was determined by ONP absorbance at 420 nm. The stability of nitrocefin and HP-nitrocefin was determined by absorbance at 390 and 486 nm, respectively.

**Outer and Inner Membrane Permeability.** Melittin from bee venom (85% purity) was used as a positive control in the membrane permeability experiments. Melittin is a small peptide, constituted of 26 amino acids.[38] This peptide is active on biological membranes and shows antimicrobial activity;[37] it is especially known to form pores in bacterial membranes and to permeabilize the outer and inner membranes.[33,39]

Melittin and lysozyme (Liot, Annezin, France) activity against the outer and inner membranes was measured. The sample solutions to measure their activity contained either 0.015 mg/mL melittin or 0.05 mg/mL up to 10 mg/mL of lysozyme. The sample solutions were inoculated with $10^7$ or $10^8$ CFU/mL of *E. coli* ML-35p. A negative control sample consisted of solutions prepared as described above, but without melittin or lysozyme.

To test outer membrane permeability, 0.015 mg/mL nitrocefin (substrate of *β*-lactamase) was added to the sample solutions. When the outer membrane was permeabilized, the periplasmic *β*-lactamase came into contact with its substrate nitrocefin, resulting in HP-nitrocefin release (Figure 1). HP-nitrocefin absorbance was measured at 486 nm, at 37 °C under stirring.

This reaction could result from nitrocefin entrance into the bacteria and/or from *β*-lactamase leakage. To test the assumption of *β*-lactamase leakage in the presence of lysozyme, a 0.25 mg/mL lysozyme solution was inoculated with $10^7$ CFU/mL *E. coli* ML-35p and incubated at 37 °C for 5 h before centrifugation (5000*g*, 10 min); the *β*-lactamase activity was then measured in the supernatant by adding 0.05 mg/mL nitrocefin. HP-nitrocefin absorbance was measured at 486 nm at 25 °C under stirring.

To test inner membrane permeability, 0.7 mg/mL ONPG (substrate of *β*-galactosidase) was added to the sample solutions. When the inner membrane was permeabilized, the cytoplasmic *β*-galactosidase came into contact with its substrate ONPG, resulting in ONP release (Figure 1). ONP absorbance was measured at 420 nm at 37 °C under stirring.

Similarly to what was described above, to test the assumption of *β*-galactosidase leakage from bacteria cells in the presence of lysozyme, a 0.25 mg/mL lysozyme solution was inoculated with $10^7$ CFU/mL *E. coli* ML-35p and incubated at 37 °C for 5 h before centrifugation (5000*g*, 10 min); the *β*-galactosidase activity was then measured in the supernatant by adding 1 mg/mL ONPG. ONP absorbance was measured at 420 nm at 25 °C under stirring.

**Quantification of Membrane Permeabilization.** The absorbance responses versus time, resulting from experiments of outer and inner membrane permeabilization, were analyzed to quantify the antibacterial efficiency of melittin and lysozyme. Absorbance curves for both outer and inner membrane permeabilization are the result of an enzymatic reaction between a substrate and the respective enzyme, which becomes accessible when the membranes are permeabilized.

For outer membrane permeabilization, the maximal slope ($\Delta AU_{486\ nm}$/30 min) was considered to quantify the velocity of the enzymatic reaction. Because the substrate concentration was fixed, this velocity was relied only on the concentration of accessible enzyme; an increase of the accessible enzyme quantity was indicative of a more severe membrane disruption. Then, the higher is the maximal slope, the more intense is the outer membrane permeabilization.

For inner membrane permeabilization, the maximal slope ($\Delta AU_{420\ nm}$/30 min) was considered to quantify the intensity of the inner membrane disruption, in a similar way as described above. Moreover, the lag time before the absorbance signal increase was considered indicative of the delay between outer and inner membrane permeabilization. The lag time was determined as the intersection between the baseline and the tangent of the curve at the maximal slope (Figure 2).

**Statistical Analysis.** All experiments were at least performed in triplicates. Statistical analysis was performed with R 2.15.2. Significance levels were at least 95%. Data from the normal distribution and with equal variances were treated with parametric tests. In this case, for the


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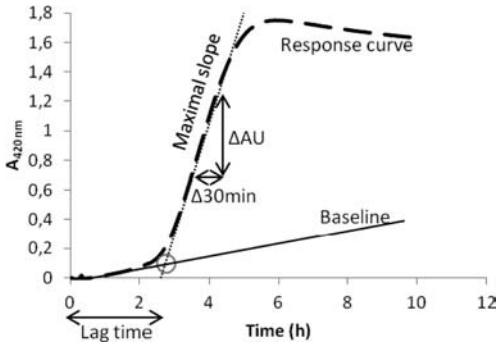


**Figure 2.** Quantification of inner membrane permeabilization using maximal slope and lag time.

comparison of means the Student *t* test was used. Data from other distributions or with unequal variances were treated with nonparametric tests. In this case, for the comparison of means the Wilcoxon rank sum test was used.

## RESULTS

**Preliminary Protocol Improvements for Reliable Measurements of Outer and Inner Membrane Disruption for Long Durations.** Extension of Lehrer's method to long durations implies that substrates and products of *β*-lactamase and *β*-galactosidase are time-stable. Nitrocefin and ONPG absorbances were both stable at 37 °C for durations as long as 10 h (data not shown). On the contrary, the absorbances of HP-nitrocefin (Figure 3A) and ONP (Figure 3B) were not stable.

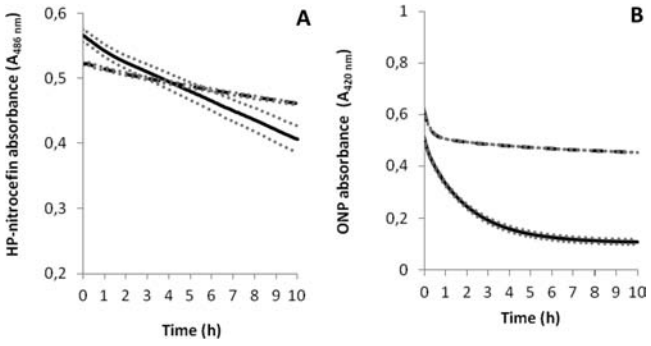


**Figure 3.** Absorbance stability of the reaction products of *β*-lactamase and *β*-galactosidase in nonsealed (full line) and sealed (dashed line) microplates. (A) HP-nitrocefin is detected by absorbance at 486 nm. (B) ONP is detected by absorbance at 420 nm. Standard deviation was calculated from triplicates (gray dotted line). Results were not corrected with a reference measurement, meaning that the absorbance values include the absorbance of the microplate and the buffer solution.

Sealing the microplate improved ONP stability from an 80% decrease to only 26% decrease of absorbance. Similarly, HP-nitrocefin absorbance decreased only 12% under sealed conditions compared to 28% under nonsealed conditions.

To prove the relevance of sealing microplates, melittin has been used as a reference antibacterial agent. When the outer membrane permeabilization was measured by melittin, the maximum absorbance was higher under sealed conditions, compared to nonsealed ones (Figure 4A). Moreover, a higher maximal slope was observed with a sealed microplate (Figure 4C).

When the inner membrane permeabilization was measured (Figure 4B), the absorbance signal corresponding to ONP release was dramatically different, depending on whether the microplate was sealed or not. Especially, during the first 3 h, the slope of the absorbance curve was much higher under sealed conditions. Because of this initial discrepancy, and despite an equivalent maximal slope between 3 and 5 h, the maximum absorbance was lower under nonsealed conditions (Figure 4B).

With regard to the latter results, experiments with lysozyme will be performed only with sealed microplates.

**Lysozyme Activity against Outer and Inner Membranes of *E. coli*.** Lehrer's method was applied to test HEWL activity, including the modifications as described above. The results exhibited that 0.25 mg/mL HEWL disturbed the outer membrane of *E. coli* because *β*-lactamase activity was detectable after around 0.5 h of incubation, whereas no absorbance was measured in the negative control, that is, without lysozyme (Figure 5A). Despite HP-nitrocefin content subsequently increasing in the negative control, it remained much lower than in the presence of 0.25 mg/mL lysozyme, throughout the 10 h experiment. Moreover, the supernatant of the bacterial suspension treated with lysozyme contained *β*-lactamase activity, unlike the supernatant of the negative control (Figure 5C).

During the first 2 h, a slight *β*-galactosidase activity was also measured, but in a similar way in samples with and without lysozyme (Figure 5B). On the contrary, when the experiment was extended to durations as long as 2.7 h and longer, *β*-galactosidase activity was much more extensive in the presence of 0.25 mg/mL HEWL, compared to the negative control (Figure 5B). However, the supernatant of the bacterial suspension treated with lysozyme did not contain *β*-galactosidase activity (data not shown).

**Membrane Permeabilization Depending on Lysozyme Concentration and *E. coli* Inoculum.** When $10^7$ CFU/mL *E. coli* was inoculated, *β*-lactamase activity, that is, outer membrane permeabilization, remained unchanged whatever the lysozyme concentration was, from 0.05 to 10 mg/mL (maximal slope around 0.0025 $\Delta AU_{486\ nm}$/min; Figure 6A). On the contrary, when the inoculum was $10^8$ CFU/mL, the intensity of outer membrane permeabilization increased when lysozyme concentration increased from 0.05 to 0.5 mg/mL; above 0.5 mg/mL HEWL, the intensity of outer membrane permeabilization decreased when the lysozyme concentration increased (Figure 6A).

For both inocula levels, *β*-galactosidase activity, that is, inner membrane permeabilization, increased when lysozyme concentration increased (higher maximal slope; Figure 6B). Moreover, the higher inoculum showed systematically higher maximal slopes. Considering the lag time, when $10^7$ CFU/mL was inoculated, lag time strongly decreased when lysozyme concentration increased (Figure 6C). On the contrary, with $10^8$ CFU/mL inoculum, the lag time first remained stable between 0.05 and 0.5 mg/mL HEWL and then slightly decreased when lysozyme concentration increased over 0.5 mg/mL HEWL (Figure 6C). Lag time was systematically shorter with $10^8$ CFU/mL inoculum compared to $10^7$ CFU/mL.

## DISCUSSION

Membrane permeabilization is a major mechanism involved in the activity of many antimicrobial molecules, especially antimicrobial peptides and proteins.[9,10] Most of the studies aiming to highlight such bacterial membrane disruption are limited to short-time experiments (<2 h). However, it is conceivable that membrane permeabilization could sometimes need more time, especially when proteins are concerned. Indeed,

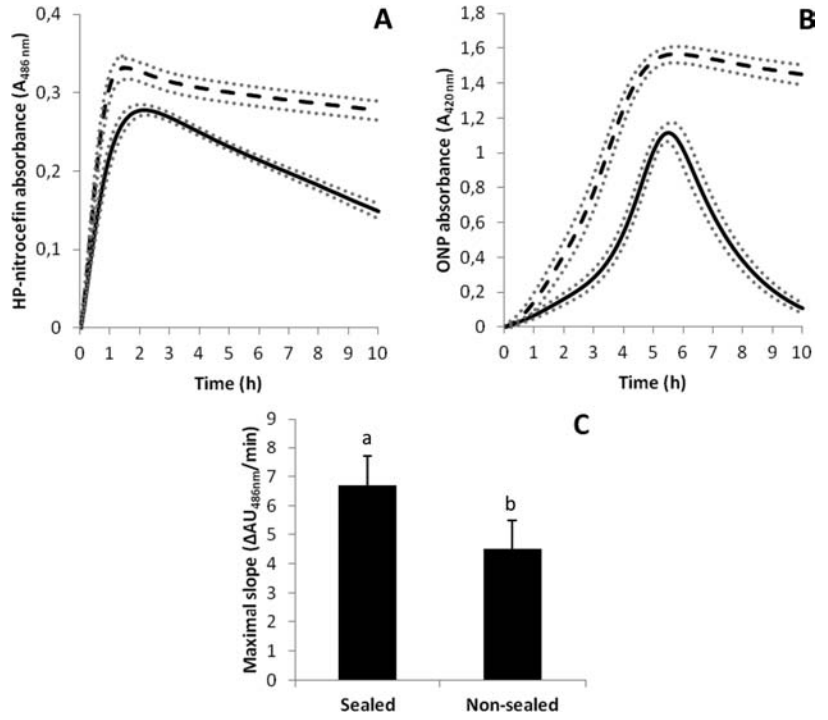


**Figure 4.** Permeabilization of the outer membrane (A) and inner membrane (B) of *E. coli* ML-35p ($10^7$ CFU/mL) by melittin (15 $\mu$g/mL) in nonsealed (full line) and sealed (dashed line) microplates, as evidenced by HP-nitrocefin and ONP release, respectively. Standard deviations were calculated from nine replicates (gray dotted line). Results were corrected with a reference absorbance including the absorbance of the microplate and the buffer solution. (C) Comparison of the maximal slopes for outer membrane permeabilization with and without sealing. Different letters (a, b) indicate significant difference ($p < 0.05$, Student $t$ test).

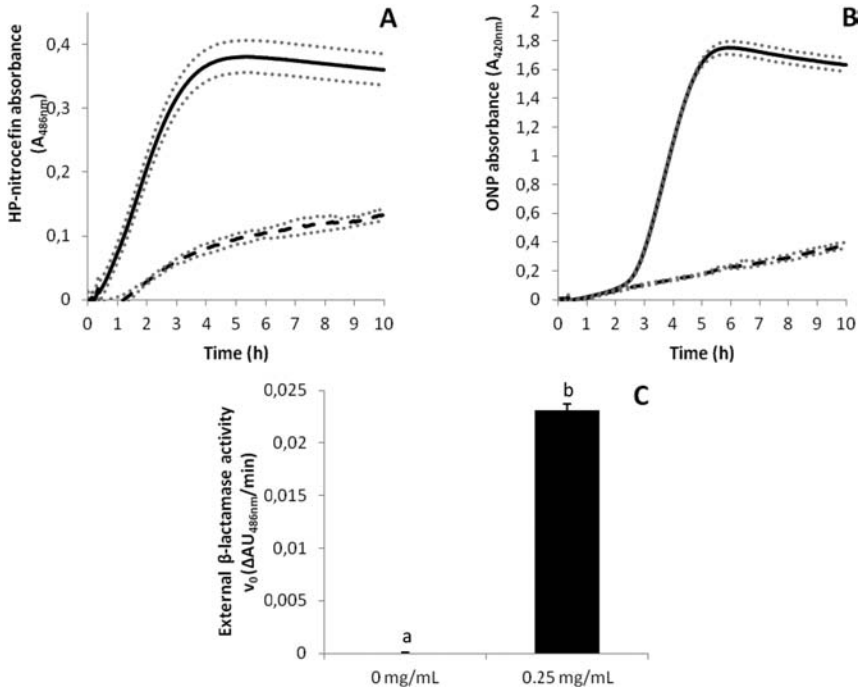


**Figure 5.** Permeabilization of outer membrane (A) and inner membrane (B) of *E. coli* ML-35p ($10^7$ CFU/mL) for 10 h, in the presence of 0.25 mg/mL HEWL (full line), and without lysozyme (dashed line). Standard deviations were calculated from triplicates (gray dotted line). Results were corrected with a reference absorbance including the absorbance of the microplate and buffer solution. (C) Externalization of $\beta$-lactamase in the absence and presence of 0.25 mg/mL of lysozyme measured by supernatant enzyme activity. Results stem from six replicates. Different letters (a,b) indicate significant difference ($p < 0.01$, Wilcoxon rank sum test).

mechanisms described to explain the antimicrobial activity of peptides and the interaction between lysozyme and lipid bilayers generally suppose conformational modifications.[21,36] Then, protein structure changes at the water/cell membrane interface might be slower than with peptides, because proteins are generally much more rigid molecules compared to peptides.


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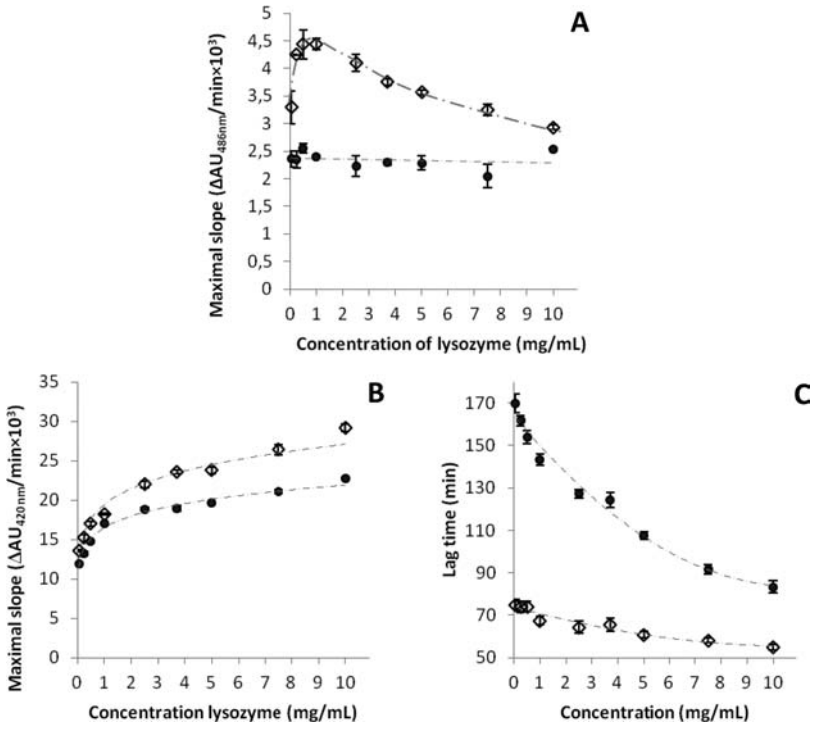


**Figure 6.** Membrane permeabilization as a function of lysozyme concentration for $10^7$ CFU/mL (●) and $10^8$ CFU/mL (◇) inocula: (A) outer membrane permeabilization as evidenced by $\beta$-lactamase activity; inner membrane permeabilization as evidenced by (B) $\beta$-galactosidase activity and (C) lag time between outer and inner membrane permeabilization. Standard deviations were calculated from triplicates.

Especially, HEWL is known as a particularly rigid protein cross-linked by four disulfide bridges.[37] The extension to long durations of the traditional methods to investigate bacterial membrane permeabilization by proteins is then a relevant challenge. The popular and simple spectrophotometric method developed by Lehrer has here been selected for such an adaptation.[32]

**Sealing Microplates Is an Efficient Way To Improve the Reliability of Lehrer's Method for Long Experiments.** To extend Lehrer's method to durations longer than 2 h, the substrates (ONPG and nitrocefin) and the products (ONP and HP-nitrocefin) of both enzymatic reactions need to be stable at 37 °C. This condition was not fulfilled for ONP, even for short-time experiments (Figure 3B), and to a lesser extent for HP-nitrocefin (Figure 3A). ONP, which results from ONPG hydrolysis by $\beta$-galactosidase, turns out to be especially unstable when nonsealed plates are used (Figure 3B). This is likely the result from the high volatility of this compound at 37 °C, because the ONP signal decrease is largely limited with sealed microplates. In these conditions, only a slight decrease is observed at the very beginning of the experiment (Figure 3B), probably due to the partial evaporation of ONP in the gas phase, between the liquid phase and the film, until the gas/liquid equilibrium was reached for this chemical compound. This observation suggests that a minimal headspace between the liquid phase and the film should be preferred; however, it cannot be reduced to zero, because of practical considerations such as sealing difficulty and risk of cross-contamination between adjacent wells. Although much less significant than for ONP, the HP-nitrocefin signal also decreases throughout the 10 h experiment when performed without sealing; this decrease is smaller when microplates are previously sealed (Figure 3A). Sealing microplates as proposed in this study appears then to be an easy and efficient way to improve the reliability of Lehrer's method when time extension up to 10 h is needed. Moreover, sealing avoids cross-contamination between wells, which can happen because of microplate stirring.

When the modified method (sealing microplates) was performed to measure the melittin antibacterial activity against *E. coli*, the results were significantly improved compared to the original method: higher initial rates were measured for both outer and inner membrane permeabilization (Figure 4). This indicates that the technical adjustments proposed in this study solve the underestimation induced by the original protocol. This underestimation is quite moderate for outer membrane permeabilization (Figure 4A), but a huge difference exists for the measurement of the inner membrane permeabilization (Figure 4B). In the latter case, the use of sealed microplates appears absolutely necessary, even for short-time experiments. Indeed, even in the very first moments of the test, because an extensive and quick disappearance of ONP occurs simultaneously with ONP enzymatic release, the initial rate of permeabilization is underestimated by 80% when nonsealed microplates are used.

**HEWL Disrupts Outer and Inner Membranes of *E. coli*.** The method adjustments proposed above enabled the investigation of lysozyme membrane activity for durations as long as 10 h. With such long experiments, the ability of HEWL to permeabilize both outer and inner membranes of *E. coli* was demonstrated. Indeed, both $\beta$-lactamase and $\beta$-galactosidase activities were detected when HEWL (0.25 mg/mL) was added to an *E. coli* culture, as indicated by HP-nitrocefin and ONP release, respectively (Figure 5). The weak absorbance signals obtained with the negative control likely result from the spontaneous lysis of bacteria that occurs during the 10 h experiments. However, both $\beta$-lactamase and $\beta$-galactosidase activities were higher when HEWL was added, undoubtedly proving the membrane permeabilization induced by lysozyme.

Outer membrane permeabilization has already been described by Wild et al. and Pelligrini et al. for a similar HEWL concentration.[7,40] These authors observed the outer membrane permeabilization using electron microscopy and Lehrer's membrane permeabilization assay, respectively. The original Lehrer method enabled this because outer membrane permeabilization occurs after around 30 min, for 0.25 mg/mL lysozyme. However, these authors conclude that no inner membrane permeabilization occurs due to the direct action of HEWL.

The present study highlights that, when Lehrer's method is extended to long durations, HEWL induces inner membrane permeabilization, too, but this is only detectable after 2.7 h of incubation with 0.25 mg/mL HEWL and $10^7$ CFU/mL *E. coli* inoculum. It is then a slow phenomenon, compared to what is usually observed with antibacterial peptides. The delay necessary for the detection of the inner membrane permeabilization could be explained by the succession of hurdles that HEWL has to get over: passing through the outer membrane, peptidoglycan hydrolysis or diffusion through the peptidoglycan network,[41] and finally disturbance of the inner membrane.

To ensure that the inner membrane permeabilization is not the result of cell lysis caused by peptidoglycan disintegration, the presence of $\beta$-galactosidase was investigated in the supernatant of the *E. coli* cells ($10^7$ CFU/mL) treated with 0.25 mg/mL lysozyme (as explained under Outer and Inner Membrane Permeability). $\beta$-Galactosidase would be present in the supernatant when peptidoglycan disintegration and thus cell lysis occur.[42] However, no $\beta$-galactosidase activity could be measured in the supernatant, demonstrating that this enzyme was not leaking out of the cytoplasm; then, there was no cell lysis, and the $\beta$-galactosidase activity detected when *E. coli* cells are in the presence of lysozyme resulted from the diffusion of ONPG into the cell. This confirms that HEWL directly acts on the inner membrane of *E. coli*, modifying its permeability, and independent of the lysozyme enzymatic activity on peptidoglycan.

It is noticeable that, in opposition to $\beta$-galactosidase, $\beta$-lactamase activity was measured in the supernatant of *E. coli* cells treated with 0.25 mg/mL lysozyme (Figure 5C). This proves that HEWL disrupts the outer membrane in such a way that this enzyme of 28.9 kDa can leak out of the periplasm. Large size pores inside the outer membrane are thus induced by HEWL.

**HEWL Acts by a Two-Step Process: Saturation of the Outer Membrane before Entrance into the Cell and Permeabilization of the Inner Membrane.** The extent of the outer membrane permeabilization, quantified by the $\beta$-lactamase activity (Figure 6A), was unchanged between 0.05 and 10 mg/mL HEWL with inoculation of $10^7$ CFU/mL. On the contrary, the outer membrane permeabilization increased when HEWL concentration increased from 0.05 to 0.5 mg/mL with inoculation of $10^8$ CFU/mL. This suggests that a critical ratio for outer membrane saturation should be 0.05 mg/mL HEWL:$10^7$ CFU/mL *E. coli*. Indeed, $\beta$-lactamase activity did not increase when >0.05 mg/mL lysozyme was added to $10^7$ CFU/mL. Moreover, the maximum $\beta$-lactamase activity when $10^8$ CFU/mL was inoculated was reached at a ratio 0.5 mg/mL lysozyme:$10^8$ CFU/mL, that is, the same ratio as 0.05 mg/mL HEWL:$10^7$ CFU/mL.

As far as the inner membrane is concerned, a dose−response effect occurred for 0.05−10 mg/mL HEWL at both *E. coli* inocula ($10^7$ and $10^8$ CFU/mL). The extent of the inner membrane permeabilization increased when HEWL concentration increased (Figure 6B). The maximal slopes obtained with $10^8$ CFU/mL inoculum were systematically higher than those obtained with $10^7$ CFU/mL. This is consistent with the higher quantity of $\beta$-galactosidase potentially accessible to ONPG when the bacterial inoculum was higher. Simultaneously, the lag time decreased when the HEWL concentration increased, for both *E. coli* inocula (Figure 6C). Because the lag time is the delay needed for the release of detectable quantities of ONP, a lag time decrease indicates a faster increase of ONP concentration, related to a higher $\beta$-galactosidase activity. Therefore, the lag time is consistently shorter with $10^8$ CFU/mL *E. coli* compared to $10^7$ CFU/mL.

When the inoculum was $10^7$ CFU/mL *E. coli*, the lag time regularly and strongly decreased when HEWL increased from 0.05 to 10 mg/mL. This suggests that the higher the HEWL concentration in the bulk, the higher the quantity of HEWL reaching the inner membrane. The lag time decrease is then consistent with the assumption of the outer membrane saturation with HEWL concentration of 0.05 mg/mL or higher and $10^7$ CFU/mL *E. coli*. Indeed, because of such a saturation, each additional HEWL molecule added in the bulk remains "free" (not entrapped into the outer membrane), able to cross over the disrupted outer membrane and to reach the inner membrane.

When $10^8$ CFU/mL *E. coli* was inoculated, the lag time was constant between 0.05 and 0.5 mg/mL HEWL. Because the outer membrane would not be saturated with HEWL molecules in these conditions, as suggested above, each additional HEWL molecule added in the bulk would then be essentially entrapped inside the outer membrane and then unavailable for deeper penetration into the bacteria cell. On the contrary, with HEWL concentrations >0.5 mg/mL, meaning when outer membrane saturation is achieved, the lag time decreased when HEWL concentration increased, similarly to what was observed when $10^7$ CFU/mL *E. coli* was inoculated; in these conditions, each additional HEWL molecule remains "free" and able to enter into the cell. However, even with the highest lysozyme concentration, that is, 10 mg/mL, the lag time remained >50 min; this could be the minimal delay for lysozyme entrance into the cell and interaction with the inner membrane.

**Outer Membrane Permeabilization Is Reduced When *E. coli* Inoculum and HEWL Concentration Are Simultaneously High.** At high inoculum levels, quorum sensing can play a major role in bacterial resistance against antimicrobial agents.[43] Quorum sensing is a cell-to-cell communication between bacteria by excretion of signal molecules, which can be detected by other bacteria of the same or other species.[44] In *E. coli* K12, AI-2 is one of those signal molecules that up-regulates several genes related to the outer membrane such as *rfaY*; *rfaY* controls the LPS-core biosynthesis. Stress induction of AI-2 has been demonstrated due to the addition of glucose, $Fe^{3+}$, NaCl, and dithiothreitol.[45,46] Thus, quorum sensing can be a stress-induced phenomenon.

In the present study, it is noticeable that *E. coli* outer membrane permeabilization decreased when HEWL concentration exceeds 1 mg/mL and when $10^8$ CFU/mL was inoculated, but this was not observed when inoculum was $10^7$ CFU/mL (Figure 6A). The assumption of a lysozyme stress ($\geq$1 mg/mL) could be proposed. This stress could activate quorum sensing between bacterial cells, in a dose-dependent manner. In these conditions, that is, high inoculum and high HEWL concentration, the outer membrane permeabilization of some bacterial cells may induce the expression of signal molecules, which could activate defense mechanisms by the sister cells. These defense mechanisms could include changes in the outer

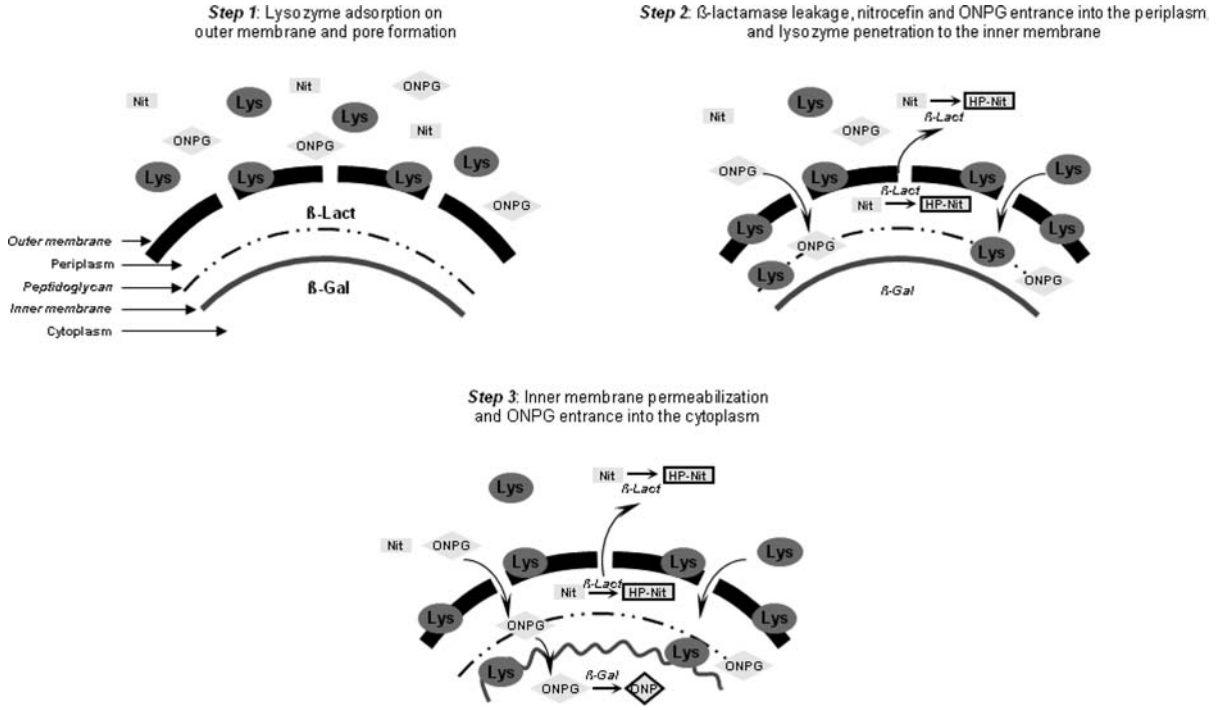


**Figure 7.** Hypothetical sequential events explaining the action of HEWL on outer and inner membranes of *E. coli*. Lys, lysozyme; *β*-Lact, *β*-lactamase; *β*-Gal, *β*-galactosidase; Nit, nitrocefin, and HP-Nit, HP-nitrocefin, substrate and product of *β*-lactamase, respectively; ONPG and ONP, substrate and product of *β*-galactosidase, respectively.

membrane composition, thus decreasing permeabilization by lysozyme. However, more investigations are needed to prove such hypothetical mechanisms.

This study then demonstrated that HEWL is able to permeabilize the outer and inner membranes of *E. coli*. A sequential event is proposed (Figure 7): first, entrapping of HEWL molecules inside the outer membrane, inducing its disruption with large size pore creation; then, transfer of "free" HEWL into the cell, to the inner membrane having increased permeability, but without massive cytoplasm leakage. Whereas the first step is quite rapid, the second one is a much longer phenomenon, depending on the quantity of "free" HEWL and then depending on the initial HEWL concentration in the bulk. Experiments are in progress to investigate the interactions between HEWL and *E. coli* membranes.

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## ABBREVIATIONS USED

HEWL, hen egg white lysozyme; LPS, lipopolysaccharide; NPN, 1-*N*-phenylnaphthylamine; $DiSC_3$, 3,3-dipropylthiadicarbocyanine iodide; ONPG, *o*-nitrophenylgalactoside; ONP, *o*-nitrophenol; HP-nitrocefin, hydrolysis product of nitrocefin

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