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Antimicrobial Activity Screening of Recombinant and Synthetic Varasin a Defensin from the Hard Tick *Dermacentor variabilis* Against Various Bacteria

Julia A. Sharp
Old Dominion University

ANTIMICROBIAL ACTIVITY SCREENING OF RECOMBINANT AND SYNTHETIC VARISIN, A DEFENSIN FROM THE HARD TICK *DERMACENTOR VARIABILIS*, AGAINST VARIOUS BACTERIA

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Julia A. Sharp
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Approved by:

Wayne L. Hynes (Director)

Roland Cooper (Member)

Alex Greenwood (Member)

ABSTRACT

ANTIMICROBIAL ACTIVITY SCREENING OF RECOMBINANT AND SYNTHETIC VARISIN, A DEFENSIN FROM THE HARD TICK *DERMACENTOR VARIABILIS*, AGAINST VARIOUS BACTERIA

Julia A. Sharp
Old Dominion University, 2007
Director: Dr. Wayne L. Hynes

Hematophagous arthropods, such as ticks and mosquitoes, rely on their innate immune system for defense against pathogens ingested in a blood meal as well as those acquired through injury. In response to pathogen recognition, the production of antimicrobial peptides, such as defensin, is typically up-regulated. Varisin, a defensin, is thought to be a key component in the immuno-competence of the hard tick *D. variabilis* against *Borrelia burgdorferi*, the causative agent of Lyme disease. To study the antimicrobial effects of varisin, recombinant varisin was expressed by both insect cells and *E. coli*. Purification of the protein followed by enterokinase treatment yielded a protein identified by Western blotting as varisin. However, neither recombinant protein was bacteri-cidal against *M. luteus*, a bacterium with known susceptibility to varisin. The lack of activity is possibly the result of varisin aggregates forming prior to their interaction with the target cell. Synthetic varisin was shown to inhibit the growth of 13 out of 14 Gram-positive bacteria and 2 out of 20 Gram-negative bacteria via well-diffusion and microtiter inhibition assays. Studies of varisin are essential to further our understanding of the innate immune response as well as providing possible insight into treatment or preventative measures for bacterial illness.

This thesis is dedicated to my children, Evan and Sophie. You are my inspiration.

ACKNOWLEDGMENTS

First and foremost, I would like to acknowledge Dr. Wayne L. Hynes for his guidance and understanding through the course of my research. I am most grateful for the time, consideration, and patience that he has given me during my research and educational endeavors. I would also like to thank Dr. Roland Cooper and Dr. Alex Greenwood for their advice and support. Furthermore, I would like to extend my appreciation to Stephanie M. Todd for her instruction during my early days in the lab.

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INTRODUCTION

Arthropods are frequently exposed to microorganisms and parasites, but rarely become infected. Through the combined action of hemocytes and the production of antimicrobial peptides, their innate immune system, although not adaptive, offers protection against many types of pathogens (14, 16, 25, 27). While not known for ticks, it is assumed that the innate immune system is initiated by the recognition of pathogen-associated molecular patterns (PAMPs) (7), such as β-1,3-glucan of fungi, phosphoglycan of parasites, and the bacterial cell components lipopolysaccharide and lipoteichoic acid (14, 22). Recognition of PAMPs triggers a cascade of events which leads to changes in gene expression or altered cell function. This response may involve the coordinated action of several different hemocytes, specialized blood cells of the arthropod hemolymph (circulatory fluid), through mechanisms such as phagocytosis, encapsulation, nodulation, and the production of antimicrobial peptides.

Phagocytosis, a widely conserved cellular response to small invaders such as bacteria, is a function of hemocytes (14, 18, 24). Plasmatocytes, which are hemocytes with functional similarity to mammalian macrophages (24), are the primary phagocytic cells. Granular cells (granulocytes) are also known to be phagocytic, as shown in *Ixodes ricinus* (1) and Lepidoptera members (18). Phagocytosis begins with the receptor-mediated hemocyte:pathogen attachment followed by endocytosis, which involves cytoskeletal rearrangements in the

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hemocyte. The engulfed invader is typically destroyed within a phagosome (14, 24).

If the invader is too large to be phagocytosed, encapsulation may occur via the action of both hemocytes and plasma proteins (18, 23). The attachment of hemocytes to the pathogen initiates the release of chemotactic molecules that attract additional hemocytes to the site of infection. Cellular encapsulation in the soybean looper *Pseudoplusia includens*, Lepidoptera spp., for instance, initiates with the attachment to and aggregation of granulocytes around the invader. Plasmatocytes then surround the layer of granulocytes, with an additional layer of granulocytes completing the capsule (23). It is thought that cell-adhesion molecules (e.g., integrins) enhance the ability of hemocytes to adhere to invaders (14, 23).

The process of melanization, a humoral type response, generally follows encapsulation and requires the proteolytic cleavage of the zymogen prophenoloxidase to its activated form phenoloxidase. Cleavage occurs via the action of the enzyme prophenoloxidase-activating proteinase. This activation system is dependent on a cascade of serine proteases that are comparable to vertebrate complement proteins (14, 23, 24). Phenoloxidase catalyzes the synthesis of melanin via the production of quinones from the oxidation of tyrosine-derived phenols. Pathogens are destroyed during melanization as both melanin and its intermediates are cytotoxic (24). Encapsulation and melanization generally occur in response to protozoan or metazoan parasitic invasion. Insect

orders such as Lepidoptera and Diptera are known to respond to parasites in this fashion (14, 23).

Large quantities of extracellular bacteria may be ensnared by the process of nodulation which involves the formation of hemocytic aggregates. These nodules may attach to tissues within the arthropod and may ultimately become encapsulated (14, 18). Nodule formation can be induced following the recognition of bacterial cell components such as LPS or some glycoproteins (14). The hard tick *Dermacentor variabilis*, for example, employs nodulation followed by encapsulation to control *E. coli* infections (6). Nodules may also undergo melanization as a means of destroying the bacteria (14).

As part of the humoral type response to invasion, the production of antimicrobial peptides by hemocytes or the fat body, which has comparable function to the mammalian liver, is upregulated (4, 6, 14, 22). Antimicrobial peptides are effective against a variety of microorganisms including fungi, protozoa, enveloped viruses, and bacteria (8, 14, 16, 19). A synergistic defense is also possible when more than one antimicrobial peptide is present (4). The type of antimicrobial peptide produced depends on the invading pathogen and the host species, and may include cecropin, lectin, attacin, drosomycin, or defensin (8, 14).

Antimicrobial peptides possess several common characteristics. Typically, they have a molecular weight below 5 kDa and may contain amphipathic α-helices (4), which allow for an interaction with microbial membranes (13), β sheets with a hairpin arrangement, or a mixture of the two

(4). Having a net positive charge at physiological pH provides them with a binding preference for bacterial membranes (10).

Defensins are broadly distributed throughout both the animal and plant kingdoms and possess antibacterial, antifungal, and antiviral properties (27), but are most effective against Gram-positive bacteria (4, 8, 31). Defensins are cationic peptides of approximately 4kDa in size that contain between 29-46 amino acids, with most having six cysteine residues (2, 8, 27, 31). Defensins have three distinct regions: an amino-terminal loop, an amphipathic α-helix, and a C-terminal anti-parallel β-sheet (8) (Fig. 1). Most defensins contain three disulfide bridges; however, defensins with four disulfide bonds have been reported in the mussel *Mytilus galloprovincialis* (15, 30). In addition to providing stability, disulfide bonds are thought to be instrumental in the antimicrobial action of the peptide, as disruption of these bonds results in loss of activity (8).

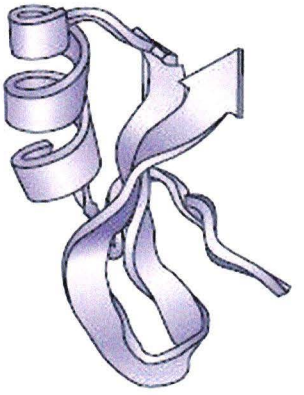

FIG. 1. Structure of an insect defensin. This representative structure shows the α-helix and C-terminal anti-parallel β-sheet common to arthropod defensins (13).

Defensins are produced as precursor molecules that possess a signal sequence and a propeptide region. Both the amino acid and nucleotide sequences that encode the signal sequence are highly conserved among defensin families. Defensins become active in their mature form following cleavage of the pro-region (9, 16) with mature defensins exerting their antimicrobial effect by interacting with elements of the negatively charged bacterial cell membrane (Fig. 2). Attracted by electrostatic forces, the peptides aggregate near the membrane surface causing a distortion in the membrane; this leads to the formation of multimeric pores (8, 30) facilitated by the amphiphatic α-helix (13). Against Gram-positive bacteria, defensins cause a disruption in the permeability of the cytoplasmic membrane resulting in partial depolarization of the cell due to a loss of potassium; this leads to reduced respiration (8, 30) and protein synthesis, and decreased levels of RNA, DNA (12), and ATP (8, 30). Against Gram-negative bacteria, defensins target the LPS layer and displace the divalent cations Ca^{2+} or Mg^{2+} that bridge the LPS components (9, 30). Once defensins gain access to the inner membrane, aggregation and pore formation occurs similarly to the cytoplasmic membrane disruption of Gram-positive bacteria (30).

Hematophagous arthropods, such as ticks, mosquitoes, and sand flies, rely on their innate immune system for protection against pathogens ingested in a bloodmeal in addition to those acquired through septic injury. Once induced, defensins are rapidly produced and released. When produced constitutively,

defensins are stored and released following microbial challenge (4, 14, 27, 30, 33).

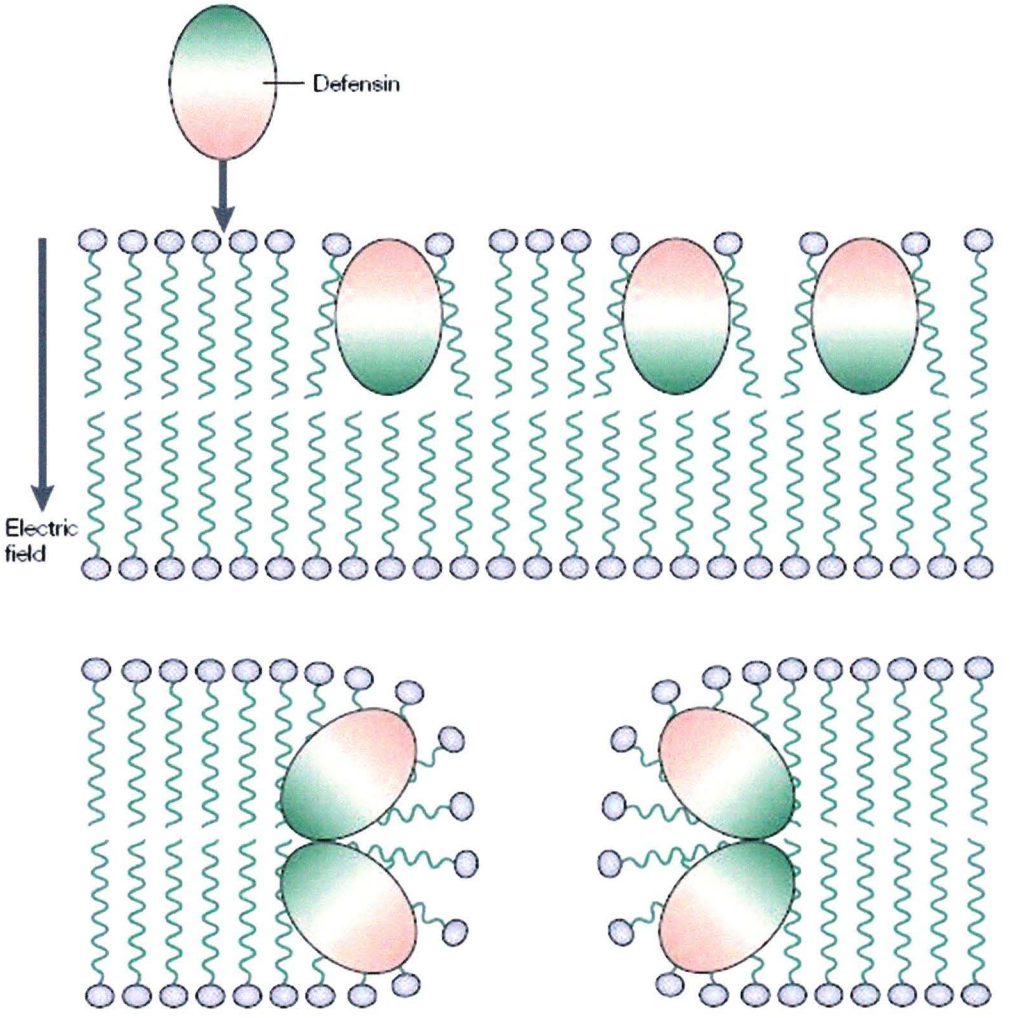

FIG. 2. Defensin mechanism of action. Defensins are attracted by electrostatic forces to the negatively charged bacterial cell membrane. As defensins possess both hydrophilic and hydrophobic regions, they are able to interact with the target cell membrane. Once inserted into the membrane, defensins aggregate which leads to multimeric pore formation and subsequent cell lysis (13).

The soft tick *Ornithodoros moubata* constitutively expresses defensin in the midgut, fat body, and the reproductive tract (26). Similarly, defensin

expression is detected in the midgut, fat body, and the ovaries of the cattle tick *Boophilus microplus* (11). Following bacterial challenge, the production of defensin is upregulated in the hemolymph of adult *Anopheles gambiae* mosquitoes with continued constitutive expression evident in the midgut (35). Due to the potential for pathogen contact, the midgut and hemolymph are primary sites for antimicrobial peptide secretion (2, 27, 33).

The hard tick *Haemaphysalis longicornis* is a major vector for the parasitic infection babesiosis of humans and domestic animals. Tsuji et al. (2007) report the discovery of a unique parasiticidal peptide, longicin, which possesses antimicrobial properties similar to those exerted by defensin homologues. As with other defensins, longicin is constitutively expressed, with an upregulation following a blood meal. Longicin expression occurs primarily in the midgut epithelium of *H. longicornis*, with secretion into the gut lumen suggested. As visualized by fluorescent confocal microscopy, longicin was shown to be effective against the merozoite stage of equine *Babesia equi* by binding to the parasite membrane with subsequent membrane lysis at 44 h post incubation. *In vivo* analysis of the hemoparasiticidal activity of longicin against *B. microti*, a murine *Babesia* parasite, showed that the rate of parasitemia in *B. microti*-infected mice was significantly reduced following intravenous treatment with longicin. Moreover, longicin appeared stable *in vivo*, perhaps due to the lack of murine proteases specific for this peptide, with efficacy of longicin treatment superior to treatment with current antibabesial pharmaceuticals (34).

Johns et al. (2001) studied the fate of the spirochete *Borrelia burgdorferi*, the causative agent of Lyme disease, following hemocoel inoculation of the hard ticks *Ixodes scapularis* and *D. variabilis*. *I. scapularis*, the competent vector for Lyme disease, although possessing the defensin gene scapularisin (17), was unable to clear the infection (21). Direct Immunofluorescence Assay (DFA) of the hemolymph, salivary glands and ovaries detected numerous intact spirochetes 1 h post infection. At 24 h post infection, DFA revealed few intact spirochetes in the hemolymph with spirochete fragments in or adjacent to hemocytes. However, both the salivary glands and the ovaries contained intact spirochetes. Conversely, *D. variabilis*, an incompetent vector of Lyme disease, was able to lyse most of the spirochetes within 1 h. The combined action of antimicrobial proteins, including the defensin varisin, and phagocytosis are suggested as the dual mechanism for spirochete control in *D. variabilis* (21).

Varisin has a molecular weight of 4.2 kDa with its first 30 amino acids sharing an 83% sequence similarity to the defensin of the scorpion *Leiurus quinquestriatus*, suggesting high conservation. RT-PCR of RNA from the hemocytes of *B. burgdorferi*-challenged *D. variabilis* resulted in a 624 bp amplicon with a 225 bp region translating to a preprodefensin of 74 amino acids (7).

Studies of the antimicrobial properties of partially purified varisin revealed its marked effects against the growth of *Bacillus subtilis* (20) and *M. luteus* (32), in addition to its antiborrelial activity, as discussed (20, 32). It is thought that varisin is stored in the hemocytes and released into the hemolymph in response

to bacterial challenge (7). To date, mature varisin is not thought to be produced in the midgut, although varisin transcript has been detected in midgut-cell lysates of *D. variabilis*. The presence of varisin transcript in unchallenged *D. variabilis* fat-body lysates implicates the fat body as a potential storage site for varisin. Whether or not the midgut and fat body store and release varisin is unknown (7).

A recent study by Ceraul et al. (2007) identifies a second defensin isoform produced by *D. variabilis* with different tissue distribution patterns seen between varisin (Defensin-1) and the new isoform (Defensin-2). Whereas hemocytes are the principal site for varisin expression, Defensin-2 is primarily expressed, as determined by presence of transcript, by the midgut, fat body and ovaries, with only slight expression by hemocytes. These findings may suggest that *D. variabilis* has tissue-specific antimicrobial gene expression profiles adapted to defend against various stages of pathogen invasion. The authors indicate that further investigations into the local versus systemic antimicrobial expression in response to Rickettsial challenge will further elucidate the importance of this finding (5).

Currently, research efforts involving the detection of varisin, such as with Western blotting, are difficult when using rabbit serum that contains antibodies that bind non-specifically to tick proteins. Synthetic varisin and its complementary antibody work well together but are expensive. Therefore, the production of purified recombinant varisin would provide the means for large-scale purified antibody production, be more cost-effective, and would greatly improve analysis efforts that require the detection of varisin. Although studies of

the antimicrobial effects of partially purified varisin have been assessed (20, 32), the production of pure varisin and analysis of its antimicrobial activity have not been reported. Likewise, assessment of the antimicrobial activity of synthetic varisin would greatly enhance our knowledge of the capabilities of this defensin.

Since defensins are produced by many types of organisms, including mammals, studies of the antimicrobial activity of varisin will provide information with the potential to give insight into the properties and action of other defensins. With the knowledge that varisin is particularly effective against Gram-positive bacteria and that it is also borreliacidal, studies of this peptide may elucidate possible treatment or preventative approaches to bacterial illnesses such as Lyme disease and perhaps those caused by antibiotic resistant strains such as Methicillin-resistant *Staphylococcus aureus* (MRSA).

This project was designed to assess the antimicrobial activity of both recombinant varisin (mature) and synthetic varisin against various Gram-positive and Gram-negative bacteria. Insect cells were chosen as the primary expression system that should facilitate the appropriate folding pattern of the peptide, thereby mimicking what would be the natural conformation of varisin. Due to the possibility of post-translational modification, such as glycosylation, through expression via insect cells, recombinant varisin was also expressed by bacterial cells (*E. coli*). Generally, proteins expressed via prokaryotic cells do not form intramolecular disulfide bonds, which are central to the activity of defensin (8). Therefore, a strain of *E. coli* with mutations in genes for thioredoxin reductase

and glutathione reductase was selected for expression studies due to their ability to form disulfide bonds in the cytoplasm.

It is hypothesized that both the recombinant and synthetic peptides would have bactericidal effects upon the Gram-positive bacteria assayed, in particular against *M. luteus*, as this bacterium is known to be highly susceptible to varisin (31). Conversely, neither peptide was expected to inhibit the growth of the Gram-negative bacteria assayed in this study, due to the complexity of their membranes.

As summarized in Fig. 3, the insect cells and bacteria were transfected or transformed, respectively, with a corresponding plasmid containing the mature varisin insert and expression was induced. Purification of the proteins was conducted via metal chelation chromatography specific for a 6xHis-tag region. Additional manipulation of the recombinant proteins involved recombinant enterokinase treatment to remove the His-tag region from the protein, thereby yielding a pure protein. The sf9-expressed protein was also treated with various denaturing or reducing agents to disrupt disulfide bonds. Both recombinant proteins were assessed for molecular weight and His-tag or varisin-specific regions using silver staining of an SDS-PAGE gel and Western blotting, as appropriate. The antimicrobial activity of recombinant varisin (both insect-cell and bacterial cell expressed) against *M. luteus* was evaluated by well-diffusion assay. Antimicrobial activity of synthetic varisin against various bacteria was determined using both well-plate diffusion and microtiter inhibition assays.

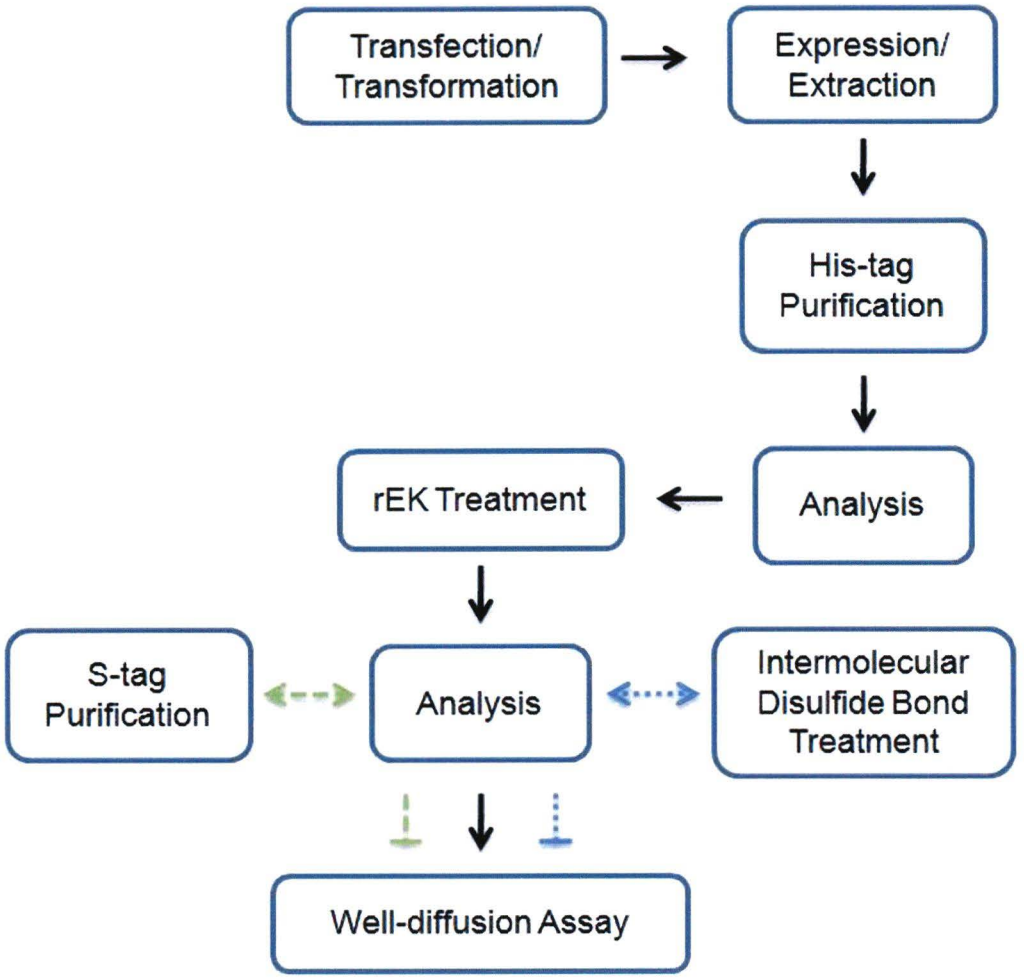

FIG. 3. Experimental flow chart. This chart represents the basic steps involved in recombinant varisin production, isolation, treatment, and screening. Solid arrows: main flow; dashed or dotted arrows: variances from the main flow (Sf9-expressed protein only); blunt-ended arrows: step not conducted. Analysis included SDS-PAGE, Western blotting, and/or silver staining.

MATERIALS AND METHODS

Insect cell culture. Sf9 insect cells (Novagen) derived from *Spodoptera frugiperda* were grown in Graces Insect Medium (GIBCO) supplemented with 10% fetal bovine serum (Cambrex Bio Science) at 28°C. Cells were grown to approximately 90% confluency, as assessed under a light microscope, then subcultured.

Insect cell transfection. Prior to transfection, cells were collected in BacVector medium (Novagen) and viable cells were determined using the Trypan Blue exclusion method. A cell density of 10^7 cells per 8 ml media was used for each transfection. Sf9 insect cells were transfected using the InsectDirect (Novagen) method, per manufacturer's instructions, using the pIEX-3 plasmid (Novagen) containing the mature varisin coding sequence insert (pIEX-3vsn; sequenced and verified for correct orientation by Lisa J. Fellman, ODU; Fig. 4 and 5). Briefly, 20 μg plasmid DNA in 1 ml of BacVector medium was added dropwise to 1 ml of BacVector medium containing 100 μl of InsectGene Juice Transfection Reagent (Novagen). Following 15 min incubation at room temperature, the mixture was added to an Erlenmeyer flask containing the Sf9 cells. The transfection mixture was placed in a 28°C shaking incubator, 150 rpm, for 48 h to allow for expression.

Extraction of Sf9-expressed protein. Following the transfection and 48-h incubation period, extraction of the target protein was performed by the addition of 0.05 x culture volume of Insect PopCulture Reagent (Novagen) followed with

0.4 μl of benzonase nuclease (Novagen) per ml of original culture volume. After gentle mixing and 15 min incubation at room temperature, the mixture was frozen at -20°C for storage.

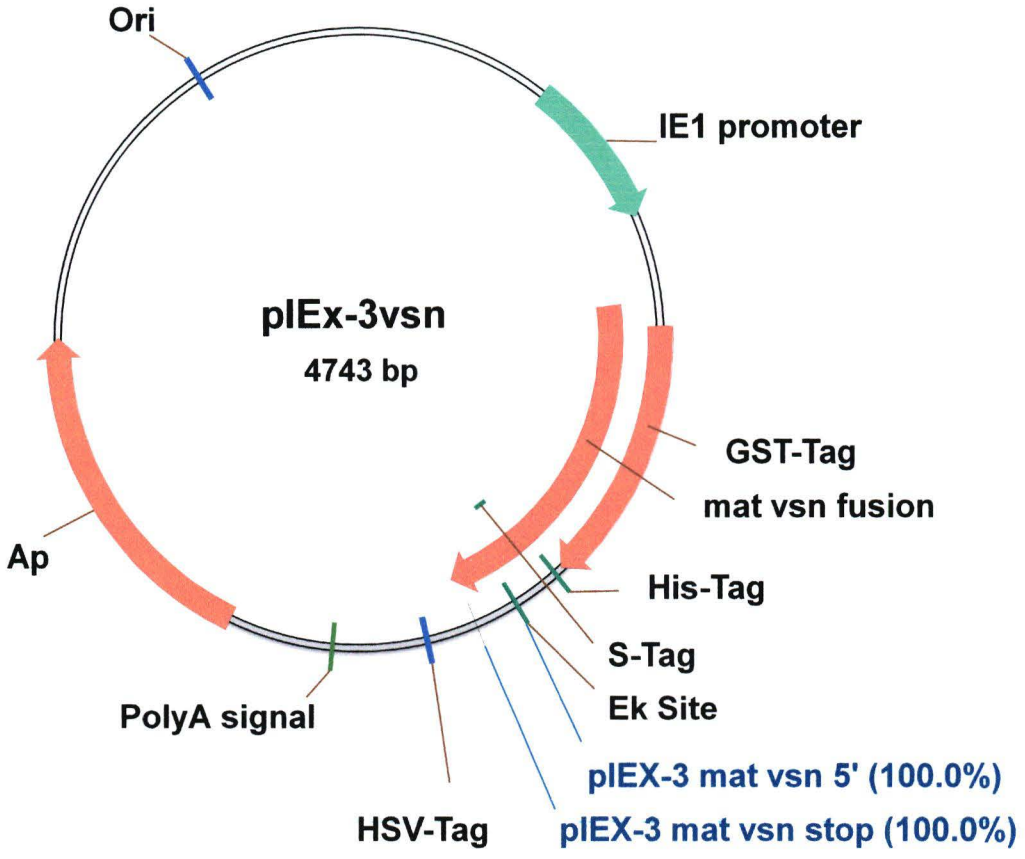

FIG. 4. pIEx-3vsn plasmid map. pIEx-3 plasmid (Novagen) with mature varisin insert.

Purification of Sf9-expressed protein. Isolation of recombinant varisin was performed following extraction from cells. The pIEx-3 plasmid contains a fusion-to-protein 6xHis-tag and an S-tag region which are useful for purification purposes (Fig. 4 and 5). The 6xHis-tag can be detected using Western-blot

analysis. The molecular weight of the detected protein can be compared to the expected molecular weight of recombinant varisin (Fig. 6).

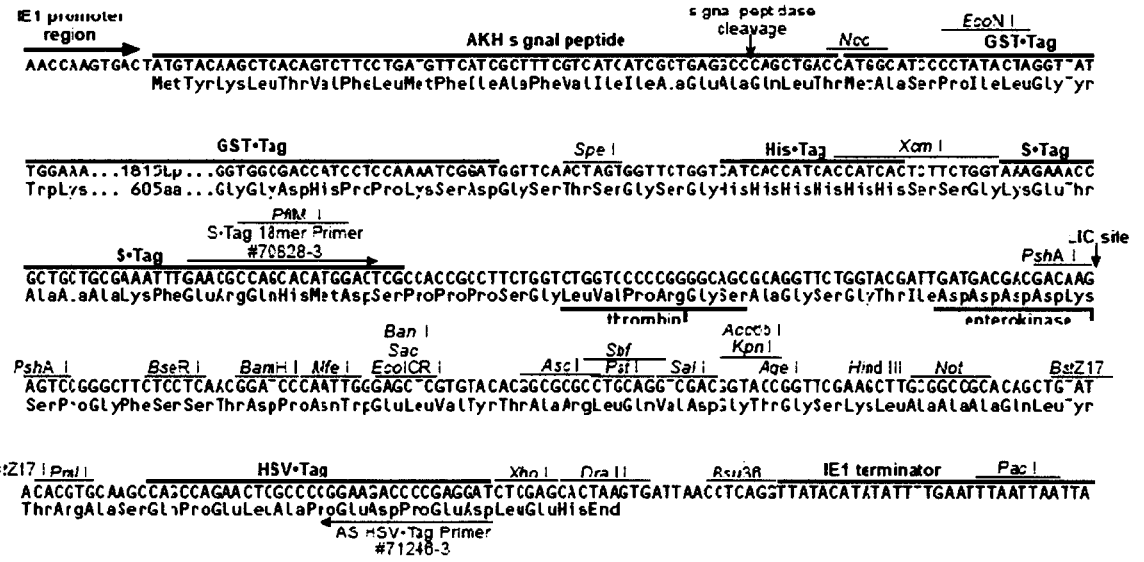

FIG. 5. pIEx-3 cloning/expression region (Novagen).

```
  1  MYKLTVFLMF  IAFVIIAEAQ  LTMASPILGY  WKIKGLVQPT  RLLLEYLEEK
 51  YEEHLYERDE  GDKWRNKKFE  LGLEFPNLPY  YIDGDVKLTQ  SMAIIRYIAD
101  KHNMLGGCPK  ERAEISMLEG  AVLDIRYGVS  RIAYSKDFET  LKVDFLSKLP
151  EMLKMFEDRL  CHKTYLNGDH  VTHPDFMLYD  ALDVVLYMDP  MCLDAFPKLV
201  CFKKRIEAIP  QIDKYLKSSK  YIAWPLQGWQ  ATFGGGDHPP  KSDGSTSGSG
251  HHHHHHSSGK  ETAAAKFERQ  HMDSPPPSGL  VPRGSAGSGT  IDDDDK▼GFGC
301  PLNQGACHNH  CRSIRRRGGY  CSGIIKQTCT  CYRN*
```

Molecular weight: 38077.58; Length: 335 amino acids; * stop; ▼Enterokinase cleavage

FIG. 6. Translation of recombinant varisin (pIEx-3vsn). Signal peptide is indicated by the underlined region and is cleaved during translocation to the extracellular environment (supernatant); mature varisin is indicated by the bolded region.

His-tag purification of the supernatant was performed using Ni-NTA His Bind Resin (Novagen) that binds to the 6xHis-tag sequence via metal chelation chromatography. This system provides a non-denaturing method for purification. Flowthroughs and eluant samples of the column were assessed using SDS-PAGE (4-12% Bis-Tris and 10% Tris-Gly; Invitrogen); silver staining (SilverXpress Silver Staining Kit, Invitrogen), per manufacturer's instructions; and Western blotting using His-tag monoclonal antibody (Novagen). Samples positive for the His-tag were combined, then dialyzed (12-14K MWCO tubing) with 500 ml (x2) of 1x wash/bind buffer (200 mM Tris-HCl pH 7.5, 1.5 M NaCl, 1% Triton X-100) necessary for S-tag purification.

S-tag purification of the His-tag-purified sample was performed using S-protein agarose beads (included in the S-Tag rEK Purification Kit, Novagen) which bind S-tag fusion proteins, per manufacturer's instructions. Elution was accomplished using 3 M magnesium chloride. The eluted sample was dialyzed (12-14K MWCO tubing) with 500 ml (x2) of 20 mM Tris, pH 8.0. Samples of the purified protein were concentrated by lyophilization.

Recombinant enterokinase (rEK) treatment of Sf9-expressed protein. Removal of both the His- and S-tags from the purified protein was accomplished via elution with rEK (S-Tag rEK Purification Kit, Novagen), as per manufacturer's instructions using S-tag specific agarose beads. Use of the beads enabled the desired protein fragment (varisin without the His-tag region) to be released into solution while the tag region remained bound to the beads via the S-tag. Additionally, rEK treatment without S-tag-specific agarose beads was performed

to allow the molecular weight of both fragments to be compared. Samples were then dialyzed (3K MWCO tubing) with 500 ml (x2) 10 mM Tris, pH 8.0.

Concentration of Sf9-expressed protein. Using Micron Centrifugal Filter Device with 3,000 MWM, samples of His-tag purified eluant (both rEK-treated and nontreated) were concentrated to facilitate detection of protein. Briefly, 500 µl of each sample was placed in a separate filter device, capped, and centrifuged at 14,000 *x g* for 80 min. The supernatant (concentrated protein) and the flowthroughs were analyzed via silver staining of a 4-12% Bis-Tris SDS-PAGE (Invitrogen).

Intermolecular disulfide bond disruption of Sf9-expressed protein. Disruption of the intermolecular disulfide bonds of the rEK-treated protein was attempted via treatment with either 4.76 M or 8 M guanidine thiocyanate (GdSCN, FischerSci); 7.14 M or 10 M urea (FischerSci) in 50 mM phosphate buffer, pH 7.0; 20 mM Beta mercaptoethanol (BME, FischerSci; $\delta = 1.14$); or 1% sodium dodecylsulfate (SDS, Sigma). Following 10 min incubation at room temperature, the samples were boiled at 95°C for 10 min. Analysis of samples via SDS-PAGE gel (4-12% Bis-Tris; Invitrogen) with silver staining (SilverXpress Silver Staining Kit, Invitrogen) was performed.

Purification of pET-32vsn plasmid. *E. coli* cells containing pET-32vsn plasmid (pET-32 plasmid (Novagen) plus mature varisin insert; Fig. 7 and 8), previously transformed by Lisa J. Fellman (ODU), were grown overnight at 37°C on Luria Bertani (LB) agar containing 100 µg/ml of ampicillin. Extraction of

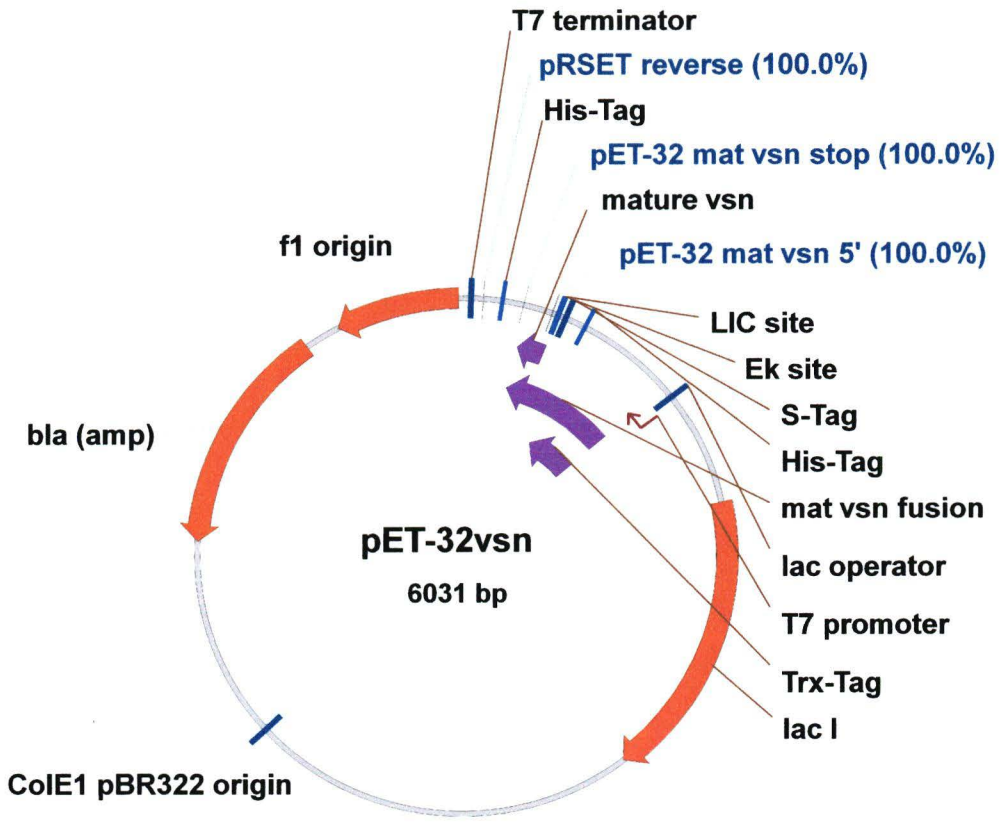

FIG. 7. pET-32vsn plasmid map. pET-32 plasmid (Novagen) with mature varisin insert.

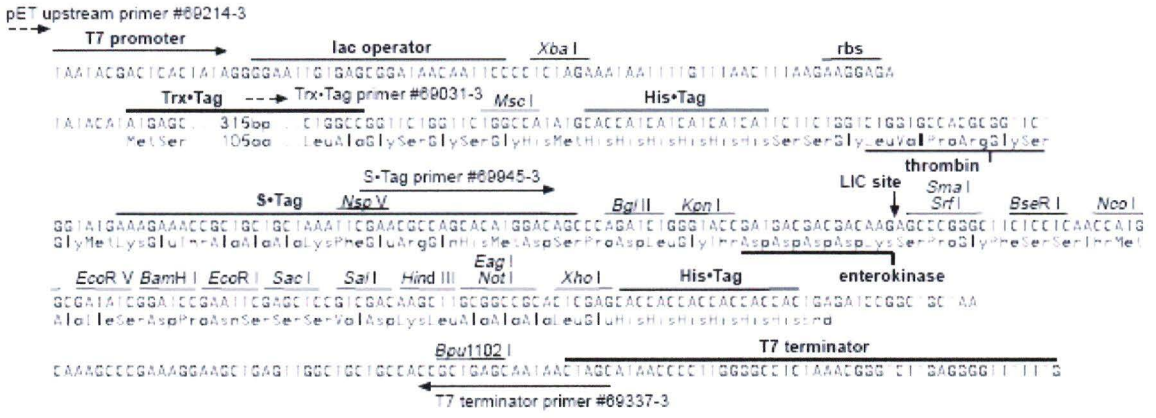

FIG 8. pET-32 cloning/expression region (Novagen).

plasmid DNA was accomplished using the Wizard® Plus SV Minipreps DNA Purification System (Promega) per manufacturer's instructions. Briefly, tubes with 2 ml LB broth containing 100 μg/ml ampicillin were inoculated and incubated overnight at 37°C with shaking. The overnight cultures were pelleted via centrifugation and resuspended in Cell Resuspension Solution. Cell Lysis solution was added to lyse the cells, Alkaline Protease Solution was added to degrade proteins, and Neutralization Solution was added to neutralize the culture. Following centrifugation, the cleared lysate was transferred to a spin column and centrifuged to bind the plasmid DNA to the spin column. The column was washed twice with Wash Solution and eluted with 100 μl nuclease-free water.

Sequencing of purified pET-32vsn plasmid. Sequencing was done using the BigDye® Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) and the ABI PRISM 3130xL Genetic Analyzer, per manufacturer's instructions. The sequencing reactions contained 2.0 μl primer (pRSET or T7 Promoter), 2.6 μl Terminator Ready Reaction, 4 μl purified plasmid DNA, 5.4 μl 5x Sequencing Buffer, and sterile water to a final volume of 20 μl. PCR reactions were performed to incorporate dye terminators with 25 cycles of the following: 30 s at 96°C (denaturation); 15 s at 50°C (annealing); and 4 min at 60°C (extension) with ramping at 1°C/s. The samples were then precipitated via 15 min incubation with 0.125 M EDTA, 3 M sodium acetate, and 100% ethanol, to remove unincorporated dye terminators. Following 20 min centrifugation at 14,000 rpm, the resultant pellets were washed with 70% ethanol, dried at 90°C

for 1 min, then resuspended in 20 μl Hi-Dei formamide. Samples were denatured by heating at 95°C for 2-4 min then placed on ice prior to loading on an ABI PRISM 3130xL Genetic Analyzer. Analysis of sequences was accomplished using Vector NTI Suite (Invitrogen).

Transformation of pET-32vsn into *E. coli* and pilot expression. One Shot® BL21(DE3) pLysS cells (Invitrogen) were transformed with the purified pET-32vsn plasmid. Briefly, purified plasmid DNA was added to a vial of cells, mixed gently, and incubated on ice for 30 min. The cells were heat shocked at 42°C for 30 s then placed on ice. Following the addition of 250 μl of room temperature SOC, the cells were incubated at 37°C with shaking for 30 min. The transformation reaction was added to 10 ml LB broth containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol and grown overnight at 37°C with shaking.

A 500 μl sample of the overnight culture was added to 10 ml LB broth containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. Following 2-h incubation at 37°C with shaking (OD_{600} 0.5-0.8), the culture was divided into two 5-ml cultures. To one culture, isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, while the other culture remained the control (uninduced). An aliquot of 500 μl was taken from each culture (time point 0), centrifuged, supernatant aspirated, and resultant pellet frozen. The cultures continued incubating at 37°C with shaking and 500 μl time-point samples were taken each hour for 6 h and processed as described. Pellets were resuspended in 80 μl 1X LDS sample buffer, boiled at 90°C for 5 min, then briefly mixed via

centrifugation. Samples were loaded on a 4-12% Bis-Tris SDS-PAGE gel (Invitrogen) and ran at 200V for 35 min, followed by Western-blot analysis using His-tag monoclonal antibody (Novagen).

Transformation of pET-32vsn into Origami™ 2(DE3) pLysS cells. Origami™ 2(DE3) pLysS cells (Novagen) were transformed with purified pET-32vsn plasmid DNA. Briefly, purified plasmid DNA was added to 20 μl of cells, mixed gently, and incubated on ice for 5 min. The cells were then heat shocked at 42°C for 30 s then placed on ice for 2 min. Following the addition of 80 μl of room temperature SOC, the cells were incubated at 37°C with shaking for 60 min. Various volumes (1 μl, 10 μl, and 89 μl) of the transformation reaction were spread on LB plates containing 100 μg/ml ampicillin, 34 μg/ml chloramphenicol, 12.5 μg/ml tetracycline, and 50 μg/ml streptomycin and grown overnight at 37°C.

Expression/induction of transformed Origami™ 2(DE3) pLysS cells. Briefly, 2 ml LB broth containing 100 μg/ml ampicillin, 34 μg/ml chloramphenicol, 12.5 μg/ml tetracycline, and 50 μg/ml streptomycin were inoculated with a single colony and incubated overnight at 37°C with shaking. An aliquot of 1 ml of the overnight culture was added to 10 ml LB broth containing 100 μg/ml ampicillin, 34 μg/ml chloramphenicol, 12.5 μg/ml tetracycline, and 50 μg/ml streptomycin and grown for 6 h at 37°C with shaking (OD_{600} 0.5-0.8). Expression was induced via the addition of IPTG to a final concentration of 1 mM. After growth at 37°C for 3 h with shaking, bacterial cells were harvested via centrifugation for 10 min at 3000 *x g*.

Pellet processing/cell lysis of transformed *E. coli*. Cells were lysed by resuspending pellets in BugBuster (Novagen) and Benzonase Nuclease (Novagen) (5 ml and 1 μl per 1 g of wet pellet, respectively). Following 20 min incubation at room temperature with rotation, the samples were centrifuged for 20 min at 16000 *x g* at 4°C. The supernatant was purified via His-tag metal chelation chromatography (Ni-NTA His Bind Resin, Novagen) as previously described. Eluant samples were dialyzed (12-14K MWCO tubing) with 500 ml of 10 mM Tris, pH 8.0.

Recombinant enterokinase (rEK) treatment of *E. coli*-expressed protein. Removal of the His-tag region from *E. coli*-expressed recombinant varisin was accomplished via elution with rEK (S-Tag rEK Purification Kit, Novagen), as per manufacturer's instructions; however, this was done without using S-tag specific agarose beads to allow both fragments to be free in solution for molecular weight comparison (Fig. 9).

```
  1  MSDKIIHLTD  DSFDTDVLKA  DGAILVDFWA  EWCGPCKMIA  PILDEIADEY
 51  QGKLTVAKLN  IDQNPGTAPK  YGIRGIPTLL  LFKNGEVAAT  KVGALSKGQL
101  KEFLDANLAG  SGSGHMHHHH  HHSSGLVPRG  SGMKETAAAK  FERQHMDSPD
151  LGTDDDDK▼GF  GCPLNQGACH  NHCRSIRRRG  GYCSGIIKQT  CTCYRN*
```

Molecular weight: 21398.89; Length: 197 amino acids; * stop; ▼ Enterokinase cleavage site

FIG. 9. Translation of recombinant varisin (pET-32vsn). Mature varisin is indicated by the bolded region.

Analysis of purified and rEK-treated protein. Both Sf9-expressed and *E. coli*-expressed proteins were assessed using SDS-PAGE (4-12% Bis-Tris,

Invitrogen); silver staining (SilverXpress Silver Staining Kit, Invitrogen), per manufacturer's instructions; and Western blotting using His-tag monoclonal antibody (Novagen) or varisin antibody (PA0422, PA0421; OpenBiosystems).

Crude activity screening of recombinant varisin against *M. luteus*. A well-diffusion assay was conducted to assess the antimicrobial activity of both His-tag purified and rEK-treated recombinant varisin produced by both Sf9 cells and Origami™ 2(DE3) pLysS cells against *M. luteus*. An overnight culture of *M. luteus* was grown in Todd Hewitt (TH) broth at 37°C. Using a TH agar plate with wells punched, various volumes of recombinant varisin (10 μl, 20 μl, 40 μl, or 60 μl) and 10 μl of synthetic varisin (PepSyn, LLC) as a control (100 μg/ml) were added to individual wells and allowed to air dry. The plate surface was exposed to chloroform vapor for 20 min to sterilize the agar surface, then aired. After application of a lawn of *M. luteus* taken from the overnight culture, the plate was incubated at 37°C overnight. Zones of growth inhibition, evident by clearing around a well, were measured.

Antimicrobial activity screening of synthetic varisin against various bacterial species. Well-diffusion assays were used to indicate antimicrobial activity of synthetic varisin against several bacterial species, both Gram-positive and Gram-negative. Briefly, overnight cultures of the bacteria of interest were grown overnight in Tryptic Soy Broth (TSB). Using TSA plates with wells punched, 10 μl of synthetic varisin (125 μg/ml, 200 μg/ml, or 1 mg/ml) was added to each well, air dried, then the plate surface was exposed to chloroform vapor for 20 min. After airing, a lawn of bacteria taken from an overnight culture was

applied and the plate incubated overnight at the appropriate temperature (25°C, 37°C, or 55°C, depending on the growth requirement of the bacteria).

For those bacterial species sensitive to synthetic varisin, as determined by well-diffusion assay, microtiter inhibition assays were performed. Briefly, overnight cultures of bacteria grown in TSB were diluted 1:100 in sterile saline (0.9%). Using a 96-well microtiter plate containing 10 μl of two-fold serial dilution of synthetic varisin per well, 10 μl of bacteria was added and mixed via pipette. The highest concentration of synthetic varisin tested was 500 μg/ml, with two-fold serial dilutions in sterile saline. Wells that contained sterile saline and bacteria but no synthetic varisin were used as controls. Following 2.5-h incubation at the appropriate temperature (37°C or 55°C), individual well contents were mixed with 50 μl of sterile saline, spread on TSA plates, and incubated at the appropriate temperature for 1 or 2 days. Colonies were counted, as feasible.

Glycosylation site prediction. Due to the possibility of glycosylation via insect-cell expression, the amino acid sequence for mature varisin (Fig. 6) was analyzed for predicted glycosylation sites using the Center for Biological Sequence Analysis' online NetOGlyc 3.1 server and YinOYang 1.2 server.

RESULTS

Detection of the His-tag labeled Vsn from Sf9-expressing cells via SDS-PAGE and Western blot. Analysis of the soluble components of the extraction revealed a band at the approximate size of 38 kDa (Fig.10A, Lane 4). This is consistent with the expected size of the target protein (mature varisin plus the plasmid tag region; Fig. 6). The positive control (Fig. 10A, Lane 2) consisted of supernatant from pIEx-3vsn transfection/extraction that was not purified. This control had previously been verified to possess the His-tag via Western blot and to be the appropriate size via SDS-PAGE and silver stain analyses (SilverXpress Staining Kit, Invitrogen; results not shown). Lane 4 contained the eluant fraction from the Ni-NTA His Bind Resin column (Novagen) which was expected to contain the target protein. Due to the extraction involving cell lysis, the sample contained a mixture of intracellular and extracellular proteins. Since intracellular proteins contain a signal sequence, and therefore have a slightly higher molecular weight than extracellular proteins, this explains the double band observed in the eluant fraction (Fig. 10A, Lane 4), as shown via Western blot.

Silver staining (SilverXpress Staining Kit, Invitrogen) of an SDS-PAGE gel with comparable samples to the Western blot (Fig. 10B) confirmed the presence of a protein with the appropriate size (38 kDa; Fig. 10B, Lanes 2 and 4). However, Lane 4 (Fig. 10B), which contained the eluant fraction from the Ni-NTA His Bind Resin column (Novagen), showed evidence of additional proteins of non-target molecular weight.

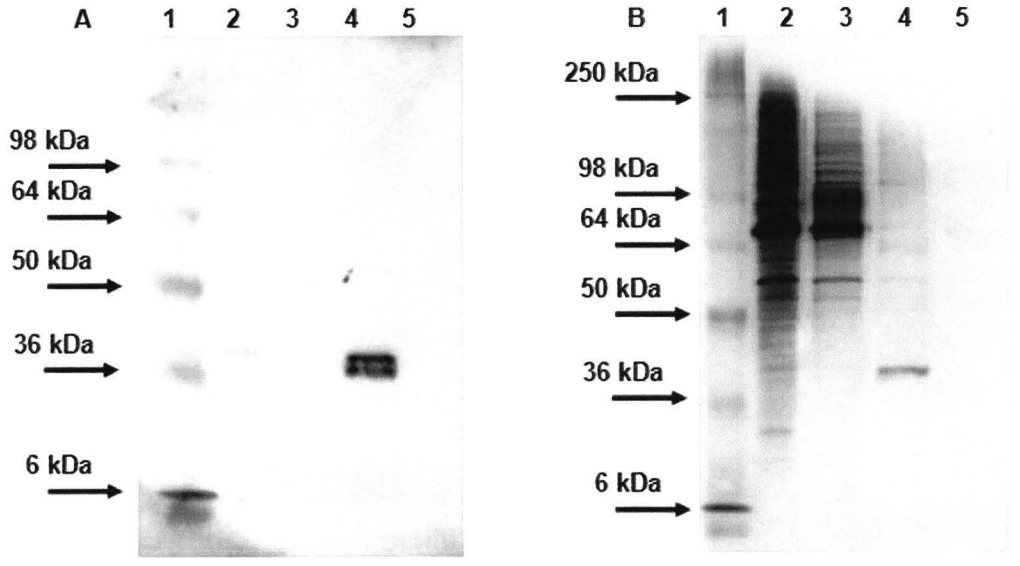

FIG. 10. Western blot and silver stain of His-tag-purified Sf9-expressed protein. 10% Tris-Gly SDS-PAGE; His-Tag mAb (Novagen). Lane 1: SeeBlue Plus2 Pre-Stained Protein Standard (Invitrogen); Lane 2: Positive Control (non-purified supernatant from pIEx-3vsn transfection/extraction); Lane 3: 1st flowthrough from His-bind resin column (Novagen); Lane 4: Eluant from His-bind resin column (Novagen); Lane 5: Wash from His-bind resin column (Novagen). A) Western blot; B) Silver stain.

Silver-stain analysis of S-tag purified Sf9-expressed protein. As shown in Fig.11, proteins evident in the S-tag purified fraction (Lane 3) are comparable to those seen in the His-tag purified fraction (Lane 2).

Analysis of rEK-treated Sf9-expressed protein without S-tag binding. Treatment of the His-tag purified protein with rEK without the use of S-tag specific agarose beads allowed the cleavage products to be free in solution for molecular weight comparison. As shown in Fig. 12 (Lane 3), rEK treatment yielded fragments of approximately 17 and 34 kDa.

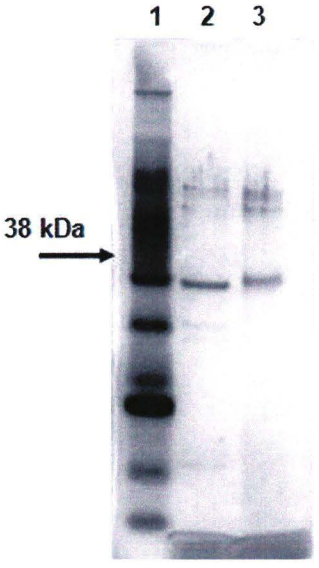

FIG. 11. Silver stain of His-tag and S-tag purified proteins. 4-12% Bis-Tris SDS PAGE (Invitrogen). Lane 1: SeeBlue Plus2 Pre-Stained Protein Standard (Invitrogen); Lane 2: His-tag purified in 20 mM Tris; Lane 3: S-tag purified, lyophilized, resuspended in sterile H_20.

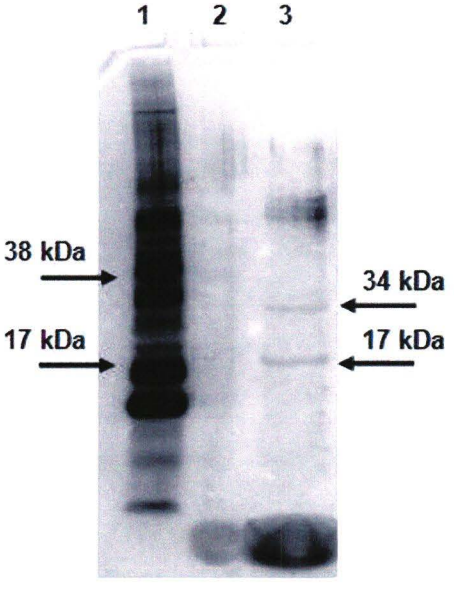

FIG. 12. Silver stain of rEK-treated Sf9-expressed protein without S-tag binding. 4-12% Bis-Tris SDS PAGE (Invitrogen). Lane 1: SeeBlue Plus2 Pre-Stained Protein Standard (Invitrogen); Lane 2: 1x buffer; Lane 3: rEK-treated protein (fragments in solution).

Analysis of rEK-treated Sf9-expressed protein and synthetic varisin via silver stain. As expected, treatment with rEK resulted in a protein of significantly less molecular weight (Fig. 13, Lanes 3 and 9) as compared to the non-rEK treated protein (Fig. 13, Lanes 2 and 7). However, the predicted weight of 4.2 kDa was not observed for the rEK-treated protein, unlike the synthetic varisin (Fig. 13, Lanes 4 - 6). The more concentrated of the rEK treated proteins tested (Lane 9) revealed a ladder of bands of 8, 17, 28, and 51 kDa.

Similarly, the various concentrations of synthetic varisin (Fig. 13, Lanes 4 - 6) revealed a steady ladder of bands. These ladders began with the expected 4.2 kDa protein with its aggregates at higher molecular weights. Lanes 8 and 10, which represent flowthroughs from the Micron Filter concentration, appear blank. This indicates that the Micron filters were successful in concentrating and maintaining the protein above the filter.

Analysis of intermolecular disulfide bond disruption treatment of Sf9-produced protein via silver stain. Disruption of the intermolecular disulfide bonds of the rEK-treated protein was attempted via treatment with GdSCN (4.76 M or 8 M), urea (7.14 M or 10 M), 1% SDS, or 20 mM BME. As shown in Fig. 14, Lanes 3 - 9, all samples reveal a band at approximately 17 kDa which corresponds to the molecular weight of a varisin tetrameter, similar to that shown in Fig. 13, Lanes 3 and 9. Multiples of these aggregates can also be detected at 51 kDa (triplicate of 17 kDa, Lane 5) and higher. These results indicate that this treatment was not successful at disrupting disulfide bonds.

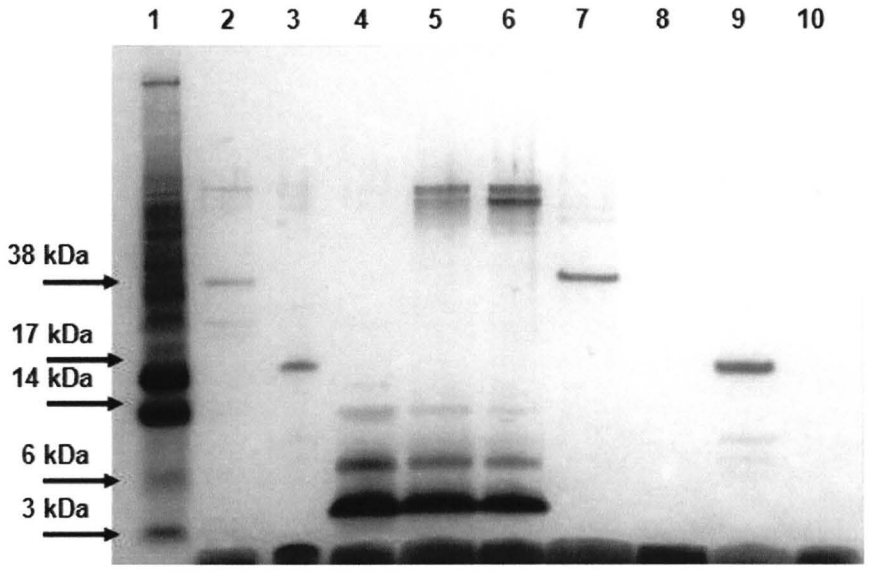

FIG. 13. Silver stain of rEK-treated, non-rEK-treated Sf9-produced protein and synthetic varisin. 4-12% Bis-Tris SDS PAGE (Invitrogen). Lane 1: SeeBlue Plus2 Pre-Stained Protein Standard (Invitrogen); Lane 2: Eluant in 1x Wash/Bind Buffer; Lane 3: rEK-treated eluant in 10 mM Tris; Lanes 4 - 6: Synthetic varisin (50 µg/ml, 31.25 µg/ml, 25 µg/ml, respectively); Lane 7: eluant (concentrated); Lane 8: Flowthrough of eluant from lane 7; Lane 9: rEK-treated eluant (concentrated); Lane 10: Flowthrough of rEK-treated eluant from lane 9.

Sequencing of purified pET-32vsn plasmid. Using pRSET and T7 Promoter primers, sequences were obtained. Sequence analyses were performed using Vector NTI Suite of programs which revealed 100% consensus with the known nucleotide sequence for varisin as well as verifying that the insert was in the correct orientation (results not shown).

Expression/induction analysis of transformed One Shot® BL21(DE3) pLysS cells. Western-blot analysis of the lysed cells was performed using His-tag monoclonal antibody (Novagen). As expected, all of the induced samples were positive for expression of the protein, while all of the uninduced samples were negative (results not shown). Based on these results, a 3 h time point was selected for future inductions.

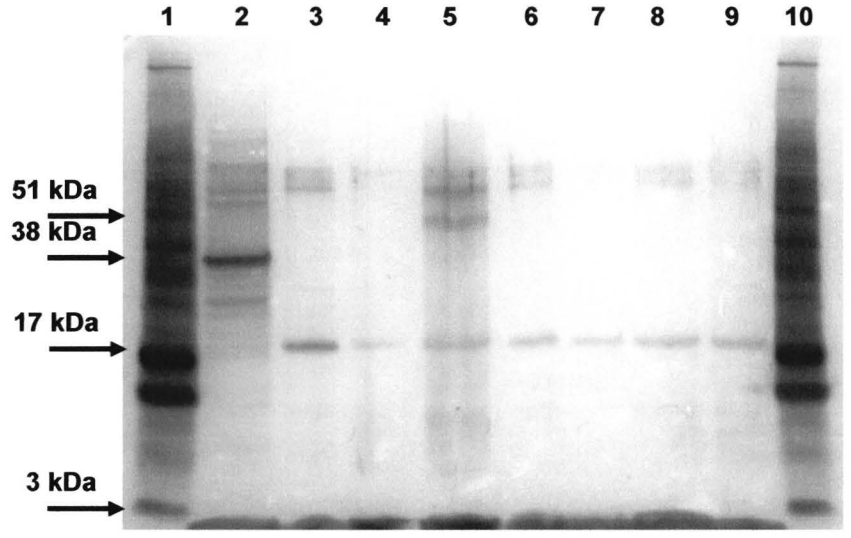

FIG. 14. Silver stain of intermolecular disulfide bond disruption treatment of Sf9-produced protein. 4-12% Tris-Bis SDS PAGE (Invitrogen). Lanes 1 and 10: SeeBlue Plus2 Pre-Stained Protein Standard (Invitrogen); Lane 2: His-purified eluant in Bind/Wash buffer; Lane 3: rEK-treated His-purified eluant in Bind/Wash buffer; Lanes 4 and 5: sample + 4.76M GdSCN or 8M GdSCN, respectively; Lane 6: sample + 7.14M urea; Lane 7: sample + 10M urea; Lane 8: sample + 1% SDS; Lane 9: sample + 20mM BME. *sample = rEK-treated His-purified rVsn from Sf9 cells in 10 mM Tris.

Analysis of rEK-treated *E. coli*-produced protein. Following cell lysis and His-tag purification, the supernatant of both One Shot® BL21(DE3) pLysS and Origami™ 2(DE3) pLysS cells were individually treated with rEK. Since S-tag purification was not performed, the rEK-treated samples were expected to contain both the plasmid-expressed protein and varisin separate in solution. As indicated in Fig. 9, the molecular weight of non-rEK-treated recombinant varisin is 21.4 kDa, making the plasmid-expressed protein 17.2 kDa, with varisin at 4.2 kDa.

Fig. 15 shows silver stain and Western-blot analyses of rEK-treated and non-rEK-treated One Shot® BL21(DE3) pLysS-expressed protein. The silver

stain showed many bands of protein for both treated and non-treated samples, with a band apparent at the target weight of approximately 4.2 kDa in all samples tested. The Western blot (His-tag mAb) revealed significantly less bands for the treated protein compared to the non-treated protein. Bands were evident at approximately 14, 15, 17, and 24 kDa for the treated protein (Lanes 2 and 3).

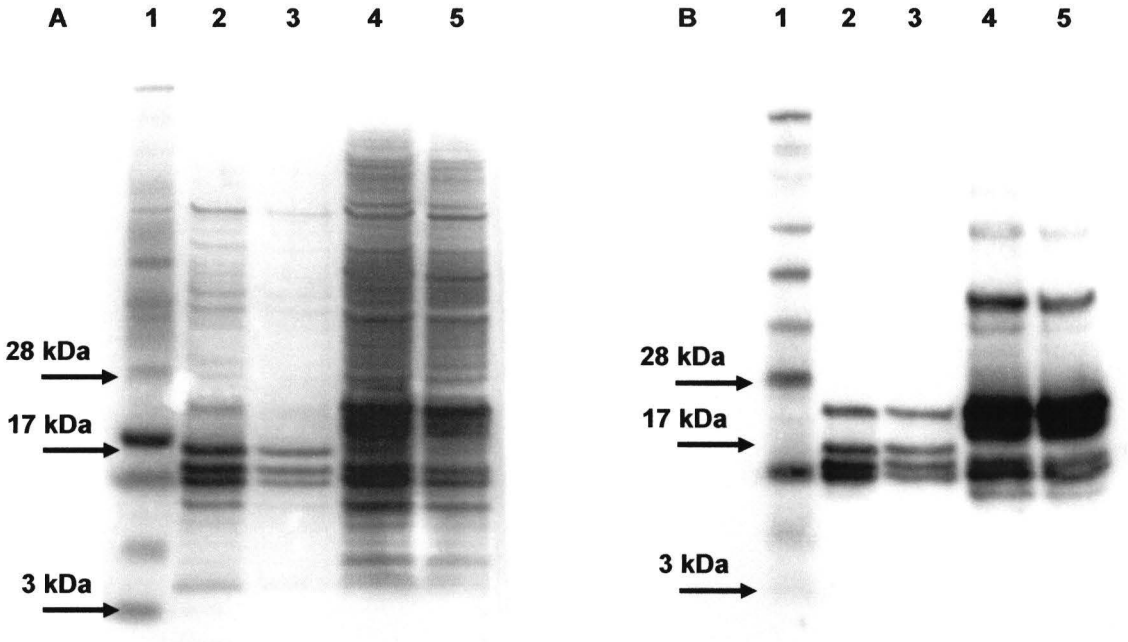

FIG. 15. Silver stain and Western blot of rEK-treated and non-rEK-treated One Shot® BL21(DE3) pLysS-expressed protein. 4-12% Bis-Tris SDS PAGE (Invitrogen); His-tag mAb (Novagen). Lane 1: SeeBlue Plus2 Pre-Stained Protein Standard (Invitrogen); Lanes 2 and 3: rEK-treated eluant (1:4 and 1:8 dilution, respectively); Lanes 4 and 5: non-rEK treated eluant (1:4 and 1:8 dilution, respectively). A) Silver stain; B) Western blot.

Fig. 16 shows a silver-stain analysis of rEK-treated and non-rEK-treated Origami™ 2(DE3) pLysS cell-expressed protein with similar results to the silver stain in Fig. 15. An anti-His-tag Western blot was not performed on these samples.

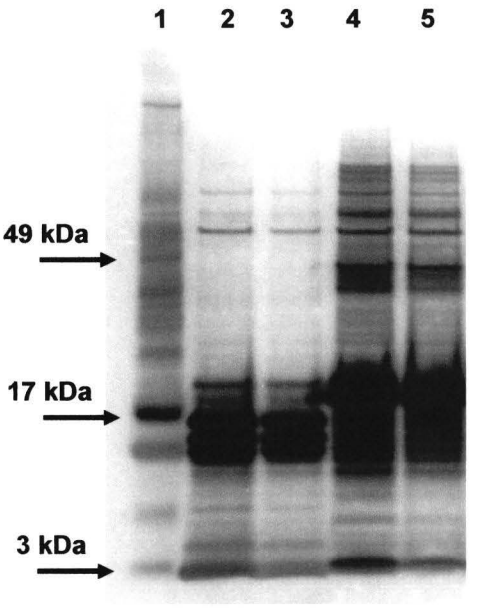

FIG. 16. Silver stain of rEK-treated and non-rEK-treated Origami™ 2(DE3) pLysS cell-expressed protein. 4-12% Bis-Tris SDS PAGE (Invitrogen). Lane 1: SeeBlue Plus2 Pre-Stained Protein Standard (Invitrogen); Lanes 2 and 3: rEK-treated eluant (1:4 and 1:8 dilution, respectively).

Anti-varisin Western-blot analysis of rEK-treated Sf9-expressed and *E. coli*-produced protein. Using an antibody specific to synthetic varisin, Western-blot analysis was performed on both insect-cell-produced and *E. coli*-produced recombinant varisin including a sample of each treated with rEK. As shown in Fig. 17, the antibody to synthetic varisin showed specificity to all recombinant varisin samples tested (Sf9- and Origami2-produced). This analysis revealed that varisin was produced by both types of expression cell.

Fig. 17 also shows that non-rEK treated and rEK treated samples of the Origami2-expressed protein (Lanes 4 and 5, respectively) are comparable to the non-rEK treated and rEK treated samples of One Shot® BL21(DE3) pLysS-expressed protein (Fig. 15B, Lanes 2-3 and 4-5, respectively).

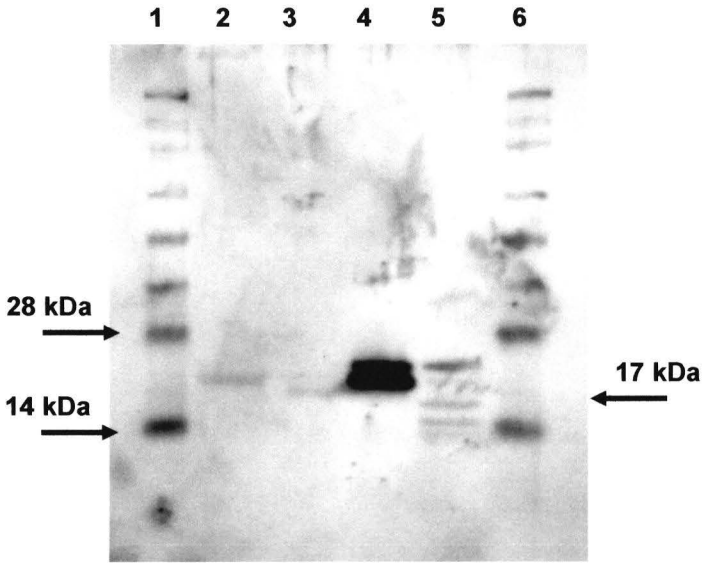

FIG. 17. Anti-varisin Western blot of recombinant varisin. 4-12% Tris-Bis SDS-PAGE; Antibody to synthetic varisin (Ab PA0422, OpenBiosystems). Lanes 1 and 6: SeeBlue Plus2 Pre-Stained Protein Standard (Invitrogen); Lane 2: rEK-treated protein (from Sf9 cells); Lane 3: non-rEK treated protein (from Sf9 cells); Lane 4: non-rEK treated protein (from Origami2 cells); Lane 5: rEK-treated protein (from Origami2 cells).

Crude activity screening of recombinant varisin against *M. luteus*. All volumes of recombinant varisin (Sf9-produced and Origami2-produced) tested via this method were negative for activity as evidenced by no obvious clearing around the wells. Synthetic varisin (10 µl of 100 µg/ml) was used as a control and was positive for activity (results not shown).

Glycosylation site prediction. Due to the possibility of glycosylation via insect-cell expression which may interfere with protein activity, the amino acid sequence for mature varisin (Fig. 6) was analyzed for predicted glycosylation sites. No O-linked or N-linked glycosylation sites were predicted using the Center for Biological Sequence Analysis' online NetOGlyc 3.1 server and YinOYang 1.2 server.

Antimicrobial activity screening of synthetic varisin against various bacteria via well-diffusion assay. Well-diffusion assays were performed similarly to the crude activity assays. Concentrations of synthetic varisin assessed began with 125 µg/ml. If no activity was observed at this concentration, the concentration was increased to 200 µg/ml then finally to 1 mg/ml, if necessary. As shown in Table 1, synthetic varisin (125 µg/ml) was effective against all but two of the Gram-postive bacteria assayed, with *M. luteus* being the most sensitive (22 mm diameter zone of growth inhibition). Synthetic varisin did not inhibit the growth of *B. cereus* (ATCC 14579) at any concentration tested.

Two out of 20 Gram-negative bacteria tested were senstive to synthetic varisin: in response to 125 µg/ml synthetic varisin, *Pseudomonas denitrificans* revealed a 9 mm-zone of growth inhibition; *Klebsiella pneumoniae* (ATCC 13885) was susceptible to 200 µg/ml synthetic varisin with a small zone of growth inhibition (5 mm).

Antimicrobial activity screening of synthetic varisin against various bacteria via microtiter inhibition assay. For those species susceptible to the activity of synthetic varisin, micotiter inhibition assays were performed with concentrations of varisin beginning at 500 µg/ml with subsequent 2-fold serial dilutions. Bacteria with sterile saline and no synthetic varisin were used as controls. To assess the potency of synthetic varisin against these bacteria after treatment, the well contents were spread on plates, incubated, and colonies counted. As expected, bacteria sensitive to synthetic varisin (as revealed via

well-diffusion assay, Table 1) were sensitive with the microtiter inhibition assay (Table 2). Unlike the well-diffusion assay, *C. xerosis* was the bacterial species most sensitive to synthetic varisin with no growth observed for wells containing concentrations up to 7.813 µg/ml of synthetic varisin; 1 and 6 colonies were counted for wells containing synthetic varisin concentrations of 3.907 and 1.95 µg/ml, respectively. Although the control sample revealed a low bacterial count of 32, *C. xerosis* appears to be highly sensitive to the activity of synthetic varisin. As expected, *M. luteus* was highly suscpetible to synthetic varisin with no growth observed for concentrations above 15.63 µg/ml of synthetic varisin.

Although *E. faecalis, M. phlei, M. smegmatis,* and *S. aureus* were too numerous to count in all their respective spread plates, there were noticeably less colonies evident in plates representing treatment with 500 µg/ml synthetic varisin compared to the control (no varisin).

TABLE 1. Antimicrobial activity of synthetic varisin via well-diffusion assay.

	Microorganism	Synthetic varisin concentration		
		125 µg/ml	200 µg/ml	1 mg/ml
		Zone of inhibition diameter (mm)		
Gram-positive bacteria	*Bacillus cereus* NSC 5050	7	N/A	N/A
	Bacillus cereus ATCC 14579	None	None	None
	Bacillus megaterium	9	N/A	N/A
	Bacillus subtilis ATCC 6051	10	N/A	N/A
	Corynebacterium kutscheri ATCC 15677	15	N/A	N/A
	Corynebacterium xerosis ATCC 373	15	N/A	N/A
	Enterococcus faecalis ATCC 19433	7	N/A	N/A
	Micrococcus luteus ATCC 14698	22	N/A	N/A
	Mycobacterium phlei NSC 3285	13	N/A	N/A
	Mycobacterium smegmatis NSC 3250	9	N/A	N/A
	Staphylococcus aureus ATCC 25928	6	N/A	N/A
	Staphylococcus epidermidis ATCC 12228	11	N/A	N/A
	Staphylococcus saprophyticus UMRL 1227	17	N/A	N/A
	Streptococcus pyogenes ATCC 19616	8	N/A	N/A
Gram-negative bacteria	*Acinetobacter calcoaceticus* ATCC 19605	None	None	None
	Alcaligenes faecalis NSC 3025	None	None	None
	Citrobacter freundii ATCC 8598	None	None	None
	Enterobacter aerogenes ATCC 13018	None	None	None
	Enterobacter cloacae ATCC 23359	None	None	None
	Escherichia coli ATCC 25922	None	None	None
	Escherichia coli B	None	None	None
	Escherichia coli C	None	None	None
	Klebsiella pneumoniae 25965	None	None	None
	Klebsiella pneumoniae ATCC 13885	None	5	N/A
	Proteus mirabilis CDC-S-117	None	None	None
	Proteus vulgaris ATCC 23315	None	None	None
	Pseudomonas aeruginosa ATCC 27853	None	None	None
	Pseudomonas denitrificans ATCC 18867	9	N/A	N/A
	Pseudomonas fluorescens NSC 5265	None	None	None
	Salmonella typhimurium ATCC 14094	None	None	None
	Serratia marcescens ATCC 8150	None	None	None
	Serratia marcescens CB	None	None	None
	Shigella flexneri ATCC 12022	None	None	None
	Shigella sonnei ATCC 25954	None	None	None

N/A, Did not test.

TABLE 2. Antimicrobial activity of synthetic varisin via microtiter inhibition assay.

Microorganism	Synthetic Varisin Concentration (µg/ml) 500	250	125	62.5	31.25	15.63	7.813	3.907	1.95	0
B. cereus NSC 5050	1	545	TNTC	TNTC	TNTC	TNTC	TNTC	N/A	N/A	TNTC
B. megaterium	0	0	127	TNTC	TNTC	TNTC	TNTC	N/A	N/A	TNTC
B. subtilis ATCC 6051	1	1	9	TNTC	TNTC	TNTC	TNTC	N/A	N/A	TNTC
C. kutscheri ATCC 15677	0	0	0	0	148	381	TNTC	N/A	N/A	TNTC
C. xerosis ATCC 373	0	0	0	0	0	0	0	1	6	32
E. faecalis ATCC 19433	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	N/A	N/A	TNTC
M. luteus ATCC 14698	0	0	0	0	0	0	111	309	N/A	TNTC
M. phlei NSC 3285	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	N/A	N/A	TNTC
M. smegmatis NSC 3250	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	N/A	N/A	TNTC
S. aureus ATCC 25928	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	N/A	N/A	TNTC
S. epidermidis ATCC 12228	4	174	381	TNTC	TNTC	TNTC	TNTC	N/A	N/A	TNTC
S. saprophyticus UMRL 1227	204	491	TNTC	TNTC	TNTC	TNTC	TNTC	N/A	N/A	TNTC
S. pyogenes ATCC 19616	0	0	0	0	1	TNTC	N/A	N/A	N/A	TNTC
K. pneumonia ATCC 13885	17	28	25	69	71	97	131	N/A	N/A	107
P. denitrificans ATCC 18867	0	0	0	0	0	34	52	N/A	N/A	85

Colony Count

N/A, Did not test; TNTC, too numerous to count.

DISCUSSION

Hematophagous arthropods, such as ticks and mosquitoes, lack an adaptive immune system and must rely on their innate defense mechanisms, such as defensins, for protection against pathogens ingested in a blood meal as well as those acquired through septic injury. The properties of these antimicrobial peptides vary from species to species with levels of protection likely contributing to immunocompetence of the arthropod. For example, the immunocompetence of the hard tick *D. variabilis* against *B. burgdorferi*, the causative agent of Lyme disease, is thought to be due in part to the production of varisin, a defensin (21).

In addition to its apparent antiborrelial activity, studies on the antimicrobial properties of partially purified varisin have shown its inhibitory effects on the growth of *B. subtilis* (20) and *M. luteus* (32). However, further investigations into the potency of varisin had not been reported. This thesis represents the first report of the antimicrobial activity of synthetic varisin against various bacteria.

The production of recombinant varisin was accomplished by both insect cells and *E. coli*. Since the correct folding of defensin, which includes intramolecular disulfide bond formation between three cysteine pairs, is central to its activity (8), insect cells were chosen as the principal expression system that should mimic the natural conformation of varisin.

Both plasmids (pIEx-3 and pET-32; Novagen) contained His- and S-tag regions which were important for purification purposes due to the selective

binding properties of the tagged regions. Through His-tag purification, any non-target proteins would be removed from solution; this included any insect defensin that the Sf9 cells inherently expressed. Following His-tag purification, proteins expressed by both Sf9 cells and *E. coli* were assessed via SDS-PAGE (silver stained) and Western blotting (anti His-tag mAb). These analyses revealed proteins of the molecular weight consistent with the size of varisin plus the plasmid tag region. The double-band observed in the Sf9-expressed protein (Fig. 10A, Lane 4), with an approximate 2-kDa difference in protein size, is attributed to the signal sequence remaining on non-translocated (intracellular) proteins which were included in the sample due to cell lysis.

rEK treatment of recombinant varisin did not result in a protein of the expected size (4.2 kDa); rather, a ladder of proteins was detected via SDS-PAGE (silver stained). It is thought that the multitude of detected proteins represent varisin aggregates. Aggregate formation is a likely occurrence due to the properties of the cysteine residues which can form both intramolecular and intermolecular disulfide bonds.

Disruption of the disulfide bonds was attempted via both denaturing and reducing agents; however, analysis via SDS-PAGE (silver stained) did not indicate a difference in protein size of any sample-agent complex (Fig. 14). This result is puzzling and suggests that the probable bonds formed by recombinant varisin aggregates are extremely strong and are able to withstand the denaturing conditions used in this study. Nevertheless, the analysis of the fragments formed as the result of rEK activity (Fig. 12) on the Sf9-expressed recombinant varisin

(initial size 38 kDa) revealed proteins the size of 34 kDa and 17 kDa, in addition to heavier bands thought to be varisin aggregates. When compared to the 38 kDa expected size of recombinant varisin, a fragment of 34 kDa would logically leave another at approximately 4 kDa, the size of varisin. This suggests that the 17 kDa band likely represents a tetramer of varisin. Additional studies such as mass spectroscopy or amino-acid sequencing are required to confirm this possibility.

Western blot analysis of rEK-treated *E. coli*-expressed protein (Fig. 15B, Lanes 2 and 3) using His-tag mAb, showed bands at 14, 15, 17, and 24 kDa. Since treatment with rEK is expected to liberate varisin from the plasmid-expressed protein (which contains the His-tag), proteins less than 17 kDa would not be expected to bind the His-tag mAb and, therefore, would not be observed on the Western blot. The proteins at 14 and 15 kDa did not follow this expectation and may be the result of imprecise cleavage or cleavage by an unknown proteolytic enzyme expressed by *E. coli*.

As evidenced via Western blot using an anti-varisin antibody (Fig. 17), both Sf9- and Origami2 expression systems produced recombinant varisin. The most evident was that of the Origami2 with a strong band at approximately 21 kDa (non-rEK-treated) correlating to a ladder of bands in the rEK-treated sample. The Sf9 rEK-treated sample was positive for varisin at approximately 21 kDa, which may indicate an aggregate of varisin. The non-treated sample was positive for varisin at approximately 17 kDa, which was unusual and not seen in prior analyses. The reason for a protein of uncharacteristic size in this sample is

unknown. Perhaps the protein became unstable during storage or subsequent processing.

Antimicrobial activity of both insect-cell and *E. coli*-produced proteins was assessed against *M. luteus*, a bacterium known to be highly susceptible to the antimicrobial effects of varisin (32). Neither form of recombinant varisin was effective at inhibiting the growth of *M. luteus*. The lack of activity may be due to the sheer size of the aggregates that appear to form intermolecularly. This solution is plausible as defensin molecules are small in size (2, 8, 27, 31) enabling their ease of movement toward a target cell, followed by membrane insertion. Due to a change in conformation resulting from aggregation, the active sites of varisin may be obscured thereby hindering its ability to exert its lytic effects on target cell membranes. Moreover, intermolecular configurations may impede movement via electrostatic forces as the inherent attractiveness of varisin to the negatively charged membrane of bacteria may be altered due to a difference in charge. Although defensins are known to aggregate, a property needed for crossing the target-cell membrane, it is thought that aggregation occurs at the target-cell membrane (8, 30), not before. Of course, defensin molecules must form the correct combination of disulfide bonds (cysteines 1-4, 2-5, 3-6 for varisin (5)) to be active (8). Improper pairing of the cysteine residues would also result in loss of activity.

Using insect cells as an expression system also lends itself to the possibility of glycosylation of expressed proteins, which would also modify the structure of the protein and likely interfere with activity. However, amino-acid

sequence analysis of recombinant varisin via two Center for Biological Sequence Analysis servers (NetOGlyc 3.1 and YinOYang 1.2) predicted no glycosylation sites (N-linked or O-linked). Furthermore, an increase in the molecular weight of the recombinant protein due to post-translational changes such as glycosylation was not indicated in assessment of the non-rEK-treated protein. Therefore, glycosylation is not likely.

Comparative analysis of recombinant varisin (Sf9-expressed) and synthetic varisin via SDS-PAGE (silver stained) showed that synthetic varisin also formed aggregates (Fig. 13); unlike recombinant varisin however, varisin monomers (4.2 kDa) were the predominant species. This indicates that the synthetic protein did not fully convert to aggregation while in solution. Therefore, it was not surprising that the synthetic peptide was able to inhibit growth of *M. luteus*.

Although recombinant varisin was shown to be expressed via Western blot (Fig. 17), the lack of activity greatly lessened the expectations of this study. Perhaps future efforts using an alternate expression system would yield an active protein. For example, Boulanger et al. (2004) produced a recombinant sand-fly defensin that was effective against filamentous fungi and yeast using *Saccharomyces cerevisiae* as an expression vector (3).

Antimicrobial activity screening of synthetic varisin via well-diffusion assay demonstrated its ability to inhibit the growth of 13 of the 14 Gram-positive bacteria tested (Table 1). *M. luteus* was the most susceptible to synthetic varisin followed by *S. saprophyticus*, *C. kutscheri, C. xerosis, M. phlei, S. epidermidis, B.*

subtilis, B. megaterium, and *M. smegmatis* with growth-inhibition diameters measuring 22, 17, 15, 15, 13, 11, 10, 9, and 9 mm, respectively. Although *S. pyogenes*, *E. faecalis*, *B. cereus* (NSC 5050), and *S. aureus* were sensitive, their corresponding growth-inhibition diameters were decidedly smaller (8, 7, 7, and 6 mm, respectively), indicating less sensitivity to the antimicrobial effects of synthetic varisin at this concentration. Surprisingly, synthetic varisin had no inhibitory effect on the growth of *B. cereus* (ATCC 14579) at any concentration tested (up to 1 mg/ml); the reason for which is unknown and unexpected due to the sensitivity of strain NSC 5050.

Conversely, synthetic varisin showed little ability to inhibit the growth of Gram-negative bacteria; only two out of the 20 tested showed susceptibility (Table 1). *P. denitrificans* was the only Gram-negative bacteria affected by 125 µg/ml synthetic varisin, as indicated by a 9 mm growth-inhibition diameter. *K. pneumoniae* (ATCC 13885) was not susceptible to this concentration; however, a growth-inhibition diameter of 5 mm was evident at a higher concentration (200 µg/ml). None of the other Gram-negative bacteria showed susceptibility to any concentration of synthetic varisin tested. Due to the differences between the membranes of Gram-negative and Gram-positive bacteria, with Gram-negative bacteria having a more complex membrane to penetrate, the disparity of inhibitory effects exerted by synthetic varisin was expected.

Microtiter inhibition assays confirmed the susceptibility of each species shown sensitive by well-diffusion assay. However, *C. xerosis* appeared to be the most susceptible species, not *M. luteus*, in this type of assay. Although assayed

cultures were treated the same, bacterial counts prior to incubation with synthetic varisin were not ascertained which likely led to disproportional assay conditions (i.e., bacteria to varisin ratio). Furthermore, it is probable that differing growth rates of the assayed bacteria contributed to variances noted in the effectiveness of synthetic varisin as slower growth rates likely lead to increased susceptibility due to the opportunity for greater diffusion of the peptide. However, to account for some of these pitfalls, controls which did not contain synthetic varisin were included in all studies.

When comparing synthetic varisin treated bacteria with those left untreated, the potency of synthetic varisin is evident (Table 2). However, apparent dissimilar sensitivity levels are to be expected due to variation between bacterial species. For example, resistance to human defensin has been reversed via mutagenic methods for both *Salmonella* and *Staphylococci* by disrupting the regulatory system PhoP/PhoQ of *Salmonella* (28), or the virulence factor Mprf in *Staphylococci* (27). It is likely that other bacteria have mechanisms that similarly offer protection from defensin. These variances coupled with differences between the membranes of Gram-negative and Gram-positive bacteria may explain the disparity of inhibitory effects exerted by synthetic varisin.

Most of the bacteria assayed can be found in soil and water, others on skin; some are typically linked to humans (e.g., *S. pyogenes, S. aureus,* and *S. epidermidis*). Keeping in mind that varisin is a tick defensin, it is expected that varisin would be most effective against bacteria that the tick may encounter.

Although, no antimicrobial protein would be expected to be effective against a wide range of bacteria, the scope of bacteria that are inhibited by synthetic varisin is impressive.

Studies of varisin and other such defensins will provide information necessary for our understanding of the innate immune system, its evolution, and potentially shed light on disease transmission. Since defensins are highly conserved, and therefore produced by many types of organisms, including humans, this preliminary data may also help direct investigations to the potential discovery of antimicrobial remedies or preventative approaches for bacterial illness, especially against those bacteria that have developed antibiotic resistance.

CONCLUSION

Arthropods, which lack an adaptive immune system, must rely on their innate immune response for protection against pathogens. Following microbial challenge, the production of antimicrobial peptides, such as defensins, is up-regulated. Defensins are small, cysteine rich, cationic peptides that disrupt the permeability of their target cell, causing cell lysis; they are most effective against Gram-positive bacteria. The immunocompetence of *D. variabilis* to *B. burgdorferi* is thought to be augmented by the action of varisin, a defensin.

This project focused on the expression, isolation, and subsequent antimicrobial activity screening of recombinant varisin as compared to its synthetic peptide. Recombinant varisin was produced by both insect cells (Sf9) and *E. coli*. Protein purification followed by enterokinase treatment yielded a protein identified by Western blotting as varisin. However, neither recombinant protein was bactericidal against *M. luteus*, a bacterium with known susceptibility to varisin. This inactivity is likely due to the premature formation of varisin aggregates which hindered the peptide's interaction with the target cell membrane. As expected, synthetic varisin was effective against Gram-positive bacteria (inhibited 13 out of 14); however, its effectiveness against 2 of the 20 Gram-negative bacteria was surprising. With such a wide-range of bacteria affected by this peptide, the notion that defensins are potential candidates for antimicrobial therapy is plausible. Studies of defensins not only provide information to further our understanding of the innate immune system, but also

offer conceivable avenues to follow in the hopes of developing effective treatments to bacterial illness.

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VITA

Julia A. Sharp

Department of Biological Sciences
Old Dominion University
Norfolk, VA 23529

Education

Old Dominion University, Norfolk, VA

- **MS Biology** December 2007 GPA 3.93
- **BS Biology** (Chemistry Minor) December 2003 GPA 4.0

Thomas Nelson Community College, Hampton, VA

- **AS Computer Science** May 1998 GPA 3.93
- **AS Science** December 1996 GPA 3.91

Related Experience

Old Dominion University, Norfolk, VA
Graduate Teaching Assistant August 2005 – July 2007

- Human Anatomy and Physiology (lab), Biology 250
- Human Anatomy and Physiology (lab), Biology 251
- Life Science (lab), Biology 108

Presentation

American Society of Microbiology – Virginia Branch, 2006 Annual Meeting, November 2006, Virginia Military Institute, Lexington, VA

Professional Membership

American Society of Microbiology – Virginia Branch

Award

Outstanding Scholar Award, College of Sciences, Old Dominion University, December 2003