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Elucidation of the Molecular Mechanisms of Action of the Natural Antimicrobial Peptide Subtilosin Against the Bacterial Vaginosis-associated Pathogen *Gardnerella vaginalis*

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Abstract

Subtilosin A is a 35-amino acid long cyclical peptide produced by *Bacillus amyloliquefaciens* that has potent antimicrobial activity against a variety of human pathogens, including the bacterial vaginosis-related *Gardnerella vaginalis*. The specific mode of action of subtilosin against *G. vaginalis* was elucidated by studying its effects on the proton motive force's (PMF) components: transmembrane electric potential ($\Delta\Psi$), transmembrane pH gradient (ΔpH), and intracellular ATP levels. The addition of subtilosin to *G. vaginalis* cells caused an immediate and total depletion of the ΔpH , but had no effect on the $\Delta\Psi$. Subtilosin also triggered an instant but partial efflux of intracellular ATP that was twofold higher than that of the positive control bacteriocin, nisin. Taken together, these data suggest that subtilosin inhibits *G. vaginalis* growth by creating transient pores in the cells' cytoplasmic membrane, leading to an efflux of intracellular ions and ATP and eventually cell death.

Keywords

Bacteriocin; Subtilosin; Mode of action; Vaginal pathogen

Introduction

Bacterial vaginosis (BV) is a common condition characterized by an imbalance in the vaginal microflora, where healthy lactobacilli are replaced by a proliferation of facultative and anaerobic microorganisms, most notably *Gardnerella vaginalis* and *Prevotella*, *Peptostreptococcus*, *Porphyromonas*, and *Mobiluncus* spp. [9,10,22,33]. It has been estimated that between 10 and 30% of women in North America are afflicted by this ailment, frequently prompting them to seek medical attention [33]. Although BV often remains asymptomatic [1], the unrestricted growth of these organisms has been demonstrated to have pathogenic effects, particularly in pregnant women. BV is associated with the development of pelvic inflammatory disease [14], as well as a variety of pregnancy-related complications, including low fetal birth weight [18], pre-term births with an elevated risk of infant death [28], intra-amniotic infections leading to fetal brain damage [11,27], and spontaneous abortion [8,26]. Also of great concern is the well-established connection between BV infection and sexually transmitted diseases (STDs). Bacterial vaginosis, and *G. vaginalis* in particular, has been shown to increase the probability of contracting HIV and to stimulate its proliferation in multiple cell lines [15,16,29,37].

BV is typically treated by administering the antibiotics metronidazole and clindamycin orally or intravaginally. Although effective, these drugs do not specifically target the pathogens involved in BV, causing widespread inhibition of the healthy vaginal microbiota. In turn, this leads to a high (~20%) recurrence rate of BV shortly after cessation of treatment [40], often with newly arisen developed antibiotic resistances [3,21,23]. As such, it is critical that new treatments target the pathogens without affecting the host's healthy vaginal microflora.

Bacteriocins are ribosomally synthesized peptides produced by bacteria that have antimicrobial activity against organisms closely related to the producer species [7]. Bacteriocins have garnered much attention for their use as safe, natural food preservatives, as well as their potential in medical applications. One bacteriocin, subtilisin A, has strong potential for inclusion in alternative BV therapies. Produced by both *Bacillus subtilis* [2,34] and *Bacillus amyloliquefaciens* [35], subtilisin A (commonly referred to as subtilisin) has a cyclical, cross-linked structure unique among characterized bacteriocins. It has demonstrated antimicrobial activity against a wide variety of human pathogens [30], including *G. vaginalis* [35], and was recently shown to possess potent spermicidal activity while remaining completely nontoxic to human vaginal epithelial cells and healthy vaginal lactobacilli [30,35,36]. However, the inclusion of subtilisin in products aimed at BV prophylaxis or treatment requires a more detailed understanding of its specific mechanism of action against *G. vaginalis*, one of the primary pathogens involved in BV.

The primary mechanism of action for many bacteriocins is permeabilization of their target cell's cytoplasmic membrane. Electrostatic interactions between the membrane and bacteriocin provide a temporary linkage that allows the hydrophobic peptide to insert itself into the membrane [31]. In many cases, these transient pores produce leakage of intracellular ions, amino acids, and other low molecular weight molecules and eventually cause a total depletion of the cell's transmembrane ion potential (ΔpH) [5]. The disruption of this component of the proton motive force (PMF) may then lead to either a subsequent degradation of or efflux of intracellular ATP and/or disruption of the transmembrane electric potential ($\Delta\Psi$) [5,6]. In vitro studies conducted by Thennarasu et al. [38] demonstrated that at high concentrations, subtilisin is able to bind to lipid bilayers, although this binding is modulated by lipid composition. Due to its cyclical, cross-linked structure and uncommon net anionic charge [24], subtilisin can partially penetrate the hydrophobic core of lipid bilayers, disrupting their structure and thereby causing membrane permeabilization. Based on this knowledge, we hypothesized that subtilisin would selectively inhibit the growth of *G. vaginalis* by depleting cells' ATP levels and by dissipating one or more components of the proton motive force (PMF).

Materials and Methods

Bacterial Strains and Growth Conditions

Stock cultures of *G. vaginalis* ATCC 14018 were kept at -80°C in BHI broth (Difco, Sparks, MD) supplemented with 3% horse serum (JRH Biosciences, Lenexa, KS) and 15% glycerol. Cultures of *G. vaginalis* were grown anaerobically in BHI broth + 3% horse serum at 37°C without shaking. *B. amyloliquefaciens* cultures were grown overnight in MRS broth (Difco) at 37°C without shaking. The initial cultures were subcultured multiple times before use in experimental testing.

Preparation of Antimicrobial Solutions

The partially purified preparation of subtilisin was prepared as previously described [35]. The purity of this preparation was confirmed via PAGE analysis, with a single protein band

evident on the gel. Nisin (Sigma–Aldrich, St. Louis, MO; 100 AU/mL) was prepared according to the protocol given by Turovskiy et al. [39].

ATP Efflux Assay

The effect of subtilisin on ATP depletion in *G. vaginalis* cells was assessed by the previously established bioluminescence method [13] and modifications of Turovskiy et al. [39] using an ATP Bioluminescent Assay Kit (Sigma–Aldrich) and a Luminoskan™ single-tube luminometer (Labsystems, Helsinki, Finland). This kit correlates ATP release with relative fluorescence as a result of oxidation of the D-luciferin molecule by the firefly luciferase enzyme in the presence of ATP and Mg^{2+} .

G. vaginalis cells were grown overnight in 15-mL BHI broth supplemented with 3% horse serum to an $OD_{660} \approx 0.6$. Once they reached the appropriate growth stage, cells were centrifuged for 15 min at 4500 g (Hermle Z400K; LabNet, Woodbridge, NJ) at room temperature and then washed once with 50 mmol/L MES buffer (pH 6.5). The cells were then maintained at room temperature for 5 min prior to an energization period, in which the cells were resuspended in half their original volume of 50 mmol/L MES buffer (pH 6.5) with 0.2% glucose and held at room temperature for 20 min. Following energization, the cells were collected by centrifugation under the aforementioned conditions and resuspended in half their original volume in 50 mmol/L MES buffer (pH 6.5). This suspension was aliquoted in 100- μ L volumes into sterile 1.5-mL microcentrifuge tubes, to which 20 μ L of the appropriate treatment was added. Subtilisin was used at a final concentration of 2 μ g/mL, while the positive control (bacteriocin nisin) reached a final concentration of 1.5 μ g/mL, as per Winkowski et al. [41]. Subtilisin diluent (ddH₂O) and nisin diluent (0.02 M hydrochloric acid, pH 1.7) were used as negative controls. Each sample then remained at room temperature for 5 min prior to recording bioluminescent measurements.

The total ATP concentration in *G. vaginalis* cells was measured by combining 20 μ L of the final cell suspension with 4.9 mL of ice-cold ddH₂O and 80 μ L of DMSO. DMSO was chosen for its known ability to completely lyse bacterial cells, thus releasing all intracellular ATP. The data obtained for the negative controls were extremely uniform, allowing all other results to be normalized to their average and expressed as a percentage value.

Effect of Subtilisin on Proton Motive Force (PMF) in *G. vaginalis*

$\Delta\Psi$ Dissipation Assay—The ability of subtilisin to affect the transmembrane electric potential ($\Delta\Psi$) of *G. vaginalis* cells was assessed according to the protocol given by Sims et al. [32] and the modifications outlined by Turovskiy et al. [39].

Briefly, *G. vaginalis* cells were grown as previously described to an OD_{600} of 0.6, harvested, then washed once, and resuspended in $1/100$ of their original volume of fresh medium. The $\Delta\Psi$ of the cells was monitored as a function of the fluorescent intensity of the probe 3,3'-dipropylthia-dicarbocyanine iodide [DiSC₃ (5)] (Molecular Probes, Eugene, OR) at 22 °C using a PerkinElmer LS-50B spectrofluorometer (PerkinElmer Life and Analytical Science, Inc., Boston, MA) with a slit width of 10 nm and excitation and emission wavelengths of 643 and 666 nm, respectively. Initially, 5 μ L of probe was added to 2 mL of fresh BHI broth supplemented with 3% horse serum in quartz cuvettes (10 mm light path) at a final concentration of 5 μ mol/L. This was followed by addition of 20 μ L of cell suspension, which caused an immediate decrease in fluorescence. Once the signal had equilibrated, the cells were exposed to 2 μ L of 5 mM nigericin (Sigma) in order to convert the ΔpH into $\Delta\Psi$. After stabilization of the signal, subtilisin (2 μ g/mL), the positive control nisin (1.5 μ g/mL), or the negative control nisin diluent (HCl at pH 1.7) was added. Finally, any remaining $\Delta\Psi$ was dissipated by the addition of 2 μ L of 2 mmol/L valinomycin (Sigma).

Δ pH Dissipation Assay—The ability of subtilisin to affect the transmembrane pH gradient (Δ pH) of *G. vaginalis* cells was analyzed according to the protocol given by Molenaar et al. [25] and the modifications described by Turovskiy et al. [39].

Initially, *G. vaginalis* cells were grown overnight to an OD₆₀₀ of 0.6, harvested, then washed twice, and resuspended in a hundredth of their original volume of 50 mmol/L potassium phosphate buffer (PPB, pH 6.0). The cells were then exposed for 5 min to the pH sensitive probe BCECF-AM (MP Biomedicals, Inc., Solon, OH) at ambient temperature to allow the probe to diffuse into the cytoplasm. Following exposure, the cells were washed twice with 1 mL of 50 mmol/L PBS (pH 6.0) and resuspended in 200 μ L of the same. To measure dissipation of the transmembrane pH gradient, quartz cuvettes containing 2 mL of PPB (pH 7.0) were treated with 10 μ L of the cell suspension. Fluorescence was read using a PerkinElmer LS-50B spectrofluorometer with slit widths of 5 nm for excitation and 15 nm for emission, and wavelengths of 502 and 525 nm, respectively. After signal stabilization, the cells were energized with 4 μ L of 2.2 mmol/L glucose; the fluorescence subsequently rises as a result of an increase in intracellular pH. After again allowing for the signal to even out, 2 μ L of 5 μ mol/L valinomycin was added to convert the $\Delta\Psi$ component of the PMF into Δ pH. The cells were then treated with either subtilisin (2 μ g/mL), the positive control nisin (1.5 μ g/mL), or the negative control nisin diluent (HCl at pH 1.7). Two microliter of 2 μ mol/L nigericin was added to dissipate any remaining Δ pH.

Results

Subtilisin Causes an Efflux of ATP from *G. vaginalis* Cells

The effect of subtilisin on intracellular ATP levels in *G. vaginalis* cells was assessed as a function of bioluminescence, via the oxidation of the luminescent D-luciferin molecule by luciferase in the presence of extracellular ATP and Mg²⁺. Subtilisin caused an efflux of intracellular ATP, denoted by the increased intensity of the luminescence in Fig. 1a. On the other hand, the positive control (nisin) did not cause an efflux of ATP but instead triggered internal hydrolysis of the molecule, evidenced by a decrease in the luminescence in the total ATP sample (Fig. 1b). It was not possible to determine the effect of exposure to subtilisin and the controls past the single 5-min time point as the fastidiously anaerobic *G. vaginalis* cells poorly tolerated prolonged aerobic conditions (data not shown).

Subtilisin has no Effect on *G. vaginalis* Transmembrane Electrical Potential ($\Delta\Psi$)

The ability of subtilisin to dissipate the transmembrane electrical potential ($\Delta\Psi$) in *G. vaginalis* cells was observed using the fluorescent probe 3,3'-dipropylthiadicarbocyanine iodide [DiSC₃ (5)]. The ionophore nigericin (a K⁺/H⁺ exchanger) was added to the *G. vaginalis* cells in growth medium in order to convert the Δ pH to $\Delta\Psi$. The addition of nisin caused an instantaneous increase in the fluorescent signal of the probe as a result of the cellular membrane being depolarized by the bacteriocin (Fig. 2b). Subsequent introduction of the ionophore valinomycin had little effect, indicating nisin caused a complete collapse of this PMF component. However, the addition of subtilisin or the negative controls (nisin diluent and ddH₂O) did not cause an elevation in the probe's fluorescence, signifying they had no effect on the $\Delta\Psi$ (Fig. 2a, b). For both nisin diluent and subtilisin, subsequent addition of valinomycin fully depleted the $\Delta\Psi$, resulting in a fluorescence increase comparable to that seen after the addition of nisin (Fig. 2a, b). Unlike the positive control nisin, which caused a complete dissipation of the $\Delta\Psi$, subtilisin does not cause *G. vaginalis* cell damage by depleting this component of the PMF.

Subtilisin Causes an Immediate Depletion of the Transmembrane pH Gradient (ΔpH)

Subtilisin had an immediate impact on the transmembrane pH gradient (ΔpH). Prior to exposure to subtilisin and the controls, the ionophore valinomycin was introduced to convert all $\Delta\Psi$ to ΔpH . Addition of subtilisin caused an instant drop in the signal intensity of the pH dependent, fluorescent probe BCECF-AM, indicating an immediate intracellular decrease in pH in the *G. vaginalis* cells (Fig. 3a). Nisin also caused a decrease in the fluorescent signal, although at a slower, more gradual rate (Fig. 3b). Since the assay buffer was designed to have a pH lower than the intracellular pH of *G. vaginalis* cells [39], the decrease in intracellular pH is due to a depletion of the ΔpH . Adding nigericin to deplete any remaining ΔpH did not cause a further drop in fluorescence for either sample, indicating both nisin and subtilisin caused a total depletion of the ΔpH (Fig. 3a, b) through formation of transmembrane pores.

Discussion

In this study, we clarified the molecular mechanism of action of the bacteriocin subtilisin against the vaginal pathogen *G. vaginalis*. To the best of our knowledge, this is the first report detailing subtilisin's mode of action against a specific target microorganism.

Our results clearly show that subtilisin acts by fully depleting the transmembrane pH gradient (ΔpH) and causing an immediate efflux of intracellular ATP, but has no effect on the transmembrane electric potential ($\Delta\Psi$). The fact that subtilisin does not affect both portions of the PMF is not surprising, as several other bacteriocins are known to selectively dissipate only one PMF component. For example, enterocin P is able to dramatically decrease intracellular ATP concentrations and membrane potential ($\Delta\Psi$) in *Enterococcus faecium* T136 without affecting the ΔpH [17]. In contrast, pediocin PD-1 creates pores in target cell membranes that allow for leakage of K^+ ions but not ATP and other essential metabolites [4]. Taken together, the current results strongly suggest that the changes in the PMF brought about by subtilisin are due to the formation of transient pores in the cytoplasmic membrane of *G. vaginalis*. To our knowledge, this is the first report of the mode of action of subtilisin conducted on live cells. The in vivo results from this study support those of Thennarasu et al. [38], who demonstrated via an in vitro, cell-free system that subtilisin binds and inserts itself into the lipid bilayer of their target cell membrane. Their work showed that only the hydrophobic region of the cyclic peptide is submerged in the bilayer, while the anionic portion of the molecule remains free above the membrane. Further, they found that membrane permeabilization occurred at subtilisin concentrations well above the MIC level for the tested strain of *E. coli*, a phenomenon that has also been reported for the bacteriocins nisin and mersacidin [19,20]. At these high concentrations, aggregation occurs that creates multimeric units of subtilisin, thereby greatly increasing destabilization of the cell membrane. Thus, this destabilization event likely leads to subsequent formation of transient pores, which then cause the ΔpH dissipation and ATP efflux seen in the current investigation.

Our previous research [35,36] established the antimicrobial activity of subtilisin against a variety of pathogens involved in human health, as well as its safety for human tissues. However, the possibility of its inclusion in personal care products aimed at the treatment and/or prophylaxis of BV required that its specific mode of action against the target pathogen, *G. vaginalis*, be fully characterized. The data gathered in this study will allow for an intelligent design of subtilisin-based formulations for the effective control of BV-associated microorganisms. The growing problem of bacterial resistance to common antimicrobials is often due to prolonged exposure that allows the microorganisms to develop resistance. This can be avoided or delayed through the use of multiple compounds with differing modes of action that synergize to more effectively control the growth of the

pathogen. The target cells will have less time to adapt to the various stresses, and it is far less likely that resistant organisms will appear. Now that subtilisin's mode of action has been clarified, future research will focus on evaluating potential synergies with other natural antimicrobials that possess differing mechanisms of action in a multiple-hurdle approach [12] against *G. vaginalis*. Furthermore, model studies will be performed using membrane vesicles and liposomes derived from *G. vaginalis*' cell wall in order to fully ascertain whether a cell wall receptor molecule plays a role in subtilisin binding and eventual cell death. These assays will provide a final key piece of information in the intelligent design and formulation of a novel, safe product containing subtilisin for the treatment of BV.

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Fig. 1.

Subtilisin causes an efflux of intracellular ATP from *G. vaginalis* cells. *Closed bars* represent the total ATP content (intracellular + extracellular), while *open bars* represent extracellular ATP. Subtilisin (**a**) caused an efflux of ATP approximately 1.5-fold higher than that of nisin (**b**) and 2-fold higher than the negative control. Total ATP levels for nisin (**b**) were 20% lower than that of subtilisin (**a**) and both negative controls (**a, b**), indicating intracellular hydrolysis of ATP

Fig. 2.

Subtilisin has no effect on transmembrane electric potential ($\Delta\Psi$) in *G. vaginalis* cells. Two $\mu\text{mol/L}$ of nigericin (Nig) was used to convert the ΔpH portion of the PMF into $\Delta\Psi$ (**a, b**). Subtilisin (Sub, **a**) caused no fluctuation in the fluorescent signal, indicating it has no effect on the $\Delta\Psi$. Addition of nisin (Nis, **b**) caused a corresponding spike in fluorescence, due to release of the DiSC₃ (5) probe and dissipation of the $\Delta\Psi$. In both cases, the negative controls (control (-)) subtilisin diluent (**a**) and nisin diluent (**b**) had no effect on the $\Delta\Psi$. Two $\mu\text{mol/L}$ of valinomycin (Val) was used to dissipate any remaining $\Delta\Psi$ (**a, b**). There was no increase in fluorescence in the nisin sample (**b**), demonstrating that nisin caused a total depletion of the $\Delta\Psi$ portion of the PMF

Fig. 3. Subtilisin immediately depletes the transmembrane pH gradient (ΔpH) in *G. vaginalis* cells. Cells were energized with 2.2 mM glucose at start of fluorescence readings. Two $\mu\text{mol/L}$ of valinomycin (Val) was used to transform the $\Delta\Psi$ of the PMF into ΔpH (a, b). Subtilisin (Sub, a) caused an immediate decrease in the fluorescent signal, indicating a depletion of the ΔpH . Nisin (Nis, b) triggered a slower, gradual decrease in fluorescence. Both negative controls, (control (-)) subtilisin diluent (a) and nisin diluent (b), had no effect on the ΔpH . Two $\mu\text{mol/L}$ of nigericin (Nig) was used to dissipate any remaining ΔpH (a, b). For both subtilisin and nisin, there was no subsequent drop in fluorescence, signifying that both bacteriocins totally depleted the ΔpH portion of the PMF