**ORIGINAL ARTICLE**

**The species-specific mode of action of the antimicrobial peptide subtilosin against *Listeria monocytogenes* Scott A**

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**Abstract**

**Aims:** To elucidate the molecular mechanism of action of the antimicrobial peptide subtilosin against the foodborne pathogen *Listeria monocytogenes* Scott A.

**Methods and Results:** Subtilosin was purified from a culture of *Bacillus amyloliquefaciens*. The minimal inhibitory concentration of subtilosin against *L. monocytogenes* Scott A was determined by broth microdilution method. The effect of subtilosin on the transmembrane electrical potential (ΔΨ) and pH gradient (ΔpH), and its ability to induce efflux of intracellular ATP, was investigated. Subtilosin fully inhibited *L. monocytogenes* growth at a concentration of 19 μg ml⁻¹. Subtilosin caused a partial depletion of the ΔΨ and had a similar minor effect on the ΔpH. There was no significant efflux of intracellular ATP.

**Conclusion:** Subtilosin likely acts upon *L. monocytogenes* Scott A by perturbing the lipid bilayer of the cellular membrane and causing intracellular damage, leading to eventual cell death. Subtilosin’s mode of action against *L. monocytogenes* Scott A differs from the one previously described for another human pathogen, *Gardnerella vaginalis*.

**Significance and Impact of the Study:** This is the first report on the specific mode of action of subtilosin against *L. monocytogenes* and the first report of a bacteriocin with a species-specific mode of action.

**Introduction**

*Listeria monocytogenes* is a Gram-positive, non-spore-forming bacterium. The organism is ubiquitous in the environment and is associated with the serious foodborne disease listeriosis, which may cause gastroenteritis, sepsis, meningitis, perinatal infections, spontaneous abortion and even death (Allerberger and Wagner 2010). Listeriosis is mainly of danger for persons with a suppressed immune system, like pregnant women, the elderly, AIDS patients or those with other immune-compromising diseases (Donnelly 2001; McLauchlin et al. 2004; Warriner and Namvar 2009). Epidemiological studies show that many different food products can become contaminated by the organism, although dairy and meat products are primarily associated with outbreaks of listeriosis (Farber and Peterkin, 1991; Allerberger and Wagner 2010). However, because *L. monocytogenes* can survive extreme environmental conditions, it is difficult to control in the food industry. For example, the pathogen grows in a wide range of temperatures (4–50°C), pH levels (4–9), salt concentrations (up to 25% NaCl) and water activities (as low as 0.91) (Farber and Peterkin, 1991; Tienungsung et al. 2000; Donnelly 2001; Gandhi and Chikindas 2007; Warriner and Namvar 2009). Disturbingly, *L. monocytogenes* can survive and multiply at refrigeration temperatures until it reaches levels high enough to cause disease (Chan and Wiedmann 2009). Recently, it was discovered that increasing numbers of *Listeria* strains have become resistant to the sanitizers and disinfectants used to clean processing plants in the food industry (for a full review, see Gandhi and Chikindas 2007).

A variety of new, effective measures are clearly needed to combat this micro-organism and prevent its
contamination of food products. One option often mentioned in the literature is bacteriocins (Dykes 1995; Moll et al. 1999; Cleveland et al. 2001; Chen and Hoover 2003; Joerger 2003; Galvez et al. 2007). Bacteriocins are generally defined as ribosomally synthesized antimicrobial compounds of proteaceous nature. Bacteriocins can be active against both closely related and more evolutionarily distant species (Dykes 1995; Moll et al. 1999; Cleveland et al. 2001; Chen and Hoover 2003; Joerger 2003; Galvez et al. 2007). Nisin is the only bacteriocin regulated and used in the food industry, but nisin-resistant bacterial strains can spontaneously develop (Gravesen et al. 2002, 2004), which is a concern for food product safety. Therefore, other bacteriocins should be considered for use as food preservatives.

Subtilosin was originally isolated by Babasaki et al. (1985) from Bacillus subtilis and was later found in Bacillus amyloliquefaciens (Sutyak et al. 2008a). The molecule is a cyclic peptide in which three cross-links exist between sulfur atoms of cysteine and the α-position of two phenylalanines and threonine (Kawulka et al. 2004). The spectrum of subtilosin’s antimicrobial activity against bacterial pathogens was reported by Shelburne et al. (2007), although these authors dismissed subtilosin as having limited usefulness as a human therapeutic. Furthermore, it was shown to have spermicidal activity against human, boar, rat, bovine and horse spermatozoa (Silkin et al. 2008; Sutyak et al. 2008b). More recently, Sutyak et al. (2008a) observed the antimicrobial effect of subtilosin against L. monocytogenes and several other human pathogens. However, the molecular mode of subtilosin’s action against L. monocytogenes is yet to be elucidated. Understanding how subtilosin inhibits listerial growth will aid in the development of optimal food preservation techniques. Sutyak Noll et al. (2011) reported subtilosin’s mode of action against the vaginal pathogen Gardnerella vaginalis; they discovered that it kills the target cells by depleting the transmembrane pH gradient (ΔpH) portion of the proton motive force (PMF) and by causing an efflux of intracellular ATP. Gardnerella vaginalis has an extremely unique cell wall structure that causes variable Gram-stain results (for review, see Turovskiy et al. 2011). Because of these properties, it is possible that subtilosin may act differently on L. monocytogenes, a true Gram-positive organism. Thus, we proposed to study subtilosin’s mode of action on L. monocytogenes by measuring differences in the PMF. The PMF consists of two components: a transmembrane electrical potential (ΔΨ) and the pH gradient (ΔpH) (Montville et al. 1995). In principle, if a compound interacts with and damages the membrane, changes will occur in either the ΔΨ, the ΔpH or both. Furthermore, as the energy status of a cell is generally considered to be an indication of the cell’s wellbeing, increases in extracellular ATP induced by membrane damage will also be measured. In this study, the mode of action of subtilosin will be studied against L. monocytogenes by measuring differences in the PMF and ATP metabolism.

Materials and methods

Production and extraction of subtilosin

The production and purification of subtilosin were conducted as described by Sutyak et al. (2008a). Briefly, a crude extract of subtilosin was obtained through ammonium sulfate precipitation of the cell-free supernatant from a B. amyloliquefaciens culture. Column chromatography was performed to further purify the peptide, with 90 and 95% methanol used to elute the protein from the columns. Methanol was removed from the active fractions, and they were concentrated to dryness using a Savant SC110 Speed Vac and UVS400 Universal Vacuum System (Savant Instruments, Farmingdale, NY, USA), and the peptide was resuspended in sterile ddH2O. As previously reported (Sutyak et al. 2008a), the purity of the sample has been verified through SDS-PAGE gels that indicate a single protein band corresponding to the known size of subtilosin (Sutyak et al. 2008a). The protein concentration of the sample was determined using a protein kit (Micro BCA® Protein Assay Kit; Thermo Scientific, Rockford, IL, USA) according to the manufacturer’s instructions.

Minimal inhibitory concentration (MIC) determination

Listeria monocytogenes Scott A (laboratory collection of micro-organisms) was grown overnight in tryptic soy broth (Difco™, Detroit, MI, USA) supplemented with 0.6% yeast extract (TGY) (Difco) at 30°C. Prior to experimentation, the overnight culture (c. 10⁶ CFU ml⁻¹) was diluted 100-fold in fresh TGY broth to a final concentration of c. 10⁴ CFU ml⁻¹. Stock solutions of subtilosin in sterile ddH₂O were prepared in such a way that the end concentration in the wells was 75, 56, 37.5, 30, 18.75, 9.375 or 4.69 μg ml⁻¹. The positive control nisin (2.5% bacteriocin preparation; Sigma, St Louis, MO, USA) was prepared as a 10 mg ml⁻¹ stock solution in nisin diluent (0.02 mol L⁻¹ hydrochloric acid, pH 1.7), of which 50 μl was added to wells containing 150 μl of the diluted L. monocytogenes culture. Uninoculated TGY broth was used as a negative control, while wells containing 200 μl of the L. monocytogenes dilution without antimicrobial served as a positive control for microbial growth. Each plate was covered with a clear thermal adhesive sealing film (lot no. 204786; Fisher, Pittsburgh, PA, USA) to pre-
vent evaporation of the contents. The plates were observed using a Molecular Devices THERMOMax Microplate Reader (Sunnyvale, CA, USA) with temperature control. The optical density of each well was measured at 595 nm every 30 min, with shaking 5 s prior to each reading, for 24 h at 30°C. The results were analysed using Microsoft Excel. Each experiment was performed at least twice in duplicate.

$\Delta \Psi$ measurement

The effect of bacteriocin treatment on transmembrane electrical potential ($\Delta \Psi$) was assessed as described by Bennik et al. (1998) with the following modifications. *Listeria monocytogenes* Scott A was grown in TGY broth at 30°C until an approximate OD$_{600}$ nm = 0.6 was reached. The cells were harvested by centrifugation for 15 min at 4500 g at 4°C (Hermle Z400K; LabNet, Woodbridge, NJ, USA), washed twice with 50 mmol l$^{-1}$ K-HEPES buffer at pH 7.0 (Sigma-Aldrich, St Louis, MO, USA) and concentrated in 1/100 of their original volume in the same buffer. During the experiment, cells were stored on ice. The $\Delta \Psi$ of the cells is observed in relation to the fluorescence of the probe DiSC$_3$(5) (3,3¢-dipropylthiadicarbocyanine iodide; Molecular Probes, Eugene, OR, USA). The experiment was performed using a PerkinElmer LS50B spectrofluorometer (PerkinElmer Life and Analytical Science, Inc., Boston, MA, USA) at excitation and emission wavelengths of 643 and 666 nm, respectively, and a slit width of 10 nm. Two millilitres of 50 mmol l$^{-1}$ K-HEPES buffer (pH 7.0) was used to equilibrate the fluorometer and acted as the starting point for the measurement of fluorescence. The reagents were added in the following order as soon as the fluorescent signal had stabilized from the previous step: 5 µl of 2 mmol l$^{-1}$ DiSC$_3$(5) (final concentration of 5 µmol l$^{-1}$), 10 µl of concentrated *L. monocytogenes* Scott A, 20 µl of 20% glucose, 2 µl of 5 µmol l$^{-1}$ nigericin dissolved in ethanol (Molecular Probes), an appropriate amount of bacteriocin or control to reach the desired end concentration, and 2 µl of 2 µmol l$^{-1}$ valinomycin in ethanol (Molecular Probes). The final concentration of subtilosin used was 19.56 µg ml$^{-1}$ (MIC, data not shown). A similar amount of sterile ddH$_2$O was used as a negative control while nisin (32 µg ml$^{-1}$) was used as a positive control. Each assay was performed at least twice in duplicate.

$\Delta$pH measurements

$\Delta$pH measurements were conducted as described by Molenaar et al. (1991) with the following modifications. *Listeria monocytogenes* Scott A was grown overnight in 30 ml of TGY broth until an OD$_{600}$ nm of 0.6 was reached. The cells were harvested by centrifugation for 15 min at 4500 g at 4°C (Hermle Z400K; LabNet, Woodbridge, NJ, USA), washed twice with 50 mmol l$^{-1}$ KPi buffer at pH 6.0 (Sigma-Aldrich) and concentrated 100-fold by resuspending them in 300 µl of the same buffer. Ten microlitres of BCECF-AM (2',7'-bis-(2-carboxylethyl)-5-(and-6)-carboxyfluorescein; Molecular Probes) was added to 200 µl of the concentrated cells, which were then incubated for 5 min at room temperature to allow the probe to be internalized. After this incubation, 1 ml of 50 mmol l$^{-1}$ KPi buffer (pH 6.0) was added and the cells were harvested by centrifugation as previously described. The cells were washed twice with 1 ml of 50 mmol l$^{-1}$ KPi buffer (pH 6.0) and resuspended in 200 µl of the same buffer. $\Delta$pH measurements were performed using a PerkinElmer LS50B spectrofluorometer at excitation and emission wavelengths of 502 and 252 nm, respectively, and slit widths of 5 nm for excitation and 15 nm for emission. The experiment was started by measuring the fluorescence of 2 ml of 50 mmol l$^{-1}$ KPi buffer (pH 6.0) in quartz cuvettes to provide a baseline fluorescence signal prior to adding the BCECF-loaded cells. As soon as the fluorescent signal stabilized, the compounds were added in the following order: 3 µl of BCECF-loaded cell suspension, 20 µl of 20% glucose (in water), 2 µl of 5 µmol l$^{-1}$ valinomycin in ethanol, an appropriate amount of bacteriocin or control to reach the desired end concentration and 2 µl of 2 µmol l$^{-1}$ nigericin in ethanol. Subtilosin was used at a concentration of 19.56 µg ml$^{-1}$ (MIC). A similar amount of water was used as a negative control, and nisin (32.61 µg ml$^{-1}$) was used as a positive control. Each experiment was performed at least twice in duplicate.

ATP measurements

The effect of bacteriocin treatment on intracellular ATP levels was determined using an ATP Bioluminescent Assay Kit (Sigma-Aldrich) and as described by Guihard et al. (1993) with the following modifications. All data measurements were taken using a Luminoskan™ single-tube luminometer (Lumineskan, Helsinki, Finland). An ATP standard curve was generated prior to each assay. Dilutions of ATP from 10$^{-8}$ to 10$^{-12}$ mol l$^{-1}$ were prepared using the ATP stock solution (3.79 × 10$^{-7}$ moles ml$^{-1}$). One hundred microlitres of these dilutions were mixed with 100 µl of the ATP assay mix (luciferin, luciferase, MgSO$_4$, DTT, EDTA, bovine serum albumin and tricine salt buffer salts), and the luminescence was measured. The concentrations were plotted against luminescence expressed in relative luminescence units.

*Listeria monocytogenes* Scott A was grown in TSB broth to a final OD$_{600}$ nm of 0.6. The cells were washed with
50 mmol l\(^{-1}\) 2-morpholinoethanesulfonic acid (MES) buffer (pH 6.5) and maintained on ice. The cells were then equilibrated at room temperature for 5 min. After this, the cells were energized by suspending them in half their original volume of 50 mmol l\(^{-1}\) MES buffer (pH 6.5) with 0.2% glucose and holding them at room temperature for 20 min. Two different measurements were performed: external ATP and total ATP (internal + external ATP). Prior to both measurements, 100 µl of the cell suspension was mixed with 100 µl of ATP assay mix. For total ATP levels, 20 µl of the cell mixture including subtilosin (final concentration 3 µg ml\(^{-1}\)) or water (2 µl) was mixed with 80 µl of DMSO and 4.9 ml of cold water. For the external ATP measurements, 100 µl of the cell mixture including the compound of interest or control was utilized as the positive control as it is known to create pores in target cell membranes (Moll et al. 1999). Nisin (final concentration 1:5 µg ml\(^{-1}\)) and DMSO were used as positive controls for total ATP. For the external ATP determination, water, nisin (final concentration 1:5 µg ml\(^{-1}\)) and MES buffer were used as controls. The final concentration of subtilosin used in the external ATP assay was 3 µg ml\(^{-1}\). The results were compared with the ATP standard curve. Each experiment was performed at least twice in duplicate.

**Results**

**Subtilosin production**

The procedure to obtain subtilosin was based on that described by Sutyak et al. (2008a). After partial purification, the protein content of the sample was measured and found to be 149:5 µg ml\(^{-1}\).

**Minimal inhibitory concentration**

The MIC was considered as the lowest concentration at which subtilosin completely inhibited the targeted microorganism. Subtilosin was found to completely inhibit the growth of the pathogen at a concentration of 19:56 µg ml\(^{-1}\). All MIC determination assays were conducted at least twice in duplicate. There was no variability between the data, resulting in no SD of the MIC.

**Proton motive force assays**

Subtilosin causes a partial dissipation of the transmembrane electrical potential (ΔΨ). Figure 1 represents the ΔΨ measurements resulting from exposure of *L. monocytogenes* Scott A to subtilosin. The ionophore nigericin was added to initially convert all ΔpH into ΔΨ, which does not influence fluorescence. After this step, subtilosin (final concentration 19:56 µg ml\(^{-1}\)) was added. The resultant increase in fluorescence indicates that an interaction with the cell membrane occurred. The ionophore valinomycin was then added to completely dissipate the ΔΨ. Valinomycin caused an increase in fluorescence as result of complete depolarization of the cells. This increase signifies that subtilosin does not completely dissipate the ΔΨ in *L. monocytogenes* cells when used at the MIC (19:56 µg ml\(^{-1}\)). Based on the total fluorescence increase caused by the addition of valinomycin, it is estimated that subtilosin dissipates 30% of the ΔΨ. As expected, addition of water, the negative control, did not influence fluorescence (data not shown). The bacteriocin nisin was utilized as the positive control as it is known to create pores in target cell membranes (Moll et al. 1999). Nisin caused a rapid increase in fluorescence after addition to the probe loaded cells. Further addition of valinomycin caused no increase in fluorescence, indicating that nisin completely dissipates the ΔΨ (data not shown).

Subtilosin has no effect on the ΔpH portion of the PMF. Figure 2 shows the effect of subtilosin on the ΔpH in *L. monocytogenes* Scott A cells. After the addition of subtilosin, the bioluminescence partially decreases (18.4%) before the signal equilibrates. Subsequent addition of nigericin caused an immediate and total depletion of the remaining ΔpH, confirming that subtilosin has only a partial effect on this portion of the PMF. Nisin caused a complete dissipation of the ΔpH. Thus, subtilosin has only a minor effect, and dissipation of the ΔpH is not subtilosin’s main mode of action against *L. monocytogenes* Scott A.

**ATP measurements**

ATP levels were determined using an ATP bioluminescence assay. The negative controls, water and MES buffer, resulted in an extremely low level of luminescence, signifying that the controls did not cause ATP efflux and that the *L. monocytogenes* cells were not leaking ATP prior to bacteriocin treatment (data not shown). Nisin, the positive control, caused an immediate efflux of ATP equivalent to 76.5% of the total ATP release. Conversely, subtilosin caused an efflux equivalent to 6.3% of the total ATP. Therefore, there is no efflux of ATP as subtilosin is probably unable to form pores in the membrane of *L. monocytogenes* Scott A cells.

**Discussion**

In this study, we have investigated the in vivo molecular mode of action of the antimicrobial peptide subtilosin against *L. monocytogenes* Scott A. Confirming the previously published reports (Sutyak et al. 2008a; Amrouche...
et al. 2010), subtilosin was shown to have potent antimicrobial activity against this pathogen with an MIC of just 19 µg ml⁻¹. Furthermore, Amrouche et al. (2010) found that subtilosin was even active against nisin-resistant strains of *Listeria*. Although its antimicrobial activity against *Listeria* is promising, it is necessary to determine exactly how the peptide works against the susceptible cells before it can successfully be used as a food preservative or protective packaging agent.

A detailed understanding of the precise mechanism of action is gained by examining the effect of the bacteriocin on the integrity of the cellular membrane, specifically by monitoring the PMF and efflux of intracellular ATP. As evident from the performed PMF assays (Figs 1 and 2), subtilosin has only a minor effect on the ΔΨ, and no effect on the ΔpH. This is in direct contrast to the results of Sutyak Noll et al. (2011), who showed that subtilosin inhibits the vaginal pathogen *G. vaginalis* by fully depleting the ΔpH, although it also had no effect on the ΔΨ. Subtilosin also caused a partial efflux of intracellular ATP in *G. vaginalis*, while we demonstrated that it did not cause an efflux of intracellular ATP from *L. monocytogenes* cells. Sutyak Noll et al. (2011) hypothesized that the effects of subtilosin were because of its ability to form transient pores in the *G. vaginalis* cellular membrane. This concurred with previous research that showed that in an *in vitro*, cell-free environment, subtilosin can cause damage by binding and inserting itself into the lipid bilayer (Thennarasu et al. 2005; Yamamoto et al. 2010). Indeed, the elucidated mode of action against *L. monocytogenes* (partial dissipation of ΔpH, no effect on intracellular ATP or ΔΨ) is in direct contrast to that described for *G. vaginalis* (full dissipation of ΔpH, partial efflux of ATP, no effect on ΔΨ).

To the best of our knowledge, this is the first report describing a species-specific mode of action for a bacteriocin. As Thennarasu et al. (2005) described, there are three possible mechanisms of subtilosin’s action involving bacterial...
cell membranes. Besides forming transient pores, it may interact with membrane-associated receptors or translocate across the membrane in an ion-mediated manner, thereby interrupting cellular processes and resulting in cell death. Specifically, many anionic peptides like subtilosin require zinc and complex with it for optimal activity; Brogden et al. (2003) speculated that zinc may therefore form a salt bridge that allows the peptide to overcome the overall negative charge on the bacterial cell’s surface. Thus, the peptide is able to penetrate the outer membrane, where it may then attach to ribosomes, inhibiting ribonuclease activity and initiating cell death. The changes in PMF observed in this study suggest that instead of forming pores, subtilosin is likely binding to the cellular membrane, perhaps via a membrane-bound receptor molecule, and inserting itself this study suggest that instead of forming pores, subtilosin is likely binding to the cellular membrane, perhaps via a membrane-bound receptor molecule, and inserting itself this study suggest that instead of forming pores, subtilosin is likely binding to the cellular membrane, perhaps via a membrane-bound receptor molecule, and inserting itself the lipid bilayer as Thennarasu et al. (2005) described previously. Huang et al. (2009) recently reported that a single amino acid substitution (T6I) in subtilosin greatly enhanced its haemolytic activity and demonstrated that altering the hydrophobicity of the molecule drastically affected its ability to bind the erythrocyte membrane. Clearly, interaction of subtilosin with the target cell membrane is essential for it to retain activity. Thus, it is feasible that perturbation of the membrane could disrupt cellular processes enough to cause eventual cell death without the dramatic or noticeable effects visualized with G. vaginalis. Consequently, it can be hypothesized that subtilosin has the unparalleled ability to kill its target organisms by a variety of mechanisms that are unique to the specific species.

The investigation into bacteriocins’ mechanism of action is always challenging; while bacteriocins’ MIC values are effective at killing micro-organisms and causing various measurable effects on the cells, much higher concentrations are often used to evaluate the bacteriocin activity on various artificial systems such as membrane vesicles, liposomes, etc. Although we recognize the limitations of using significantly elevated concentrations, it will still be interesting to investigate the mechanistic effect of subtilosin on L. monocytogenes cellular structures/functions when used in concentrations above its MIC. Our future studies will also focus on identifying other natural antimicrobials that inhibit L. monocytogenes and evaluating them for synergistic activity with subtilosin. This research will provide valuable information that can aid in the design and implementation of smarter, more effective food safety measures.

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References


Guillard, G., Bénédicti, H., Besnard, M. and Letellier, L. (1993) Phosphate efflux through the channels formed by colicins and phage T5 in Escherichia coli cells is responsible


