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Recombinant DNA procedures for producing small antimicrobial cationic peptides in bacteria

(Defensin; cecropin; inclusion bodies; fusion protein; expression)

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SUMMARY

Natural polycationic antibiotic peptides have been found in many different species of animals and insects and shown to have broad antimicrobial activity. To permit further studies on these peptides, bacterial expression systems were developed. Attempts to produce these peptides with an N-terminal signal sequence were unsuccessful due to the lability of the basic peptides. Therefore, a number of different fusion protein systems were tested, including fusions to glutathione-S-transferase (GST) (on plasmid pGEX-KP), *Pseudomonas aeruginosa* outer membrane protein OprF (on plasmid pRW5), *Staphylococcus aureus* protein A (on plasmid pRIT5), and the duplicated IgG-binding domains of protein A (on plasmid pEZZ18). In the first three cases, stable fusion proteins with the defensin, human neutrophil peptide 1 (HNP-1), and/or a synthetic cecropin/melittin hybrid (CEME) were obtained. In the course of these studies, we developed a novel method of purifying inclusion bodies, using the detergent octyl-polyoxyethylene (octyl-POE), as well as establishing methods for preventing fusion protein proteolytic breakdown. Cationic peptides could be successfully released from the carrier protein with high efficiency by chemical means (CNBr cleavage) and with low efficiency by enzymatic cleavage (using factor X_a protease). Fusions of protein A to cationic peptides were secreted into the culture supernatant of *S. aureus* clones and after affinity purification, CNBr digestion and column chromatography, pure cationic peptide was obtained. CEME produced by this procedure had the same amino acid (aa) content, aa sequence, gel electrophoretic mobility and antibacterial activity as CEME produced by protein chemical procedures.

INTRODUCTION

Over the past decade, more than twenty natural peptide antibiotics have been discovered that have a broad range of antimicrobial activities. These peptides usually range between 15 and 34 aa in length, contain a minimum

of four Lys and/or Arg residues, and are abundant in the organisms or cells in which they are found. They can be divided into subsets of molecules such as defensins (DEF; reviewed in Ganz et al., 1990), cecropins (Hultmark et al., 1980; Hultmark et al., 1982), magainins (Zasloff, 1987), melittin (Habermann and Jentsch, 1967) and others,

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Abbreviations: A, absorbance; aa, amino acid(s); Ap, ampicillin; AU, 8 M acid urea; bp, base pairs; CEME, synthetic cecropin/melittin hybrid; Cm, chloramphenicol; DEF, defensin(s); *E.*, *Escherichia*; FPLC, fast protein liquid chromatography; GST, glutathione-S-transferase; HNP-1, human neutrophil peptide 1; IPTG, isopropyl-β-D-thio

galactopyranoside; kb, kilobase(s) or 1000 bp; LPS, lipopolysaccharide; MIC, minimal inhibitory concentration(s); nt, nucleotide(s); octyl-POE, octyl-polyoxyethylene; oligo, oligodeoxyribonucleotide; OprF, *P. aeruginosa* outer membrane protein FPA, protein A in either plasmid constructions (e.g., PPA-CEME) or fusion protein (e.g., PA-CEME); *P.*, *Pseudomonas*; PAGE, polyacrylamide-gel electrophoresis; PCR, polymerase chain reaction; PepRPC, peptide reverse-phase chromatography; *S.*, *Staphylococcus*; SDS, sodium dodecyl sulfate; SP, signal peptide; TFA, trifluoroacetic acid; TST, 50 mM Tris pH 7.6/150 mM NaCl/0.05% Tween 20; [], denotes plasmid-carrier state.

including some synthetic hybrid peptides (Wade et al., 1990; Andreu et al., 1992), all with specific characteristics that differ from each other. They are interesting antibacterial agents that are able to kill (Ganz et al., 1990), permeabilize the outer membranes of (Sawyer et al., 1988; Lehrer et al., 1989) and bind to the endotoxin of (Sawyer et al., 1988) a variety of important medical pathogens.

To further study these molecules, we felt it important to develop a bacterial expression system that would allow for large scale purification. In the past, the only ways to obtain these small cationic peptides were to isolate them from the host organism, which requires large amounts of material and yields small amounts of protein, or to synthesize them by protein chemical methods, which can be very expensive. The advantages of producing these peptides in a bacterial expression system include: (1) the relative ease with which the system can be scaled up, (2) cost effectiveness, (3) the ability to make variants using site-directed mutagenesis or to clone in entirely new peptides and utilize optimized isolation procedures, and (4) the opportunity to incorporate heavy atoms to assist in structural studies.

Recently, groups have attempted to produce various peptides in different biological systems. Cecropin A has been produced in two different baculovirus expression systems (Andersons et al., 1991; Hellers et al., 1991), and insect defensin A from *Phormia terranova* has been expressed in yeast and purified (Reichhart et al., 1992). The only example of an antimicrobial cationic peptide to be expressed in bacteria is a scorpion insectotoxin (Pang et al., 1992). This peptide was expressed in *E. coli*, but due to improper processing, had extra aa at the N terminus, and no biological activity was recovered. Here, we have attempted to produce HNP-1 and CEME (C1-8M1-18; Wade et al., 1990) in a number of different bacterial expression systems. These peptides were chosen because they represent two different families of antimicrobial peptides and are both active against *P. aeruginosa*. We have shown that these peptides can be produced in different expression systems, and that in at least one of them, pure, biologically active peptides can be obtained.

RESULTS AND DISCUSSION

(a) Expression systems for cationic peptides

Direct production of HNP-1 (Fig. 1) preceded by the SP for *E. coli* alkaline phosphatase (Chang et al., 1986) was attempted using plasmids pT7-5 and pT7-7 (Tabor and Richardson, 1985) in *E. coli*. These constructs did not result in production of a 4-kDa protein as determined by SDS-PAGE of whole cell lysates (Table I). Northern blots (results not shown) indicated that a transcript of

about 300 nt present in IPTG-induced cultures, was absent in uninduced cultures.

Therefore, attempts were made to produce cationic peptides as fusion proteins with the capability of releasing the peptide from the carrier molecule using enzymatic or chemical methods (Table I). Three different fusion protein expression systems were tried in preliminary studies, involving fusions to GST on plasmid pGEX-KP [a derivative of pGEX-3X (Pharmacia) in which the *Bam*HI-*Sma*I-*Eco*RI multiple cloning site was changed to *Sph*I-*Hind*III-*Eco*RI by PCR], to the N-terminal 188 aa of *P. aeruginosa* outer membrane protein OprF on plasmid pRW5 (Finnen et al., 1992; R. Wong and R.E.W.H., unpublished), and to the duplicated IgG-binding domains of protein A on pEZZ18 (Nilsson et al., 1987). In all cases, a synthetic oligo encoding a cationic peptide was inserted 3' to the sequence encoding the fusion partner, and production of the fusion proteins was easily observable (Table I, Fig. 2). However, in four of these constructs proteolysis of the fusion protein occurred such that the apparent M_r of the fusion protein was similar to M_r of the fusion protein partner without the appended cationic peptide (Table I; Fig. 2A, lane 5). To overcome problems with proteolysis in the pGEX system, we inserted between the fusion partner and the cationic peptides a synthetic pre-pro defensin sequence (Fig. 1), encoding the aa sequence found prior to the DEF-encoding gene of eukaryotic cells (Daher et al., 1988). This resulted in complete protection of the fusion protein from proteolytic degradation, presumably due to secondary structure formed between the anionic pre-pro defensin sequence and the cationic peptide sequence. Protection from proteolysis was observed for both the homologous HNP-1 DEF sequence and the heterologous CEME sequence. In addition, a fusion of the protease-resistant outer membrane protein OprF with CEME was protease resistant (Table I).

Fusion proteins derived from pGEX derivatives were purified from inclusion bodies by conventional techniques (Sambrook et al., 1989) except for an additional extraction of the insoluble pellet with 3% octyl-POE before urea solubilization, resulting in a very pure preparation of the fusion proteins (e.g., Fig. 2B, lane 1). To release the cationic peptides from the GST carrier molecule, two systems were tested. These were a specific factor X_a protease cleavage site and a CNBr sensitive Met, which were engineered adjacent to the cationic peptide-encoding sequence (Fig. 1A-C). In our hands, factor X_a cleavage was inefficient, requiring 60 h at 37°C with an enzyme to substrate ratio of 1:25. However, it did yield a band of HNP-1 as confirmed by its mobility on SDS-PAGE and N-terminal aa sequencing (Fig. 2B, lane 2). An alternative strategy involved solubilization of GST-

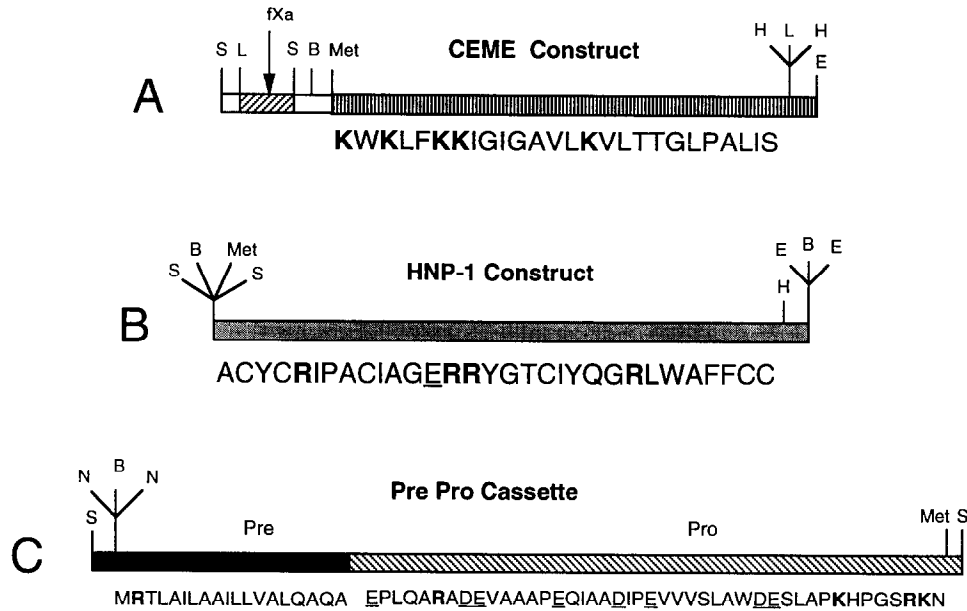


Fig. 1. Schematic diagrams of the synthetic oligos (A-C), and their corresponding aa sequence, that were constructed or modified for use in these studies. Only restriction sites that were pertinent are shown: S, *Sph*I; H, *Hind*III; E, *Eco*RI; B, *Bam*HI; L, *Sall*; N, *Nde*I; fX_a, factor X_a recognition site; Met, methionine residue. **Methods:** The aa sequences of HNP-1 (Selsted et al., 1985) and CEME (Wade et al., 1990) and the pre-pro region of HNP-1 (Daher et al., 1988) were used to design oligos encoding genes for these peptides. The HNP-1- and CEME-encoding genes were inserted between the *Sph*I and *Hind*III sites of pGEX-KP [constructed from the Pharmacia plasmid pGEX-3X as described in section a] to give pGEX-HNP-1 and pGEX-CEME, respectively. These inserts were sequenced to ensure correct oligo synthesis. The nt sequence encoding the pre-pro region of HNP-1 (Daher et al., 1988) was synthesized with *Sph*I cohesive ends and inserted into pGEX-HNP-1 and pGEX-CEME that had been digested with *Sph*I, resulting in pGEX-proHNP-1 and pGEX-proCEME, respectively. The CEME-encoding gene was cloned into the pEZZ vector using a 5' *Bam*HI and a 3' *Hind*III site. To clone the CEME- and HNP-1-encoding genes into pRIT5, the 5' and 3' restriction sites of each had to be changed. In the case of CEME, the oligos 5'-CGTCGACATCGAAGGTCGTGCATG and 5'-CACGACCTTCGATGTCGACGCATG were annealed together and inserted into the 5' *Sph*I site to convert it to a *Sall* site. (A factor X_a site was included to provide the possibility of releasing the peptide from the fusion protein.) This construct was sequenced to confirm correct insertion orientation. The 3' end was also converted to a *Sall* site by insertion of the self-annealing oligo 5'-AGCTTGTCGACA-3' into the *Hind*III site. The resulting *Sall* fragment (A) was inserted into pRIT5 to give pPA-CEME. The HNP-1-encoding gene was modified in a similar way using an *Sph*I-to-*Bam*HI adaptor (5'-CGGATCCATGGCATG and 5'-CCATGGATCCGCATG) on the 5' end and an *Eco*RI-to-*Bam*HI adaptor (5'-AATTCGGATCCG-3') on the 3' end. Using *Bam*HI, the HNP-1-encoding gene was cloned into pRIT5 to give pPA-HNP-1, which now also contained a Met codon to allow peptide release with CNBr (B). The pre-pro DEF sequence was inserted into the remaining *Sph*I site of pPA-HNP-1 to give pPA-proHNP-1. All DNA techniques were carried out according to either Sambrook et al. (1989) or Ausubel et al. (1987) unless otherwise stated. DNA sequencing was performed using a kit from Applied Biosystem, an Ericomp thermocycler, and Applied Biosystems sequencer. Oligos were synthesized on an Applied Biosystems oligo synthesizer (model 380B) using phosphoramidite chemistry.

proCEME inclusion bodies in 70% formic acid containing 1 M CNBr and incubating for 18–24 h at 25°C. This yielded a band corresponding to the migration of CEME produced by classical peptide synthesis techniques (data not shown), however the existence of twelve Met residues in GST made subsequent purification complex and poorly efficient. Thus, although the above methods could be used to prepare cationic peptides by recombinant DNA techniques, they were rather inefficient.

(b) Expression and purification of protein A fusion proteins

E. coli DH5α[pRIT5] (Nilsson et al., 1985) or DH5α[pPA-CEME] were grown and whole cells analyzed by Western immunoblot for the production of fusion proteins. As with the pEZZ fusion proteins, the results indicated that the fusion proteins were being degraded (Fig. 3, lanes 1 and 2). Plasmid pRIT5 possesses

the protein A signal sequence preceding the gene for protein A, allowing the fusion protein to be exported to the external medium when grown in *S. aureus*. Since it was reasoned that this would be an advantage in preventing proteolytic degradation of the fusion protein, pRIT5, pPA-CEME, pPA-HNP-1 and pPA-proHNP-1 were electroporated into *S. aureus* RN4220 (Kreiswirth et al., 1983). Culture supernatants of cells containing the various recombinant plasmids were analyzed by Western immunoblot, which demonstrated that the heterologous proteins were stably produced (Fig. 3, lanes 3–6). Culture supernatant from RN4220[pPA-CEME] was passed over an IgG Sepharose column and the fusion protein eluted to give a relatively pure preparation (Fig. 3, lane 7). After CNBr digestion, the protein was passed over a Bio-Gel P100 gel sieving column and fractions analyzed by AU-PAGE. All fractions containing the CEME peptide were pooled, lyophilized, and subjected to reverse-phase FPLC

TABLE I
Expression systems for cationic peptide production

Expression vector ^a	Fusion partner ^b	Cationic peptide ^c	Expression			
			Production ^d	Proteolysis observed ^e	Cellular location ^f	Fusion protein purified ^g
pT7-5 ^h and pT7-7 ^h	None	HNP-1	-	++	N.D.	-
pGEX-HNP-1	GST	HNP-1	++	++	IB	+
pGEX-CEME	GST	CEME	++	+	IB	+
pGEX-proHNP-1	GST-prepro	HNP-1	++	-	IB	+
pGEX-proCEME	GST-prepro	CEME	++	-	IB	+
pOprF-CEME	OprF	CEME	+	-	OM	-
pEZZ-CEME	IgG binding site	CEME	+	++	C	-
pPA-CEME	Protein A	CEME	+	++	C	-
pPA-HNP-1 (<i>S. aureus</i>)	Protein A	HNP-1	+	-	S	+
pPA-CEME (<i>S. aureus</i>)	Protein A	CEME	+	-	S	+
pPA-proHNP-2 (<i>S. aureus</i>)	Protein A-prepro	HNP-1	+	-	S	-

^aExpression vectors were in *E. coli* unless otherwise indicated (see *S. aureus*).

^bFusion partners are described in section a.

^cIndicates the product of the gene which is fused to the fusion partner.

^d++, major cell protein; +, band easily observable; -, no band observed. All + and ++ were confirmed immunologically with Western blots.

^e++, proteolysis of >50% of protein; +, proteolysis of <50% of protein; -, little or no proteolysis of protein.

^fIB, inclusion bodies; OM, outer membrane; C, soluble cytoplasmic form; S, extracellular supernatant; ND, not determined.

^g+, purified; -, not purified.

^hTabor and Richardson (1985).

on a PepRPC column (Fig. 4), leading to homogeneously pure CEME that comigrated with chemically synthesized CEME (Fig. 4, inset). A sample of the purified peptide was analyzed for aa content and the N-terminal aa sequence. In both cases, the results confirmed that the purified peptide was indeed CEME.

A similar purification scheme was utilized with PA-HNP-1. The fusion protein was isolated, cleaved with CNBr, and passed over a P100 column. This resulted in a partially purified preparation of HNP-1, as determined by comigration with purified HNP-1 (a gift from M. Selsted) on AU-PAGE (data not shown).

(c) Activity of peptides

Although the peptide had the primary aa sequence of CEME, it had to be determined whether or not its antibacterial activity was retained throughout the purification process. Samples of isolated PA-CEME fusion protein, CNBr-digested PA-CEME, purified CEME from the PepRPC column, chemically synthesized CEME, and melittin were subjected to AU-PAGE (Fig. 5A) and tested for activity using a bacterial overlay assay, with *E. coli* DC2 (Richmond et al., 1976) as the test organism. Fig. 5B shows that the CEME produced by recombinant DNA techniques had antibacterial activity comparable to melittin

and the CEME produced by chemical synthesis, while the intact PA-CEME showed no activity. To quantitate this activity, minimum inhibitory concentration (MIC) assays were performed on melittin, biologically produced CEME and chemically produced CEME using *P. aeruginosa* strains K799 and Z61 (Angus et al., 1982), a clinical isolate and its antibiotic supersusceptible derivative respectively. Biological CEME and chemical CEME had similar MIC of 2.5–5 μ M for K799 and 1.2–2.5 μ M for Z61. Melittin gave MIC values of 2.5 μ M and 1.2 μ M for K799 and Z61, respectively.

The partially purified HNP-1 isolated using this system was also tested for antimicrobial activity using the gel overlay assay and MIC. It was shown that in both of these tests, the HNP-1 preparation had no detectable antibacterial activity. This is not surprising considering that defensins are inactive without proper disulfide bonding (Selsted and Harwig, 1989), and there was no attempt made in the present study to refold the disulfide bonds which were probably incorrectly linked during synthesis in bacteria.

We have successfully produced CEME in the protein A system, purified it, and shown that it retains biological activity. This appears to be the first example of the biological production of an active, cationic, antimicrobial pep-

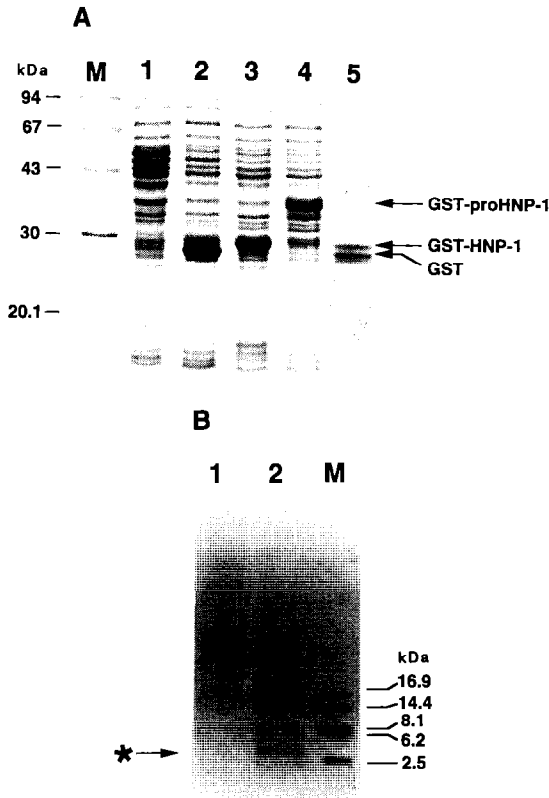


Fig. 2. Production of HNP-1 in the pGEX system. Lanes M: M, markers (BRL) in kDa. (A) SDS-PAGE in which lanes 1–4 represent whole cell lysates of induced *E. coli* DH5 α containing: lane 1, no plasmid; lane 2, control plasmid pGEX-KP; lane 3, pGEX-HNP-1; lane 4, pGEX-proHNP-1. Lane 5 is a sample of the fusion protein that was eluted from a glutathione agarose affinity column as previously described (Smith and Johnson, 1988). The positions of GST, GST-HNP-1, and GST-proHNP-1 are indicated. 1% SDS–10% PAGE was performed as described by Hancock and Carey (1979). (B) 0.1% SDS–12–25%-gradient PAGE showing purified GST-HNP-1 inclusion bodies that were incubated at 37°C for 60 h without (lane 1) or with (lane 2) factor X_a protease. The asterisk indicates the Coomassie stained protein band in which the HNP-1 peptide was found. **Methods:** Cells of *E. coli* strain DH5 α containing the various pGEX plasmids (Table I) were grown at 37°C to mid-log phase ($A_{600}=0.5$) and IPTG (BRL) added to a final concentration of 0.2 mM to induce fusion protein expression. Growth was allowed to continue for 3 h before cells were harvested by centrifugation at 3000 \times g for 10 min.

tide in bacteria. Pang et al. (1992) were able to express a scorpion insectotoxin in *E. coli* using a secretion vector which exports the peptide to the external medium. The isolated peptide, however, had no biological activity, again indicating that direct expression of these peptides is not feasible. We have also successfully purified a variant of CEME that has two additional basic residues using the protein A fusion protein system (data not shown). This peptide also retained its antimicrobial activity, thus demonstrating that the system is accommodating to different peptides. The production of small peptides as fusion proteins has a number of advantages. First, the

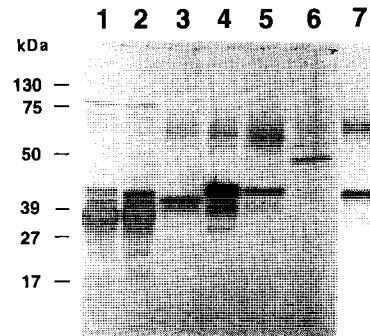


Fig. 3. Protein A fusion protein production. Western immunoblot showing the production of various fusion proteins from the protein A system. Lanes 1 and 2 are whole cell lysates of *E. coli* DH5 α containing lane 1, pRIT5 and lane 2, pPA-CEME. Lanes 3–6 are samples of extracellular medium from cultures of *S. aureus* RN4220 containing: lane 3, control plasmid pRIT5; lane 4, pPA-CEME; lane 5, pPA-HNP-1; lane 6, pPA-proHNP-1. Lane 7 represents PA-CEME fusion protein that was purified on an IgG Sepharose (Pharmacia) affinity column. The blot was probed with a rabbit polyclonal serum raised against GST-CEME. The upper band in lanes 3–7 is probably native protein A produced by *S. aureus* RN4220. **Methods:** Plasmids pRIT5, pPA-CEME, pPA-HNP-1 and pPA-proHNP-1 were electroporated into *S. aureus* strain RN4220. *S. aureus* RN4220 cells were electroporated as previously described (Compagnone Post et al., 1991) except that Lennox medium was used instead of CY-GP media. Electroporation was carried out using a Bio-Rad Gene Pulser, 0.1 cm electroporation cuvettes, and settings of 200 ohms, 25 μ F and 1.8 kV. Transformants were selected on Lennox medium agar plates containing 10 μ g Cm/mL. *S. aureus* cells harbouring the various plasmids were grown at 37°C to an A_{600} approx. 1.5 to 1.7 before the cells were removed by centrifugation.

peptides can be protected from proteases when exported to the external medium, as shown in the case of PA-CEME, PA-HNP-1 and PA-proHNP-1 in *S. aureus*. Second, many proteins that are used as fusions have an affinity for some molecule (Sassenfeld, 1990) and thus can be purified in a single step. Third, the presence of a fusion molecule may prevent the antimicrobial peptide from being active against the host organism, as is the case for PA-CEME. Fourth, the heterologous protein can be used to elicit antibodies without needing to conjugate it to a hapten (Löwenadler et al., 1987). Fifth, the fusion partner can be manipulated to improve peptide stability, a critical feature in the case of polycationic peptides. Sixth, the peptide can be fused to the carrier protein in such a way that it can be released by chemical or specific proteolytic cleavage without leaving any extra aa on the N terminus, which is not always the case with direct expression (Pang et al., 1992). This is important with respect to functional studies using the peptide, since the addition of one or more aa can alter its biological activity (Bessalle et al., 1992).

There are several features of our systems that allow for flexibility when attempting to express a peptide. Since most antimicrobial peptides are short, the complimentary DNAs that encode them can be synthesized as overlap-

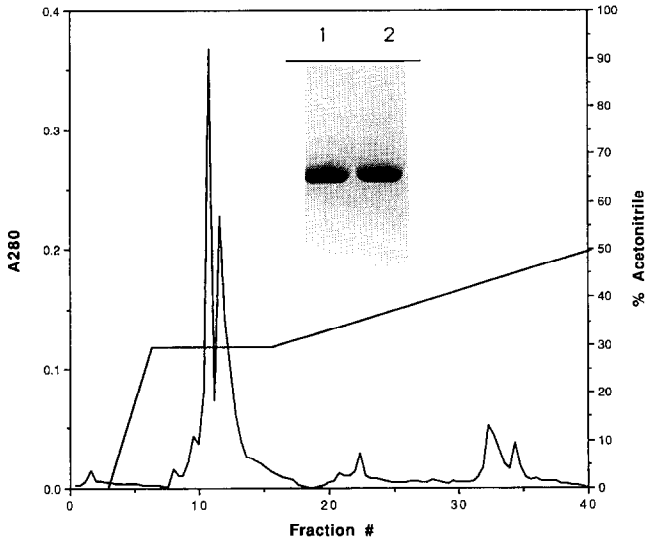


Fig. 4. Reverse-phase purification of CEME. **Methods:** To isolate PA-CEME, culture supernatants from *S. aureus*[pPA-CEME] were adjusted to pH 7.6 with NaOH and passed over an IgG Sepharose column previously equilibrated with TST buffer. The column was washed sequentially with 10 vols. of TST and 5 vols. of 5 mM ammonium acetate pH 5.0. The fusion protein was eluted with 0.5 M acetic acid pH 3.4, lyophilized, and cleaved with CNBr as described in section a. The CNBr-cleaved fusion protein was resuspended in 1% acetic acid and passed over a 2 × 90 cm Bio-Rad P100 column at a flow rate of 10 mL/h. Two mL fractions were collected, lyophilized and analyzed using 8 M acid urea-15% PAGE (Panyim and Chalkey, 1969). Fractions from P100 chromatography containing CEME were pooled, lyophilized and resuspended in 0.1% TFA. Samples (1 µg) were applied to a PepRPC HR 5/5 (Pharmacia) column at a flow rate of 0.7 mL/min and eluted with a 3 mL 0–30% and 20 mL 30–50% acetonitrile/0.1% TFA gradient. Fractions (0.5 mL) were collected, lyophilized and analyzed by 8 M acid urea-15% PAGE (inset) showing chemically synthesized CEME (lane 1) and a sample of purified CEME from fraction 33 (lane 2). A sample of CEME was electoblotted onto Immobilon membrane using the manufacturer's method and analyzed for aa content using an ABI aa sequencer (model 470) and N-terminally sequenced using an ABI sequencer (model 420).

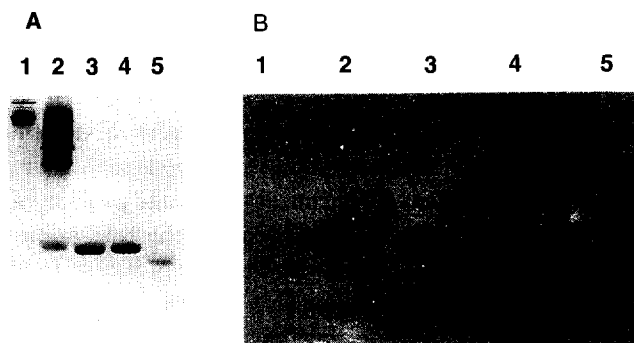


Fig. 5. Antibacterial activity of CEME. Various samples were analysed in duplicate by 8 M acid urea-15% PAGE, and either stained with Coomassie blue (A) or overlaid with *E. coli* DC2 (B) as described by Hultmark et al. (1980). In panel B, samples were loaded in every other lane. Lane 1, purified PA-CEME fusion protein; lane 2, CNBr-cleaved PA-CEME; lane 3, pure CEME; lane 4, chemically synthesized CEME; lane 5, melittin (Calbiochem).

ping oligos. This allows for flexibility in terms of which restriction endonuclease sites are to be used, as well as to the mode of peptide release. For example, we have engineered a Met codon directly 5' to the peptide-encoding genes in order to release the peptide with CNBr. Another feature that can be utilized in the systems that have been developed is the pre-pro DEF cartridge. We have shown that in some cases such as GST-CEME, the fusion protein is still susceptible to proteolysis. The inclusion of the pre-pro cartridge to produce GST-proCEME was shown to stabilize the fusion protein, thus allowing its purification.

(d) Conclusions

(1) To permit expression in bacteria of small cationic peptides with intrinsic antibacterial activity, two major barriers must be overcome. These include the potential ability of the cationic peptide to kill the producing strain and the susceptibility of the cationic peptides to proteolytic degradation. Use of fusion protein expression systems overcame such barriers, although it was found that additional stabilization with the anionic pre-pro DEF sequence was required in some cases.

(2) Four fusion protein expression systems were examined involving fusion to GST, OprF, protein A, and the duplicated IgG-binding domains from protein A. In the first three systems, production of fusion proteins with the cationic peptides HNP-1 and/or CEME were demonstrated in cells containing the appropriately constructed plasmids. In two cases an engineered Met adjacent to the cationic peptides permitted release of the peptide with CNBr. Use of a factor X_a cleavage site for peptide release was generally less successful.

(3) CEME was purified from culture supernatants *S. aureus* expressing a plasmid encoding a PA-CEME fusion protein, after CNBr cleavage. The purified peptide was identical to chemically synthesized CEME with respect to antibacterial activity, gel mobility, and aa sequence.

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