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Production and isotope labeling of antimicrobial peptides in *Escherichia coli* by means of a novel fusion partner that enables high-yield insoluble expression and fast purification

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A method is presented that allows efficient production of antimicrobial peptides in bacteria by means of fusion to the histone fold domain of the human transcription factor TAF12. This small fusion partner drives high-level expression of peptides and leads to their accumulation in an entirely insoluble form, thereby eliminating toxicity to the host. Using the antimicrobial peptide LAH4 as an example, we demonstrate that neither affinity purification of the TAF12 fusion protein nor initial solubilization of inclusion bodies in denaturing buffers is required. Instead, crude insoluble material from bacteria is directly dissolved in formic acid for immediate release of the peptide through chemical cleavage at a unique Asp-Pro site. This is followed by purification to homogeneity in a single chromatographic step. Because of the elevated expression levels of the histone fold domain and its small size (8 kDa), this straightforward purification scheme produces yields in excess of 10 mg active peptide per liter of culture. We demonstrate that TAF12 fusion allows expression of a wide range of antimicrobial peptides as well as efficient isotope labeling for NMR studies. **Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.**

Keywords: antimicrobial peptide; bacterial expression; NMR; high-throughput

Introduction

Escherichia coli (E. coli) is arguably the most convenient microorganism available to date for the large-scale production of recombinant polypeptides *in vivo* [1]. The bacterium is also ideally suited for uniform labeling strategies to enable structural and biophysical studies, as it can be grown in well-defined synthetic media containing appropriately labeled nutrients. Polypeptides labeled with ^{15}N, ^{13}C, or seleno-methionine can be produced very cost-effectively in this manner and as a result have become standard tools in NMR and x-ray crystallography [2].

Unfortunately, expression of foreign genes in *E. coli* often fails due to vector instability, resulting from toxicity of the molecule of interest towards the host. This problem is particularly common in the case of antimicrobial peptides, natural defense molecules involved in the innate immune system of metazoans, which kill bacteria by permeating and disrupting their membranes [3]. Investigation of the structural properties and dynamics of such peptides by heteronuclear NMR is of considerable biomedical interest, as the emergence of resistance traits in pathological bacteria calls for rational design of new antibiotics [4]. Although native antimicrobial peptides can be produced synthetically, uniform isotope labeling by chemical means would in most cases be prohibitively expensive. Consequently, many laboratories have sought to establish methods for the production of antimicrobial peptides in *E. coli*, in spite of their inherent toxicity towards bacteria [5].

The lethal effects of antimicrobial peptides on *E. coli* can often be circumvented by expressing the molecule of interest as part of a fusion protein. Although this approach allows expression of some peptides in a soluble form (e.g. in conjunction with glutathione S-transferase (GST) [6,7] or thioredoxin [8–11]), most successful cases involve fusion proteins that accumulate entirely in inclusion bodies [12–19]. This suggests that protein aggregation provides a powerful means of neutralizing toxicity of antimicrobial peptides towards the host.

Antimicrobial peptides may spontaneously form inactive aggregates if fused to soluble partners such as ubiquitin [12] or if multimerized [13,14]. Nevertheless, it would be advantageous to develop a robust, generally applicable method that allows targeting of any toxic peptide of interest to inclusion bodies. To this end, several laboratories have identified highly insoluble proteins that can be used as fusion partners, such as ketosteroid isomerase [15], baculoviral polyhedrin [16,17], RepA [18], and an artificial polypeptide encoded by portions of the *E. coli* Trp operon [19]. Although expression levels of the corresponding fusion proteins tend to be satisfactory, all the aforementioned partners have the disadvantage that they are relatively large (typically 20 kDa or more). This diminishes the efficiency of the expression system, as the peptide

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of interest is usually as small as 2–3 kDa and thus comprises only a modest percentage of the material that is expressed.

An additional problem is the fact that obtaining pure peptide from an insoluble fusion protein preparation is not as straightforward as it would be in the case of a soluble fusion protein. The main reason for this is that the denaturing conditions required for initial purification of the fusion preclude the use of proteases to release the peptide. Instead, most protocols published to date make use of chemical cleavage either by cyanogen bromide (following a methionine residue) or by formic acid (which cleaves the sequence Asp-Pro). Although the latter method leaves a proline at the *N*-terminus of the peptide of interest, it is more specific (chances of an Asp-Pro site occurring within a peptide of interest are smaller than that of an internal methionine residue) and has the additional advantage that formic acid is less toxic than cyanogen bromide. Moreover, the acid is readily eliminated, for instance by evaporation. Nevertheless, peptide isolation remains a costly and time-consuming procedure that typically involves purification of the denatured fusion protein by metal affinity chromatography, exchange of the solvent by dialysis, chemical cleavage, and final chromatographic purification of the peptide itself. The use of large fusion partners further complicates matters, as in this case multiple internal chemical cleavage sites are likely to be present. This means numerous contaminating fragments are generated, some of which may be difficult to separate chromatographically from the peptide of interest.

Here, we describe a fast and highly efficient alternative for the expression and purification of antimicrobial peptides, which is based on fusion to the histone fold domain (HFD) of the human transcription factor TAF12. This protein domain is considerably smaller than traditional fusion partners (8 kDa) yet highly expressed in an entirely insoluble form in the absence of its natural dimerization partner [20–22]. The HFD is therefore ideally suited as a fusion partner for insoluble expression, and we show that it is capable of neutralizing the toxicity of a wide range of antimicrobial peptides. Only one internal Asp-Pro site is present in the HFD itself, which we have mutated to simplify peptide purification. Using the antimicrobial peptide LAH4 as an example, we demonstrate that affinity purification of the TAF12 fusion protein and even initial solubilization in buffers containing 8 M urea or 6 M guanidinium chloride before cleavage can be avoided. Instead, crude inclusion body preparations are directly dissolved in formic acid to set up cleavage reactions, whereupon a single HPLC purification step suffices to purify the peptide of interest to homogeneity. We show that this remarkably simple procedure readily produces large quantities of fully active LAH4 and demonstrate its usefulness for rapid production of the milligram amounts of uniformly ^{15}N-labeled material required to perform NMR studies.

Materials and Methods

Construction of Expression Vectors

The naturally occurring Asp-Pro site in the gene encoding the human TAF12 HFD [20] was changed to Asp-Ala by PCR-mediated mutagenesis [23]. Expression vectors pTIPX-1 and pTIPX-2 were constructed by insertion of this mutated gene plus oligonucleotides corresponding to a novel polylinker and an enterokinase site (pTIPX-2 only) into pET-15b (Novagen), using standard cloning techniques [24]. The vectors pTIPX-3 and pTIPX-4 were subsequently generated by transfer of the BglII–Bpu1102I fragment from, respectively, pTIPX-1 and pTIPX-2 to pET-28b (Novagen). All expression vectors (Figure 1 and Table 1) were verified by DNA sequencing.

Synthetic DNA encoding several antimicrobial peptides (Table 2) was purchased from Geneart AG, Regensburg. Individual genes were amplified by PCR and cloned in between the BamHI and Sac I or SacII sites of the expression vectors. All constructs were verified by DNA sequencing.

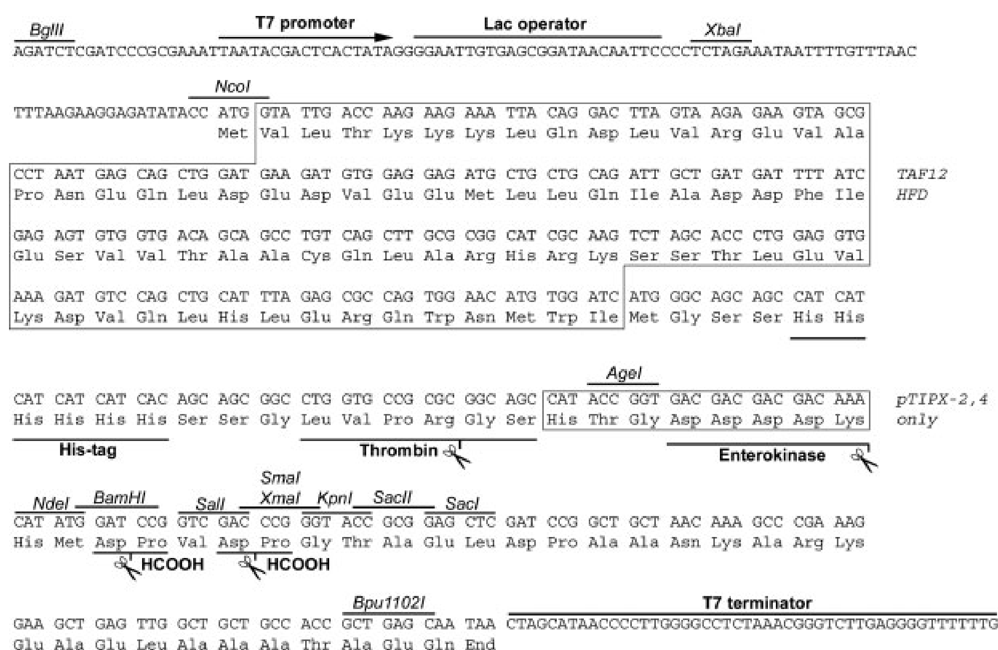

Figure 1. Schematic representation of the TAF-fusion expression vectors used in this study. Translation as well as cleavage sites in the expressed fusion protein have been indicated. All restriction sites shown are unique. NdeI and BamHI (or SalI) sites can be used for cloning of the sequence of interest directly downstream of an Asp-Pro sequence (for formic acid cleavage), or a Met codon (for CnBr cleavage), respectively. The boxed sequence containing the enterokinase site is only present in pTIPX-2 and pTIPX-4.

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Table 1. Properties of the pTIPX expression vectors used in this study. The pTIPX vectors differ from the original pET vectors in the region between the BglII and Bpu1102I restriction sites, as shown in Figure 1

Expression vector	Selection marker	Enterokinase site	Derived from
pTIPX-1	Ampicillin	No	pET-15b
pTIPX-2	Ampicillin	Yes	pET-15b
pTIPX-3	Kanamycin	No	pET-28b
pTIPX-4	Kanamycin	Yes	pET-28b

Expression of TAF12 Fusion Proteins

Overnight precultures from single colonies of transformed BL21 (DE3) were diluted 1 : 100 in Luria Bertani (LB) medium containing the appropriate antibiotic (100 μg/l ampicillin or 20 μg/l kanamycin) and 1% glucose (weight/volume) where indicated, incubated in a shaker for 2–3 h at 37 °C, and induced by addition of isopropyl β-D-1-thiogaLactopyranoside (IPTG) to a final concentration of 1 mM at an optical density (600 nm) of 0.4–0.6. After an additional 3-h incubation, cells were collected by centrifugation (10 min at 4000 × *g*, 4 °C), resuspended in 30 ml of lysis buffer (50 mM Tris–HCl pH 8.0, 100 mM NaCl, 5 mM EDTA, 0.5% Triton X-100) per liter of culture and stored at −80 °C until further use. Uniformly ^{15}N-labeled material was produced identically, but instead of LB medium M9 minimal medium [24] was used that contained 0.5 g/l $^{15}NH_4Cl$ as the sole nitrogen source.

Peptide Purification

Bacteria were thawed and lysed by sonication on ice for 5 min (Bandelin Sonopuls HD200 with SH213G booster horn and MS72/D tip, 20% pulse intervals at 25% power), followed by centrifugation of the lysate for 30 min at 4 °C, 10 000 × *g*. The insoluble fraction was resuspended in wash buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM EDTA), followed by sonication for 1 min and centrifugation as before. The resulting pellet was then dissolved in 5–10 ml of 100% formic acid per liter of culture. This solution was mixed with de-ionized water so as to arrive at a final formic acid concentration of 50% (volume/volume) and incubated for 24 h at 50 °C. Upon evaporation of all solvent under vacuum using a Rotavapor R-200 (Büchi) at 50 °C, the cleaved peptide was dissolved in de-ionized water (5 ml per liter of culture), followed by centrifugation (30 min at room temperature, 16, 000 × *g*) to remove water-insoluble bacterial debris. The peptide was then purified to homogeneity by HPLC (Prontosil 300-5-C4) using a 10–80% acetonitrile gradient in 0.1% TFA. The purified peptide was dried (Rotavapor) and dissolved in 4% acetic acid and lyophilized to remove remaining TFA.

Analysis of Protein Samples

Protein samples were analyzed by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) using 15% gels [24]. Peptide samples (Figure 4(a)) were analyzed by means of tricine SDS-PAGE [32]. All gels were stained using Coomassie Brilliant Blue. LAH4 yield was assessed directly by determining the mass of the purified and lyophilized peptide using a microbalance. The LAH4 fusion protein expression level lower limit was calculated on the basis of the final peptide yield, assuming no losses. Expression levels of all other fusion proteins were estimated by comparison with the LAH4 fusion protein using Coomassie-stained gels (Figure 3). Antimicrobial activity of LAH4 was measured using *E. coli*, BL21 (DE3), essentially as described by Vogt and Bechinger [33].

NMR Spectroscopy

For the $^{1}H^{15}N$-HSQC spectrum shown in Figure 5, 1.8 mg of uniformly ^{15}N-labeled LAH4 was dissolved to a final concentration of 1 mM in a 1 : 1 mixture of deuterated TFE and H_2O. Measurements were performed at 298 K on a Bruker Avance 500 MHz spectrometer equipped with a broadband inverse (BBI) probe, employing a 3.9-19 watergate sequence [34] with 8 scans and 512 increments, and referenced with respect to water (^{1}H) and liquid NH_3 (^{15}N). Data were processed using the program NMRnotebook (NMRtec, Illkirch Graffenstaden, France).

Results

Design of Expression Vectors

Four related vectors were constructed for the expression of target genes fused to the insoluble HFD of TAF12. An overview of these pTIPX (*T*AF12-mediated *I*nsoluble *P*eptide e*X*pression) vectors is given in Figure 1 and Table 1. In addition to the HFD, all vectors encode a histidine tag for optional binding to metal affinity resin, and have multiple cloning sites for seamless insertion of the gene of interest directly downstream of an Asp-Pro site for formic acid cleavage, or following a Met codon for CnBr cleavage. The naturally occurring Asp-Pro site within the TAF12 HFD itself was changed to Ala-Pro by PCR-mediated mutagenesis. This facilitates purification following formic acid cleavage, because only two fragments are generated, that is, the peptide of interest and the remainder of the fusion protein. Plasmids are maintained via either an ampicillin (pTIPX-1, pTIPX-2) or a kanamycin (pTIPX-3, pTIPX-4) selection marker.

Expression of Antimicrobial Peptides

We tested the pTIPX vectors using LAH4, an artificial antimicrobial peptide developed in our laboratory [25]. This molecule is also an efficient transfection agent for eukaryotic cells and a potential gene therapy vector [35]. Because of its pronounced antibacterial activity, earlier attempts to express LAH4 as part of soluble fusion proteins had failed (our unpublished data). On the contrary, fusion of LAH4 to TAF12 using the expression vector pTIPX-4 results in a highly expressed, entirely insoluble protein (Figure 2(a)).

Expression results for all four pTIPX vectors are shown in Figure 2(b). In the presence of 1% glucose (right panel) for catabolite repression during the growth phase [36], each of the vectors produces significant amount of the insoluble fusion protein. However, optimal results are obtained with pTIPX-2 and pTIPX-4. This suggests that the enterokinase site in these vectors abolishes slight residual toxicity, originating either from trace amounts of soluble fusion protein or from soluble degradation products. Indeed, work by Lee *et al.* [13] indicated that a sequence of negatively charged residues directly preceding a cationic antimicrobial peptide can significantly reduce its toxicity, presumably by neutralizing the overall positive charge of the molecule. Our results are consistent with this hypothesis, and suggest that the highly negatively charged enterokinase site (DDDDK) is an efficient inhibitory sequence.

In the presence of 1% glucose (Figure 2(b), right panel), selection by ampicillin and kanamycin appears equally effective (compare

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Table 2. Antimicrobial peptides expressed in this study by means of pTIPX-4

Peptide [Reference]	Origin	Structure	Sequence
LAH4 [25]	Designed	α-Helix	KKALLALALHHLAHLALHLALALKKA
Thanatin [26,27]	Insect	β-Sheet	GSKKPVPIIYCNRRTGKCQRM
MGD-1 [28]	Mussel	Mixed α/β	GFGCPNNYQCHRHCKSIPGRCGGYCGGWHRLRCTCYRCG
RK-1 [29]	Rabbit	Mixed α/β	MPCSCKKYCDPWEVIDGSCGLFNSKYICCREK
Distinctin 1 [30,31]	Frog	4-Helix bundle	ENREVPPGFTALIKTLRKCKII
Distinctin 2 [30,31]	Frog	4-Helix bundle	NLVSGLIEARKYLEQLHRKLKNCKV

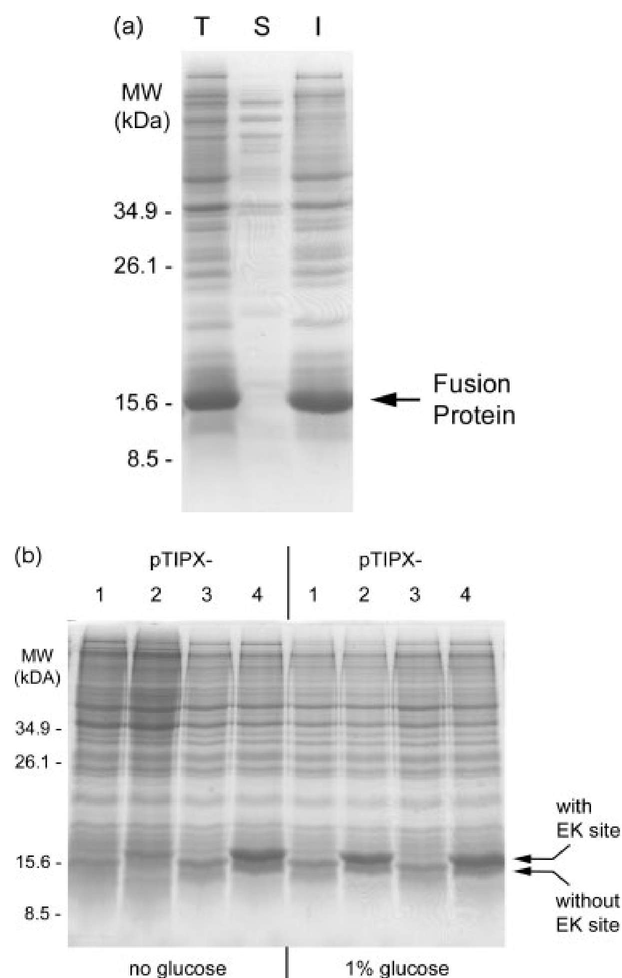

Figure 2. Expression of the antimicrobial peptide LAH4. Samples analyzed correspond to 100 μl of bacterial culture. (a) Insoluble expression of LAH4 using pTIPX-4: total lysate (T), soluble fraction (S), insoluble fraction (I). (b) Effect of expression vector used and catabolite repression by glucose. Only insoluble fractions are shown here.

pTIPX-2 and pTIPX-4), indicating that under these conditions there is sufficient repression of peptide expression and toxicity to allow unhindered propagation of each plasmid. Without glucose-mediated repression, however, higher levels of leaky expression are to be expected, which in the case of ampicillin selection (pTIPX-2) and in the absence of other precautionary measures (such as regularly refreshing the culture medium) might lead to reduced plasmid stability [36]. Indeed, without glucose addition (Figure 2(b), left panel), we observe higher expression levels for pTIPX-4 than for pTIPX-2. Even though catabolite repression – at least in the case of LAH4 – provides a simple means of restoring pTIPX-2 expression levels, we conclude that to assure maximally robust production of highly toxic cationic antimicrobial peptides, pTIPX-4 is preferable. We, therefore, decided to use this vector, which combines TAF-fusion, an enterokinase site, and kanamycin selection, for all subsequent experiments. We also continued to use media supplemented with 1% glucose for enhanced repression.

In order to investigate if pTIPX-4 is also an efficient vector for the production of antimicrobial peptides other than LAH4, we attempted expression of a series of representative molecules (listed in Table 2). These peptides, originating from a variety of organisms and belonging to several different structural and functional classes [3], were all found to be highly expressed (Figure 3). Fusion protein production levels (lanes 2–6) are equal to or even exceeding those observed for LAH4 (lane 1). Assuming comparable purification efficiencies (*vide infra*), this means that a yield of 10–20 mg of the peptides can be obtained per liter of culture. Growth of transformed bacteria is in all cases uninhibited, that is, doubling times measured are identical to those for nontransformed bacteria. These results indicate that pTIPX-4 efficiently neutralizes toxicity of a wide range of antimicrobial peptides and enables their high-level expression.

Peptide Purification

Because TAF12 fusion proteins accumulate to relatively high-purity levels in inclusion bodies and only two products (the peptide of interest and the fusion partner) are generated by formic acid cleavage, we attempted to simplify the traditional

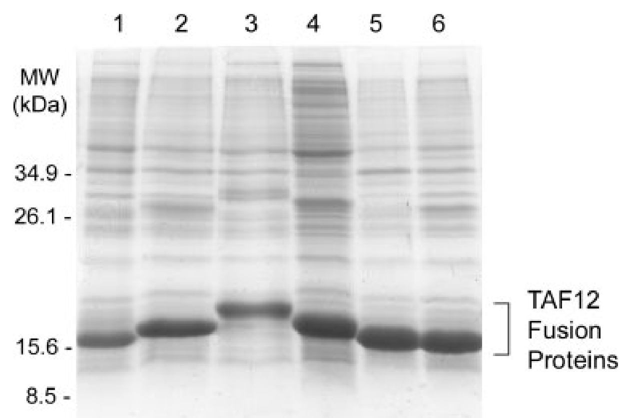

Figure 3. Expression of a variety of antimicrobial peptides using pTIPX-4: LAH4 (lane 1), thanatin (lane 2), MGD-1 (lane 3), RK-1 (lane 4) and the individual chains 1 and 2 of the distinctin heterodimer (lanes 5 and 6, respectively). Only insoluble fractions are shown, corresponding to 130 μl of bacterial culture. Further details concerning the peptides are given in Table 2.

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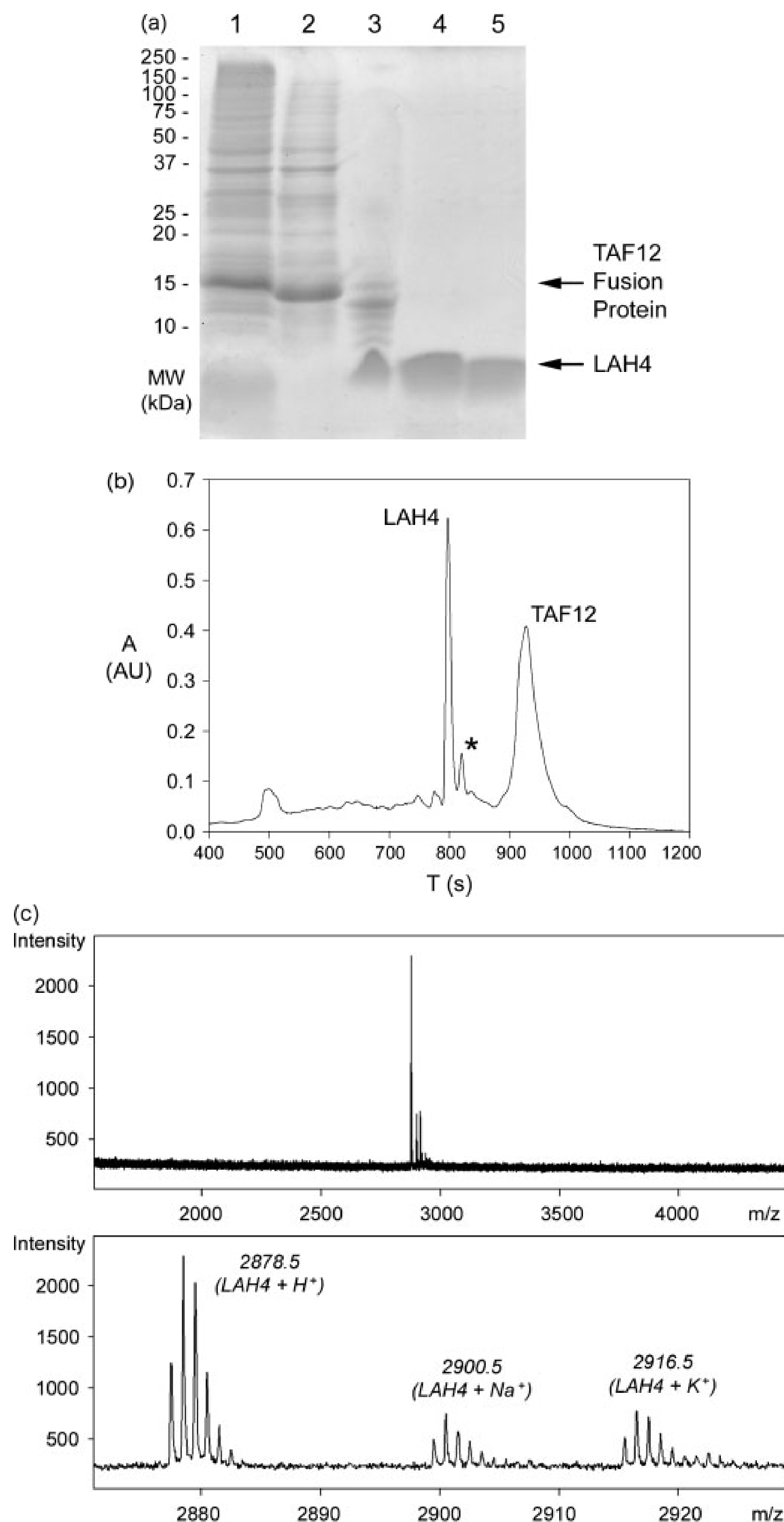

Figure 4. Purification of LAH4. (a) Results of individual purification steps: total cell lysate (lane 1), crude insoluble material (lane 2), and formic acid cleavage products (lane 3), each corresponding to 100 µl of bacterial culture. Lane 4 shows 10 µg of HPLC-purified end product; lane 5 shows 10 µg of synthetic LAH4 for comparison. (b) HPLC profile of material from the LAH4 cleavage reaction. The peak assigned * presumably corresponds to a formic acid adduct of LAH4 (see main text). (c) MS (MALDI) analysis of purified LAH4. Intensities are expressed in arbitrary units. The lower panel shows a detailed view of the peaks in the upper panel. Three masses can be identified, which correspond to LAH4 alone or associated to a sodium or potassium ion originating from the MALDI sample preparation procedure.

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purification scheme for insoluble fusion proteins. Thus, we found that initial purification of the fusion protein (e.g. by metal affinity chromatography under denaturing conditions) and even solubilization in buffers containing 8 M urea or 6 M guanidinium chloride can be avoided. Instead, the insoluble fraction of the bacterial lysate is washed once with buffer and dissolved directly in pure formic acid. This solution is then diluted with water to set up a cleavage reaction in 50% formic acid, which releases the peptide from the fusion partner [37]. Following incubation for 24 h at 50 °C, the solvent is evaporated under vacuum. The cleaved peptide is then dissolved in water and purified to complete homogeneity by HPLC. The effectiveness of this extremely simple procedure is illustrated for LAH4 in Figure 4(a) and (b). Additional experimental details are given in the Materials and Methods section.

Analysis by MS confirmed that the sharp peak in the HPLC elution profile shown in Figure 4(b) indeed corresponds to LAH4 (Figure 4(c), an observed mass of 2877.5 Da, in good agreement with the expected value of 2877 Da). Notably, small amphipatic peptides such as LAH4 are readily separated from the hydrophobic fusion partner, which elutes very late in the acetonitrile gradient. The minor peak directly following LAH4 was also analyzed using mass spectrometry (not shown), and found to correspond to a molecule with a molecular mass of 2905.5 Da, that is, exceeding that of the main product by 28 Da. We observed that this peak increased when the formic acid incubation time was augmented from 24 to 48 h (data not shown). Taken together, these observations suggest that the additional peak corresponds to a formic acid adduct of LAH4, formed during the cleavage reaction. This underlines the importance of avoiding incubation times in excess of 24 h. However, even such minor side products are easily removed by HPLC and the peptide obtained is homogeneous as evidenced by SDS-PAGE (Figure 4(a)) as well as mass spectrometry (Figure 4(c)). The final yield obtained is 10 mg LAH4 per liter of culture.

The antimicrobial activity of the purified peptide, which differs from standard LAH4 in that it contains an *N*-terminal proline residue remaining from the formic acid cleavage site, was subsequently investigated. We found that the bacterially expressed peptide inhibits *E. coli* growth as efficiently as does chemically synthesized LAH4, the latter either with or without additional *N*-terminal proline. Measured LD50 concentrations are around 10 μg/l for all three of the peptides (bacterially expressed peptide, chemically synthesized peptide, and chemically synthesized peptide with an *N*-terminal proline residue), in good agreement with values reported earlier for synthetic LAH4 [33]. The additional N-terminal proline residue therefore does not interfere with the antimicrobial activity of LAH4.

Using M9 minimal medium containing $^{15}NH_4Cl$ as the only nitrogen source, and the fast purification scheme outlined above, we succeeded in rapidly producing milligram amounts of isotope-labeled LAH4 for use in NMR studies. Figure 5 shows the resulting $^{1}H^{15}N$-HSQC spectrum of the peptide in 50% TFE, indicative of a homogeneous sample and efficient uniform ^{15}N-labeling.

Discussion

Here, we have described a procedure that allows rapid expression and purification of peptides that are normally toxic to *E. coli*, and demonstrated its effectiveness for the production of the antimicrobial peptide LAH4 in native as well as uniformly ^{15}N-labeled form. Compared with earlier methods that use insoluble fusion partners to neutralize peptide toxicity towards the host, our approach has a number of significant advantages. In the first place, the fusion partner we use, TAF12, is not only expressed in an entirely insoluble form and at very high levels (typically more than 40 mg per liter of culture), but in addition is smaller than the fusion partners typically used for this purpose. This increases efficiency, as the peptide of interest constitutes a larger percentage of the protein that is produced. Moreover, we demonstrated that TAF12 fusion allows high-level expression of a series of entirely unrelated antimicrobial peptides, suggesting that the fusion is quite generally applicable.

Importantly, the high expression levels and the absence of internal Asp-Pro sites in the fusion partner enabled us to bypass a number of commonly used purification steps and directly treat the insoluble fraction of *E. coli* with formic acid, followed by single-step HPLC purification of the peptide of interest. This simplification of the traditional purification scheme leads to a considerable gain in time and eliminates losses in the course of intermediate chromatography steps. In addition, purification costs are significantly reduced because no affinity resin is required and only relatively inexpensive chemicals are used.

We established our method primarily with the aim of facilitating high-throughput expression and purification of active, uniformly labeled antimicrobial peptides for structural and biophysical studies, in particular by NMR. However, the procedure is extremely straightforward, fast and inexpensive and could be developed

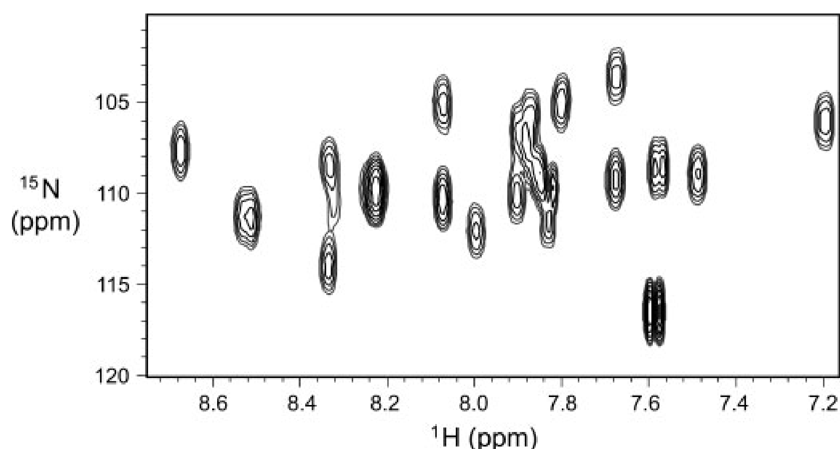

Figure 5. Heteronuclear single quantum coherence (HSQC) spectrum of bacterially expressed and uniformly ^{15}N-labeled LAH4 in 50% TFE.

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into an interesting alternative for large-scale chemical synthesis of native peptides as well. Moreover, the method clearly has advantages (in particular in terms of cost-effectiveness and speed) over soluble expression techniques and may therefore be of interest for the *in vivo* production of nontoxic peptides and possibly proteins that are amenable to post-purification renaturation.

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References

1. Sørensen HP, Mortensen KK. Advanced genetic strategies for recombinant protein expression in *Escherichia coli*. *J. Biotechnol.* 2005; **115**: 113–128.
2. Yokoyama S. Protein expression systems for structural genomics and proteomics. *Curr. Opin. Chem. Biol.* 2003; **7**: 39–43.
3. Jenssen H, Hamill P, Hancock REW. Peptide antimicrobial agents. *Clin. Microbiol. Rev.* 2006; **19**: 491–511.
4. Yacoby I, Benhar I. Targeted anti bacterial therapy. *Infect. Disord. Drug Targets* 2007; **7**: 221–229.
5. Ingham AB, Moore RJ. Recombinant production of antimicrobial peptides in heterologous microbial systems. *Biotechnol. Appl. Biochem.* 2007; **47**: 1–9.
6. Moon JY, Henzler-Wildman KA, Ramamoorthy A. Expression and purification of a recombinant LL-37 from *Escherichia coli*. *Biochim. Biophys. Acta* 2006; **1758**: 1351–1358.
7. Capparelli R, Ventimiglia I, Palumbo D, Nicodemo D, Salvatore P, Amoroso MG, Iannaccone M. Expression of recombinant puroindolines for the treatment of staphylococcal skin infections (Acne Vulgaris). *J. Biotechnol.* 2007; **128**: 606–614.
8. Huang L, Wang J, Zhong Z, Peng L, Chen H, Xu Z, Cen P. Production of bioactive human Beta-defensin-3 in *Escherichia coli* by soluble fusion expression. *Biotechnol. Lett.* 2006; **28**: 627–632.
9. Xu Z, Peng L, Zhong Z, Fang X, Cen P. High-level expression of a soluble functional antimicrobial peptide, human Beta-defensin 2, in *Escherichia coli*. *Biotechnol. Prog.* 2006; **22**: 382–386.
10. Xu Z, Zhong Z, Huang L, Peng L, Wang F, Cen P. High-level production of bioactive human Beta-defensin-4 in *Escherichia coli* by soluble fusion expression. *Appl. Microbiol. Biotechnol.* 2006; **72**: 471–479.
11. Xu X, Jin F, Yu X, Ji S, Wang J, Cheng H, Wang C, Zhang W. Expression and purification of a recombinant antibacterial peptide, cecropin, from *Escherichia coli*. *Protein Exp. Purif.* 2007; **53**: 293–301.
12. Moon WJ, Hwang DK, Park EJ, Kim YM, Chae YK. Recombinant expression, isotope labeling, refolding, and purification of an antimicrobial peptide, piscidin. *Protein Exp. Purif.* 2007; **51**: 141–146.
13. Lee JH, Minn I, Park CB, Kim SC. Acidic Peptide-mediated expression of the antimicrobial peptide buforin II as tandem repeats in *Escherichia coli*. *Protein Exp. Purif.* 1998; **12**: 53–60.
14. Tian ZG, Teng D, Yang YL, Luo J, Feng XJ, Fan Y, Zhang F, Wang JH. Multimerization and fusion expression of bovine lactoferricin derivative LfcinB15-W4,10 in *Escherichia coli*. *Appl. Microbiol. Biotechnol.* 2007; **75**: 117–124.
15. Majerle A, Kidric J, Jerala R. Production of stable isotope enriched antimicrobial peptides in *Escherichia coli*: An application to the production of a 15N-enriched fragment of lactoferrin. *J. Biomol. NMR* 2000; **18**: 145–151.
16. Wei Q, Kim YS, Seo JH, Jang WS, Lee IH, Cha HJ. Facilitation of expression and purification of an antimicrobial peptide by fusion with baculoviral polyhedrin in *Escherichia coli*. *Appl. Environ. Microbiol.* 2005; **71**: 5038–5043.
17. Li L, Kim YS, Hwang DS, Seo JH, Jung HJ, Du J, Cha HJ. High and compact formation of baculoviral polyhedrin-induced inclusion body by Co-expression of baculoviral FP25 in *Escherichia coli*. *Biotechnol. Bioeng.* 2007; **96**: 1183–1190.
18. Zhang L, Falla T, Wu M, Fidai S, Burian J, Kay W, Hancock REW. Determinants of recombinant production of antimicrobial cationic peptides and creation of peptide variants in bacteria. *Biochem. Biophys. Res. Commun.* 1998; **247**: 674–680.
19. Pazgier M, Lubkowski J. Expression and purification of recombinant human α-defensins in *Escherichia coli*. *Protein Exp. Purif.* 2006; **49**: 1–8.
20. Werten S, Mitschler A, Romier C, Gangloff Y-G, Thuault S, Davidson I, Moras D. Crystal structure of a subcomplex of human TFIID formed by TBP-associated factors hTAF4 (hTAFII135) and hTAF12 (hTAFII20). *J. Biol. Chem.* 2002; **277**: 45502–45509.
21. Fribourg S, Romier C, Werten S, Gangloff YG, Poterszman A, Moras D. Dissecting the interaction network of multiprotein complexes by pairwise coexpression of polypeptides in *E. coli*. *J. Mol. Biol.* 2001; **306**: 363–373.
22. Gangloff YG, Werten S, Romier C, Carré L, Poch O, Moras D, Davidson I. The human TFIID components TAF(II)135 and TAF(II)20 and the yeast SAGA components ADA1 and TAF(II)68 heterodimerize to form Histone-like Pairs. *Mol. Cell. Biol.* 2000; **20**: 340–351.
23. Higuchi R. *PCR Protocols, a Guide to Methods and Applications*. Academic Press: San Diego, CA, 1990; 177–183, Chapter 22.
24. Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989.
25. Bechinger B. Towards membrane protein design: pH-sensitive topology of Histidine-containing polypeptides. *J. Mol. Biol.* 1996; **263**: 768–775.
26. Fehlbaum P, Bulet P, Chernysh S, Briand JP, Roussel JP, Letellier L, Hetru C, Hoffmann JA. Structure-activity analysis of thanatin, a 21-residue inducible insect defense peptide with sequence homology to frog skin antimicrobial peptides. *Proc. Natl. Acad. Sci. U.S.A.* 1996; **93**: 1221–1225.
27. Mandard N, Sodano P, Labbe H, Bonmatin JM, Bulet P, Hetru C, Ptak M, Vovelle F. Solution structure of thanatin, a potent bactericidal and fungicidal insect peptide, determined from proton Two-dimensional nuclear magnetic resonance data. *Eur. J. Biochem.* 1998; **256**: 404–410.
28. Yang YS, Mitta G, Chavanieu A, Calas B, Sanchez JF, Roch P, Aumelas A. Solution structure and activity of the synthetic Four-disulfide bond mediterranean mussel defensin (MGD-1). *Biochemistry* 2000; **39**: 14436–14447.
29. McManus AM, Dawson NF, Wade JD, Carrington LE, Winzor DJ, Craik DJ. Three-dimensional structure of RK-1: a novel Alpha-defensin peptide. *Biochemistry* 2000; **39**: 15757–15764.
30. Batista CV, Scaloni A, Rigden DJ, Silva LR, Rodrigues Romero A, Dukor R, Sebben A, Talamo F, Bloch C. A novel heterodimeric antimicrobial peptide from the Tree-frog phyllomedusa distincta. *FEBS Lett.* 2001; **494**: 85–89.
31. Raimondo D, Andreotti G, Saint N, Amodeo P, Renzone G, Sanseverino M, Zocchi I, Molle G, Motta A, Scaloni A. A Folding-dependent mechanism of antimicrobial peptide resistance to degradation unveiled by solution structure of distinctin. *Proc. Natl. Acad. Sci. U.S.A.* 2005; **102**: 6309–6314.
32. Schägger H, von Jagow G. Tricine-sodium dodecyl Sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 1987; **166**: 368–379.
33. Vogt TCB, Bechinger B. The interactions of Histidine-containing amphipathic helical peptide antibiotics with lipid bilayers. The effects of charges and pH. *J. Biol. Chem.* 1999; **274**: 29115–29121.
34. Sklenár V, Piotto M, Leppik R, Saudek V. Gradient-tailored water suppression for 1H-15N HSQC experiments optimized to retain full sensitivity. *J. Magn. Reson., Ser. A* 1993; **102**: 241–245.
35. Kichler A, Mason AJ, Bechinger B. Cationic amphipathic Histidine-rich peptides for gene delivery. *Biochim. Biophys. Acta* 2006; **1758**: 301–307.
36. Zhang Y, Taiming L, Liu J. Low temperature and glucose enhanced T7 RNA Polymerase-based plasmid stability for increasing expression of Glucagon-like peptide-2 in Escherichia coli. *Protein Exp. Purif.* 2003; **29**: 132–139.
37. Li Y, Li X, Wang G. Cloning, expression, isotope labeling, and purification of human antimicrobial peptide LL-37 in *Escherichia coli* for NMR studies. *Protein Exp. Purif* 2006; **47**: 498–505.

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