Available online at www.sciencedirect.com
SCIENCE DIRECT
Food Control 18 (2007) 173–178
FOOD CONTROL
www.elsevier.com/locate/foodcont

Antimicrobial peptides derived from hen egg lysozyme with inhibitory effect against *Bacillus* species

Adham M. Abdou [b,*], S. Higashiguchi [a], A.M. Aboueleinin [b], M. Kim [a], Hisham R. Ibrahim [c]

[a] *Research Department, Pharma Foods International Company Ltd., 24-5 Donoato-Nishimachi, Kisshoin-Ishihara, Minami-Ku, Kyoto 601-8357, Japan*
[b] *Department of Food Control, Benha University, Moshtohor 13736, Kaliobyia, Egypt*
[c] *Department of Biochemistry and Biotechnology, Kagoshima University, Kagoshima 890-0065, Japan*

Received 27 August 2004; received in revised form 17 September 2005; accepted 19 September 2005

Abstract

In food industry, *Bacillus* species are encountered in deteriorating many food products thus shortening their shelf-life. Moreover, *Bacillus cereus* and the *subtilis* group (*Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus pumilus*) have been recognized as food poisoning agents. Lysozyme peptides preparation (LzP) is a commercially available as a natural food preservative. Although, LzP derived from lysozyme yet it showed only 11% of the lysozyme lytic activity. LzP at a concentration of 100 μg ml^{-1} completely inhibited *B. subtilis, B. licheniformis, B. megaterium, B. mycoides, B. pumilus, B. coagulans, B. amyloliquefaciens, B. polymexa* and *B. macerans*. However, *B. cereus* and *B. stearothermophilus* showed a slightly higher resistance. Interestingly, LzP at concentration $\geqslant 10$ μg ml^{-1} showed inhibitory effect on both vegetative and spore forms of *B. subtilis*. Moreover, LzP was stable at 95 °C for 30 min and at different pH values (4.5–7). In conclusion, LzP may be useful to control growth of *Bacillus* spoilage organisms.

Keywords: Lysozyme; Lysozyme peptide; *Bacillus* species; Natural food preservative

1. Introduction

Bacillus species are widely distributed in nature and have a remarkable ability to survive strong environmental stresses (Sheath, Mair, Sharpe, & Holt, 1986). In food industry, *Bacillus* contamination might originate from soil, water, processing equipment and processing environment. They are known as aerobic spore formers and act as food pathogens or food spoilage microorganisms. Their spores are highly resistant to heat treatment and drying of foods; hence, subsequent spore germination and outgrowth of vegetative cells can occur (Mansour, Amri, Bouttefroy, Linder, & Milliere, 1999). Thermo resistance, spore forming and psychrotrophic properties of *Bacillus* species are the main deteriorating factors that would spoil food products and shorten their shelf-life (Carlin et al., 2000). Moreover, *Bacillus cereus* is a hazardous food poisoning organism being incriminated in many foodborne outbreaks (Granum, 1997). Recently, the *subtilis* group, which compromising *Bacillus subtilis, Bacillus licheniformis*, and *Bacillus pumilus,* has been associated with incidents of foodborne gastroenteritis (Lund, 1990; Rowan, Caldow, Gemmell, & Hunter, 2003).

Bacillus species were encountered in deteriorating of large varieties of food products including milk, dairy products, meat products, bakery products, fermented soy beans, mashed potato products, vegetable purees, pasta products, coca herbs and spices (Kimura, Inatsu, & Itoh, 2002; Nissen, Holo, Axelsson, & Blom, 2001; te Giffel, Beumer, Leijendekkers, & Rombouts, 1996). As the food industries are

* Corresponding author. Present address: Research Department, Pharma Foods International Company Ltd., 24-5 Donoato-Nishimachi, Kisshoin-Ishihara, Minami-Ku, Kyoto 601-8357, Japan. Tel.: +81 75 693 8607; fax: +81 75 693 8608.
E-mail address: dradham@yahoo.com (A.M. Abdou).

0956-7135/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodcont.2005.09.010

more involved with extended shelf-life products, the problem with spore forming bacilli will become a main concern.

Nowadays, there is a growing pressure on the food industry to reduce its reliance on synthetic chemical preservatives. Consequently, manufacturers are urged to develop alternative preservatives that are based on natural compounds. Lysozyme is well known as an antimicrobial protein and has been attracted considerable interest as a natural food preservative. However, its antimicrobial activity is limited to certain Gram-positive bacteria through its muramidase enzyme that can hydrolyze the polypeptidoglycan layer in the cell wall of the Gram-positive bacteria (Jollès & Jollès, 1984). Recently, the antimicrobial properties of lysozyme have been subjected to many studies. Several authors have proposed a novel antibacterial mechanism of action of lysozyme that is independent of its muramidase activity (Ibrahim et al., 1996b; Laible & Germaine, 1985; Pellegrini, Thomas, von Fellenberg, & Wild, 1992). These reports observed that the denatured lysozyme deprived of muramidase activity has unique and potent microbicidal properties (Ibrahim, Higashiguchi, Juneja, Kim, & Yamamoto, 1996a; Ibrahim, Matsuzaki, & Aoki, 2001a). Furthermore, other researches could identify some bactericidal peptides derived from hen egg lysozyme that have an exaggerated and broad-spectrum microbicidal activity (Ibrahim, Thomas, & Pellegrini, 2001b; Pellegrini et al., 1997).

Lysozyme peptides (LzP) powder is commercially available antimicrobial peptides preparation produced by limited protease hydrolysis of hen egg lysozyme in order to model its structure to novel antimicrobial peptides.

The aim of this work was undertaken to evaluate the activity of LzP as a new nutrapreservative against the most encountered *Bacillus* species in the food industry.

2. Materials and methods

2.1. Microbial strains and culture conditions

Bacillus species were obtained from NBRC (NITE Biological Resource Center, Chiba, Japan). All species strains (11 strains) were rehydrated in the recommended fluid medium (rehydration fluid 702: polypeptone 10 g, yeast extract 2 g, $MgSO_4 \cdot 7H_2O$ 1 g, distilled water 1 L and pH 7.2) and then cultivated in the recommended medium (growth medium 802: polypeptone 10 g, yeast extract 2 g, $MgSO_4 \cdot 7H_2O$ 1 g, distilled water 1 L, Agar 15 g, and pH 7.0). Cultures were maintained in trypticase soy broth with 10% glycerol at −20 °C. A single colony of each bacterial strain was grown on trypticase soy agar plates, then inoculated in 50 ml trypticase soy broth and grown overnight at the appropriate temperature (30 °C for all strains except *Bacillus stearothermophilus* at 50 °C, *Bacillus coagulans* and *Bacillus amyloliquefaciens* at 37 °C). One milliliter of pre-cultured bacterial suspension was diluted (1:50) in 10 mM sodium phosphate buffer pH 7.0. Bacteria were harvested by centrifugation at 3000*g* for 10 min, washed and resuspended in trypticase soy broth.

2.2. Preparation of LzP

LzP (as Lysozyme peptides powder) was obtained from Pharma Foods International Co., Ltd (Kyoto, Japan). It was produced by partial enzymatic hydrolysis from native lysozyme using pepsin enzyme. LzP powder is a mixture composed of 50% active peptides and 50% glycin. It was dissolved in 1.0% wt./vol in sterilized distilled water. The solution was stirred slightly to avoid foaming, filtered and kept refrigerated as a stock solution at 4 °C (up to three days).

2.3. Antibacterial assay

LzP working solution was prepared to give a final concentration of 400 μg ml^{-1}. As previously described (Ibrahim et al., 2001b), aliquots (400 μl) of trypticase soy broth were mixed with 200 μl of the bacterial suspension (adjusted to a final concentration of 5 log cfu ml^{-1} of cells), and then 200 μl of LzP working solution was added (final concentration will be 100 μg ml^{-1} according to manufacturer's recommendation). A control was prepared without the addition of LzP. The suspensions were incubated at the given temperature for 1, 7, and 14 days, serially diluted in sodium phosphate buffer, pH 7.0, and plated on trypticase soy agar. Colony-forming units were obtained after incubation of the plates at the specified temperature for 2–3 days. All assays were performed in triplicates and the results are the means of three independent experiments.

2.4. Effect of LzP on B. subtilis

2.4.1. Effect of various concentrations on vegetative and spore forms

The antimicrobial assay was performed as described above using two sets containing different LzP concentrations adjusted to a final concentration of 0, 5, 10, 20, 50, 100 μg ml^{-1}. In the first set, *B. subtilis* vegetative cells were added to give a final count of 10^5 cfu ml^{-1}. A spore-only suspension of *B. subtilis* was prepared according to Setlow et al. (2002). The suspension was heated at 80 °C for 10 min and quickly cooled to 5 °C. It was then centrifuged for 20 min at 4000*g* at 5 °C and washed twice with sterile distilled water. Finally the spores were resuspended in 0.85% NaCl and then added to the second set (10^3 spores ml^{-1}).

2.4.2. Effect of incubation time

LzP at 100 μg ml^{-1} was incubated with vegetative forms of *B. subtilis* for a period up to four weeks. Counts were monitored by standard plate count. Control mixtures were prepared without the addition of LzP.

2.4.3. Influence of pH

In order to detect the antibacterial activity of LzP at different pH, two series of tubes were adjusted to different pH values (4.5, 5, 5.5, 6, 6.5, and 7). The first one did not contain LzP (control), while the other containing LzP (100 μg ml^{-1}). Bacterial suspension was added in both ser-

ies to give a final concentration of 10^5 cfu ml^{-1} and incubated at 30 °C for two weeks.

2.4.4. Antimicrobial activity of heated LzP

LzP (1% solution wt./vol in distilled water) was heated at 95 °C for 30 min or autoclaved at 121 °C for 15 min. The antibacterial assay was carried out as described above against vegetative form of *B. subtilis*.

2.5. Muramidase activity of LzP

The lytic activity of LzP and lysozyme against *Micrococcus lysodeikticus* cells (Sigma Chemical Co., St. Louis, MO) was determined according to a turbidimetric method, previously reported (Ibrahim et al., 1996a), based on the decrease in turbidity of 1.9 ml cell suspension (170 μg dry cells ml^{-1}) in 50 mM potassium phosphate buffer (pH 6.0) following the addition of 100 μl portion of lysozyme (Inovatech Inc., Canada) or LzP solutions after equilibration to achieve constant absorbance. The decrease in absorbance at 450 nm was monitored using a Shimadzu UVmini-1240 spectrophotometer (Shimadzu, Kyoto, Japan). One unit of muramidase activity is the amount of lysozyme or LzP that produce 0.001 changes per min. The activity is expressed in U mg^{-1} of protein or as a percentage relative to native lysozyme.

3. Results and discussion

3.1. Effect of LzP against Bacillus species

There are several routes by which *Bacillus* species can contaminate and deteriorate a large variety of foods. We have examined the effect of LzP against the most encountered *Bacillus* strains in different foods. LzP at a concentration of 100 μg ml^{-1} was incubated with 11 *Bacillus* strains for 1, 7, and 14 days as shown in Table 1, complete inhibition was observed for 9 strains (*B. subtilis*, *B. licheniformis*, *B. pumilus*, *B. mycoides*, *B. coagulans*, *B. amyloliquefaciens*, *B. megaterium*, *B. polymexa*, and *B. macerans*). Meanwhile, *B. cereus* and *B. stearothermophilus* were showing slight resistance. It was reported that *B. cereus* strains were among the least sensitive bacilli against different antimicrobials including nisin, lysozyme, and nisin-monolaurin combination (Mansour & Milliere, 2001; Pirttijärvi, Wahlström, Rainey, Saris, & Salkinoja-Salonen, 2001). In agreement with our study, López-Pedemonte, Roig-Sgués, Trujillo, Capellas, and Guamis (2003) reported that the low sensitivity of *B. cereus* would be due to the use of high initial load (around 6 log cfu ml^{-1}). On the other hand, *B. stearothermophilus* is an obligatory thermophilic bacterium (50–55 °C) so the antibacterial activity of LzP may be decreased due to its prolonged incubation at such temperature.

Recognition of *Bacillus* species as a potential spoilage problem in ambient-stored foods, ability to grow in a variety of foods, resisting heat treatment, and shortening the shelf-life has been documented as challenges for food industries (Mansour et al., 1999; te Giffel et al., 1996). Moreover, *B. cereus* was recognized as a major food poisoning bacterium worldwide. Furthermore, food poisoning due to other *Bacillus* species are being increasingly recognized, particularly, the *subtilis* group (*B. licheniformis*, *B. subtilis*, and *B. pumilus*) has been associated with incidents of food borne gastroenteritis (Lund, 1990; Rowan et al., 2003). The work presented here suggests a promising strategy to control some strains of *Bacillus* species through the addition of LzP.

3.2. Effect of concentration, pH, and incubation time on B. subtilis

Total inhibition by 100 μg ml^{-1} of LzP was achieved against high microbial load of *B. subtilis*, and this inhibition lasted for four weeks at 30 °C (Fig. 1). Mansour and Milliere (2001) recorded an immediate reduction of *Bacillus*

Table 1
Bacillus species used in this study and their reduction by incubation with LzP[a] in TSB[b] for 1, 7, and 14 days at the appropriate temperature

Organism	Source	Control[c] (log cfu ml^{-1})			LzP (log cfu ml^{-1})		
		1d	7d	14d	1d	7d	14d
B. subtilis	NBRC 3013	6.3 ± 0.2	7.8 ± 0.3	8.3 ± 0.2	ND[d]	ND	ND
B. licheniformis	NBRC 12197	6.2 ± 0.3	7.7 ± 0.2	8.0 ± 0.2	ND	ND	ND
B. cereus	NBRC 3836	6.2 ± 0.2	7.8 ± 0.3	8.3 ± 0.2	3.7 ± 0.12	4.3 ± 0.15	4.2 ± 0.3
B. pumilus	NBRC 12088	5.9 ± 0.1	7.5 ± 0.2	8.0 ± 0.2	ND	ND	ND
B. mycoides	NBRC 3039	6.0 ± 0.1	7.5 ± 0.2	8.0 ± 0.2	ND	ND	ND
B. stearothermophilus	NBRC 12550	6.2 ± 0.2	7.8 ± 0.3	8.3 ± 0.2	4.0 ± 0.13	4.3 ± 0.12	4.1 ± 0.2
B. coagulans	NBRC 12583	6.4 ± 0.2	8.0 ± 0.2	8.6 ± 0.3	ND	ND	ND
B. amyloliquefaciens	NBRC 15535	5.8 ± 0.1	7.5 ± 0.2	8.0 ± 0.2	ND	ND	ND
B. megaterium	NBRC 15308	6.4 ± 0.2	8.0 ± 0.2	8.6 ± 0.3	ND	ND	ND
B. polymexa	NBRC 15309	5.8 ± 0.1	7.5 ± 0.2	8.0 ± 0.2	ND	ND	ND
B. macerans	NBRC 15307	6.2 ± 0.2	7.8 ± 0.2	8.3 ± 0.3	ND	ND	ND

[a] LzP: lysozyme peptides preparation (100 μg ml^{-1}).
[b] TSB: trypticase soy broth.
[c] Control: mixtures prepared without the addition of LzP.
[d] ND: not detected in dilution 10^{-1}.

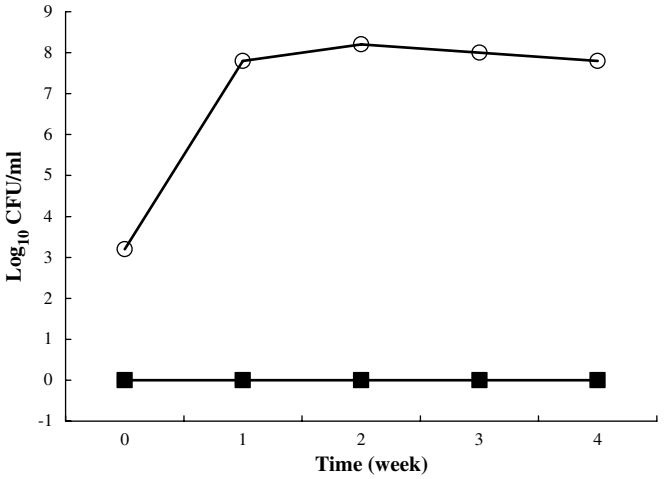

Fig. 1. Influence of lysozyme peptides preparation (LzP) at 100 μg ml^{-1} on *B. subtilis* after incubation for a period up to four weeks in trypticase soy broth at 30 °C (■). Control mixtures were prepared without the addition of LzP (○).

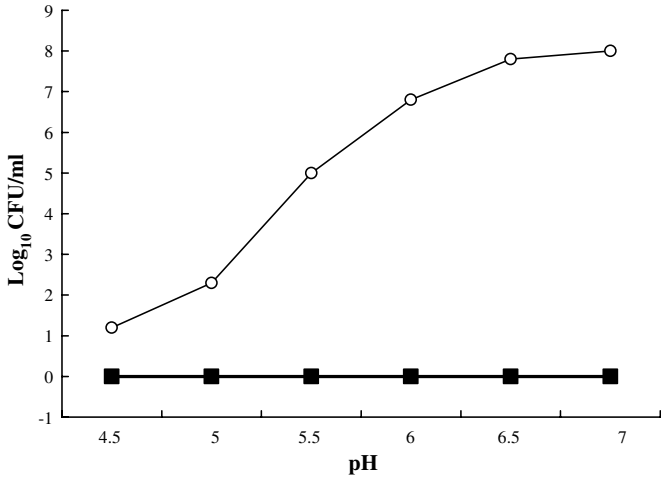

Fig. 2. Effect of Lysozyme peptides preparation (LzP) at 100 μg ml^{-1} on *B. subtilis* after incubation in trypticase soy broth adjusted to different pH from 4.5 to 7 at 30 °C for two weeks (■). Control mixtures were prepared with the same pH adjustment and without the addition of LzP (○).

species by using nisin, a widely used natural antimicrobial, monolaurin or a combination but the reduction was transient because re-growth occurs during the five days period of examination.

Fig. 2 illustrates the activity of LzP as a function of pH and shows that LzP at concentration of 100 μg ml^{-1} showed a complete inhibition of *B. subtilis* at all tested pH values. Growth of *B. subtilis* in the absence of LzP was varied according to the pH value; at pH lower than 4.5 the organism did not show any detectable growth whereas its optimum growth was at pH 6–7. A similar growth pattern of *Bacillus* species has been reported by Valero, Leontidis, Fernándes, Martinez, and Salmerón (2000). This growth pattern may exaggerate the inhibitory effect of LzP at lower pH and renders it useful as a shelf-life extender in acid foods.

Moreover, when the activity of different concentrations of LzP on vegetative and spore forms of *B. subtilis* was examined, LzP at concentration 10 μg ml^{-1} or more inhibited both vegetative and spore forms (Table 2). LzP showed very low inhibitory concentration, which may be, differ in other strains, otherwise, Komitopoulou, Boziaris, Davies, Delves-Broughton, and Adams (1999) found that nisin at considerably low levels (5 IU ml^{-1}) inhibited both vegetative and spore forms of *Alicyclobacillus acidoterrestris* (originally named *B. acidoterrestris*).

The effect of heat treatments on the antimicrobial activity of LzP was tested. LzP was subjected to heating at 95 °C for 30 min and autoclaving at 121 °C for 15 min. Heated LzP showed the same inhibitory effect against *B. subtilis*, while the autoclaved one lost about 57% of its activity (Fig. 3). The highly inhibitory effect of heated LzP gives it an important role in foods subjected to heat treatment during processing.

3.3. Muramidase lytic activity

It was found that LzP has only 11.05% (2409 u/mg) of the lytic activity of the native lysozyme (100%: 21,799 u/mg). Meanwhile, LzP was exhibited strong bactericidal activity against *Bacillus* species. Recently, it was confirmed by several investigators that denatured lysozyme lacking enzymatic activity was able to kill efficiently wide range of microorganisms (Ibrahim et al., 1996a; Ibrahim et al., 2001a; Pellegrini et al., 1992). It was suggested that the bactericidal potency of lysozyme is not only due to its muramidase activity but also to its cationic and hydrophobic properties (Hancock & Chapple, 1999; Ibrahim, Aoki, &

Table 2
Effect of different concentrations of LzP[a] on vegetative and spore forms of *Bacillus subtilis* for 1, 7, and 14 days

LzP concentration	Vegetative (log cfu ml^{-1})			Spores (log cfu ml^{-1})		
	1d	7d	14d	1d	7d	14d
0 μg ml^{-1}	6.1 ± 0.1	7.5 ± 0.2	8.0 ± 0.2	4.2 ± 0.13	4.8 ± 0.12	5.1 ± 0.2
5 μg ml^{-1}	1.1 ± 0.12	2.3 ± 0.15	3.2 ± 0.3	2.1 ± 0.12	3.3 ± 0.15	3.2 ± 0.3
10 μg ml^{-1}	ND[b]	ND	ND	ND	ND	ND
20 μg ml^{-1}	ND	ND	ND	ND	ND	ND
50 μg ml^{-1}	ND	ND	ND	ND	ND	ND
100 μg ml^{-1}	ND	ND	ND	ND	ND	ND

[a] LzP: lysozyme peptides preparation.
[b] ND: not detected in dilution 10^{-1}.

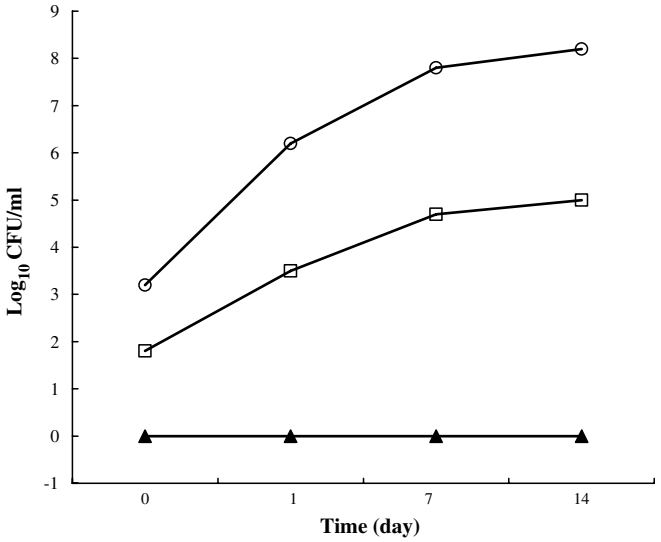

Fig. 3. Effect of heating at 95 °C for 30 min (▲) or autoclaving at 121 °C for 15 min (□) of Lysozyme peptides preparation (LzP) on its antimicrobial activity against *B. subtilis* after incubation for 1, 7, and 14 in trypticase soy broth at 30 °C. Control mixtures were prepared without the addition of LzP (○).

Pellegrini, 2002; Pellegrini et al., 1997). In addition, the possibility of synergistically acting peptides can be considered, which was demonstrated with peptides of cathepsin G (Bangalore, Travis, Onunka, Pohl, & Shafer, 1990).

LzP is a commercially available antimicrobial preparation produced from hen egg lysozyme by limited proteolysis using pepsin enzyme in order to exaggerate its antimicrobial activity. It was produced on industrial scale based on the new splendid conception that lysozyme had a powerful antimicrobial activity through its active peptides (Ibrahim et al., 2002).

Our results indicate that LzP is a new candidate to control *Bacillus* species and may eliminate spoilage caused by such species in different foods. However, the data presented here were carried out in liquid media and further studies for application in various foods are required to give insight into what may happen in food system.

References

Bangalore, N., Travis, J., Onunka, V. C., Pohl, J., & Shafer, W. M. (1990). Identification of the primary antimicrobial domains in human neutrophil cathepsin G. *Journal of Biological Chemistry, 256*, 13584–13588.

Carlin, F., Guinebretière, M., Choma, C., Pasqualini, R., Braconnier, A., & Nguyen-the, C. (2000). Spore-forming bacteria in commercial cooked, pasteurized and chilled vegetable purees. *Food Microbiology, 17*, 153–165.

Granum, P. E. (1997). *Bacillus cereus*. In M. P. Doyle, L. R. Beuchat, & T. J. Montville (Eds.), *Food Microbiology: Fundamentals and Frontiers* (pp. 136–327). Washington: ASM Press.

Hancock, R. E., & Chapple, D. S. (1999). Peptide antibiotics. *Antimicrobial Agents Chemotherapeutics, 43*, 1317–1323.

Ibrahim, H. R., Higashiguchi, S., Juneja, L. R., Kim, M., & Yamamoto, T. (1996a). A structural phase of heat-denaturated lysozyme with novel antimicrobial action. *Journal of Agricultural and Food Chemistry, 44*, 1416–1423.

Ibrahim, H. R., Higashiguchi, S., Koketsu, M., Juneja, L. R., Kim, M., Yamamoto, T., et al. (1996b). Partially unfolded lysozyme at neutral pH agglutinates and kills Gram-negative and Gram-positive bacteria through membrane damage mechanism. *Journal of Agricultural and Food Chemistry, 44*, 3799–3806.

Ibrahim, H. R., Matsuzaki, T., & Aoki, T. (2001a). Genetic evidence that antibacterial activity of lysozyme is independent of its catalytic function. *FEBS Letters, 506*, 27–32.

Ibrahim, H. R., Thomas, U., & Pellegrini, A. (2001b). A helix-loop-helix peptide at the upper lip of the active site cleft of lysozyme confers potent antimicrobial activity with membrane permeabilization action. *Journal of Biological Chemistry, 276*, 43767–43774.

Ibrahim, H. R., Aoki, T., & Pellegrini, A. (2002). Strategies for new antimicrobial proteins and peptides: lysozyme and aprotinin as model molecules. *Current Pharmaceutical Design, 2002*, 671–693.

Jollès, P., & Jollès, J. (1984). What's new in lysozyme research? *Molecular and Cellular Biochemistry, 63*, 165–189.

Kimura, K., Inatsu, Y., & Itoh, Y. (2002). Frequency of the insertion sequence IS4Bsu1 among *Bacillus subtilis* strains isolated from fermented soybean foods in Southeast Asia. *Bioscience Biotechnology and Biochemistry, 66*, 1994–2006.

Komitopoulou, E., Boziaris, I. S., Davies, E. A., Delves-Broughton, J., & Adams, M. R. (1999). *Alicyclobacillus acidoterrestris* in fruit juices and its control by nisin. *International Journal of Food Science and Technology, 34*, 81–85.

Laible, N. J., & Germaine, G. R. (1985). Bactericidal activity of human lysozyme, muramidase-inactive lysozyme, and cationic polypeptides against *Streptococcus sanguis* and *Streptococcus faecalis III*: inhibition by chitin oligosaccharides. *Infectious Immunology, 48*, 720–728.

López-Pedemonte, T. J., Roig-Sgués, A. X., Trujillo, A. J., Capellas, M., & Guamis, B. (2003). Inactivation of spores of *Bacillus cereus* in cheese by high hydrostatic pressure with the addition of nisin or lysozyme. *Journal of Dairy Science, 86*, 3075–3081.

Lund, B. M. (1990). Foodborne diseases due to *Bacillus* and *Clostridium* species. *Lancet, 336*, 982–986.

Mansour, M., Amri, D., Bouttefroy, A., Linder, M., & Milliere, J. B. (1999). Inhibition of *Bacillus licheniformis* spore growth in milk by nisin, monolaurin, and pH combinations. *Journal of Applied Microbiology, 86*, 311–324.

Mansour, M., & Milliere, J. (2001). An inhibitory synergistic effect of a nisin-monolaurin combination on *Bacillus* sp. vegetative cells in milk. *Food Microbiology, 18*, 87–94.

Nissen, H., Holo, H., Axelsson, L., & Blom, H. (2001). Characterization and growth of *Bacillus* spp. in heat-treated cream with and without nisin. *Journal of Applied Microbiology, 90*, 530–534.

Pellegrini, A., Thomas, U., Bramaz, N., Klauser, S., Hunziker, P., & von Fellenberg, R. (1997). Identification and isolation of bactericidal domain in chicken egg white lysozyme. *Journal of Applied Microbiology, 82*, 372–378.

Pellegrini, A., Thomas, U., von Fellenberg, R., & Wild, P. (1992). Bactericidal activity of lysozyme and aprotinin against Gram-negative and Gram-positive bacteria related to their basic character. *Journal of Applied Bacteriology, 72*, 180–187.

Pirttijärvi, T. S. M., Wahlström, G., Rainey, F. A., Saris, P. E. J., & Salkinoja-Salonen, M. S. (2001). Inhibition of bacilli in industrial starches by nisin. *Journal of Industrial Microbiology and Biotechnology, 26*, 107–114.

Rowan, N. J., Caldow, G., Gemmell, C. G., & Hunter, I. S. (2003). Production of diarrheal enterotoxins and other potential virulence factors by veterinary isolates of*Bacillus* species associated with non gastrointestinal infections. *Applied and Environmental Microbiology, 69*, 2372–2376.

Setlow, B., Loshon, C. A., Genest, P. C., Cowan, A. E., Setlow, C., & Setlow, P. (2002). Mechanisms of killing spores of *Bacillus subtilis* by acid, alkali and ethanol. *Journal of Applied Microbiology, 92*, 362–375.

Sheath, P. H. A., Mair, N. S., Sharpe, M. E., & Holt, J. G. (1986). *Bergey's manual of systematic bacteriology* (vol. 2). Baltimore: Williams and Wilkins.

te Giffel, M. C., Beumer, R. R., Leijendekkers, S., & Rombouts, F. M. (1996). Incidence of *Bacillus cereus* and *Bacillus subtilis* in foods in the Netherlands. *Food Microbiology, 13*, 53–58.

Valero, M., Leontidis, S., Fernándes, P. S., Martinez, A., & Salmerón, M. C. (2000). Growth of *Bacillus cereus* in natural and acidified carrot substrates over the temperature range 5–30 °C. *Food Microbiology, 17*, 605–612.