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# Different Strategies of Antimicrobial Peptides Production for Biomedical Applications

**Cristina Cantallops-Vilà[1]*, Laura Colomina-Alfaro[2]*, Pietro Riccio[3]*, Hanieh Ijakipour[3], Edwige Meurice[1], Antonella Bandiera[2], and Artemis Stamboulis[3]**

*[1]University Polytechnic Hauts-de-France, Campus Mont Houy - 59313 Valenciennes Cedex 9, FR, France; [2]University of Trieste, Department of Life Sciences, via L. Giorgieri, 1, 34127 Trieste, IT; [3]University of Birmingham, School of Metallurgy and Materials, Biomaterials Research Group, Edgbaston, Birmingham B15 2TT, UK*

## Introduction

The increase of antibiotic-resistant bacterial infections over the last decades has prompted the search for novel antimicrobial molecules. Antimicrobial peptides (AMPs), such as human defensins and cathelicidins, have become outstanding candidates to fight infection due to their properties and the low risk of developing resistance [1,2]. They can be modified by *in-silico* models to improve their biological activity and selectivity [3]. Many studies have addressed their potential but there are still limitations in the production and their effective delivery to infection sites. The synthesis of peptides can be replicated in the laboratory with techniques such as the Solid Phase Peptide Synthesis (SPPS) which allows excellent results in terms of yield, purity, and structure in a short time and with minimal use of solvents. This technique consists of the synthesis of peptides on a solid support, running from the *C*-terminal to the *N*-terminal contrary to how it occurs in nature.

On the other hand, recombinant technology offers the possibility to produce peptides on a large scale avoiding labor-intensive isolation from natural sources and costly chemical synthesis procedures [4]. This method also allows the fusion of peptides to biopolymers, which can serve as a delivery platform to the site of infection and improve their biocompatibility.

Collagen is the main structural protein of the extracellular matrix. Recent studies have found that some bacterial collagen-like proteins (Bcl) form a stable triple helix similar to mammalian collagen, making it an attractive biopolymer to increase biocompatibility and promote biointegration when used for biomedical applications [5].

Human Elastin-like Polypeptides (HELP) is a biopolymer inspired by elastin characterized by its thermo-responsive properties employed for its purification [6,7,8]. It can be tagged to peptides offering a simple purification strategy with reduced costs and high production yields [9,10].

Here, we aimed to compare different strategies to produce and purify AMPs. A peptide based on human β-defensin (P3) was produced using a chemical synthesis platform. Moreover, the human β-defensin-1 (hBD1) was produced as a fusion partner of HELP, and a cathelicidin-derived peptide, LL25, was produced as a fusion of Bcl, both produced recombinantly in the cytoplasm of *E coli*.

## Results and Discussion

AMPs were produced by chemical synthesis followed by RP-HPLC purification, and by recombinant technology fusing the AMPs to either Bcl or HELP followed by purification using IMAC or phase transition properties, respectively.

### *Chemical synthesis of P3*

A human β-defensin modified peptide (P3) was synthesised using SPPS (Rink amide resin) with Fmoc protected amino acids. The synthesis was performed using 20% piperidine in DMF as deprotection, then coupling was performed by adding the next amino acid in a basic environment. Capping was performed using acetic anhydride and DIEA (N,N-diisopropylethylamine) to avoid unwanted reactions. Finally, the cleavage from the resin was performed using 95% TFA (trifluoroacetic acid) as acid and 2.5% TIPS (triisopropylsilane) as scavenger. The mass and retention time analyses were performed using LC-MS system and the purification was done using an RP-HPLC system. Purity control, molecular mass determination, and molecular mass fingerprints were performed by MALDI-TOF mass spectrometry (Figure 1A and 1B).

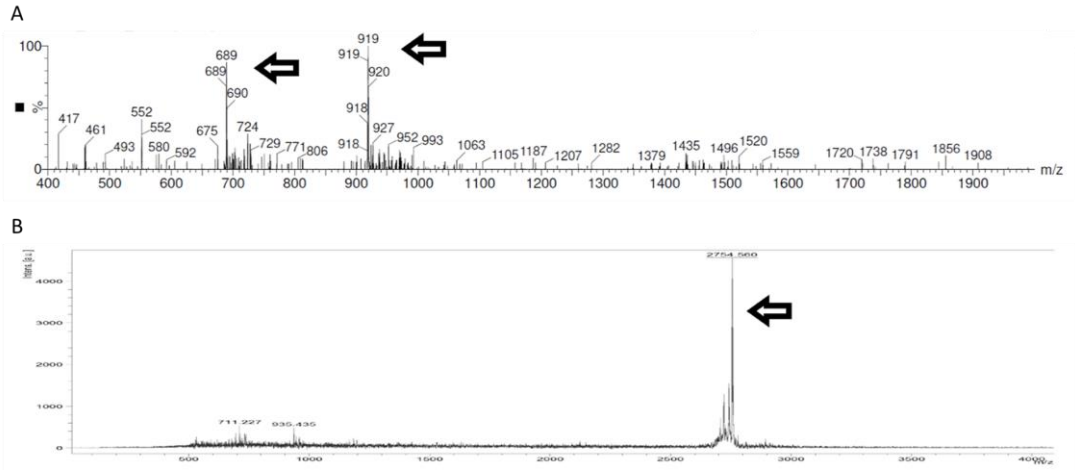


*Fig 1.* ***(A)*** *Mass analysis of the purified P3 peptide obtained by LC-MS, that eluted at 28% ACN due to the high temperature of the UPLC coupled with MS; and* ***(B)*** *spectrogram of the molecule with its molecular mass obtained by MALDI-TOF.*

### *Recombinant production of LL25 fused to Bcl*

The nucleotide sequence of the cathelicidin-derived LL25 was cloned into a pET100 plasmid fused in-frame to a Bcl protein. *E. coli* BL21(DE3) was transformed with the sequence-verified plasmid and used to express the recombinant fusion protein by induction with IPTG. Cultures were lysed to release the intracellularly produced protein, which was then recovered through IMAC purification. SDS-PAGE and Western Blot were performed to check the purity and concentration of the final product. The Western Blot analysis shown in Figure 2A revealed that, as expected, the induced culture produces significantly more recombinant Bcl protein (at around 18kDa) than the non-induced. As seen in Figure 2B in the purification by IMAC of the cell lysate, the eluted fusion protein Bcl-LL25 appears at around 20kDa in size. The presence of extra bands in the non-induced fraction of the Western Blot and in the eluted fraction of the SDS-PAGE can suggest the formation of aggregates or polymers of the recombinant protein. On the other hand, they could indicate poor efficiency of the method used due to nonspecific binding of histidine-rich proteins native to *E.coli* by the anti-His antibody or to the Nickel-charged columns, respectively [11]. This will be solved in future experiments with further purification steps such as size exclusion and ion exchange chromatographies.

### *Recombinant production of hBD1 fused to HELP*

The coding sequence of the 47 aa domain corresponding to the 22-68 region of the hBD1 (NM_005218.4) was fused in-frame to the *C*-terminus of the HELP synthetic gene. Moreover, specific endoproteinase restriction sites were introduced to precisely release the hBD1 from the fusion protein. The final construct, named HhBD1, was verified by sequencing, and the recombinant product was successfully expressed in the *E. coli* C3037 strain induced by IPTG (Figure 2C, expression lanes). The HhBD1 biopolymer retained the thermo-responsive properties of HELP, therefore, it was purified by exploiting the phase transition properties of the elastin-like domain; the final product was obtained with high purity and the yield of the HhBD1 production was 0.152 g $L^{-1}$ of bacterial culture (Figure 2C, purification lanes).

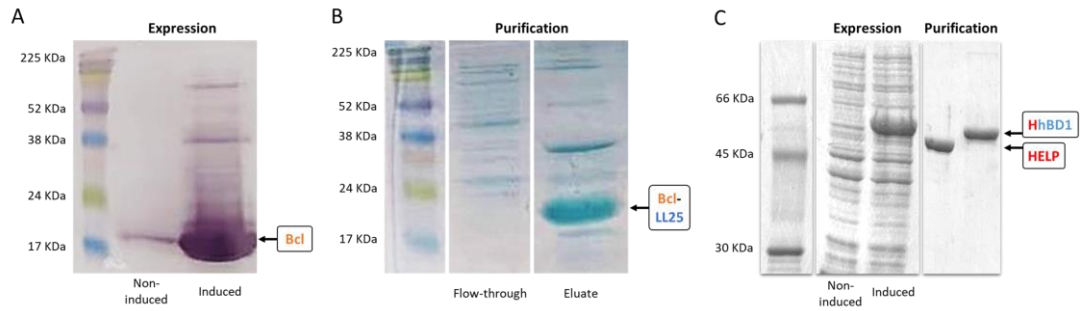


*Fig. 2.* ***(A)*** *Western Blot membrane after detection with an anti-His-tag antibody of the Bcl-producing lysed culture;* ***(B)*** *15% SDS-PAGE gel of the lysate of the Bcl-LL25-producing culture after purification through IMAC;* ***(C)*** *9% SDS-PAGE for the visualisation of the expression of the HhBD1 in E.coli C3037 induced by IPTG and the pure HhBD1obtained by phase transition cycling (purification).*

## *Antimicrobial potency of the free AMP and fusion biopolymers*

A broth inhibition assay was performed to evaluate the antimicrobial activity of peptides and biopolymers. This assay allows the determination of the minimum inhibitory concentration (MIC) of each peptide versus the pathogen tested. The pathogens used as targets were chosen among gram-positive and negative bacteria strains such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *E. coli* [12].

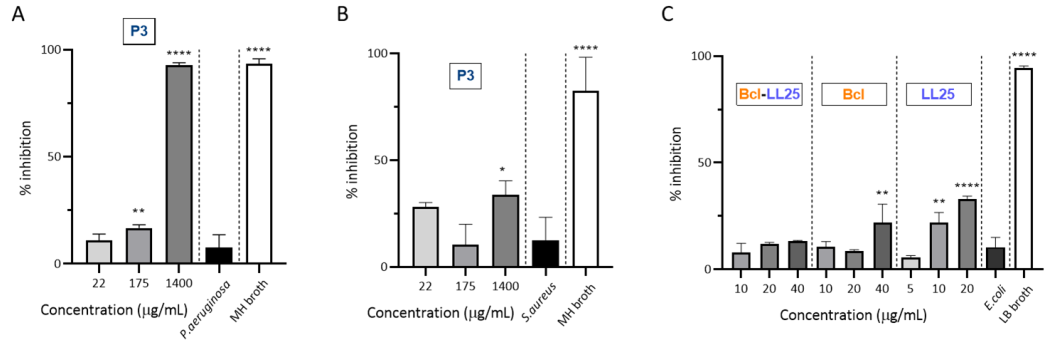


*Fig. 3.* ***(A)*** *Broth dilution assays of the peptide P3 against Pseudomonas aeruginosa PAO1, and* ***(B)*** *Staphylococcus aureus SH1000,* ***(C)*** *as well as the fusion protein Bcl-LL25, Bcl and LL25 alone, against E. coli JM8.*

The antimicrobial activity of the peptide P3 is confirmed only at high concentrations and predominantly against Gram-negative bacterial species such as *Pseudomonas aeruginosa* (Figure 3A and 3B), probably due to differences in membrane composition. Indeed, the thick peptidoglycan layer of Gram-positive bacteria does not allow the peptide to interact efficiently with the cell membrane.

The antimicrobial activity of LL25 is confirmed and concentration-dependent. Bcl-LL25 does not show significant antimicrobial potential, but an increase in growth inhibition can be observed with the concentration (Figure 3C). All in all, higher concentrations and purity of the fusion protein are expected to improve its antimicrobial activity.

MIC assay performed using HhBD1 didn't evidence any antimicrobial potency, even after the digestion with the two specific endoproteinases (data not shown), although the 36 amino acids peptide produced by incubation with Asp-N, is reported to be active towards *S. aureus*, *E. coli*, and *P. aeruginosa* [13]. Many reasons withstand behind these observations, mainly the uncontrolled

oxidation state of the biopolymer and the presence of salts. Thus, the characterization of the antimicrobial properties derived from HhBD1 is still under study.

Here, we present strategies that could successfully lead to the development of highly promising biomaterials that can be incorporated into implants reducing their failure due to bacterial infections and with a low risk of developing bacterial resistance to the material. Their biocompatibility and antimicrobial activity will be further studied to evaluate their suitability for use in biomedical applications.

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