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Recombinant expression of indolicidin concatamers in *Escherichia coli*

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Abstract Antimicrobial peptides are part of the innate immune system of vertebrates and invertebrates. They are active against gram-negative and gram-positive bacteria, fungi, and protozoa. Currently, most antimicrobial peptides are extracted from host organisms or produced by solid-phase peptide synthesis. Recombinant protein expression in *Escherichia coli* is a tool for greater production yields at a decreased cost and reduces the use of hazardous materials. We have constructed a concatamer of indolicidin and successfully expressed a fusion product with thioredoxin in *E. coli* BL21DE3. Codons for methionine residues flanking individual indolicidin genes were incorporated for cyanogen bromide cleavage of the fusion protein and liberation of active monomeric indolicidin. Peptide yields of 150 μg/l monomeric indolicidin were achieved in this first report of recombinant production of indolicidin with demonstrated antimicrobial activity.

Introduction

Small peptides that fight microbial infection are natural antibiotics and function as part of the innate immune system of vertebrates and invertebrates (Boman 1995). The antimicrobial peptide indolicidin was first discovered in the cytoplasmic granules of bovine neutrophils. One of the smallest of all naturally occurring linear antimicrobial peptides, indolicidin is 13 amino acids long and is amidated at the carboxyl terminus (Selsted et al. 1992). It possesses antimicrobial activity (in concentrations of micrograms per milliliter) against gram-negative and gram-positive bacteria (Selsted et al. 1992; Falla et al. 1996; Subbalakshmi et al. 1996), fungi (Ahmad et al. 1995; Lee et al. 2003), and protozoa (Aley et al. 1994), and it has antiviral activity against human immunodeficiency virus-1 (Robinson et al. 1998), herpes simplex virus, and Junin virus (Matanic and Castilla 2004). Circular dichroism studies of indolicidin in solution reveal an unordered structure due to its unusual composition, consisting of 39% tryptophan and 23% proline, with an insufficient peptide length to stabilize secondary structure (Falla et al. 1996). Several modes of action have been proposed, including the permeabilization of the outer membrane (Falla et al. 1996; Halevy et al. 2003), formation of ion channels (Lee et al. 2003), and inhibition of DNA and protein synthesis (Subbalakshmi and Sitaram 1998; Friedrich et al. 2001).

Due to their natural destructive behavior toward microorganisms, the production of antimicrobial peptides in heterologous hosts requires an approach that will mask their innate activity. Strategies used to enable the production of toxic peptides include the use of fusion proteins such as glutathione *S*-transferase (Piers et al. 1993), inclusion body-forming proteins (Haught et al. 1998; Lee et al. 2000), an anionic prepro region to neutralize the positive charge of antimicrobial peptides (Zhang et al. 1998), and tandem repeats of an acidic peptide–antimicrobial peptide fusion (Lee et al. 1998b). A number of antimicrobial peptides using these strategies—including cecropin A (Andersons et al. 1991; Hellers et al. 1991); defensin A (Reichhart et al. 1992); β-defensin-2 (Fang et al. 2002); CEME, a cecropin–melittin hybrid (Piers et al. 1993); apidaecin (Taguchi et al. 1994); moricin (Hara and Yamakawa 1996); magainin P2 (Haught et al. 1998; Lee et al. 1998a); buforin II (Lee et al. 1998b, 2002); bactenecin and indolicidin (Zhang et al. 1998; Metlitskaia et al. 2004); esculentin-1 (Ponti et al. 1999); MiAMP1 (Harrison et al. 1999); gaegurin 4 (Kim et al. 1997); sarcotoxin IA (Skosyrev et al. 2003); and MSI-344 (Lee et al. 2000; Hwang et al. 2001)—have been produced. A yield of active peptides produced by bacterial expression systems varies from 0.1 to 300 mg/l. Production of indolicidin has been reported using a small *Escherichia coli* replication protein, Rep21, and an anionic sequence from the human defensin prepro sequence HNP-1 fused to the N-terminus (Zhang et al. 1998). However, purification, yield, and activity were not discussed.

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In this study, genes encoding indolicidin concatamers were constructed for the production of recombinant indolicidin in *E. coli* BL21DE3 cells. We hypothesized that the multimerization of indolicidin, in conjunction with a thioredoxin fusion protein, may decrease its toxicity to *E. coli* by inducing nonnative folding and by preventing active peptides from circulating throughout the cytoplasm. Purification was achieved by affinity chromatography of the fusion protein, chemical cleavage to release monomeric indolicidin, and reverse-phase high-performance liquid chromatography (RP-HPLC) to isolate the active indolicidin. A purified recombinant peptide was characterized by electrospray ionization mass spectrometry (ESI-MS), confirming both the expected molecular weight and the peptide sequence. The antimicrobial activity of purified indolicidin was verified against *Staphylococcus aureus*, *Bacillus cereus*, and *Salmonella epidermidis* and was compared to synthetic indolicidin.

Materials and methods

Bacterial strains and plasmids

The cloning vector pUC-LINK (Prince et al. 1995) modified from pUC-18 plasmid was used for multimerization, and pET32a+ and pET28a+ (Novagen, Madison, WI) was used as the expression plasmid. Chemically competent *E. coli* XL1-Blue cells (Stratagene, La Jolla, CA) were used for the propagation of recombinant plasmids and BL21DE3 cells (Novagen) as expression hosts. Both host strains were grown in Luria–Bertani (LB) broth (Gibco Invitrogen, Carlsbad, CA) supplemented with 50 μg/ml carbenicillin at 37°C.

Cloning and concatamerization of indolicidin

A synthetic gene for indolicidin monomer (Selsted et al. 1992) was constructed from a pair of complementary oligonucleotides to include *Nhe*I and *Spe*I restriction sites at the N-terminus and C-terminus, respectively. These sites are compatible but are not synonymous, allowing concatamerization of the peptide gene. Each oligonucleotide (Biotech Resource Center, Cornell University, Ithaca, NY) was dissolved in H_2O to yield a concentration of 1 μg/μl. Ten micrograms of each oligonucleotide was phosphorylated with 100 U of T4 polynucleotide kinase (all enzymes were purchased from New England Biolaboratories, Beverly, MA) at 37°C for 1 h, and the two complementary strands were annealed by heating in 1 l of boiling H_2O and by allowing the beaker to adjust to room temperature overnight.

Concatamerization was performed using the pUC-LINK (Prince et al. 1995) vector, which contains both *Nhe*I and *Spe*I restriction sites in the multiple cloning region flanked by *Bam*HI sites. Indolicidin genes (Fig. 1a) (100 ng) were ligated to each other with 10 U of T4 DNA ligase overnight

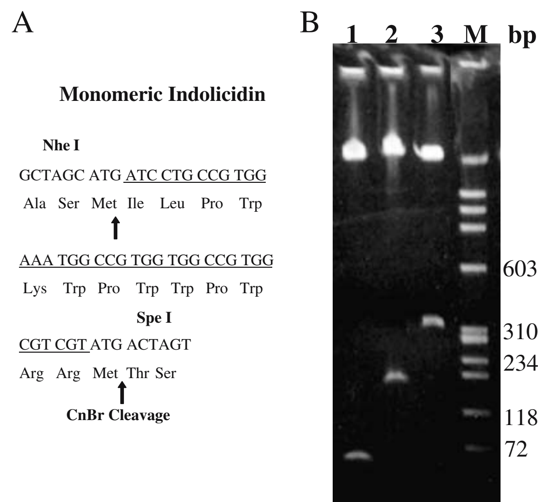

Fig. 1 Concatamerization of indolicidin monomeric genes. **a** Gene and corresponding peptide sequence for monomeric indolicidin. A *Nhe*I restriction site at the N-terminus and a *Spe*I site at the C-terminus are incorporated for concatamerization. Upon ligation, the *Nhe*I and *Spe*I sites enable orientation determination from the unique, incompatible site created (GCTAGT), which cannot be cleaved by either *Nhe*I or *Spe*I. Methionine residues are introduced for the liberation of monomeric indolicidin following cleavage with CNBr. **b** A 4–12% TBE polyacrylamide gel of *Bam*HI-digested pUC-LINK clones used to excise concatamers of the indolicidin gene. *Lane 1*, indolicidin monomer (67 bp, theoretical); *lane 2*, indolicidin trimer (169 bp); *lane 3*, indolicidin hexamer (320 bp); *M*, Φ×-174 bp marker. This represents the correct sizes of the concatamers produced

at 15°C. Concatamerized indolicidin gene was ligated with a 1–10 molar excess of insert into a dephosphorylated pUC-LINK vector and transformed into competent *E. coli* XL1-Blue cells (Sambrook et al. 1989). Individual colonies were screened by inoculation of 4 ml of LB broth containing 50 μg/ml carbenicillin and 12.5 μg/ml tetracycline and were grown overnight at 37°C and 200 rpm to propagate the plasmid. QIAprep Spin miniprep kits were used to isolate the cloned pUC-LINK DNA (Qiagen Inc., Valencia, CA).

The extent of multimerization was determined by *Bam*HI digestion of the pUC-LINK/indolicidin construct and electrophoresis on 4–20% Tris–borate–ethylenediaminetetraacetic acid (TBE) polyacrylamide gels (Sambrook et al. 1989) stained with 0.5 μg/ml ethidium bromide. Plasmids containing indolicidin dimer (Ind2), trimer (Ind3), and hexamer (Ind6) were digested with *Nhe*I and *Spe*I and run on 4–20% TBE gels to confirm the proper orientation of monomeric indolicidin genes. Concatamers ligated in the correct head-to-tail fashion (required for proper translation) were digested with *Bam*HI and ligated into a purified, dephosphorylated pET32a+ or pET28a+ expression vector. Plasmids were propagated in XL1-Blue cells and purified for DNA sequencing using Qiagen's plasmid midiprep kit. All constructs were sequenced by the BioResource Center (Cornell University) and analyzed with DNASTAR software (DNASTAR, Madison, WI).

Expression and purification

All multimers ligated into pET28a+ and pET32a+ were tested by in vitro transcription/translation according to the manufacturer's instructions (Promega, Madison, WI). The protein product was evaluated by Western blot using a streptavidin–alkaline-phosphatase conjugate (Promega) to detect biotinylated lysine residues. Only the pET32a:Ind3 clone yielded a protein product and was therefore chosen for in vivo expression.

A 4-ml starter culture of LB broth with 50 μg/ml carbenicillin was inoculated with a pET32a:Ind3 BL21DE3 glycerol stock, incubated at 37°C and 200 rpm, and grown to an OD_{600} of 0.6. This starter culture was used to inoculate a 1-l culture grown to an OD_{600} of 0.6 and induced with a final concentration of 0.1 M isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 h at 37°C and 200 rpm. Cell pellets were collected by centrifugation at 4°C and solubilized in 100 ml of 6 M guanidine hydrochloride, 100 mM NaH_2PO_4, and 10 mM Tris (pH 8.0) at room temperature. The cell lysate was centrifuged and the supernatant was removed. The supernatant was separated on a 4–12% NuPAGE polyacrylamide gel (Invitrogen Corp., Carlsbad, CA) and transferred to a polyvinylidene fluoride membrane for Western blot analysis using an S-tag alkaline phosphatase Western blot kit (Novagen).

Purification of the fusion product was achieved by affinity chromatography using the QIAexpressionist kit (Qiagen). A slurry of 50% (w/v) nickel resin in 30% ethanol was added to the lysate and incubated for 1 h with mixing at room temperature. Following incubation, the slurry was poured into a column (17.5×2.5 cm), and the flowthrough was collected. Packed resin was washed with 25 column volumes of 8 M urea, 100 mM NaH_2PO_4, and 10 mM Tris (pH 6.3). Thioredoxin:indolicidin (Trx:Ind3) protein was eluted with three column volumes of 8 M urea, 100 mM NaH_2PO_4, and 10 mM Tris (pH 4.5). All salts were removed by dialysis into water. The dialyzed sample was lyophilized and solubilized in 500 μl of 70% formic acid containing 50 mg/ml cyanogen bromide (CNBr) and incubated for 16 h at room temperature in the dark. The reaction was lyophilized and solubilized in 1 ml of 6 M guanidine hydrochloride for purification by RP-HPLC with a DeltaPak C18 column (150×3.9 mm ID, 5 μm, 100 Å; Waters, Milford, MA) and a water/acetonitrile gradient containing 0.1% trifluoroacetic acid (TFA) monitored at 230 nm. Optimal separation of the indolicidin monomer was achieved using the following gradient conditions: 0–30% acetonitrile for 10 min, 30–36% acetonitrile for 32 min, and 36–95% acetonitrile for 20 min at 1 ml/min. Purified recombinant indolicidin was quantitated by RP-HPLC using a standard curve generated with synthetic indolicidin (0.1–10 μg; Sigma-Aldrich, St. Louis, MO). Purified recombinant indolicidin was analyzed by ESI-MS (Thermo Finnigan LCQ Advantage) using direct infusion at 5 μl/min and positive ion detection. Sequencing of the indolicidin monomer was conducted by MS/MS fragmentation of the parent ion at 35 eV and was analyzed with the Bioworks 3.1 software (Thermo Electron, Woburn, MA).

Antimicrobial activity assay

Lyophilized HPLC fractions were analyzed for antimicrobial activity to identify those peaks with recombinant indolicidin. The dried fractions were solubilized in water and placed in a 96-well microplate. *S. aureus* ATCC 27217 was grown in nutrient broth (Gibco Invitrogen) and placed into each well at a final concentration of 10^5 colony-forming units (CFU)/ml. The microplate was incubated at 37°C overnight, and OD_{595} was measured after 16 h. RP-HPLC purified recombinant indolicidin and synthetic indolicidin were also tested for minimal inhibitory concentration (MIC) and minimal bacteriocidal concentration (MBC) determination. Serial dilutions of synthetic and recombinant indolicidin were placed into each microplate well in triplicate with 10^5 CFU/ml of *S. aureus* ATCC 27217 in nutrient broth, *Sa. epidermidis* 083198 in nutrient broth, or *B. cereus* 15927 in tryptic soy broth (Gibco Invitrogen). The microplate was incubated at 37°C for 16 h, and OD_{595} was measured.

Results

Concatamerization of indolicidin genes

Indolicidin was chosen for recombinant production because it has activity against a broad range of bacteria (Selsted et al. 1992; Falla et al. 1996; Giacometti et al. 1998; Subbalakshmi and Sitaram 1998; Staubitz et al. 2001). Figure 1a depicts an indolicidin monomeric gene sequence and its encoded peptide sequence. The native sequence has been altered by inserting codons for methionine residues at the start and finish of each monomeric unit, enabling the liberation of an active monomeric peptide upon chemical cleavage of the fusion product. As shown in the figure, *Nhe*I and *Spe*I restriction sites also flank the peptide gene. A unique restriction site (GCTAGT) is generated upon ligation in a head-to-tail fashion, which is not cleaved by either *Nhe*I or *Spe*I. This enables a rapid determination of the gene assembly and proper orientation required to translate indolicidin multimers. Indolicidin monomer (69 bp), trimer (169 bp), and hexamer (320 bp) genes extracted from pUC-LINK by *Bam*HI digestion are shown in a 4–12% TBE polyacrylamide gel in Fig. 1b. The expected sequences of these multimeric indolicidin genes were verified to 100% conformity by DNA sequencing (results not shown).

Protein expression and purification

The indolicidin trimer and hexamer were each ligated into pET28a+ and pET32a+ expression vectors and confirmed by DNA sequencing (results not shown). Both nonfusion (pET28a+) and fusion (pET32a+) protein vectors were chosen for expression. In vitro transcription/translation was then evaluated with the genes for Ind3 and Ind6 in both vectors. Only pET32a+:Ind3 was capable of in vitro ex-

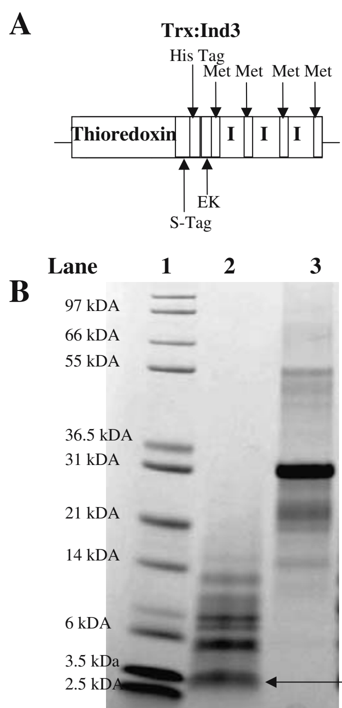

Fig. 2 Trx:Ind3 fusion product. **a** Schematic of the indolicidin trimer/thioredoxin fusion in pET32a+. An *S-tag* is included for Western blot detection, a *His tag* for affinity chromatography and Western blot detection, and an enterokinase cleavage site (*EK*) for cleavage of fusion from the concatamer. Codons for methionine (*Met*) are incorporated between each indolicidin (*I*) gene for cleavage of the fusion protein with CNBr during purification to produce an active monomeric indolicidin. **b** SDS-PAGE analysis (a 4–12% NuPAGE polyacrylamide gel stained with Coomassie blue) of nickel-purified and CNBr-cleaved Trx:Ind3. *Lane 1*, M-12 marker; *lane 2*, CNBr-cleaved Trx:Ind3 showing an indolicidin monomer (*arrow*) and a cleaved fusion protein; *lane 3*, nickel-purified Trx:Ind3 (29 kDa)

pression, producing the expected 29-kDa product (data not shown). As a result, the pET32a+:Ind3 construct was evaluated for in vivo expression.

Several host strains (BL21DE3, AD494DE3, NovablueDE3, HMS174DE3, and JM109DE3) were evaluated to determine which produced the largest quantity of recombinant Trx:Ind3 fusion protein. Only BL21DE3 and AD494DE3 successfully expressed Trx:Ind3, as confirmed by S-tag antibody detection via Western blot (results not shown).

The pET32a+:Ind3 construct (see Fig. 2a) was thus chosen for protein expression in BL21DE3 cells. The product was expressed in vivo with a 3-h induction by IPTG. Following the expression of Trx:Ind3, the cell lysate was very viscous and difficult to solubilize, requiring vortexing in four times the recommended volume of 6 M guanidine hydrochloride solution. Nondenaturing buffers could not solubilize the recombinant protein product. We were not concerned with solubilization under denaturing conditions because the secondary structure had not been demonstrated to be important for activity. In fact, indolicidin had a random structure, as confirmed by circular dichroism studies.

Nickel–agarose affinity chromatography was performed to purify the Trx:Ind3 fusion product, as shown in the Coomassie-stained gel in Fig. 2b. The enterokinase site internal to the fusion protein and multimerized peptide was not used due to the insolubility of the expressed Trx:Ind3 protein in the native buffers required for enterokinase activity. As a result, the thioredoxin fusion protein could not be cleaved prior to CNBr digestion of the recombinant indolicidin. Due to the inability to separate thioredoxin from multimerized indolicidin, several contaminating peptides were generated during CNBr digestion (see Fig. 2b). The heterogeneous nature of the sample required an additional purification step.

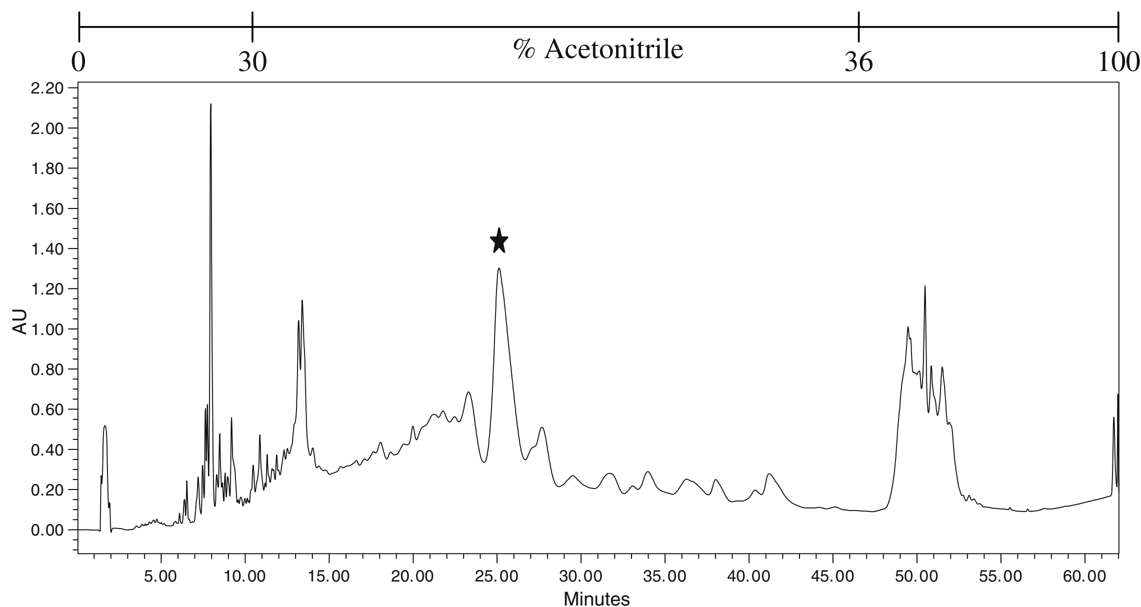

Fig. 3 RP-HPLC of CNBr-cleaved Trx:Ind3. The cleaved sample was run over a Waters DeltaPak C18 column and separated with a 30–36% acetonitrile/0.1% TFA gradient. Fractions were collected at regular intervals and tested for antimicrobial activity; the fraction marked by a *star* exhibited activity against *S. aureus* 27217

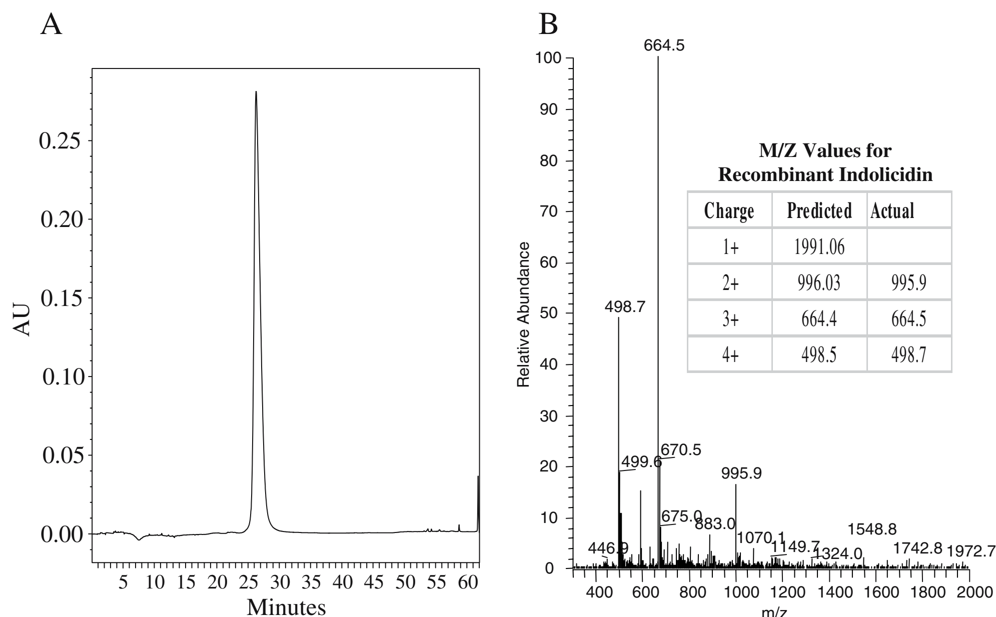

Fig. 4 Purified recombinant indolicidin and MS characterization. **a** A purified recombinant indolicidin was injected onto a Waters DeltaPak C18 column with a 0–95% 0.1% TFA/acetonitrile gradient over 62 min. The peak area was calculated to determine a purity of 93%. **b** Four micrograms of HPLC-purified recombinant indolicidin was injected by direct infusion into the LCQ ESI-MS in 50% methanol/0.05% formic acid. The most abundant peaks correspond to doubly charged (995.9), triply charged (664.5), and quadruply charged (498.7) *m/z* ratios, correlating to the expected molecular mass of the recombinant 1,991-Da indolicidin

Quantitation and characterization

The CNBr-cleaved sample was initially separated with a broad gradient (0–95% acetonitrile containing 0.1% TFA); fractions were collected at regular intervals and assayed for antimicrobial activity to identify those containing active indolicidin. Gradient conditions yielding an optimized separation of the peptide were empirically determined (see Fig. 3). Purified indolicidin was collected, lyophilized, and solubilized in H_2O for HPLC quantitation with a synthetic indolicidin standard curve. Purified recombinant indolicidin was produced at a yield of 150 µg/l and purified to approximately 93% homogeneity, as shown in the chromatogram in Fig. 4a.

Table 1 Comparison of the antimicrobial activities of recombinant indolicidin vs synthetic indolicidin

Organism	MIC (µg/ml)		MBC (µg/ml)	
	Recombinant indolicidin	Synthetic indolicidin	Recombinant indolicidin	Synthetic indolicidin
S. aureus 27217	2	2	6	6
B. cereus 15927	4	2	6	4
Sa. epidermidis 083198	8	6	10	8

Each value was determined after five independent experiments performed in triplicate. Antimicrobial activity was tested by broth microdilution method to determine the MIC and MBC of recombinant indolicidin and synthetic indolicidin. These results conclude that recombinant indolicidin retained activities comparable to synthetic indolicidin

MIC Minimum inhibitory concentration, *MBC* minimum bacteriocidal concentration

MS determined recombinant indolicidin to be 1,991-Da, as predicted, with detection of doubly, triply, and quadruply charged species (see Fig. 4b). Upon fragmentation (MS/MS) of the triply charged molecule, the predicted fragment masses were identified to confirm the correct amino acid sequence using the Bioworks software (data not shown).

Antimicrobial activity

Purified recombinant indolicidin was tested for activity against *S. aureus*, *Sa. epidermidis*, and *B. cereus* in a microplate assay and compared to the activity of synthetic indolicidin. Recombinant indolicidin had a biological activity similar to that of synthetic indolicidin, resulting in an MIC of 2 µg/ml for *S. aureus*, 4 µg/ml for *B. cereus*, and 8 µg/ml for *Sa. epidermidis*, as shown in Table 1. The MBC of recombinant indolicidin was also very similar to synthetic indolicidin at 6 µg/ml for both *B. cereus* and *S. aureus* and at 10 µg/ml for *Sa. epidermidis*.

Discussion

The high cost of synthetic peptide synthesis and low yields from the natural host led to the exploration of recombinant bacterial expression for the production of antimicrobial peptides. We have demonstrated the successful expression and purification of active recombinant indolicidin produced in *E. coli*, overcoming the peptide's inherent toxicity and difficulty of production due to its unusual structure. This was achieved by concatamerizing the natural indolicidin gene and expressing it with a thioredoxin fusion protein. Whereas many other antimicrobial peptides have been produced using similar methodologies, this work demonstrates the antimicrobial activities of the purified

recombinant indolicidin peptide and is significant because of the unique concatamerization method.

The use of a fusion protein for toxicity reduction, CNBr cleavage for peptide release, and purification methodology is comparable to the expression of other antimicrobial peptides in *E. coli*. Earlier attempts in our laboratory to produce the indolicidin peptide in vitro in a nonfusion protein vector were unsuccessful, leading to the selection of thioredoxin as a fusion protein. Lee et al. (1999) expressed buforin II concatamers without a fusion protein at very low expression levels—believed to be due to peptide interactions with DNA by inhibiting transcription—as confirmed by in vitro transcription/translation and gel retardation. Thus, in our studies, an N-terminal fusion protein was chosen for indolicidin to prevent DNA interaction and proteolytic degradation, as demonstrated by Skosyrev et al. (2003) using an N-terminal fusion for the expression of sarcotoxin IA.

A significant yield must be achieved for large-scale production of recombinant peptides. We have determined the yield of active, purified monomeric indolicidin to be 150 μg/l. This is not optimized for production, but reflects results from other investigators using a 1-l shake flask culture (Haught et al. 1998; Skosyrev et al. 2003) grown to a maximum OD_{600} of 1.5. Despite the lack of optimization, the expression yields fall within the lower range of 0.1–310 mg/l reported for other antimicrobial peptides (Hara and Yamakawa 1996; Haught et al. 1998; Lee et al. 1998b, 2002; Hwang et al. 2001; Skosyrev et al. 2003). Fermentation strategies, which include an optimized scale-up by establishing a high cell density, report yields of 107 mg/l for purified buforin II (Lee et al. 1998b), 5.5 mg/l for moricin (Hara and Yamakawa 1996), and 310 mg/l for MSI-344 (Hwang et al. 2001). We hypothesize that a high cell density fermentation in defined media may also result in an increase in indolicidin expression, assuming that peptide production on a per-cell basis is not reduced. In addition, overexpression of tryptophan tRNA may assist in meeting the cellular requirements for high concentrations of tryptophan. Overall, it is difficult to directly compare peptide yield from our system with other published works due to the variety of reported fermentation strategies and antimicrobial peptide sequences, and, more importantly, the purity of sample and methodology used to quantitate the peptides. Furthermore, many investigators who report the expression of antimicrobial peptides do not report yield, but only sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses.

Originally, it was thought that the concatamerization of the indolicidin genes alone would mask its toxicity. Although this proved unsuccessful, merging the concatamers with a thioredoxin N-terminal fusion protein to reduce toxicity resulted in the expression of a soluble protein product with reasonable yields. Our concatamerization strategy used a unique internal restriction site (*Nhe*I and *Spe*I digestion) strategy, which elegantly enabled a rapid screening of ligated monomeric genes for the determination of orientation necessary for proper peptide translation. This approach enabled concatamers to be produced, screened, and subcloned into expression vectors rapidly. Lee et al. (1996) also developed a multimerization strategy for antimicrobial peptides using a class IIS restriction enzyme, *Bsp*I, which cleaved the gene by four bases following the restriction site. This methodology generated asymmetric cohesive ends that prevented the cleavage of monomeric peptide genes following concatamerization, requiring all constructs to be sequenced to confirm proper orientation. In a follow-up study, buforin II (Lee et al. 1998b) was expressed as a concatamer of an acidic peptide fusion protein/peptide complex, where only 50% of the fusion product was buforin II. Our concatamerization method encompassed a 3:1 indolicidin/fusion protein ratio that resulted in a more efficient expression system.

Antimicrobial activities of recombinant indolicidin have not been reported prior to this study. Zhang et al. (1998) reported the production of indolicidin in an *E. coli* system, but data such as SDS-PAGE analysis, yield, or antimicrobial activity were not presented to support these claims. Metlitskaia et al. (2004) expressed a nonnative variant of indolicidin by inserting charged amino acids into the sequence, enhancing solubility with minimal effect on antimicrobial activity. We have demonstrated that the antimicrobial activities of recombinant and synthetic indolicidin against *S. aureus* 27217, *Sa. epidermidis* 083198, and *B. cereus* 15927 are comparable. Both synthetic indolicidin and natural indolicidin have previously shown activities against *E. coli* ML-5, *S. aureus* 502A (Selsted et al. 1992), *E. coli* UB1005, *E. coli* DC2, *Pseudomonas aeruginosa* Z61, *Salmonella typhimurium* MS7953s, *S. aureus* RN4220, *Staphylococcus epidermidis* C621 (Falla et al. 1996), *S. aureus* ATCC 8530 (Subbalakshmi et al. 1996), *S. aureus* ATCC 25923 (Falla and Hancock 1997), *S. aureus* Newman (Staubitz et al. 2001), and *E. coli* ATCC 25922 (Giacometti et al. 1998) at quantities of 2–64 μg/ml. Finally, whereas the native indolicidin and synthetic indolicidin are amidated at the C-terminus (Selsted et al. 1992; Falla et al. 1996), our recombinant indolicidin is not. Falla et al. (1996) demonstrated that amidation is not required for antimicrobial activity, but C-terminal modification does increase its activity for both gram-positive and gram-negative bacteria. Even in the absence of a C-terminal amidation, the antimicrobial activity of the recombinant peptide is directly comparable to the activity of the amidated synthetic peptide. A homoserine group at the C-terminus of our recombinant indolicidin peptide may, however, play a role in blocking the C-terminus and enhancing the antimicrobial activity.

Toxicity to bacterial cells, unique amino acid compositions, and modifications to the natural sequence of antimicrobial peptides pose problems in many recombinant systems. These factors were overcome by a concatamerization of the antimicrobial peptide gene, attachment of a soluble fusion protein, and production in a protease-resistant bacterium. This is the first report on the production and purification of active recombinant indolicidin in *E. coli*, which may lead the way to larger-scale production. Concatamerization of indolicidin, in conjunction with a

fusion protein for masking toxicity, may aid in the successful expression of many other antimicrobial peptides.

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