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A chromatography-focused bioprocess that eliminates soluble aggregation for bioactive production of a new antimicrobial peptide candidate

Xiang Li, Susanna Su Jan Leong*

School of Chemical and Biomedical Engineering, Nanyang Technological University, 62 Nanyang Drive, Singapore 637459, Singapore

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ABSTRACT

Human beta defensins (hBDs) are an important class of antimicrobial peptides (AMPs), which provide the host with innate protection from bacteria, fungi and viruses. Human β-defensin-25 (hBD25) is a new hBD variant which has been recently discovered in the male genital tract. Since its discovery, hBD25 was hypothesized to play a key role in protection against genital tract infection, which has significantly increased mortality rates in the last decade. However, further studies to confirm the role of hBD25 are hindered by the lack of sufficient amounts of pure hBD25 for clinical studies. This study reports the first successful development of an efficient and low cost chromatography-oriented bioprocess for production of hBD25. hBD25 was expressed predominantly as soluble aggregates although the peptide was co-expressed with a Maltose Binding Protein (MBP) fusion tag in *E. coli*. The soluble aggregates were disrupted by denaturation–reduction of the hBD25, followed by an in vitro size exclusion chromatography refolding step which readily yielded bioactive and purified hBD25 peptides at 90% purity. The refolded hBD25 showed antimicrobial activity against *E. coli* K12 at a minimal inhibitory concentration of 60 μg/mL. With an overall hBD25 bioprocess yield of 48% obtained, this bioprocess will open the way for detailed clinical studies of hBD25, and serve as a generic platform for efficient recovery of other 'fusion protein'-derived peptides that inevitably exist as soluble aggregates.

1. Introduction

Antimicrobial peptides (AMPs) have attracted great attention from researchers and clinicians alike in recent years due to their high antimicrobial performance, low susceptibility to incur antimicrobial resistance and good biocompatibility properties [1–4]. AMPs which are usually 12–50 amino acids long, are cationic and amphiphilic in nature [5,6]. AMPs are routinely produced by many organisms as elements of first wall of defense against microbial infection [7]. Despite being ancient host defense molecules with an essential role in the innate immune system [8], proper understanding of their mechanisms of action is still largely hindered by the lack of substantial amounts of purified peptides for detailed characterization and clinical studies.

An appealing member of AMPs is defensins, which are found in higher order organisms, including plants, animals and humans [9]. In humans, defensins are epithelial-derived, cationic and cysteine-rich. Recently, interest in human defensins has increased because of their expanding roles in innate and adaptive immunity, and their recently discovered anti-viral properties [10,11]. Up to now, more than twenty variants of human β-defensins have been isolated and their coding nucleotide sequences identified by genome analysis [12]. Human β-defensin-25 (hBD25) is one of the recently discovered hBD variants which has 47 amino acid residues and was first found in the male genital tract [13]. The possible role of hBD25 in protection against genital tract infection, a commonly occurring infection, however, remains to be elucidated and established. The extremely low peptide concentration present in natural sources makes peptide extraction a difficult and costly task. To accurately characterise the antimicrobial role of hBD25 and extend its use as an antimicrobial coating material in urinary catheters, for example, an efficient bioprocess to rapidly and cost-effectively produce purified and bioactive hBD25 is needed.

Compared with traditional chemical synthesis methods [14], bio-production of peptides in microbial cell factories is potentially cheaper, necessitates a simpler process and is more environmental friendly [15]. Among all the expression systems, *E. coli* is often favoured as a host cell system because it has a well-characterised genome and grows rapidly in cheap medium [16]. Due to toxicity towards the expression host, AMPs must be expressed as fusion proteins in the *E. coli* host. In addition to eliminating host cell toxicity, fusion tags can improve soluble protein expression yield and simplify protein purification [17–21]. The general hypothesis is that fusion tags comprising highly soluble proteins such as maltose binding protein (MBP), glutathione-S-transferase (GST) and thioredoxin A (TrxA) function as chaperones to improve cor-

* Corresponding author. Tel.: +65 6316 8775; fax: +65 6794 7553.
E-mail address: Sleong@ntu.edu.sg (S.S.J. Leong).

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rect foldability and enhance solubility of the expressed fusion protein [22,23]. Cleavage of the fusion protein to recover the target peptide can, however, significantly reduce yield and necessitates post-cleavage purification, if not adequately optimized. Although chemical reagents such as cyanogen bromide, hydroxylamine or 1-cyano-4-dimethylaminopyridinium tetrafluoroborate are commonly employed to cleave fusion proteins [22,23], chemical cleavage suffers from the disadvantage of non-specificity and can often result in chemical modification of the target proteins [24]. In recent years, the use of more site-specific proteases like enterokinase, factor Xa thrombin, and tobacco etch virus protease (TEVp) are preferred to enhance the selectivity of peptide cleavage [25]. TEVp is a cysteine protease which has an exceptionally high degree of cleavage specificity and high activity rate [26], shows resistance against many protease inhibitors, and is not easily degraded [27]. Based on these highly desirable properties, TEVp was chosen as a fusion protein cleavage protease for selective recovery of hBD25 in this work.

In this study, the use of a MBP–hBD25 fusion protein construct resulted in the expression of hBD25 fusion proteins predominantly as soluble aggregates in *E. coli*. The development of a 'chromatography refolding'-based bioprocess successfully addressed the problem of soluble aggregation and recovered highly purified hBD25 peptides with good bacteria killing activity. This reported bioprocess is expected to serve as a generic platform for the biomanufacture of 'soluble aggregate'-originating products.

2. Experimental

Kanamycin sulfate, Chloroamphenicol, isopropylthio-D-galactoside (IPTG), urea, L-arginine, tris(hydroxymethyl) aminomethane (Tris), ethylenediamine-tetraacetic acid (EDTA), sodium chloride (NaCl), dithiothreitol (DTT) and reduced (GSH) and oxidized glutathione (GSSG), hydrochloric acid (HCl) and maltose were purchased from Sigma–Aldrich. Tryptone and yeast extract were purchased from Becton, Dickson and Co. (USA), and USB Corporation (USA), respectively. All chromatography purification and refolding work were conducted using an AKTA Explorer 100 Fast Protein Liquid Chromatography (FPLC) system (GE Healthcare, Singapore) at room temperature (24 °C).

2.1. Plasmids

Three pET-48b(+) plasmids harbouring the gene sequences of (i) GST-6His-TEVp-hBD25, (ii) MBP-6His-TEVp-hBD25 and (iii) TrxA- 6His-TEVp-hBD25 (N- to C-terminus) were designed, where TEVp represents the TEVp cleavage site. The plasmid pRK793 containing TEVp was used for TEVp production. The amino acid sequence of TEVp is as follows: GHHHHHHHGES LFKGPRDYNPISSTICHLTNESDGHTTSLYGIGFGPFIITNKHLFRRNNG-TLLVQSLHGVFKVKNTTTLQQHLIDGRDMIIIRMPKDFPPFPQKLKF-REPQREERICLVTTNFQTKSMSSMVSDTSCTFPSSDGIFWKHWIQTKD-GQCGSPLVSTRDGFIVGIHSASNFTNTNNYFTSVPKNFMELLTNQEAQ-QWVSGWRLNADSVLWGGHKVFMVKPEEPFQPVKEATQLMNRRRRR. These plasmid constructs were synthesized by the Protein Expression Facility, University of Queensland.

2.2. hBD25 fusion protein expression in E. coli

Following heat shock transformation of the pET-48b(+) vector into competent *E. coli* BL21-CodonPlus (DE3)-RIL cells, the transformed cells were grown overnight in LB medium (10 g/L Bacto-tryptone, 5 g/L yeast extract, 10 g/L NaCl) containing 30 μg/mL kanamycin sulfate and 34 μg/mL chloroamphenicol at 37 °C under shaking conditions. 1% (v/v) of the overnight culture was inoculated into 50 mL LB medium and hBD25 fusion protein expression was induced with IPTG at OD_{600} = 0.7–0.8. After 4 h post-induction, the cells were harvested by centrifugation at 10,000 × *g* for 30 min. The cell pellets were washed with PBS, and centrifuged at 10,000 × *g* for 30 min.

2.3. Cell lysis for hBD25 recovery

The washed cell pellets were resuspended in 10 mL 50 mM Tris–HCl (pH 8.0) and disrupted by sonication using a digital sonifier (Branson Sonifier Cell Disrupter, Connecticut, USA). Sonication was performed for 30 cycles at 5 s/cycle, followed by 10 s cooling after each sonication cycle. The lysed cells were centrifuged at 10,000 × *g* for 30 min, and soluble protein expression was determined by SDS–PAGE analysis.

2.4. hBD25 fusion protein purification

The MBP–hBD25 fusion protein was purified from the cell lysate by amylose affinity chromatography on FPLC. The supernatant of the cell lysate (from Section 2.3) was filtered with a syringe filter (0.45 μm Acrodisc, Pall Corporation) and loaded into a 5 mL MBP Trap column (GE Healthcare, Singapore), which was equilibrated with buffer A (50 mM Tris–HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4) at a flow rate of 2.5 mL/min. The column was washed with 5 CV buffer A to remove unbound proteins, and two step elutions at 0.4% and 40% buffer B (50 mM Tris–HCl, 200 mM NaCl, 1 mM EDTA, 10 mM maltose, pH 7.4) were performed to recover purified MBP–hBD25. MBP–hBD25 concentration and purity were determined by Bradford assay and SDS–PAGE analysis.

2.5. hBD25 recovery by fusion protein cleavage using TEVp

E. coli BL21-CodonPlus (DE3)-RIL transformed with the pRK-793 plasmid encoding the sequence of an N-terminal poly-his-tagged and C-terminal poly-Arg-tagged S219 v TEVp was used to express the TEVp [26]. Recombinant TEVp was prepared as described in our previous study [28].

Cleavage of the MBP–hBD25 fusion protein to release the hBD25 peptide was performed at a TEVp to hBD25 mol ratio of 1:10 in an optimised cleavage buffer (50 mM Tris–HCl, pH 8.0). The post-cleavage mixture was incubated for 12 h at room temperature. MBP–hBD25 cleavage efficiency was determined by SDS–PAGE analysis.

2.6. Fusion protein size characterization using size exclusion chromatography

Size exclusion chromatography (SEC) was performed on the FPLC at a constant mobile phase flow rate of 0.5 mL/min for size characterization of the (i) 'MBP affinity chromatography'-purified fusion proteins, and (ii) cleaved hBD25 peptides. 0.5 mL fractions from (i) and (ii) were introduced into the SEC column (Superdex 200 10/300 GL, GE Healthcare, Singapore) which was equilibrated with 2 CV buffer C (50 mM Tris–HCl, 150 mM NaCl, pH 8.0). After sample loading, the protein was eluted in buffer C over 1.5 CV.

2.7. Refolding and purification of hBD25 by SEC

The post-cleavage mixture was denatured–reduced in 8 M urea and 10 mM DTT for 2 h, and then introduced into a SEC column (Superdex 200 10/300 GL, GE Healthcare, Singapore) using a 0.5 mL sample loop for refolding. The SEC column was equilibrated with 2 CV refolding buffer (2 M urea, 0.5 M arginine, 150 mM NaCl, pH 8.0, 1 mM to 10 mM GSH and 1 mM GSSG) at a flow rate of 0.5 mL/min. The hBD25 peptide was eluted in refolding buffer over 1.5 CV.

Table 1
Soluble expression yields of MBP–hBD25 and TrxA–hBD25 fusion proteins under optimised culturing conditions.

Protein	Condition			
	Medium	Temperature (°C)	Post induction time (h)	Fusion protein concentration (mg/L)
TrxA–hBD25	LB	26	4	100
MBP–hBD25	2×YT	37	2	120

2.8. Antibacterial activity assay of hBD25

Lyophilized refolded hBD25 peptides were solubilized in distilled H_2O to a final concentration of 150 μg/mL and filtered through a 0.22 μm filter. *E. coli* K12 was used as the model strain for the antimicrobial activity assay and bacteria inhibition test in liquid culture to determine the minimum inhibitory concentration of hBD25 to fully inhibit microbial growth in liquid culture (i.e. MIC_{100}). *E. coli* K12 was first grown overnight in Mueller Hinton Broth (MHB) (Fluka, Spain). 1% (v/v) of the *E. coli* cells were inoculated into fresh MHB medium, grown at 37 °C and 200 rpm until mid-log phase (i.e. OD_{600} = 0.8–1.0). The antimicrobial assays were conducted in quarter-strength MHB medium in a 96-well microplate. 25 μL MHB was added to each well to sustain cell growth. Stock hBD25 peptides in distilled H_2O were then added to each well at different volumes to obtain final peptide concentrations ranging from 0 to100 μg/mL. 10 μL of diluted bacteria culture (10^5 CFU/mL) was added into each well to achieve a final cell concentration of 10^4 CFU/mL. Distilled H_2O was then added to each well to make up a total sample volume of 100 μL for each well. The blank control comprised only the culture medium, while the negative control comprised bacteria culture without hBD25 peptides.

2.9. Analyticals

SDS–PAGE analysis was performed using precast 4–12% gradient and 12% polyacrylamide gels (NuPAGE® Novex Bis-Tris gels, Invitrogen). To determine protein concentration using Bradford assay, 20 μL of the peptide sample was added to 1 mL of the Bradford reagent (B6916, Sigma) and mixed for 5 min, followed by sample absorbance measurement at 595 nm. Protein concentration was estimated using an absorbance versus mass calibration curve based on bovine serum albumin standards.

3. Results and discussion

3.1. hBD25 fusion protein expression

In this study, three different constructs harbouring the hBD25 nucleotide fused to the nucleotide sequences of three different fusion tags (i.e. GST, MBP and TrxA) were synthesized and studied for soluble hBD25 fusion protein expression. The choice of fusion tags was directed at eliminating host cell toxicity and enhancing soluble expression of hBD25 [22,23]. The soluble expression yields from the three hBD25 fusion protein constructs were compared following optimization of culture conditions such as temperature, media composition and induction conditions (Fig. 1 and Table 1). It is clear that the TrxA–hBD25 and MBP–hBD25 constructs expressed hBD25 fusion proteins in the soluble form. The presence of the MBP tag in the MBP fusion protein construct and the poly-histidine (6His) tag in the TrxA fusion protein construct facilitated hBD25 purification by affinity chromatography using an amylose affinity chromatography column (i.e. MBP Trap) and immobilized metal affinity chromatography (IMAC) column (i.e. His Trap), respectively. Although both MBP–hBD25 and TrxA–hBD25 fusion proteins

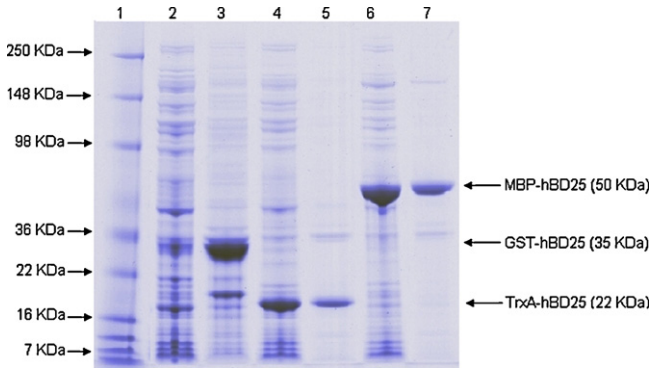

Fig. 1. SDS–PAGE analysis showing the expression profiles of different hBD25 fusion protein constructs under optimized culture conditions; lane 1: protein molecular weight marker, lanes 2 and 3: soluble and insoluble fractions of cell lysate after GST–hBD25 expression, respectively, lanes 4 and 5: soluble and insoluble fractions of cell lysate after TrxA–hBD25 expression, respectively, lanes 6 and 7: soluble and insoluble fractions of cell lysate after MBP–hBD25 expression, respectively.

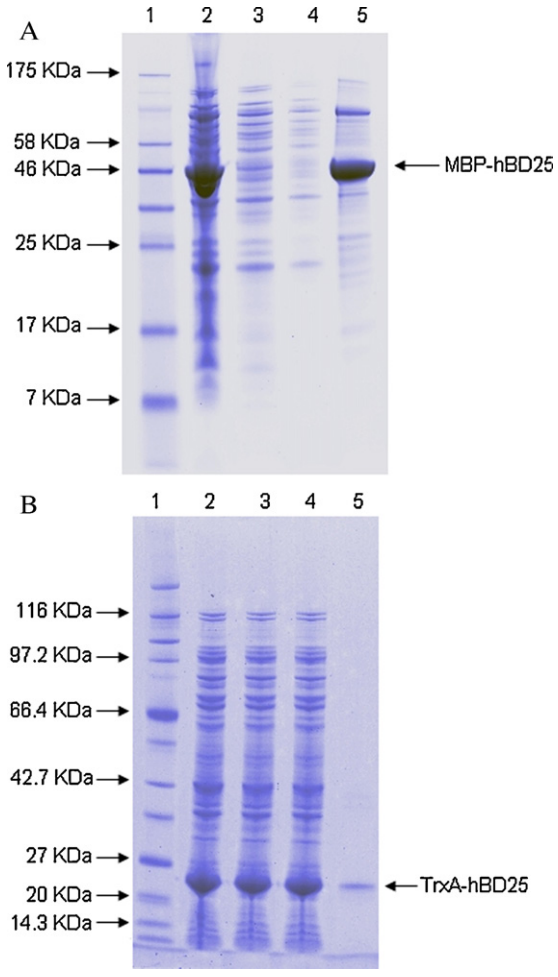

Fig. 2. SDS–PAGE analysis of (A) MBP–hBD25 and (B) TrxA–hBD25 purification by amylose and IMAC chromatography, respectively. (A) Lane 1: protein molecular weight marker, lane 2: load (supernatant of cell lysate), lanes 3 and 4: flow through, lanes 5: eluted fractions; (B) Lane 1: marker, lane 2: load (supernatant of cell lysate), lanes 3 and 4: flow through, lanes 5: eluted fractions.

were recovered at >70% purity (Fig. 2A and B), there was a huge discrepancy in recovery yields, where 87% of the MBP–hBD25 fusion protein was recovered following amylose affinity purification while only 8% of the TrxA–hBD25 fusion protein could be recovered following IMAC purification, with most of the fusion protein lost to the

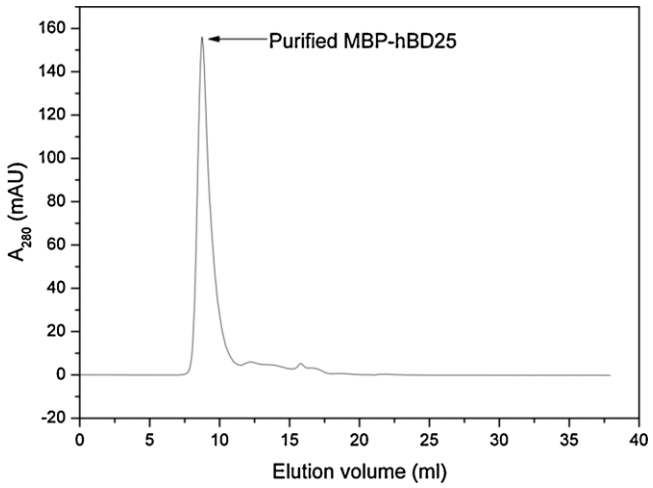

Fig. 3. Size exclusion chromatogram of 'affinity chromatography'-purified MBP–hBD25.

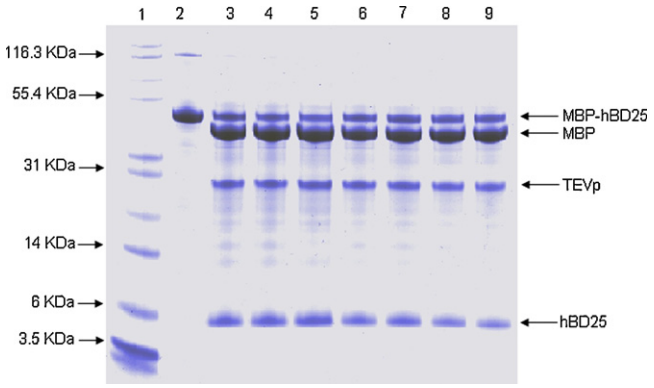

Fig. 4. SDS–PAGE analysis of TEV protease cleavage efficiency under different cleavage buffer composition at pH 8; lane 1: protein marker, lane 2: purified MBP–hBD25 fusion protein before cleavage, lane 3: post-cleavage mixture in 50 mM Tris–HCl, lane 4: post-cleavage mixture in 50 mM Tris–HCl, 5 mM DTT, lane 5: post-cleavage mixture in 50 mM Tris–HCl, 2 M Urea, lane 6: post-cleavage mixture in 50 mM Tris–HCl, 0.5 mM EDTA, lane 7: post-cleavage mixture in 50 mM Tris–HCl, 10 mM NaCl, lane 8: post-cleavage mixture in 50 mM Tris–HCl, 30 mM NaCl, lane 9: post-cleavage mixture in 50 mM Tris–HCl, 150 mM NaCl.

flow through fractions. This large difference in hBD25 fusion protein recovery yield was hypothesized to be associated with steric constraints that limit the accessibility of the 6His residues to the Ni^{2+} chromatography resin in the case of TrxA–hBD25 purification. MBP–hBD25 was subsequently employed for hBD25 bioprocess development studies for improved process productivity.

3.2. Characterisation of MBP–hBD25 fusion protein by SEC

It is important to acknowledge that soluble expression of proteins does not guarantee bioactivity of the expressed protein, as evident in several proteins which were expressed as soluble aggregates [29,30]. For example, fusion protein MBP-E6 was found to be predominantly misfolded and aggregated despite being expressed in the soluble form, where the target protein, E6, precipitated following fusion tag cleavage as a result of incorrect folding of E6 in vivo [31]. To determine monomericity of MBP–hBD25, the 'amylose affinity chromatography'-purified MBP–hBD25 fusion protein was subjected to SEC analysis. Fig. 3 shows that 'amylose affinity'-purified MBP–hBD25 eluted from SEC at an elution volume which corresponded to a molecular weight of ≥600 kDa (from calibration against protein standards of known molecular mass), which indicated the existence of MBP–hBD25 fusion proteins as soluble aggregates. IMAC-purified TrxA–hBD25 was also found to be predominantly expressed as soluble aggregates after SEC analysis (data not shown). It is therefore entirely possible that aggregate formation of the TrxA fusion proteins increases steric shielding of the 6His residues (located in between the N- and C-terminus), thus contributing to the low IMAC protein purification yield as discussed earlier. Since the MBP tag is located in the N-terminus, steric effect may be significantly minimized in the case of MBP–hBD25 purification by amylose affinity chromatography and hence the higher purification yield observed. Dynamic binding curves will be required to assess the possibility of this occurrence.

3.3. MBP–hBD25 fusion protein cleavage and characterization

MBP–hBD25 soluble aggregate formation did not hinder the ability of TEVp to cleave the fusion proteins, where 80% cleavage efficiency was readily achieved, as determined by SDS–PAGE analysis and Bradford assay. This outcome suggests that the formation of fusion protein soluble aggregates will not impact the final peptide yield. So far, only one other study has reported the same observation [32], which suggests that soluble aggregate formation does not necessarily result in loss of product. However, we acknowledge that this phenomenon may be difficult to predict because the mode of interaction of the aggregating components and hence the extent of steric hindrance associated with aggregate formation is likely to influence the accessibility of the cleavage protease to the aggregates. Post-cleavage SEC analysis of the protein fractions, however, revealed that the cleaved hBD25 peptides continued to exist as aggregates, which is undesirable. To identify the cause of the observed non-specific peptide interaction which leads to soluble aggregation, different additives (i.e. 5 mM DTT, 2 M urea, 0.5 mM EDTA and 150 mM NaCl) were added into the cleavage buffer to study the effects of these additives on cleavage yield. The reducing agent, DTT, was added to reduce intra- and inter-molecular disulfide bonds, thus eliminating covalent-induced aggregation. 2 M urea was aimed at reducing intra- and inter-molecular hydrophobic interaction between the peptides, while the addition of NaCl was hypothesized to eliminate non-specific electrostatic interaction. EDTA was added to reduce His-mediated assembly of the protein fragments caused by adventitious metal ion chelation. SDS–PAGE and densitometry analyses showed that none of these additives affected TEVp cleavage efficiency, which remained approximately constant at 80% (Fig. 4). The post-cleavage mixtures treated with the different additives were each analysed by SEC. From the SEC chromatograms obtained, none of the additives appeared to reduce hBD25 aggregation, where all the cleaved hBD25 peptides were still eluted from the SEC column as large molecular mass aggregates. Fig. 5A compares the SEC elution profile of the post-cleavage mixture with and without 150 mM NaCl, while Fig. 5B shows the protein composition of the eluted fractions. The SEC elution chromatograms of the post-cleavage mixture incubated with other additives showed a comparable profile to that obtained for 150 mM NaCl. Following the ineffectiveness of non-denaturing additives to overcome hBD25 aggregation, high concentrations of urea and DTT were subsequently employed to disrupt the aggregates, which necessitated an in vitro refolding step to recover hBD25 in a bioactive form.

3.4. Purification and refolding of hBD25 by SEC

After denaturation–reduction of the post-cleavage mixture in 8 M urea and 10 mM DTT, followed by 12 h of incubation, the cleavage mixture was subjected to refolding in a SEC column equilibrated in refolding buffer. The use of a SEC refolding platform is advantageous due to its simultaneous purification and refolding capability

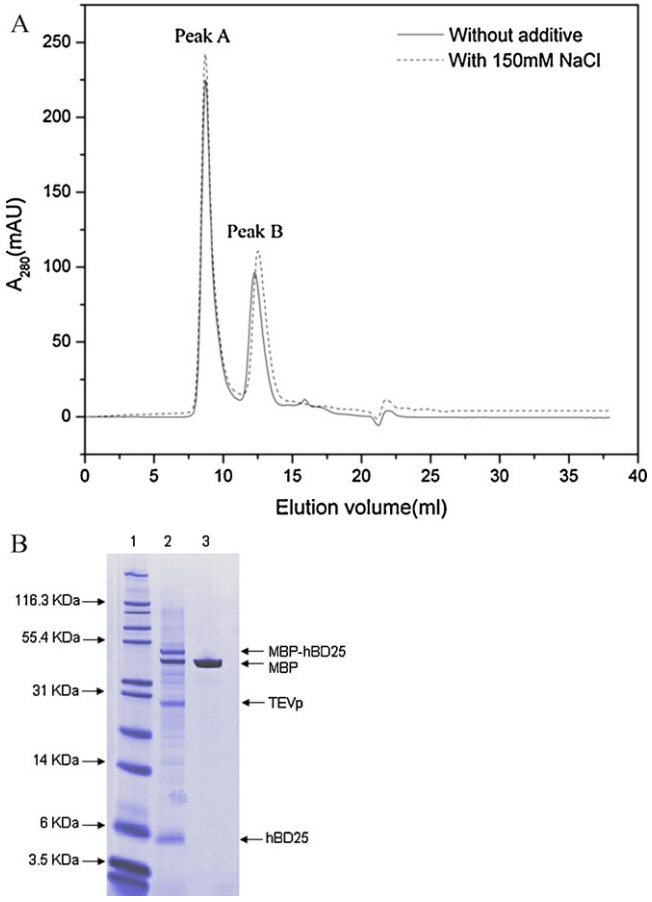

Fig. 5. (A) SEC chromatogram of post-cleavage mixture in 50 mM Tris–HCl containing no additive and 150 mM NaCl; (B) SDS–PAGE analysis of SEC-eluted fractions in (A); lane 1: protein marker, lane 2: fraction from peak 1, lane 3: fraction from peak 2.

Table 2
hBD25 step and overall yields and purity.

Process step	Yield (%)	Purity (%)
Cell lysis[a]	100	23
Amylose affinity chromatography purification[a]	87	45
TEVp cleavage[b]	80	15
SEC refolding[b]	95	90
Overall	48	90

[a] hBD25 fusion protein.
[b] hBD25 peptide.

to simplify downstream bioprocessing [33,34]. L-Arginine was added to the refolding buffer to suppress aggregate formation in the refolding process. The SEC elution profile obtained following SEC refolding is shown in Fig. 6A, where hBD25 was eluted in a monomeric form and recovered at 90% purity. SDS–PAGE analysis of the protein fractions from different purification steps in the bioprocess is shown in Fig. 6B and the step and overall bioprocess yields are given in Table 2.

These results also suggest that expressing hBD25 as inclusion bodies could be a viable alternative production route for hBD25. Inclusion body production of hBD25 would be advantageous in eliminating the need for co-expression of hBD25 with a fusion tag, where the insoluble expression will protect the host cell against any antimicrobial action of hBD25. If inclusion body yields are comparable with that of fusion protein expression, the overall process yield achieved for the peptide could be higher in the absence of an enzymatic cleavage step.

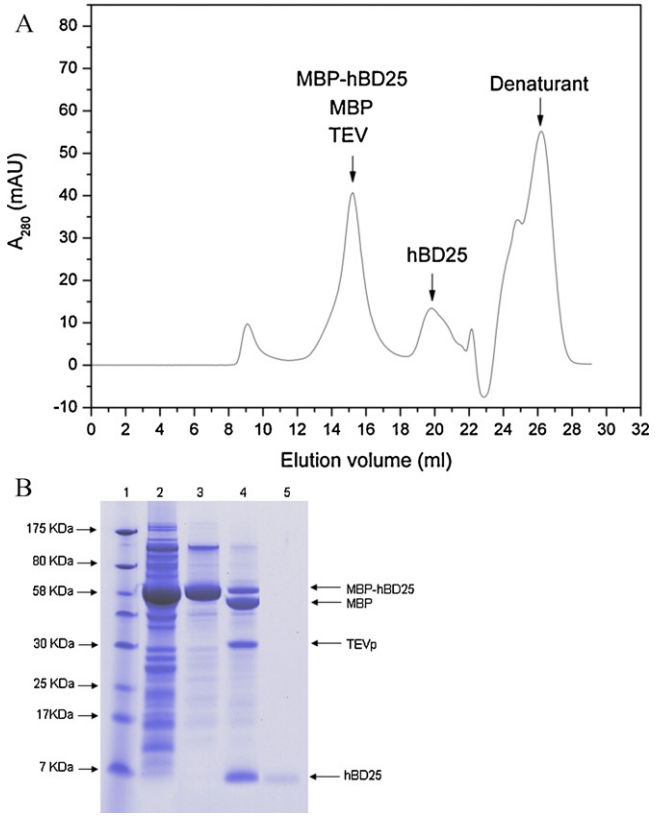

Fig. 6. SEC chromatogram showing the elution of hBD25 following SEC refolding; (B) SDS–PAGE analysis of essential bioprocess steps, lane 1: protein molecular weight marker, lane 2: cell lysate, lane 3: fusion protein after amylose affinity purification, lane 4: post-cleavage mixture, lane 5: 'SEC'-purified and refolded hBD25.

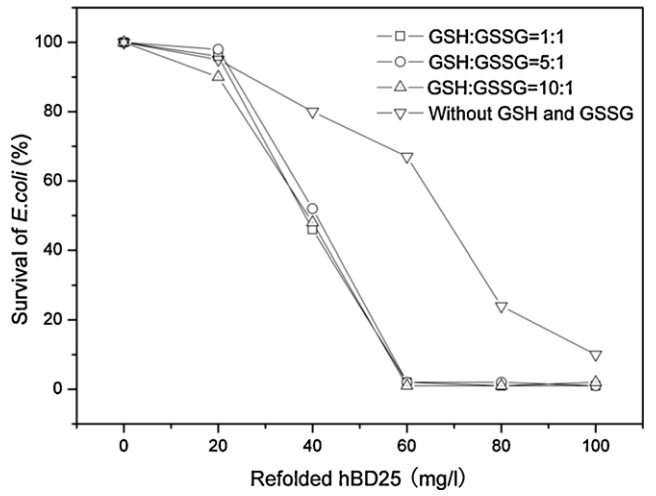

Fig. 7. Bacteria inhibition tests of refolded hBD25 in liquid culture. Survival percent is defined as the ratio of cell density (measured by OD_{600}) in the presence of hBD25 peptide to that without the hBD25 peptide.

3.5. Antimicrobial activity determination of hBD25

The antimicrobial activity of refolded hBD25 was evaluated by bacteria inhibition test using *E. coli* K12 as a model strain. Fig. 7 shows a hBD25 concentration-dependent bacteria inhibition profile, where the growth of *E. coli* K12 was significantly suppressed with increasing concentrations of refolded hBD25. 100% growth inhibition was attained at hBD25 concentration of 60 μg/mL. hBDs are cysteine-rich and disulfide bonds have been

suggested to be important for tertiary structure stabilisation of β-defensins [35–37]. Therefore, optimising the refolding buffer with respect to redox environment is considered important to enhance oxido-shuffling cysteines and hence promote correct formation of disulfide bonds [38,39]. The effect of varying GSH:GSSG ratio in the refolding buffer on the antimicrobial activity of hBD25 was investigated. MIC values of refolded hBD25 was not affected by variation in GSH:GSSG ratios within the range studied, but the antimicrobial activity of hBD25 refolded in the absence of GSH and GSSG was significantly suppressed (Fig. 7). This result suggests that correct disulfide bond formation could play an important role in hBD25's antimicrobial killing capability. Disulfide-driven stabilization of the hBD25 peptide could be instrumental in rendering an optimum distribution of positive charges on the peptide surface, thus increasing the peptides' electrostatic interaction towards the oppositely charged bacteria membrane. Having developed a robust platform that now allows rapid manufacture of purified hBD25 in sufficient amounts, ongoing studies are currently underway to elucidate hBD25 structure–activity relationship in this aspect.

4. Conclusions

A chromatography-centered bioprocess for efficient and scalable production of hBD25 is reported for the first time. The existence of MBP–hBD25 as soluble aggregates was addressed by denaturation–reduction of the post-cleavage protein mixture followed by an SEC in vitro refolding step to simultaneously renature and purify the hBD25 peptides. Soluble aggregate formation, however, did not hinder the cleavage of fusion proteins and hBD25, where 80% cleavage efficiency was readily achieved. A single SEC step was effective in recovering bioactive HBD25 at 90% purity, which demonstrated a MIC_{100} of 60 μg/mL on the model microorganism, *E. coli* K12. The successful development of a SEC refolding-based bioprocess now allows detailed structural and antimicrobial characterization of hBD25, which will potentially open the way to new hBD25-based therapy or the development of novel antimicrobial coating materials. This study provides a generic chromatography-based platform that can be widely used for bioprocessing of peptides or small molecules which inevitably exist as soluble aggregates, which until now remains an important bioprocessing challenge.

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