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Cost-effective expression and purification of antimicrobial and host defense peptides in *Escherichia coli*

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ABSTRACT

Cationic antimicrobial host defense peptides (HDPs) combat infection by directly killing a wide variety of microbes, and/or modulating host immunity. HDPs have great therapeutic potential against antibiotic-resistant bacteria, viruses and even parasites, but there are substantial roadblocks to their therapeutic application. High manufacturing costs associated with amino acid precursors have limited the delivery of inexpensive therapeutics through industrial-scale chemical synthesis. Conversely, the production of peptides in bacteria by recombinant DNA technology has been impeded by the antimicrobial activity of these peptides and their susceptibility to proteolytic degradation, while subsequent purification of recombinant peptides often requires multiple steps and has not been cost-effective. Here we have developed methodologies appropriate for large-scale industrial production of HDPs; in particular, we describe (i) a method, using fusions to SUMO, for producing high yields of intact recombinant HDPs in bacteria without significant toxicity and (ii) a simplified 2-step purification method appropriate for industrial use. We have used this method to produce seven HDPs to date (IDR1, MX226, LL37, CRAMP, HHC-10, E5 and E6). Using this technology, pilot-scale fermentation (10 L) was performed to produce large quantities of biologically active cationic peptides. Together, these data indicate that this new method represents a cost-effective means to enable commercial enterprises to produce HDPs in large-scale under Good Laboratory Manufacturing Practice (GMP) conditions for therapeutic application in humans.

1. Introduction

The increase in resistance of bacterial strains to existing antibiotics is a major public health concern (WHO (2008) World Health Statistics 2008. World Health Organization Press), and has spurred intensive efforts to develop new classes of antibiotics that are effective against antibiotic-resistant strains, with limited success [37]. The anti-infective properties of HDPs, evolutionary conserved biodefense molecules found in all species of life [43], are mediated by direct antimicrobial activities, modulation of host immune responses, or both. Although initially termed antimicrobial peptides (AMPs) due to their ability to kill microbes directly, the immunomodulatory properties associated with many of these peptides have resulted in their designation as host defense peptides (HDPs) [13]. These are characterized by being short (12–50 amino acids), having an overall positive charge of +2 to +9, and sufficient hydrophobicity to allow for interaction with or traversing of membranes of target cells [21,18].

HDPs are elements of the host innate immune defense system. The upregulation and/or release of HDPs is induced upon infection with bacteria or viruses. The more than 1000 naturally occurring HDPs are divided into 4 broad structural classes, which represent amphipathic α-helixes (e.g. cathelicidins), β-sheets with 2–4 disulfide bridges (α and β defensins and protegrins), extended structures (indolicidin), and beta-loop peptides (brevinin) [19]. Intensive research has led to the development of synthetic peptides with enhanced antimicrobial activities based on their natural counterparts as well as IDRs (innate defense regulators), which do not need to kill microbes, but rather stimulate the host immune defenses to facilitate clearance of an infection in vivo [12,13,20,36].

HDPs have received increased interest as antimicrobial therapeutics, because of their antimicrobial activity against a variety of pathogenic bacteria, including those that are resistant to

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conventional antibiotics [2]. HDPs display minimal inhibitory concentrations (MIC) as low as 0.25 μg/mL in vitro [28], and their capacity to engender resistance is lower than that of conventional antibiotics. This likely occurs either because HDPs nonspecifically interact with bacterial membranes or because they act on multiple targets [13,19,41]. Nevertheless, constitutive or induced resistance to HDPs has been reported in several pathogenic bacteria, and can be recapitulated in vitro (for an overview see [22,32,41]).

Several groups have taken advantage of advances in modeling and QSAR (*Q*uantitative *S*tructure *A*ctivity *R*elationships) methods [9,33], as well as an understanding of how endogenous peptides affect innate immune defenses [6,11,42], to develop synthetic HDPs and IDRs not found in Nature. QSAR is a mathematical relation between the biological activity of a molecular system and its geometric and chemical characteristics. Most structure–function studies provide a working conceptual model of bioactive models. In summary, QSAR studies attempt to find a consistent relationship between biological activity and molecular properties, so that these "rules" can be used to evaluate the activity of new compounds.

Synthetic peptides with antimicrobial activity and/or immunomodulatory capabilities have proven remarkably effective when used in animal models of infection against diverse pathogens including MRSA and malaria among others [1,5,30].

Despite their promise as therapeutics, clinical trials with endogenous or synthetic HDPs have been limited, with none yet approved for use in humans [2], although other peptide drugs have been approved such as the HIV fusion inhibitor Fuzeon. One important contributing factor is that the costs of manufacturing are high, making the price per dose quite expensive. Current production methodologies center on solid phase peptide synthesis, but require expensive precursor components. Several reports have introduced methods for producing HDPs in bacteria or yeast [8,17,26,31,44], but to date, scale-up has not proven cost-effective. Even when production is possible, purification requires multiple biochemical steps that are expensive and significantly reduce yield.

Here, we describe a procedure to produce HDPs on a large-scale in bacteria. We have chosen seven cationic peptides for expression:

LL-37 (# 1) is a 37-amino acid human cathelicidin peptide, which has strong immunomodulatory activity and weaker direct antimicrobial activity due to inhibition by physiological salt concentrations. By contrast, CRAMP (# 2) is the mouse homolog of human LL-37 with 60% identity and somewhat more potent direct antimicrobial activity. IDR-1 (# 3) is the prototype of the IDR peptide class since it has no direct antimicrobial activity but protects in animal infections by modulating the innate immune response and is now in Phase I clinical trials (seq: KSRIVPAIPVSLL) [36].

MX-226 (# 4) is the most clinically advanced antimicrobial peptide that has shown statistically significant efficacy in Phase III clinical trials as an antimicrobial and in Phase II trials as an anti-inflammatory.

E5 (also called sub2, # 5) and E6 (also called sub3, # 6) are 12-amino acid peptides optimized through substitutions into the bovine bactenecin peptide [15] while HHC-10 (# 7) is a promising broad spectrum 9-amino acid candidate that emerged from a QSAR modeling approach [9].

The technique for HDP production in bacteria relies on generating a fusion protein between the peptide and SUMO that both protect the bacteria from the toxicity of the peptides, and the peptides from host proteolytic enzymes. The sumoase protease Ulp1, which specifically cleaves after the C-terminal Gly–Gly residues in SUMO is then used to release HDP sequences at the C-terminus of the fusion protein [4,27,38]. Finally, we have devised a simple two-step purification protocol. The procedure is readily amenable to cost-effective industrial-scale GMP production of HDPs and IDRs.

2. Materials and methods

2.1. Bacterial strains and plasmids

Escherichia coli strain BL21 (DEplys) was used as a host for cloning and gene expression of various smtp3-HDP fusion constructs. *E. coli* was grown at 37 °C in LB media for cloning and at 30 °C in LB media for expression; kanamycin (25 μg/mL) was added during growth of plasmid containing strains. The high-density fermentation media was prepared as described [24].

The vector pET28b was used for cloning and expression of the target genes. The vector allows for expression of exogenous proteins and peptides under the control of the T7 promoter (Novagen, Madison, WI). Sumo-pET28a and sumoase-pET28b were kindly provided by Dr. X. Cheng (Emory University). All recombinant DNA manipulation was performed using standard protocols.

2.2. Construction of expression vectors for expression of cationic peptide fusions

The overall scheme for construction of SUMO-HDP fusion proteins is illustrated in Fig. 1A. Briefly, the yeast smtp3 gene, encoding sumo, including a 5′ 6xHis-linker was cloned into pET28a using the Nco1 and Nde1 sites. Peptide sequences are presented in Fig. 1C. The smtp3 gene was modified and shortened at the 3′-end to allow for a seamless fusion product after the Gly–Gly target sequence (permitting release of the peptide by sumoase Ulp1) using primers listed in Table 1.

All chosen HDP genes encode the active form of the corresponding peptides. The resulting PCR product fuses the cationic peptide gene 3′ in frame with the GG cleavage site of smtp3 (sumo), leaving no residual amino acids after cleavage (Fig. 1A). The PCR product was restricted and ligated into the restricted vector, generating his-sumo-hdp-pET28b. For LL-37 and CRAMP, the corresponding genes were cloned from a cDNA isolated either from human bone marrow cDNA library (LL-37) or from primary mast cells harvested from C57Bl/6 mice (data not shown). Starting from the cDNA, the active part of the gene for LL-37 was isolated via 2-step PCR amplification protocol with primers 1, 7, 8 and 9. The resulting PCR construct was cloned into pET28b using the generated Nco1-Nde1 fragments, and *E. coli* BL21 (DElys, RIL) were transformed with the ligated construct. Positive clones were selected after colony PCR analysis, and tested for expression.

2.3. Expression and high-density fermentation of positive strains

For small-scale expression, 5 mL overnight cultures of *E. coli* cells (BL21 DE(plys)) harboring the vector sumo-AMP/HDP-pET28a were incubated for 12 h at 37 °C. Subsequently 2 mL were used to inoculate 500 mL LB supplemented with kanamycin (25 μg/mL). The cultures were incubated at 30 °C in shaking flasks, and expression was induced at an OD_{600} of 0.5 with IPTG at a final concentration of 0.4 mM. Cells were harvested 4 h after induction and lysed in PBS + 300 mM NaCl + 10 mM imidazole using sonication. Lysed extract was cleared by centrifugation at 13,000 × *g* for 20 min. Large-scale production of fusion protein was achieved using high-density fermentation in a 10 L fermentor with high-glucose feed as described previously [24,39].

2.4. Purification of expressed fusion proteins

The catalytic domain of sumoase was purified as described previously [27] and frozen as concentrated 50% glycerol stocks in the same concentration as described. The enzyme is very specific for SUMO, and no nonspecific activity was observed; depending on the volume of the sample, 5–10 μL of sumoase, corresponding

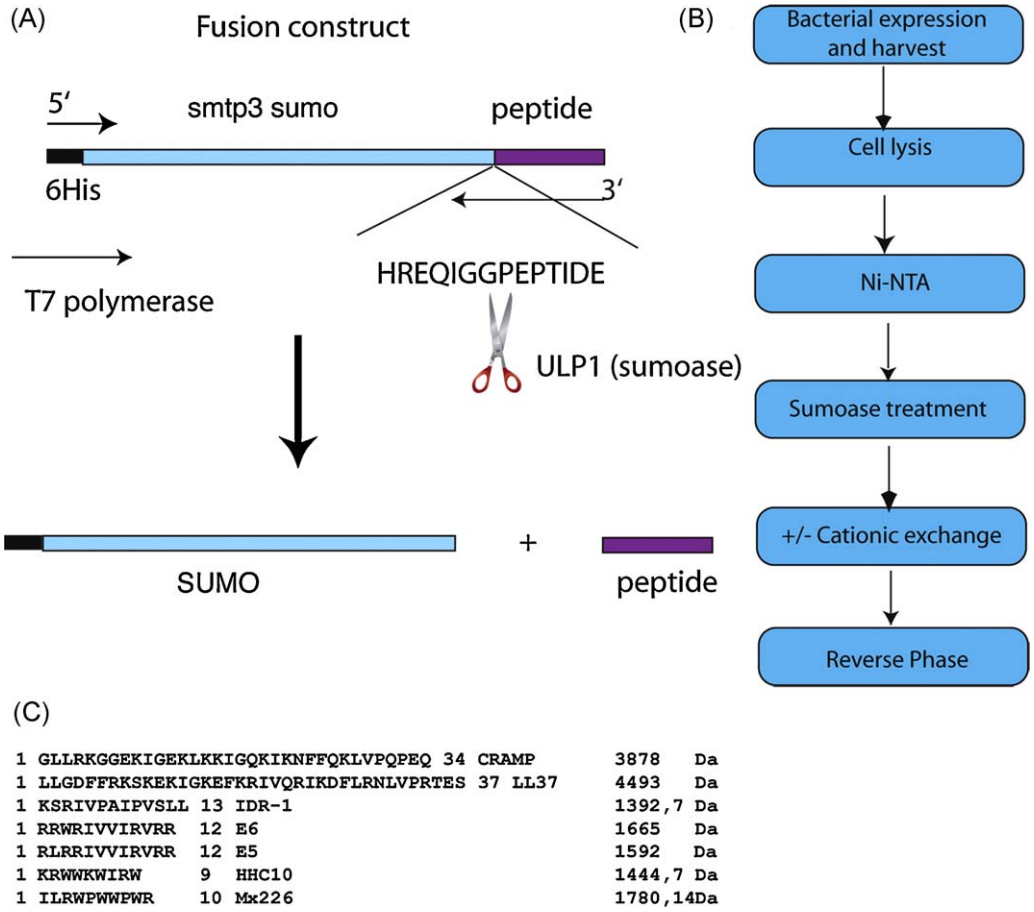


Fig. 1. (A) Schematic outline of the cloning strategy for in frame translation of the fusion product SUMO-peptide. Enhanced is the amino acid sequence at the cleavage site, showing the GG recognition sequence for sumoase followed by a generic peptide sequence. (B) Schematic outline of the purification protocol after expression of the fusion protein. Purification is achieved with 2 chromatographic steps; the cationic exchange chromatography is optional if a large RPC column size is less desired and cost-restrictive. (C) Amino acid sequence and the corresponding mass (Da) for all the peptides tested with this methodology.

to 400 U, were added for complete cleavage of the fusion protein.

After cell lysis by sonication, the cleared supernatant was applied to a Ni-NTA gravity column and allowed to pass through. The column was washed with 10× column volumes of PBS + 300 mM NaCl + 20 mM imidazole and subsequently with 10× column volumes of PBS + 300 mM NaCl + 40 mM imidazole. Bound proteins were eluted with 2–3 column volumes of PBS + 300 mM NaCl + 250 mM imidazole.

The eluted fusion protein was cleaved either overnight at 4 °C or for 1 h at RT using 400 U of sumoase and the cleaved peptide further purified and separated from its fusion partner using a C2/C18 or RESOURCE™ reversed phase chromatography column and a 0.1%TFA/acetonitrile gradient; peptides eluted between 25 and 35% acetonitrile (Fig. 1B). Purification was monitored using tricine-SDS-PAGE [35].

2.5. Characterization of the purified recombinant peptides

Purified peptides were lyophilized and compared to the synthetic peptides using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) to assess the correct size after cleavage and Edman degradation to verify the correct amino acid content. All MALDI-TOF spectra were collected in positive ion mode and the collected data was within the acceptable accuracy of the instrument's capabilities. For the antimicrobial peptides, MICs were determined in vitro using the modified microtiter broth dilution method [40] and compared to established

Table 1
Primer sequences for all the HDP genes cloned and fused to smtp3 (sumo). The restriction sites are shown in bold, the HDP gene sequence is shown in lower case and the smtp3 (sumo) fusion part is underlined.

Primer name	Primer sequence
5′-cgcg**CCATGG**GGcatcatcatcatcatcatTCGGACTCAGAAGTC-3′	5′-His-sumo-Nco1s
5′-GCGCGCC**CATATG**ctacagcagggacaccgggatcgccggcacgatgcgggatttCACCAATCTGTTC-3′	3′-Idr1-Ndeas
5′-GCGCGCC**CATATG**ctaccagcggatccatttccaccagcgtttACCACCAATC TGTTC-3′	3′-HHC10-Ndeas
5′-CGCGCC**CATATG**ctactagcggcggacgcggatgacgacgatgccgccagcggcgACCACCAATCTGTTC-3′	3′-sub3Ndeas
5′-CGCGCC**CATATG**ctactagcggcggacgcggatgacgacgatgcggcggaggcgACCACCAATCTGTTC-3′	3′-sub2Ndeas
5′-GCGCGCC**CATATG**ctatttgcggcgccacggccaccacggccagcgaaggatACCACCAATCTGTTC-3′	3′-Mx226Ndeas
5′-GAACAGATTGGTGGTctgctgggtgatttcttccgg-3′	5′-sumoLL37linker
5′-ccggaagaaatcacccagcagACCACCAATCTGTTC-3′	3′-sumoLL37linker
5′-GCGCGCC**CATATG**ctaggactctgtcctgggtac-3′	3′-LL37Ndeas

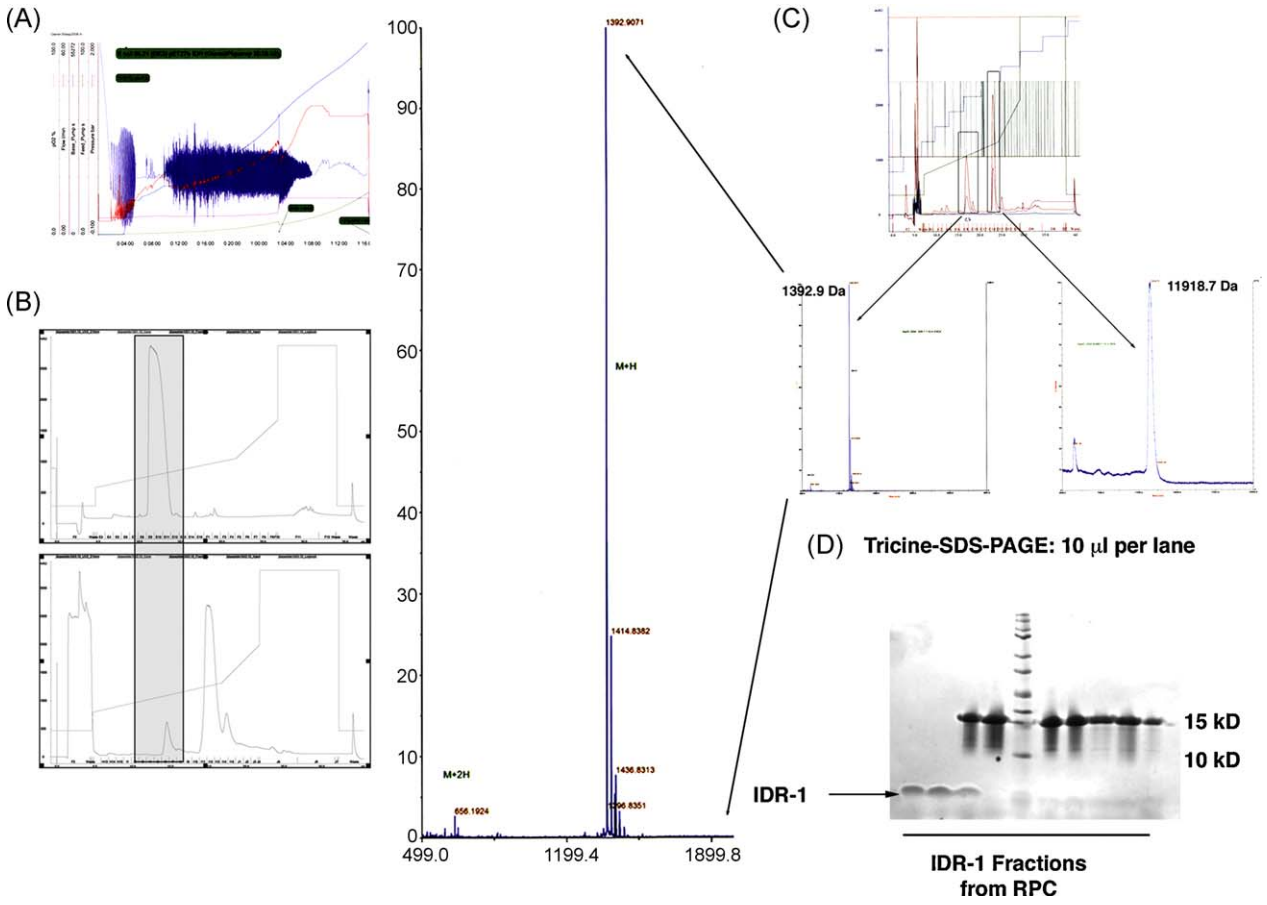


Fig. 2. (A) Fermentation profile of a 10 L high-density fermentation of SUMO-IDR-1 using glucose feed and IPTG induction for 14 h. The fermentation yielded 1.5 kg biomass. (B) Comparison of the RP-chromatography profile for both the synthetic (upper panel) and recombinant IDR-1 peptide (lower panel). Both peptides eluted at the same percent of acetonitrile. (C) MALDI analysis of the purified IDR-1 peptide as well as the remaining his-SUMO protein after cleavage. The determined mass for IDR- 1 was identical to the synthetic peptide and the correct mass for his-sumo revealed 100% cleavage using its protease sumoase. The MS profile for IDR-1 was magnified for better visualization. (D) Tricine-SDS-PAGE separation of the proteins from the recovered fractions from RP-chromatography shown in B. As can be seen in lanes 1 and 2, pure IDR-1 was recovered from fractions 11 and 12 followed by elution of SUMO in lanes 3–10. Lane 5 shows the Kaleidoscope Marker Plus (BioRAD).

MICs for the corresponding chemically synthesized peptides. For immunomodulatory peptides, induction of the cytokines TNFα and IL6 as well as the chemokine MCP-1 were assessed by enzyme linked immunoabsorbant assay (ELISA) following stimulation of human peripheral blood mononuclear cells (PBMC) with peptides for 16 h. PBMCs from different human donors were isolated as previously described [29] in accordance with University of British Columbia ethical approval and guidelines, and the cytokine induction was compared to the results with the synthetic peptide, as described previously [36]. Peptide concentration at 300 μg/mL was used in all of the ELISA assays. Bacterial contaminants were removed using the ProteoSpin kit (Norgen) according to the manufacturer's guidelines.

3. Results

3.1. Expression of cationic peptides in bacteria

We sought to develop a cost-effective means to express cationic peptides in *E. coli* with high yield and then purify them. To express peptides in *E. coli*, the cDNAs encoding them were cloned in frame and 3′ to a gene encoding His-tagged sumo. Whereas expression of the genes alone under the same promoter control resulted in few if any colonies (data not shown), adding the His-sumo to the 5′-end of the peptide gene resulted in normal bacterial growth with concomitant fusion protein expression in the soluble fraction. Therefore, we concluded that the His-SUMO served to block the antimicrobial activity of the fused peptides.

Several cationic peptides of different peptide lengths were chosen for expression using the SUMO fusion system (Fig. 1C). These included LL-37, CRAMP, MX226, IDR-1, E5 and E6 and HHC-10.

All constructs tested expressed high levels of the fusion peptide both in small scale (50 mL) as well as 1 L expression levels (data not shown). The identification of the intact fusion peptides provided evidence that the SUMO-peptide fusion was protected against cleavage by endogenous proteases. On small scale, increase in peptide length did not influence the overall expression yield of the fusion protein, but increasing peptide length will enhance the yield of the pure peptide as a result of a more favorable ratio of peptide to SUMO within the fusion construct.

3.2. High-density fermentation

Two of the peptides, one immunomodulatory peptide (IDR-1) and one antibacterial (E6) were chosen for high-density fermentation with glucose feed in a 10 L pilot scale. We decided on IDR-1 because it represents the prototype of the immunomodulatory peptides [36] and has entered phase 1 clinical trial with Inimexpharma; and on E6 as one of the first published short peptides resulting from a bioinformatics approach [15]. Both fermentations yielded around 1.5 kg wet biomass, of which 6% encompassed SUMO-IDR-1 and 4.3% SUMO-E6 (Figs. 3B and 4D). Under the conditions used, expression of IDR-1 appeared at near optimal levels based on the

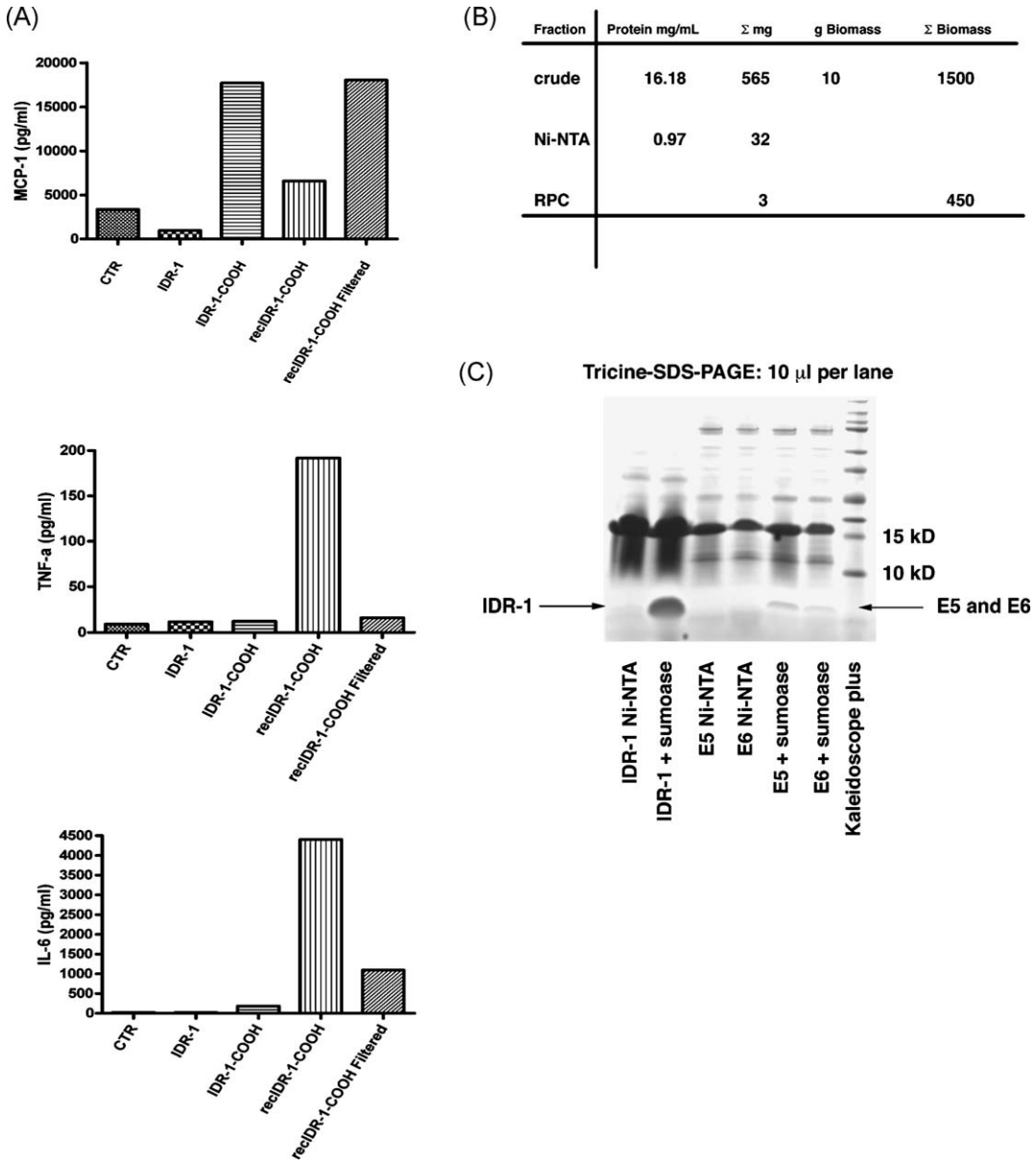


Fig. 3. (A) Cytokine release of PBMCs from different human donors after treatment with either recombinant or synthetic IDR-1. Cytokines tested are IL-6, TNFα as well as the chemokine MCP-1. (B) Purification table for IDR-1 showing the yield of pure IDR-1 after an initial pilot purification of 10 g of biomass from the fermentation as well as a projection of possible yields from processing the total biomass of this fermentation. (C) Tricine-SDS-PAGE comparing cleaved peptide yields of a high-density fermentation against a 1 L shaking flask expression. The amounts shown for E5 and E6 are representative for all the peptides tested in shaking flasks expression.

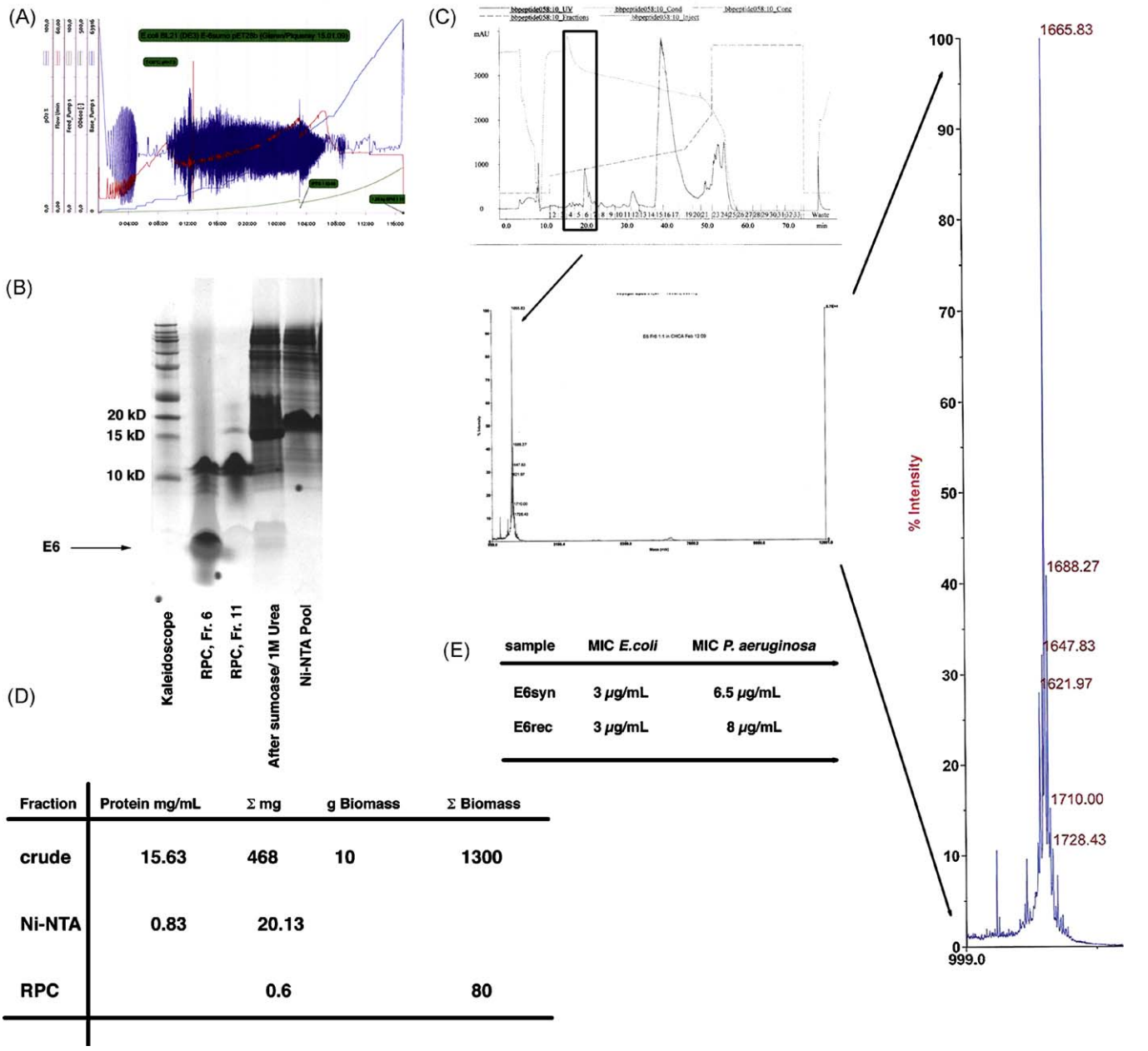
fusion protein amounts present in the soluble fraction, whereas E6 did not. Although no bacterial lysis occurred during the fermentation of either peptide, we anticipate that each construct would likely require optimization to achieve maximum yield.

3.3. Purification of peptides

In the first step, the His-SUMO fusion peptides were purified using a Ni-NTA sepharose column. The peptides were then released from the fusion by cleavage with the SUMO-specific protease sumoase, which recognizes the three-dimensional fold around a GG sequence at the SUMO-peptide boundary (Fig. 1A). The released peptide was further purified using RPC-C2/C18-FPLC or RESOURCE™ RPC-FPLC. A C8 linker on the RPC column was shown not to be effective in separating sumo from the peptide.

Purification and cleavage of the peptide was successful for all peptides tested on a small scale (see below for selected examples). As shown in Figs. 2 and 4, we achieved excellent purification from the scale-up investigation of IDR-1 and E6. To do this, 10 g of the harvested biomass from the fermenter was lysed, cleared and applied to an affinity chromatography column in purification step 1 using Ni-NTA (Fig. 1B). The fusion protein was eluted from the column with 250 mM imidazole and then cleaved with ~400 U of sumoase for 1 h at RT. SUMO-IDR-1 accounted for 6% of the total protein produced, which gave an estimated yield of 0.48 g/L fermentation (Figs. 2D and 3D). For peptides with high tryptophan content, it was necessary to add 1 M urea to the cleavage reaction to ensure precise cleavage of the peptide from the fusion partner SUMO (Figs. 4B and 5A).

Following cleavage, proteins were successfully separated using reversed phase chromatography to produce homogenously pure peptide (Figs. 2D and 4C). RP-chromatography was needed to separate SUMO and sumoase from the peptide, because repeated application onto an affinity Ni-NTA did not result in successful sepa-



Fraction	Protein mg/mL	Σ mg	g Biomass	Σ Biomass
crude	15.63	468	10	1300
Ni-NTA	0.83	20.13		
RPC		0.6		80

sample	MIC *E.coli*	MIC *P. aeruginosa*
E6syn	3 μg/mL	6.5 μg/mL
E6rec	3 μg/mL	8 μg/mL

Fig. 4. (A) Fermentation profile of a 10 L high-density fermentation of SUMO-E6 using glucose feed and IPTG induction for 14 h. The fermentation yielded 1.3 kg biomass. The total biomass is comparable to the one achieved using an immunomodulatory peptide, indicating that expression of an antimicrobial peptide is not deleterious to the bacterial host. (B) Tricine-SDS-PAGE of the purification steps used to achieve pure E6. Lane 2 showing RPC fraction # 6 corresponds to the fraction sent off for MALDI (see C). (C) RP-chromatography profile of the purification of E6 and the subsequent determination of its mass. MALDI analysis determined the correct mass of 1665 Da for this peptide with no further contamination present up to 30 kDa. The MS profile of E6 was magnified for better visualization. (D) Purification table showing the overall yield for E6 purification. (E) Direct comparison of MIC for both synthetic and recombinant E6 using *E. coli* K12 and *P. aeruginosa* PA014. The recombinant E6 showed identical MIC for *E. coli* and a slightly higher MIC for *P. aeruginosa*, again indicating that no contaminant protein is present, since then the amount of E6 would be lower than the one for the synthetic peptide.

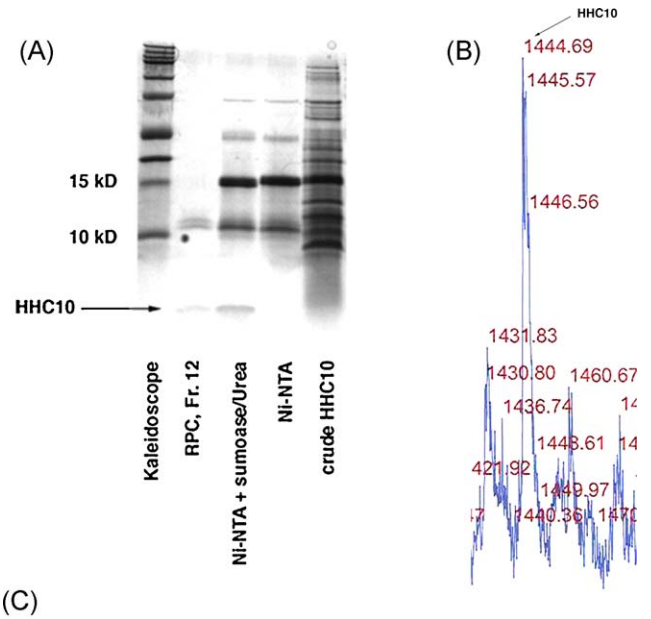
ration of the two (data not shown). Our data suggest that the overall positive charge of the peptide (between +4 and +6) may induce electrostatic interactions with negatively charged residues within SUMO (overall charge −5), perhaps annealing the two proteins during a non-hydrophobic separation procedure. Indeed it is likely that these interactions play an important role in both neutralization of biological activity and protection from endogenous proteases.

3.4. Expression and purification of other peptides

Two other cationic peptides, HHC-10 (Fig. 5A) and MX-226 (data not shown), were successfully expressed in shaking flask cultures as a fusion and successful cleavage with sumoase and purification achieved. 1 M urea was required to ensure correct cleavage at the desired position on SUMO (Fig. 5A), a procedure found necessary for Trp-containing peptides. We also successfully expressed and purified larger HDPs including CRAMP and LL-37 (36 and 37 aa long) with this methodology (data not shown).

3.5. Identification of purified peptides

The identity of the isolated peptides was confirmed by mass spectrometry (Figs. 2C and 4C) using MALDI-TOF to determine the exact mass of purified peptides. IDR-1 showed the exact molecular



(C)

sample	MIC *E.coli*	MIC *P. aeruginosa*
HHC10syn	3 μg/mL	2 μg/mL
HHC10rec	5 μg/mL	4 μg/mL

Fig. 5. Tricine-SDS-PAGE of the purification steps used to achieve pure HHC-10 from a 1 L shaking flask expression, fraction 12 in lane 2 corresponds to the sample used for mass determination. Again, no larger protein contaminants could be found, indicating that the higher bands visible in the gel are possibly aggregates of HHC-10, which could be avoided in a different solvent. (B) MALDI profile for HHC-10 showing the correct mass of 1444 Da. (C) Direct comparison of MIC for both synthetic and recombinant HHC-10 using *E. coli* K12 and *P. aeruginosa* PA014. The recombinant HHC10 showed a slightly higher MIC for *E. coli* and *P. aeruginosa*.

weight (MW) of 1391.9 Da, which corresponded to its theoretical mass (cf. Figs. 1C and 2C). N-terminal sequencing of the IDR-1 in the gel confirmed the correct first 5 amino acids of IDR-1 (KSRIV, data not shown), indicating that cleavage occurred at the right position after the Gly–Gly sequence in SUMO with no additional amino acids introduced to the peptide. The other band observed (Fig. 2C) at a molecular mass of 11,918.7 Da, was identical to the mass of the separated SUMO recovered from reverse phase chromatography (see Fig. 2C, second peak) suggesting that our sumoase cleavage protocol achieved 100% cleavage [34]. Likewise, the measured mass of 1664.83 Da for E6 corresponded to its theoretical peptide mass (Fig. 4C, see Fig. 1C for comparison). Mass determination for the short, Trp-rich peptide HHC-10 after purification showed the predicted mass of 1443.69 Da (Figs. 1C and 5B). In this case, as mentioned above, correct mass of the purified, cleaved peptide could only be achieved through addition of 1 M urea to help unfold the short hydrophobic stretch of this Trp-rich peptide adjacent to the cleavage site for sumoase. Although sumoase is thought to be highly specific [27,34], we have observed that introducing a stretch of several tryptophan residues close to the cleavage site most likely prevents access to the site through steric hindrance and causes random cleavage within the fusion protein. Addition of 1 M urea prevented such unspecific cleavage and led to the release of the full-length peptide.

3.6. Peptide yields

The amount of IDR-1 shown in Fig. 2D corresponds to 1% of the total IDR-1 recovered from a single chromatography run. With available equipment, a total of 5 runs were needed to process the 10 g biomass obtained from a single purification using the C2/C18 RPC column. However this was reduced to 2 runs using the Resource RPC column and therefore can readily be adapted to larger scale. To determine yield, the purified peptide was weighed on a fine scale after lyophilization. However, these measurements can only be considered a rough estimate, since residual salt concentrations left attached to the peptide could affect the weight. IDR-1 concentration was subsequently determined using amino acid analysis of the pure peptide and showed about 50% residual salt within the peptide, which is similar to synthetically produced peptides. Overall, 3 mg of pure IDR-1 was obtained from 10 g of biomass (Figs. 2D and 3B). In an optimized process, we expect to achieve 0.08 g/L fermentation of pure IDR-1. For E6 the purification of the fermented biomass yielded 0.6 mg from 10 g wet cells (Fig. 4D). Despite similar fermentations the heterologous expression of sumo-E6 only accounted for 4.3% of the total protein in *E. coli*. Tricine-SDS-PAGE analysis of E6 indicated several bands around 10 kDa, but thorough mass analysis up to 30 kDa did not reveal any peak other than the one for E6. These data suggest that significant aggregation might be occurring. Alternatively, LPS may have become attached to the E6 peptide, since the MW for LPS in *E. coli* is around 5–10 kDa.

3.7. Characterization of the IDR-1, E6 and HHC-10

For the HDPs E6 and HHC-10 we determined the minimal inhibitory concentration (MIC) for growth of *E. coli* and *Pseudomonas aeruginosa* PA014, and compared these values to those obtained with the corresponding synthetic peptide (Figs. 4E and 5C). Using both bacteria, MICs for the recombinant, homogenous peptide E6 were identical to the corresponding synthetic peptides, given that MICs are operatively considered to be accurate to within twofold.

For the immunomodulatory peptide IDR-1 the efficacy of the recombinant peptide was compared to the synthetic peptides using cytokine/chemokine ELISAs to determine the stimulation of a pro-inflammatory response on PBMC from a variety of human donors. TNFα was used as an indicator of inflammatory cytokine stimulation and possible contamination with bacterial components, because synthetic IDR-1 does not trigger a TNFα response. Initially, the recombinant peptide yielded higher values for IL-6 and TNFα release than two different lots of synthetic peptide. This difference could not be attributed to differences in apparent concentration between the purified and synthetic peptides. We concluded that a bacterial contaminant was still present in the IDR-1 preparation, although the amount varied strongly with the donor. We therefore further purified the recombinant peptide using a LPS removal kit (Norgen), successfully removing LPS as the contaminant and achieving the same TNFα response for the recombinant peptide compared to the synthetic peptide (Fig. 3A). On the other hand, the IL-6 response and production of MCP-1 as an indicator for chemokine induction was still higher between the recombinant and synthetic IDR-1 (Fig. 3A), so a synthetic control IDR-1 peptide lacking amidation (IDR-1-COOH) was included and indicated that the increased MCP-1 and IL6 responses were due to the lack of amidation on the recombinant peptide. Most synthetic peptides include a C-terminal amidation as part of the synthesis process, but bacteria cannot produce peptides with a C-terminal amidation, thus post-translational modifications of bacterially produced peptides via a chemical route might be necessary for therapeutic development.

4. Discussion

To explore the pharmaceutical and therapeutic potential of antimicrobial peptides, a cost-effective and scalable method for

production of active and effective HDPs is required. Procedures to express HDPs as recombinant peptides have encountered difficulties associated with cytotoxicity to the bacterial host, and, as a consequence, difficulty in scale-up and low yields following purification [8,31,44], especially when large fusion proteins are chosen [16]. Even when successful, the requirement for processing of fusion peptides (often requiring chemicals or costly enzymes) and multistep purification of peptides has shown reduced yields, has not proven cost-effective, and has met with intermittent success depending on the peptide.

In this study we have described a procedure for high yield production of several antimicrobial or immunomodulatory peptides using a fusion protein partner that has been well established to ensure high-level soluble expression of fusion proteins, even with proteins that are otherwise difficult to express (e.g. MMP13 [27]). Using this system, we have found that the fusion protein accounts for 10–25% of total protein, and yielded 3 mg of pure peptide from 10 g of biomass.

Unlike previous methods, expression of the SUMO-peptide fusion does not have to be forced into the insoluble fractions, which, on an industrial scale, can be costly due to the requirement for urea and guanidium chloride to solubilize the inclusion bodies [14,23,46]. Moreover, the fusion protein remains in the soluble fraction without lethal effects to the host bacterial cells. Thus, proteolytic degradation of the fusion protein and release of the peptide does not readily occur. Generally, there are several options available to cleave a peptide from its fusion partner, utilizing both chemical and enzymatic routes of cleavage. For chemical cleavage, two different methods have been explored. Cyanogen bromide, which cleaves C-terminally after methionine, has been used extensively, but is inefficient in its cleavage [14]. Second, cleavage of the acid-labile peptide bond between asparagines and proline using hydrochloric acid [46], leaves a proline overhang at the N-terminus of a peptide and requires neutralization of the acid, properties that are neither generally nor easily applicable on an industrial scale.

Enzymatically, several proteases, such as thrombin, Factor Xa or enterokinase, will cleave at their recognition sites, once these are introduced within the linker between the fusion protein and the peptide during cloning [45]. However, these enzymes are expensive and not feasible for industrial scale purification. Sumoase is unique in that it recognizes only residues within SUMO as its cleavage substrate and cleavage occurs precisely after the Gly–Gly in the SUMO sequence [27], leaving no unwanted amino acids at the N-terminus of the peptide. Another important advantage is that this protease is produced cheaply using the T7 driven pET system and can be easily purified with Ni-NTA affinity chromatography in a single step [27]. Also, 400 U of enzyme were used for complete cleavage [27], which seems substantial, but a 0.5 L culture yields 10,000× that amount, suggesting that the enzyme is actually quite cost-effective.

A few labs have recently used the SUMO fusion system for cost-effective antimicrobial peptide expression and demonstrated its efficacy [7,25], however, we have shown that successful endotoxin removal is critical when considering their use for therapeutic purposes. Removal of endotoxins has proven to be a challenge and several routes had to be taken until the unfavorable TNFα response was prevented. Our data indicates that the bacterially produced peptides have to be an exact match to the established synthetic peptides, if they are to be successful therapeutics. As we have demonstrated, this is especially true for the peptides' immunomodulatory potential, a peptide property that is gaining more and more importance in therapeutic applications [2]. We were able to produce peptides of varying lengths ranging from 37 amino acids for LL-37 down to 9 amino acids for HHC-10 with excellent MIC values. To the best of our knowledge, nobody has achieved bulk production of such small peptides in bacteria, especially not peptides that are already tested in clinical trials (IDR-1 (www.inimexpharma.com) and MX-226 (www.migenix.com)), whereas other recent publications have achieved expression and purification of peptides in the range of 30 amino acids and more [7,25].

Our data also indicate that posttranslational modifications of peptides can alter their cytokine profile, as demonstrated in Fig. 3A. Amidation of the C-terminus of IDR-1, as it is common practice for synthetic peptides, resulted in less cytokine response than the free C-terminus IDR-1 samples, both synthetic and recombinant. Peptide amidation can occur in mammals, since most hormonal peptides and neuropeptides are amidated through *p*eptidylglycine α-*a*midating *m*onooxygenase PAM, which is essential for their activity [3,10]. Amidation also protects from C-terminal peptide degradation, which prolongs the half-life of peptides in serum. So far, amidation has not been reported for endogenous human antimicrobial peptides.

Correct determination of peptide concentration following chromatography can only be achieved through amino acid determination, because salts used in the purification tend to attach to the peptides. This is especially crucial when using cationic exchange chromatography, where high concentrations of sodium chloride are used to elute the peptide. We have found salt to be present in the lyophilized peptide even after RP-chromatography as the last step, which does not involve salt.

Taken together, this expression system allows for large-scale production of HDPs, which retain activity similar to peptides synthesized by chemical means. The system can produce both immunomodulatory and antimicrobial peptides, apparently independent of amino acid sequence, length, or charge in industrial scale quantities. Even small peptides of just 9 amino acid in length can be produced with acceptable yields. Purification is achieved in two steps, which are easily scalable for industrial application. In short, we provide evidence that this system will enable cost-effective production of HDPs under GMP conditions in support of therapeutic applications for these molecules against infectious diseases.

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