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Inactivation of Gram-Negative Bacteria by Lysozyme, Denatured Lysozyme, and Lysozyme-Derived Peptides under High Hydrostatic Pressure

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We have studied the inactivation of six gram-negative bacteria (*Escherichia coli*, *Pseudomonas fluorescens*, *Salmonella enterica* serovar Typhimurium, *Salmonella enteritidis*, *Shigella sonnei*, and *Shigella flexneri*) by high hydrostatic pressure treatment in the presence of hen egg-white lysozyme, partially or completely denatured lysozyme, or a synthetic cationic peptide derived from either hen egg white or coliphage T4 lysozyme. None of these compounds had a bactericidal or bacteriostatic effect on any of the tested bacteria at atmospheric pressure. Under high pressure, all bacteria except both *Salmonella* species showed higher inactivation in the presence of 100 μg of lysozyme/ml than without this additive, indicating that pressure sensitized the bacteria to lysozyme. This extra inactivation by lysozyme was accompanied by the formation of spheroplasts. Complete knockout of the muramidase enzymatic activity of lysozyme by heat treatment fully eliminated its bactericidal effect under pressure, but partially denatured lysozyme was still active against some bacteria. Contrary to some recent reports, these results indicate that enzymatic activity is indispensable for the antimicrobial activity of lysozyme. However, partial heat denaturation extended the activity spectrum of lysozyme under pressure to serovar Typhimurium, suggesting enhanced uptake of partially denatured lysozyme through the serovar Typhimurium outer membrane. All test bacteria were sensitized by high pressure to a peptide corresponding to amino acid residues 96 to 116 of hen egg white, and all except *E. coli* and *P. fluorescens* were sensitized by high pressure to a peptide corresponding to amino acid residues 143 to 155 of T4 lysozyme. Since they are not enzymatically active, these peptides probably have a different mechanism of action than all lysozyme polypeptides.

High hydrostatic pressure treatment is a promising technique for cold pasteurization of foods that allows better retention of product flavor, texture, color, and nutrient content than a comparable conventional heat pasteurization (17, 24). The main obstacles that prevent a commercial breakthrough of pressure-preserved foods are the high investment cost, due to the high pressures required for efficient microbe and enzyme destruction, and the paucity of knowledge on the sensitivity of various pathogenic and spoilage microorganisms to hydrostatic pressure and on the factors affecting this sensitivity.

The application of hurdle technology has been proposed as an approach to increase the microbicidal effect of the process at lower pressures. Hurdle technology relies on the synergistic combination of moderate doses of two or more microbe-inactivating and/or growth-retarding factors (18). An interesting example of synergistic inactivation exists between high pressure and a number of antimicrobial peptides, including nisin, lysozyme, and pediocin (3, 9, 14, 19). This synergistic inactivation was observed not only in intrinsically sensitive gram-positive bacteria but also in gram-negative bacteria, which are normally insensitive to these peptides because their cellular targets are shielded by their outer membrane.

In the present work we have focused on lysozyme because it has some interesting features for application as a food preservative. First, lysozymes are naturally present in foods such as egg white (ca. 3.2 mg/ml) (1), cow milk (ca. 0.13 μg/ml) (1), and human colostrum (ca. 65 μg/ml) (20) but also in several plants, such as cauliflower (ca. 27.6 μg/ml) and cabbage (ca. 2.3 μg/ml) (22). The use of these naturally occurring lysozymes at a concentration of 10 to 100 μg/ml, as proposed in this work, should therefore not present a toxicological concern. Second, the bacteriostatic and bactericidal properties of lysozyme have been the subject of many studies, and over the last 10 years, several authors have proposed a novel antibacterial mechanism of action for lysozyme that is independent of its 1,4-β-*N*-acetylmuramidase activity. These reports are based on unique bactericidal properties observed with partially or completely denatured lysozymes having reduced or no enzymatic activity against both gram-positive and gram-negative bacteria (2, 10, 13, 16). For example, Ibrahim et al. (10, 13) demonstrated inactivation by heat-denatured lysozyme of an *Escherichia coli* K-12 strain, which was relatively insensitive to native lysozyme. Düring et al. (2) showed that native and heat-treated enzymatically inactive lysozyme from coliphage T4 caused similar inactivation levels on a particular *E. coli* strain. The antibacterial properties of denatured lysozyme have been proposed to result from the cationic nature of the peptide in combination with conformational changes leading to increased hydrophobicity. These characteristics are believed to contribute to the antimicrobial properties of several peptides (8). Specific peptides with cationic properties and without enzymatic activity derived from hen egg white and T4 lysozyme were also found to have antimicrobial activity (2, 21).

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The present work extends our previous observation that high pressure sensitizes *E. coli* for lysozyme (3, 9, 19). We have studied the effect of denatured lysozymes and lysozyme-derived peptides, in addition to native lysozyme, in combination with high pressure on a panel of six different gram-negative bacteria including several foodborne pathogens. The results of this work should provide better insight into the mode of antibacterial action of lysozyme and contribute to the development of more efficient technology for cold high-pressure pasteurization.

MATERIALS AND METHODS

Bacterial cultures and growth conditions. The bacteria used in this work are *E. coli* K-12 strain MG1655 (5), *Pseudomonas fluorescens* LMMBM07, *Salmonella enterica* serovar Typhimurium LMMBM01 (both from our laboratory collection), *Salmonella enteritidis* ATCC13076 (from the American Type Culture Collection), *Shigella flexneri* LMG10472 and *Shigella sonnei* LMG10473 (both from the Belgian Coordinated Culture Collection of Microorganisms, Ghent, Belgium). All experiments were carried out with cultures in stationary phase, obtained by growth in nutrient bouillon (Oxoid, Basingstoke, United Kingdom) for 21 h with shaking (200 rpm) at 37°C, except for *P. fluorescens*, which was incubated at 30°C.

Growth inhibition. Growth inhibition by lysozymes and lysozyme-derived peptides was determined by recording growth curves in triplicate with a Bioscreen C microbiology reader (Labsystems Oy, Helsinki, Finland). Stationary phase cultures were diluted to between 5×10^3 and 5×10^4 cells/ml in fresh medium, and 380 μl was transferred to the honeycomb plate wells of the Bioscreen C reader. The volume was then adjusted to 400 μl, either with buffer for the controls or with the appropriate solution of antimicrobial. Every 15 min the cultures were shaken at medium intensity for 1 min, and the turbidity was measured with a wide band filter. The growth curves were followed for 30 h, in which time all bacteria reached the stationary phase.

Denaturation of lysozyme and measurement of lysozyme enzymatic activity. Hen egg-white lysozyme (66,000 U/mg; Fluka, Buchs, Switzerland) was stored frozen (−20°C) as a stock solution of 1 mg/ml in potassium phosphate buffer (10 mM, pH 7.0). For heat denaturation, 100 μl of the 1-mg/ml solution was transferred to a sterile glass capillary and treated for 20 or 60 min at 80 or 100°C in a water bath. For denaturation with β-mercapto-ethanol, the 1-mg/ml lysozyme solution was incubated with 5% β-mercapto-ethanol during 1 h at 60°C. After the treatments, the lysozyme samples were put directly on ice and stored at −20°C.

The enzymatic activity of lysozyme and its derivatives was measured with lyophilized *Micrococcus lysodeikticus* (ATCC 4698) cells (Fluka) resuspended at 0.5 mg/ml in 10 mM potassium phosphate buffer (pH 7.0) as a substrate using a method adapted from Weisner (25). Thirty-microliter aliquots of different dilutions of the sample were added to 300 μl of *M. lysodeikticus* cell suspension, and the lysis of cells was measured automatically as the decrease in turbidity (optical density at 600 nm [OD_{600}]) with the Bioscreen C microbiology reader during 40 min at 20°C. The dilution resulting in a rate of turbidity decrease between 0.001 and 0.005 OD U/min was used to calculate the enzymatic activity. Enzymatic activity was expressed in units per milligram of protein or as a percentage relative to untreated lysozyme.

Synthetic peptides. Two synthetic peptides (95% purity) were purchased from Eurogentec (Herstal, Belgium). Peptide HEL96-116 (H_2N-KKI VSD GNG MNA WVA WRK RCK-COOH) is a 21-mer peptide corresponding to amino acids 96 to 116 of hen egg-white lysozyme (HEL). Peptide T4L143-155 (H_2N-PNR AKR VIT TFR T-COOH) is a 13-mer peptide and corresponds to amino acids 143 to 155 of bacteriophage T4 lysozyme (T4L).

Pressure treatment. Cells in stationary phase were harvested by centrifugation (3,800 × *g*, 5 min) and resuspended in the same volume of potassium phosphate buffer (10 mM, pH 7.0), yielding a final cell population of 5×10^8 to 5×10^9 CFU/ml. Although the pH of phosphate buffer is more pressure dependent than the pH of some other buffers (15), phosphate buffer was chosen for this study because it is widely used in inactivation studies and is not harmful to microorganisms. After the addition of lysozyme or one of its derivatives where appropriate, cell suspensions (300 μl) were sealed without air bubbles in sterile polyethylene bags and subjected at 20°C to pressures in the range of 155 to 300 MPa. Pressure treatment was done in a system with eight parallel thermostatically controlled 8-ml vessels which could be simultaneously pressurized and individually decompressed at different times (Resato, Roden, The Netherlands). The compression rate was approximately 100 MPa/min; decompression was in less than 3 s. The high-pressure transmission fluid used was Resato high-pressure fluid TP1, a mixture of glycols (Van Meeuwen, Wesp, The Netherlands). The pressurization times reported do not include the come-up and come-down time. It should also be noted that the temperature in the vessels could not be kept constant, due to adiabatic compression and decompression. Temperature measurements with thermocouples inside the pressure vessels, which had been previously conducted under identical circumstances, suggested a temperature increase to 29°C upon rapid pressurization to 300 MPa.

To measure bactericidal activities at atmospheric pressure, a part of the suspension with the additives was kept at room temperature without pressurization and was plated after the same exposure time as the pressurized samples.

Enumeration of viable cells. Appropriate dilutions in sterile potassium phosphate buffer (10 mM, pH 7.0) were surface plated with a spiral plater (Spiral System Inc., Cincinnati, Ohio) on tryptic soy agar for *E. coli* and *P. fluorescens* or on plate count agar (both media from Oxoid) for the other bacteria. The plated volume was 50 μl, and hence the detection limit was 20 CFU/ml. Colonies were allowed to develop for 24 to 48 h at the appropriate incubation temperature. Inactivation was expressed as a logarithmic viability reduction, log (N_0/N), with N and N_0 the colony counts after a treatment and in the untreated sample, respectively. For all treatments, averages ± standard deviations for at least three independent cultures of each strain are shown. Significant differences were calculated with the paired Student's *t* test.

RESULTS

Sensitization for native lysozyme. The test panel of six gram-negative bacteria including four pathogens (two *Salmonella* and two *Shigella* strains) was screened for sensitivity to native lysozyme under conditions of ambient and of elevated pressure. Two concentrations, 10 and 100 μg of lysozyme/ml, were used to investigate dose dependency. Growth curves in the presence of lysozyme revealed no inactivation or growth retardation for any of the bacteria, even at 100 μg of lysozyme/ml (data not shown). Higher concentrations of lysozyme were not tested, since these tended to cause aggregation of the bacteria, making the plate counts unreliable.

Sensitization for lysozyme by high pressure was tested by adding lysozyme to the bacterial suspensions before pressure treatment (Table 1). For each strain a pressure was chosen that, in the absence of lysozyme, caused an inactivation of at least 1 log unit. We speculated that in this way the pressure treatment would be severe enough to sensitize the cells to lysozyme. Because of their different pressure sensitivities, a uniform pressure treatment could not be used for all the bacteria. At 10 μg/ml, only *E. coli* and *P. fluorescens* were sensitized to lysozyme by high pressure, but at 100 μg/ml, *S. flexneri* and *S. sonnei* also became sensitive, and the extra logarithmic viability reduction caused by lysozyme for *E. coli* and *P. fluorescens* increased from 0.4 to 1.0 and from 1.5 to 2.5, respectively. Neither *Salmonella* strain was inactivated by lysozyme under pressure, and the presence of 100 μg of lysozyme/ml even had a protective effect against pressure inactivation for *Salmonella* serovar Typhimurium. In three treatments (*S. sonnei* with lysozyme at 10 and 100 μg/ml, and serovar Typhimurium at 100 μg/ml), standard deviations were remarkably higher than in any other treatment. These experiments were repeated in triplicate but the standard deviations remained high.

Sensitivity for lysozyme under pressure was transient, since exposure of pressure-treated cells to lysozyme (1 h at room temperature) after pressure treatment did not cause further inactivation, not even for *P. fluorescens*, which was most sensitive for lysozyme under pressure.

TABLE 1. Logarithmic viability reduction of bacterial suspensions

Organism	Pressure (MPa)	Log $N_0/N \pm SD$[a] of suspensions with:				No. of samples
		No additives (control)	Lys added[b]			
			10 μg/ml	100 μg/ml	10 μg/ml, after pressure[c]	
E. coli	300	1.6 ± 0.1 (a)	2.0 ± 0.1 (b)	2.6 ± 0.4 (b)	1.8 ± 0.2 (a)	3
P. fluorescens	155	1.6 ± 0.5 (a)	3.1 ± 0.3 (b)	4.1 ± 0.3 (c)	1.7 ± 0.1 (a)	3
S. enteritidis	270	1.9 ± 0.3 (a)	2.0 ± 0.0 (a)	1.9 ± 0.2 (a)	2.0 ± 0.1 (a)	3
Salmonella serovar Typhimurium	250	1.6 ± 0.4 (a)	1.4 ± 0.4 (a)	1.2 ± 0.9 (b)	1.5 ± 0.3 (a)	6
S. flexneri	250	3.5 ± 0.3 (a)	3.7 ± 0.4 (a)	4.6 ± 0.2 (b)	3.5 ± 0.4 (a)	3
S. sonnei	220	4.3 ± 0.3 (a)	3.8 ± 1.0 (a)	5.6 ± 1.6 (b)	4.2 ± 0.3 (a)	6

[a] Values within a row followed by different letters are significantly different ($P < 0.05$).
[b] Lys, native hen egg-white lysozyme.
[c] Pressure treatment: 15 min, 20°C, 10 mM phosphate buffer, pH 7.0.

Sensitization for partially heat- or β-mercapto-ethanol-denatured lysozyme. It has been suggested by some authors that partial or even complete heat denaturation would extend the working spectrum of lysozyme to some gram-negative bacteria that are not normally sensitive to lysozyme (2, 10, 13). We wanted to confirm these remarkable observations on our own panel of test bacteria and to compare the bactericidal efficiency of native and denatured lysozymes under high pressure. First, we tested whether lysozyme is denatured by the high pressure treatment itself. This seems not to be the case, since even after a harsh treatment at 600 MPa and 60°C during 15 min, 100% of the enzymatic activity was retained (Table 2). Lysozyme was denatured with heat in a way similar to that described by Ibrahim et al. (10, 13) and, in addition, with β-mercapto-ethanol. The different treatments of lysozyme, the designations used for each denatured product, and the corresponding remaining enzymatic activities are shown in Table 2. Two denatured forms (H100/20-lys and M-lys) were almost completely enzymatically inactive with, respectively, 0.6 and 0.5% of residual activity whereas one (H80/20-lys) still had 11.5% of lytic activity. In the high pressure experiments, H80/20-lys was applied at 10 μg/ml and the more denatured forms at 10 and 100 μg/ml.

In control experiments at atmospheric pressure, no bacteriostatic or bactericidal activity could be detected for any of the denatured forms on any of the test bacteria (data not shown). Since the solution of M-lys still contained β-mercapto-ethanol, another series of control experiments was performed in which all test bacteria were pressure treated in the presence of 5% β-mercapto-ethanol, but no enhanced lethality was observed due to the presence of this compound (data not shown). Table 3 shows the reduction factors of all the test bacteria when treated with the denatured lysozymes under high pressure. In general, denatured lysozymes were active under pressure against fewer bacteria than intact lysozyme at the same concentration. Both strongly denatured lysozymes (H100/20-lys and M-lys) were completely inactive under pressure at 10 μg/ml, although they remained active against at least one of the bacteria at 100 μg/ml. An interesting observation concerns serovar Typhimurium, which was sensitive under pressure for some of the denatured lysozymes (H80/20-lys at 10 μg/ml and H100/20-lys at 100 μg/ml), while it was not sensitive for native lysozyme even at 100 μg/ml. This remarkable behavior of serovar Typhimurium was confirmed in an experiment with six replicate samples. Serovar Typhimurium was not sensitive, however, to the β-mercapto-ethanol-denatured lysozyme (M-lys), although the latter had almost the same level of enzymatic activity as H100/20-lys. M-lys at 100 μg/ml was active only against *P. fluorescens*. This organism was also sensitive to H80/20-lys (10 μg/ml) and H100/20-lys (100 μg/ml) under pressure and was therefore the most sensitive of the tested bacteria.

Contribution of enzymatic activity to the bactericidal properties of lysozymes under pressure. In the experiment described above, the denatured lysozymes always had some residual enzymatic activity. To clarify whether enzymatic activity is necessary at all for the bactericidal effects observed with denatured lysozymes under pressure, we subjected lysozyme to heat treatment at 100°C during 60 min, obtaining a sample with undetectable enzymatic activity (H100/60-lys). The inactivation of *P. fluorescens*, as the most sensitive of all tested bacteria in the previous experiments, by H100/60-lys and H80/20-lys under pressure was subsequently compared and it was found that the completely denatured lysozyme had lost its bactericidal activity (data not shown). A further confirmation for the role of peptidoglycanolytic activity was found by microscopic observation of cell morphology after pressure treatment. The bactericidal effect caused by intact or partially denatured lysozyme under high pressure was always accompanied by a change in the morphology of the cells from rod to sphere. This change did not occur in the absence of lysozyme or with the completely denatured lysozyme and is therefore most likely due to the formation of spheroplasts as a result of the residual lytic activity of lysozyme.

TABLE 2. Residual enzymatic activity of partially denatured hen egg-white lysozyme solutions[a]

Designation	Treatment of Lys	Residual enzymatic activity	
		% of untreated Lys	U/mg
Lys	No treatment	100.0	66,000
Pressurized Lys	60°C, 15 min, 600 MPa	100.0	66,000
H80/20-lys	80°C, 20 min	11.5	7,590
H100/20-lys	100°C, 20 min	0.6	396
M-lys	5% β-mercapto-ethanol, 60°C, 60 min	0.5	330

[a] Lys, lysozyme.

TABLE 3. Logarithmic viability reduction of bacteria by denatured hen egg-white lysozymes under high pressure[a]

Organism	Pressure (MPa)	Log $N_0/N \pm SD$[b] of bacteria with:						No. of samples
		No additives (control)[c]	H80/20-lys (10 μg/ml)	H100/20-lys		M-lys		
				10 μg/ml	100 μg/ml	10 μg/ml	100 μg/ml	
E. coli	300	1.6 ± 0.1 (a)	1.9 ± 0.1 (a)	1.4 ± 0.2 (a)	1.9 ± 0.1 (a)	1.5 ± 0.2 (a)	1.9 ± 0.1 (a)	3
P. fluorescens	155	1.6 ± 0.5 (a)	3.1 ± 0.1 (c)	1.7 ± 0.2 (a)	2.5 ± 0.1 (b)	1.6 ± 0.1 (a)	2.6 ± 0.1 (b)	3
S. enteritidis	270	1.9 ± 0.3 (a)	2.0 ± 0.3 (a)	1.7 ± 0.2 (a)	2.3 ± 0.2 (a)	1.7 ± 0.2 (a)	1.9 ± 0.3 (a)	3
Salmonella serovar Typhimurium	250	1.6 ± 0.4 (a)	2.1 ± 0.3 (b)	1.6 ± 0.2 (a)	2.2 ± 0.1 (b)	1.6 ± 0.3 (a)	1.7 ± 0.2 (a)	6
S. flexneri	250	3.5 ± 0.3 (a)	3.3 ± 0.2 (a)	3.2 ± 0.2 (a)	3.8 ± 0.5 (a)	3.2 ± 0.3 (a)	4.0 ± 0.5 (a)	3
S. sonnei	220	4.3 ± 0.3 (a)	4.0 ± 0.2 (a)	3.8 ± 0.4 (a)	4.4 ± 0.4 (a)	3.8 ± 0.4 (a)	5.1 ± 0.9 (a)	3

[a] High pressure treatment: 15 min, 20°C, 10 mM phosphate buffer, pH 7.0.
[b] Values within a row followed by different letters are significantly different ($P < 0.05$).
[c] Results in this table and in Table 1 were obtained with the same bacterial cultures. Therefore, the values shown for the control treatment are taken from Table 1.

Sensitization for lysozyme-derived peptides. In a final set of experiments, two synthetic peptides derived, respectively, from hen egg-white lysozyme (HEL96-116) and *E. coli* bacteriophage T4 lysozyme (T4L143-155) were investigated for antibacterial activity. Peptide HEL96-116 is similar to the bactericidal peptide of 15 amino acids that was isolated by Pellegrini et al. (21) by digesting lysozyme with the protease clostripain but has two additional NH_2-terminal and four additional COOH-terminal amino acids from the original lysozyme sequence. This increases the cationic character of the peptide, which is known to contribute to the antibacterial activity of several antibiotic peptides (7). Peptide T4L143-155 was chosen and synthesized by Düring et al. (2) for its amphiphatic character and helicoidal structure and was also found to have bactericidal activity. The calculated isoelectric points of HEL96-116 and T4L143-155 are 10.29 and 12.40, respectively, and both peptides were completely enzymatically inactive.

These two peptides were applied to our test panel of bacteria at 100 μg/ml. At atmospheric pressure neither growth inhibition nor inactivation was observed (data not shown). Under pressure (Table 4), all bacteria were sensitized for the HEL96-116 peptide, even serovar Typhimurium and *S. enteritidis*, which were not sensitized for native lysozyme under pressure. On the other hand, peptide T4L143-155 was active under pressure against all bacteria except *E. coli* and *P. fluorescens*, two bacteria that were very sensitive to native lysozyme under pressure. HEL96-116 was more effective than T4L143-155 against all bacteria, and bactericidal activity of both peptides under pressure was not accompanied by spheroplast formation.

DISCUSSION

A first objective of this work was to investigate whether the previously reported observation that high pressure can sensitize *E. coli* to lysozyme (3, 9, 19) can be extended to other gram-negative bacteria. It was found that this is the case for some but not for all gram-negative bacteria (Table 1). Inactivation by lysozyme under pressure was concentration dependent since at 100 μg/ml more bacteria were sensitized and a higher magnitude of sensitization occurred than at 10 μg/ml. In line with what was reported earlier by Hauben et al. (9) for *E. coli*, we found that sensitization is transient. As soon as pressure was released, all bacteria immediately regained their resistance to lysozyme. In addition to this type of transient sensitization, high pressure also causes a persistant type of sensitization, for instance to the lactoperoxidase system (4). Of course, whether or not an organism gets sensitized to lysozyme by high pressure may depend on many other factors, such as pressure, temperature, pH, medium composition, and cell growth stage and history. For example, we have previously demonstrated that application of pressure pulses with brief interruptions can cause sensitization to lysozyme and nisin of bacteria that are not sensitized by a continuous pressure treatment (19).

In the present work, we explored another route to maximize the synergistic bactericidal effect of pressure and lysozyme, by replacing native lysozyme with denatured forms of lysozyme and peptides derived from lysozyme, to both of which have been ascribed certain antimicrobial effects previously.

We prepared heat-denatured lysozyme according to Ibrahim

TABLE 4. Logarithmic viability reduction of bacterial suspensions treated with high pressure[a] both without additives and with the addition of lysozyme-derived peptides

Organism	Pressure (MPa)	Log $N_0/N \pm SD$[b] with:			No. of samples
		No additives (control)	HEL96-116 (100 μg/ml)	T4L143-155 (100 μg/ml)	
E. coli	300	3.2 ± 0.5 (a)	4.4 ± 0.2 (b)	3.5 ± 0.2 (a)	3
P. fluorescens	155	1.0 ± 0.1 (a)	2.1 ± 0.4 (b)	1.3 ± 0.7 (a)	3
S. enteritidis	270	3.2 ± 0.2 (a)	4.4 ± 0.2 (b)	4.3 ± 0.2 (b)	3
Salmonella serovar Typhimurium	250	2.2 ± 1.3 (a)	3.2 ± 0.8 (b)	3.0 ± 1.2 (b)	6
S. flexneri	250	0.9 ± 0.3 (a)	2.5 ± 0.4 (b)	2.3 ± 0.1 (b)	3
S. sonnei	220	1.7 ± 1.0 (a)	3.4 ± 1.1 (b)	2.9 ± 0.9 (b)	6

[a] High pressure treatment: 15 min, 20°C, 10 mM phosphate buffer, pH 7.0.
[b] Values within a row followed by different letters are significantly different ($P < 0.05$).

et al. (10, 13), but we failed to confirm any of the bactericidal or bacteriostatic effects under atmospheric pressure that were reported by these authors. Denaturation with β-mercapto-ethanol also did not endow lysozyme with antimicrobial activity at atmospheric pressure. We believe therefore that the effects described are very strain dependent.

The experiments with the denatured lysozymes under high pressure (Tables 2 and 3) lead to two observations. One is that the enzymatic activity of lysozyme is required for it to exert a bactericidal effect under pressure. Reduction of enzymatic activity by heat or β-mercapto-ethanol denaturation clearly leads to a reduction in the observed bactericidal effect, and complete elimination of enzymatic activity by extended heat treatment (60 min, 100°C) completely eliminates the bactericidal effect. At this point, therefore, our results do not allow us to confirm the hypothesis raised by other authors that the antimicrobial activity of lysozyme and/or heat-denatured lysozyme would consist partly of a mechanism that is independent of enzymatic activity (10, 13, 21). The second observation from these experiments is that partial heat denaturation can extend the spectrum of lysozyme bactericidal activity under pressure to a wider range of bacteria. In our experiments, heat denaturation made lysozyme active against serovar Typhimurium. A threshold level of residual enzymatic activity remains a requirement also in this case, as can be deduced from the results in Table 3. The mildly denatured lysozyme H80/20-lys (with 11.5% residual activity) is active against serovar Typhimurium at 10 μg/ml under pressure, while the extensively denatured H100/20-lys (with only 0.6% residual activity) is active only at 100 μg/ml.

Taken together, these results allow us to formulate the following hypothesis about the synergistic effect of high pressure and lysozyme on the inactivation of gram-negative bacteria. At ambient pressure, lysozyme is completely inactive against most gram-negative bacteria because it cannot penetrate the outer membrane to reach its target, the peptidoglycan. Nevertheless, lysozyme has both cationic and lipophilic properties, which are known to contribute to an intimate interaction with and passage through bilayer membranes of many small peptides with antibacterial properties (8). Passage is believed to occur through the so-called self-promoted uptake mechanism (6). Interestingly, it has been demonstrated that deep rough mutants of serovar Typhimurium are sensitive to lysozyme (23), suggesting that small changes in outer membrane composition may allow self-promoted uptake of lysozyme. Conversely, a change in the structure of lysozyme by heat denaturation can also cause the enzyme to become active against strains of *E. coli* which are insensitive to native lysozyme (10, 13). Under high pressure, the ultimate mode of action of lysozyme remains the same, i.e., the peptidoglycanolytic activity. However, pressure apparently stimulates passage of lysozyme through the outer membrane of several gram-negative bacteria. We have previously named this phenomenon pressure-assisted self-promoted uptake, or, briefly, pressure-promoted uptake (19). Our present results show that pressure-promoted uptake of lysozyme is not a universal phenomenon in all gram-negative bacteria, probably because it depends on subtle properties of, and interactions between, the cell surface and the lysozyme molecule. Subtle changes in either the cell surface or lysozyme structure may change the outcome of the interaction. For example, an *E. coli* mutant has been decribed that is resistant to lysozyme under pressure (19). In the present work, mild denaturation of lysozyme allowed successful pressure-promoted uptake and bactericidal action against serovar Typhimurium, which is refractory to pressure-promoted uptake of native lysozyme. The underlying explanation may be an increase in the hydrophobicity of lysozyme by heat denaturation, since it has been shown previously that increasing lysozyme hydrophobicity by genetic fusion of a hydrophobic peptide to the NH_2 terminal of the enzyme (12) or by chemical modification of the lysyl residues with saturated fatty acids (11) enhanced the activity of lysozyme against gram-negative bacteria. An alternative mode of antibacterial action that has been proposed for lysozyme is that the binding of lysozyme to the bacterial envelope would activate the autolysins (13, 16). As a second part of this work, the bactericidal effect under pressure of two specific lysozyme-derived peptides to which antibacterial properties had been previously assigned (2, 21) was investigated. Again, we were unable to reproduce these effects with our panel of test bacteria and under our experimental conditions. However, both peptides were very effective under high pressure, in particular the peptide derived from hen egg white, which was active against all test bacteria (Table 4). For these peptides, a different mechanism of bactericidal action must be involved because they are completely devoid of enzymatic activity. The precise mechanism remains unknown, but it seems evident that, similar to lysozyme, bactericidal activity will depend on passage through the outer membrane.

The use of lysozyme, heat-denatured lysozyme, and peptides derived from lysozyme may find interesting applications in the nonthermal preservation of foods and pharmaceutical and other products by high pressure. In the presence of these additives, lower pressures can be used to achieve the desired reduction factors, making high hydrostatic pressure technology more economically feasible. For optimal performance, the working spectrum of these compounds under pressure will have to be studied in more detail, and it may be necessary to design mixtures of specific compounds to cover as wide a range of bacteria as possible. For the peptides derived from lysozyme, toxicity studies should be conducted to demonstrate their safety before they can be applied in foods.

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