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 pRWS), Staphylococcus aureus protein A (on plasmid pRITS), 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, 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, 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 terranovae 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 in C. terranovae. 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 BamHI-Smal-EcoRI multiple cloning site was changed to SphI-HindIII-EcoRI 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, protection of the fusion protein occurred such that the apparent M₆ of the fusion protein was similar to M₆ 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 Xₜ 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 Xₜ 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-
**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 SphI and HindIII 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 SphI cohesive ends and inserted into pGEX-HNP-1 and pGEX-CEME that had been digested with SphI, resulting in pGEX-proHNP-1 and pGEX-proCEME, respectively. The CEME-encoding gene was cloned into the pEZZ vector using a 5' BamHI and a 3' HindIII 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 T-CGTCGACATCGAAGGTCGTGCATG and S-CACGACCTTCGATGTCGACGCATG were annealed together and inserted into the 5' SphI site to convert it to a SalI site. (A factor Xa recognition 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'-AGCTTGTCGACAG-3' into the HindIII 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 SphI-to-BamHI adaptor (S-CGGATCCATGGCATG and 5'-CCATGGATCCGCATG) on the 5' end and an EcoRI-to-BamHI adaptor (5'-AATTCGGATCCG-3') on the 3' end. Using BamHI, 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 SphI 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 phosphoramide chemistry.

**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 A-U-PAGE. All fractions containing the CEME peptide were pooled, lyophilized, and subjected to reverse-phase FPLC.
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 anti-
bacterial activity was retained throughout the purifica-
tion 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 melit-
tin 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. aerugi-
osa 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 μM for K799 and 1.2–2.5 nM for
Z61. Melittin gave MIC values of 2.5 μM and 1.2 μM for
K799 and 261, 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 bond-
ing (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 biolog-
ical production of an active, cationic, antimicrobial pep-
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 overla-
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 x 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 containing CEME were pooled, lyophilized and analyzed using 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 electrobotted onto Immobilon membrane using the manufacturer's method and analyzed for aa content using an ABI aa sequencer (model 420).

(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 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<sub>c</sub> 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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