Redesigned pMAL expression vector for easy and fast purification of active native antimicrobial peptides

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Abstract

Aims: The aim of this study was to construct the improved pMAL expression vector to increase the efficacy of purification of small native peptides and their clear-cut separation from MBP tag. The modifications we introduced can be applied to many expression vectors.

Methods and Results: To improve the pMAL expression vector, we introduced the His₆ tag and the enterokinase cleavage site (Ek) downstream from the MBP tag and Xa cleavage site on the original vector. For cloning of a desired peptide DNA, the enterokinase site contains a unique BsaBI restriction site adjacent to the original multi-cloning site. This redesigned pMAL vector was optimized for the purification of cytoplasmic (pMALc5HisEk) and periplasmic (pMALp5HisEk) peptides. The purification of native and active peptide (P) was obtained following two-step affinity chromatography. In the first step, the entire MBP-His₆-Ek-P fusion protein is purified using the Ni-NTA agarose column. This fusion protein was cleaved with active His₆ tagged enterokinase. In the second step, the further purification was performed by column containing the mixture of amylose and Ni-NTA agarose resins. This removes both the MBP-His₆ and His₆-enterokinase leaving pure native protein in solution. These new vectors and the two-step purification protocol were successfully applied in purification of active native small antimicrobial peptides (AMPs), lactococcin A and human β-defensin.

Conclusions: We constructed the improved pMAL expression vectors and established the pipeline and optimal conditions for their use in efficient purification of large amounts of active native small peptides.

Significance and Impact of the Study: Choice of expression vector impacts on the efficiency of expression and purification of desired proteins. The idea of redesigning pMAL vector was driven by the need for rapid purification of larger amounts of active native AMPs. This newly improved pMAL vector, the cloning strategy, expression conditions and two-step purification protocol represent a unique simple approach which can be applied in every laboratory.

KEYWORDS
complete removal of tags and proteinase, His₆-enterokinase cleavage site, improved pMAL and pQE vectors, native antimicrobial peptides, two-step chromatography
INTRODUCTION

Proteins play crucial and diverse roles in all organisms. They represent the building blocks of every cell, act as enzymes, hormones, cell attachment anchors, regulators and source of amino acids, play role in signalling and defence as antibodies or antimicrobial peptides (AMPs), and contribute in many other processes. The protein over-production and purification is a powerful tool in biotechnology and basic science research and a need for a variety of purified native proteins is elevated over the last decade (Geddes et al., 2019; Vecchio et al., 2018). However, the biotechnology industry is still facing the problem in insufficient production capacity due to increasing demand for biologically active proteins, in particular those used as therapeutics (Mergulhao et al., 2004). The favourite host for recombinant protein production is an Escherichia coli, not only for its ability to grow rapidly and achieve high density on cheap media, but also because of its well-characterized genetics and the available number of cloning vectors and mutant host strains (Structural et al., 2008; Fakruddin et al., 2012; Rosano & Ceccarelli, 2014). There are many E. coli-based protein expression and purification systems and majority of corresponding expression vectors are designed to introduce the amino acid tags to the protein of interest leading to production of the tagged fusion proteins (Ki & Pack, 2020; Rosano et al., 2019; Rosano & Ceccarelli, 2014). Many of those fusion proteins retain the activity close to wild type, even in the presence of the tag. However, a significant number of tagged proteins lose the activity and so require to be purified in its native form. This is particularly true for the smaller proteins such as peptides, or more specifically, the antimicrobial peptides (AMPs). Importantly, AMPs are at the forefront of the ever-increasing antimicrobial resistance problem (Cui et al., 2021; Sinha & Shukla, 2019; Wang et al., 2016).

In this work, we redesigned the pMAL vector which is part of the protein expression and purification system typically used to purify the insoluble proteins (Nguyen et al., 2017; Sun et al., 2011; Waugh, 2016). Here, the relatively large size maltose binding protein (MBP) of ~43 kDa is fused as a tag to the protein of interest and the native protein is purified following overexpression, purification through amylose resin column, and cleavage by Xa protease which cut the fusion protein and removes the MBP tag. This system further requires the use of the size exclusion columns/filters to separate the smaller proteins from MBP. However, if the native protein/peptide of interest is too small, the chemical characteristics of size exclusion columns/filters cause the non-specific binding of small peptide to the column. Therefore, the need to develop the vector and protocol for overexpression and purification of the small native and active peptides, specifically the AMPs, was our rational to redesign the pMAL vector and accordingly change the peptide purification system itself. The effectiveness of novel vector and purification system was tested and proved using well-characterized AMPs, lactococcin A (LcnA) and human β-defensin (hBD).

MATERIALS AND METHODS

Bacterial strains and growth conditions

Escherichia coli strains DH5α (Hanahan, 1983) and ER2523 (New England Biolabs, Ltd., UK) were grown in Luria Bertani (LB) medium at 37°C with aeration (180 rpm). Lactococcus lactis BGMN1-596 was grown in M17 medium (Merck GmbH, Darmstadt, Germany) supplemented with D-glucose (0.5% w/v) (GM17) at 30°C (Kojic et al., 2006). Solid medium and soft agar were made by adding 1.5% and 0.7% (w/v) agar (Torlak, Belgrade, Serbia) to the liquid media, respectively. Ampicillin (100 µg ml⁻¹) was used for selection and maintaining of transformants. Isopropyl-β-D-1-thiogalactopyranoside (IPTG; Serva, Heidelberg, Germany) in appropriate concentrations was used for the induction of protein expression.

DNA manipulations

For plasmid isolation from E. coli transformants, a Thermo Fisher Scientific GeneJET Plasmid Miniprep kit was used according to the manufacturer’s recommendations (Thermo Scientific, Lithuania). Digestion with restriction enzymes was conducted according to the supplier’s instructions (Thermo Fisher Scientific Waltham, MA, USA). DNA was ligated using T4 DNA ligase (Agilent technologies, USA) according to the manufacturer’s recommendations. Standard heat-shock transformation method was used for transformation of E. coli with a plasmid (Hanahan, 1983).

Site-directed mutagenesis

Desired mutations were introduced as described previously by Miljkovic et al. (2016) using oligonucleotide primers (and their reverse complements) carrying mutations (see Table 1). PCR amplification of each plasmid DNA strand for site-directed mutagenesis was performed separately by adding only one primer. Amplification was conducted by Phusion High Fidelity DNA Polymerase (Thermo Fisher Scientific) one min kb⁻¹. Single strand amplicons obtained by forward and complement primers
were annealed (see below) and digested with 1 μl (10 U) of \textit{DpnI} restriction enzyme per reaction (at 37°C for 2 h) to destroy the methylated template strands. Non-digested plasmid DNA was used for transformation of \textit{E. coli} \textit{DH5}α high-competent cells by heat shock treatment. Transformants were selected on LA Petri dishes containing ampicillin. To obtain plasmid DNA from the selected colonies, the GeneJET plasmid miniprep kit was used according to the manufacturer’s recommendations (Thermo Fisher Scientific). The introduction of the desired mutations was confirmed by sequencing (Macrogen Europe, The Netherlands).

### Annealing of DNA oligonucleotides

Annealing of two single-stranded DNA oligonucleotides with complementary sequences was done by heating and cooling method. Oligonucleotides were dissolved in pure water. Annealing mixture (50 μl) composed of two oligonucleotides (with the equimolar concentration 10 μM) in annealing buffer (10 mmol l⁻¹ Tris–HCl pH 7.5, 50 mmol l⁻¹ NaCl, 1 mmol l⁻¹ EDTA) was incubated for 5 min at 95–100°C and after that slowly cool down to room temperature before transferring it on ice. Hybridized oligonucleotides were used for ligation with pre-digested vectors or stored at −20°C.

### Overexpression and two-step purification of the recombinant AMPs in \textit{E. coli} ER2523

In this study, we tested the efficacy of newly constructed (reconstructed) pMAL expression vectors pMALc5HisEk and pMALp5HisEk by expressing and purifying two AMPs, through two-step purification. We used Ni-NTA agarose and amylose resins affinity chromatographies in two different combinations. Transformants of \textit{E. coli} ER2523 were maintained overnight on LA Petri dishes containing ampicillin (100 μg ml⁻¹) and glucose (1%) at 30°C. The next day new cultures (each 200 ml of LB with 1% glucose) were inoculated using 2% sample of overnight culture and incubated at 30°C with aeration (180 rpm on rotatory shaker). Expression of recombinant peptides was carried out in LB containing ampicillin (100 μg ml⁻¹) and glucose (1%).
glucose (1%) and protein production was induced in logarithmic growth phase (OD_{600} = 0.8–1.0) with addition of 0.3 mmol l^{-1} (final) IPTG for 3 h. Bacterial cells were collected by centrifugation at 4500 × g and before purification the level of induction was tested by comparing the amount of total proteins from the same amount of induced and non-induced cells. Purification, including cell lysis, affinity chromatographies and cleavage of the fusion protein with His6-enterokinase, was performed according to the manufacturer’s instructions (amylose resin purification—according to pMAL Protein Fusion & Purification System, New England Biolabs, Ltd., UK; Ni-NTA agarose resin purification—according to The QIAexpressionist, Qiagen Gmbh, Hilden, Germany; cleavage with recombinant bovine His6-enterokinase—according to GenScript, New Jersey, USA), with the addition of cell lysis step that was performed in column buffer (CB) with 1 mg ml^{-1} lysozyme for 30 min on ice.

First, the Ni-NTA agarose resin purification step was performed and then purified fusion protein was digested in 1 X enterokinase buffer (20 mmol l^{-1} Tris–HCl, pH 7.4, 50 mmol l^{-1} NaCl, 2 mmol l^{-1} CaCl$_2$) using 10 U of Recombinant Bovine His$_6$-Enterokinase (GenScript, Piscataway, NJ, USA) overnight at room temperature. To place the purified protein in 1 X enterokinase buffer, the buffer exchange was done using Amicon Ultra 0.5 ml 30 K Centrifugal filters (Merck Millipore Ltd, Cork, Ireland). After 24 h of digestion, total cleavage of the purified protein was obtained and the second purification step using amylose and Ni-NTA agarose resins mix was performed according to the instructions (The QIAexpressionist).

Proteins from every step of expression, purification and proteolysis were analysed on 12.5% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Samples for SDS-PAGE were mixed with 2 X sample loading buffer (125 mmol l^{-1} Tris HCl pH 6.8, 10 mmol l^{-1} EDTA, 4% SDS, 25% glycerol, 5% β-mercaptoethanol, 0.07% bromophenol blue) at 1:1 ratio. Samples were denatured by heating for 5 min at 100°C before loading on the gel. Overexpression and purification of LcnA using the commercial pMALc5X vector and cleavage with Xa protease were performed as described previously (Malesevic et al., 2021). Protein concentrations were determined using Bradford assay (Bradford, 1976) with Bradford reagent (Thermos Fisher Scientific). Bovine serum albumin (BSA) solutions of known concentrations were used as protein concentration reference standards.

### Antimicrobial activity assay

A spot-on-the-lawn inhibition assay was used for testing antimicrobial activity of purified recombinant AMPs as previously described (Miljkovic et al., 2018). *L. lactis* BGMN1-596 (Kojic et al., 2006) was used as a sensitive strain in antimicrobial assay. Five microliter of the purified AMP (200 ng, final) was applied to soft agar inoculated with *L. lactis* BGMN-596. The presence of inhibition zones was examined after 24 h following incubation at 30°C. A clear zone of inhibition was taken as evidence of antimicrobial peptide activity. The antimicrobial activity assay was performed in at least two independent experiments. The His6-enterokinase and CB buffers (in which the purified AMPs were resuspended), buffer with His6-enterokinase and CB buffer with Xa protease were taken as a negative control, while the LcnA, overexpressed and purified using commercial pMALc5X expression system, was used as a positive control in an antimicrobial assay.

### RESULTS

#### Design of the improved pMAL expression vector

To meet the requirements for the fast and simple separation of the MBP tag and extra amino acids from the native AMP produced within a fusion protein, we decided to design and generate an improved version of pMAL expression vector, different from already available versions (Gnanasekaran et al., 2016). The presence of stop codons in all three reading frames, Col E1: ColE1 origin of replication, lacO: LacZ operator, RBS: Ribosome-binding site, ATG: Start codon, stop codons: Stop codons in all three reading frames, col E1: ColE1 origin of replication.

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**FIGURE 1** Construction of redesigned pMAL and pQE vectors. Circular maps and depicted redesigned nucleotide sequence of (a) pMALc/p5HisEk and (b) pQE_Ek vectors. Arrows indicate the size and direction of gene transcription. Unique restriction sites are indicated by bold letters. *malE*: Maltose-binding protein (MBP) gene, *Xa*: Xa factor cleavage site IEGR (typical Xa cleavage site is IE/DGR), 6xHis: 6xHis tag sequence, iEk: Incomplete enterokinase (Ek) cleavage site (DDDDN) to be reconstituted into complete Ek cleavage site DDDDK following cloning, MCS: Multiple cloning site with restriction sites indicated, Bla: Ampicillin resistance gene, lacI, lacPl: repressor gene, *Taq* promoter, *pBor*: pBR322 origin of replication, PTS: T5 promoter, *lacO*: Lac operon, RBS: Ribosome-binding site, ATG: Start codon, stop codons: Stop codons in all three reading frames, col E1: ColE1 origin of replication.
There is a commercially available His$_6$ tagged enterokinase protease that cuts immediately after its recognition cleavage site. This can be used to precisely release, for example, the native peptide from the protein fusion, with no additional amino acids carried on from the protease cleavage site. In addition, the Ni-NTA resin column will retain the His$_6$-enterokinase. Therefore, the idea was to use the pMAL vector and introduce the His$_6$ tag and the enterokinase cleavage site downstream from the MBP and Xa cleavage site and just upstream of the Multi Cloning Site (MCS) (Figure 1). Adjacent to the MCS and part of the enterokinase cleavage site is the BsaBI restriction site for cloning of the peptide coding DNA (see Figure 1). Based on this redesigned pMAL vector, we established the protocol for two-step affinity separation and removal of the tags (MBP and His$_6$) and the protease from the native peptide following the cleavage with the His$_6$-enterokinase.

Construction of redesigned pMAL and pQE expression vectors

We constructed the redesigned pMAL expression vectors pMALc5HisEk and pMALp5HisEk based on pMALc5X (for expression of proteins in the cytoplasm; New England Biolabs) and pMALp5X (for expression of proteins in the periplasm; New England Biolabs). Namely, the MBP at the N-terminus in pMALp5X contains the leader peptide for transfer of synthesized protein to the periplasm. The restriction enzyme recognition site sequence analysis showed that the best restriction enzyme DNA sequence that can form the enterokinase recognition site (DDDDK), and be placed upstream of the MCS 5’ end, is the one of BsaBI (GATNN/NNATC) (see Figure 1). The choice of BsaBI restriction enzyme was further supported by the fact that this BsaBI is very efficient and cuts DNA at 60–65°C with no star activity introducing blunt ends. However, the sequence analysis of pMALc5X and pMALp5X vectors revealed that they possess two BsaBI restriction sites; one in the malE gene (position 2199c/2273p, respectively) and the other at position 5422c/5497p, respectively). To destroy these sites in order to be able to introduce the BsaBI restriction site upstream of the MCS for cloning DNA fragments of desired peptides, the site-specific mutagenesis of both BsaBI restriction sites in pMAL vectors was performed using the primer pairs listed in Table 1. Since the exiting BsaBI restriction sites lay in coding genes, vital for the pMAL vector maintenance and expression, the mutations introduced by site directed mutagenesis (the codons showing the highest frequency in E. coli were selected) formed the same sense codons. The success of the restriction site mutagenesis was first checked by digestion with BsaBI restriction enzyme, and finally confirmed by sequencing. Before proceeding further, the functionality of the mutated pMAL vectors (pMALc5XΔBsaBI and pMALp5XΔBsaBI) was checked. For this, we induced the MBP expression using IPTG and detected the production of overexpressed MBP protein with the correct molecular weight on PAGE SDS electrophoresis.

The His$_6$ tag and the BsaBI restriction site were introduced upstream of the MCS in either pMALc5XΔBsaBI or pMALp5XΔBsaBI using oligonucleotides 6xHis_BsaBI-Ek-F and 6xHis_BsaBI-Ek-R (see Table 1). After annealing, double strand hybrid DNA fragment was inserted into the XmnI restriction site. In addition to BsaBI site, oligonucleotides were designed to introduce an extra SacI restriction enzyme recognition site downstream from the BsaBI (Figure 1a). The SacI digestion was used to check for the correct orientation of the inserted DNA fragment. The plasmid DNA was isolated from transformants and digested with the SacI. Clones that produced a fragment of 104bps were selected because they contain a cloned fragment in the correct orientation, which was further confirmed by sequencing. We intentionally introduced the incomplete enterokinase recognition site (iEk: DDDDN instead of complete one DDDDK) to manipulate the BsaBI restriction site, introducing asparagine (N) instead of lysine (K) (see Figure 1a). The reason for this is to emphasize the need to add an extra adenine to the 5’ end of the desired protein/peptide coding sequence (or to the forward primer used for amplification of desired protein/peptide coding sequence) to obtain both, in-frame protein/peptide synthesis with MBP tag and the constitution of complete enterokinase cleavage site (DDDDK). Two newly constructed expression vectors were named pMAL-c5HisEk and pMALp5HisEk. The inducibility and production of overexpressed MBP-His$_6$ from the redesigned pMAL vectors was checked following induction with 0.3mmol l$^{-1}$ IPTG for 3 h. The production of correct fusion MBP-His$_6$ protein was obtained as seen in Figure 2a.

In addition, following the same strategy, we constructed the pQE_Ek expression vector for the N-terminal peptide fusions. We used the BsaBI-Ek-pQE-F and BsaBI-Ek-pQE-R oligonucleotides (see Table 1) and inserted a DNA fragment carrying the incomplete enterokinase cleavage site including BsaBI restriction site into pQE30 previously digested with BamHI (Figure 1b).

Optimization of expression and purification of LcnA and hBD AMPs using redesigned pMAL vectors

To demonstrate the efficacy of redesigned pMAL vectors pMALc5HisEk and pMALp5HisEk, two AMPs were selected...
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for overexpression, purification and functionality check: lactococcin A (LcnA: KLTFIQSTAAGDLYYNTNTHKY VYQQTQAFAAANTIVNGWMGAAGGFGLHH) for expression in the cytoplasm, and human beta defensin (hBD: DHYNCVSSGGQCLYSACPIFTKIQGTCYRGKAKCCK) for expression and transport in the periplasm.

The coding sequences for LcnA and hBD were synthetic and obtained by annealing of corresponding oligonucleotides (Table 1). The codon selection for hBD was optimized for codon usage in *E. coli*. The extra adenine (A) was added upstream of coding sequences for both AMPs, while 3′ ends contained the sticky ends compatible with *HindIII* in digested vectors (Table 1). The dsDNA fragments obtained by annealing of corresponding oligonucleotides were cloned into *BsaI-HindIII* pre-digested pMALc5HisEk or pMAlp5HisEk vectors. More than 90% of analysed transformants contained plasmid carrying the desired DNA fragment. To confirm the orientation, in-frame position with the MBP-His6 tags and the presence of the correct enterokinase cleavage recognition site, we sequenced five clones from each transformation using pMalE forward primer. The newly generated pMALc5HisEk_LcnA1 and pMAlp5HisEk_hBD1 constructs were stored at −80°C in LB containing 15% glycerol and further used for the expression and purification experiments.

For overexpression of LcnA and hBD AMPs, the *E. coli* strain ER2523 was transformed with pMALc5HisEk_LcnA1 or pMAlp5HisEk_hBD1 and grown overnight at 30°C. Next day, the production of AMPs was induced in logarithmic growth phase and the level of induction was tested comparing the concentrations of total proteins from the same amount of induced and non-induced cells (Figure 2b).

Next, we demonstrated that both tags are individually efficient/active, enabling the purification of overexpressed MBP-His6-LcnA and MBP-His6-hBD. Induced cultures were divided into two equal aliquots and expressed fusion proteins were purified either via amylose resin (binding MBP tag), or via Ni-NTA agarose resin (binding His6). The
amount and purity of MBP-His$_6$-LcnA and MBP-His$_6$-hBD purified via MBP or His$_6$ tag were comparable (Figures S1, S2).

The initial strategy for two-step purification was to use the amylose resin in the first step to pull down the MBP-(Xa-His$_6$-(Ek)-AMP fusion protein. This will be followed by the step including cleavage with His$_6$-enterokinase and incubation with Ni-NTA agarose resin to remove the MBP-His$_6$-(Ek) and His$_6$-enterokinase from solution, leaving the native AMP purified. To our surprise, in the second step, there was no binding of the MBP-His$_6$ to the Ni-NTA agarose resin (Figure 3) in three independent attempts. This unexpected outcome implied that there are problems in immobilization of the MBP-His$_6$ two-tags fusion protein on Ni-NTA agarose. Although the literature suggest this might be due to irreversible change in MBP conformation (and consequently maybe the entire fusion protein) caused by the binding of the maltose to the complex fusion protein such as MBP-(Xa-His$_6$-(Ek)) AMP (Bucher, Grant, Markwick, & McCammon, 2011; Bucher, Grant, & McCammon, 2011; Mascarenhas & Kästner, 2013; Seo et al., 2014), we cannot comment on this without the additional experiments.

Before further changing the approach and doing more purification attempts, we investigated whether the digestion of complex fusion proteins by His$_6$-enterokinase is equally effective regardless of the affinity binding method used, MBP to amylase resin or His$_6$ to Ni-NTA agarose resin. The splitting of MBP-His$_6$ and AMP peptides was performed by overnight digestion of MBP-His$_6$-(Ek)-LcnA and MBP-His$_6$-(Ek)-hBD by His$_6$-enterokinase at room temperature following the purification based on either MBP or His tag (see Materials and Methods). We noted that the efficiency of the His$_6$-enterokinase protease cleavage was much higher when the fusion protein was first isolated using His tag and Ni-NTA agarose (Figure S3, lane 3). Apparently, although the proteins were completely digested after 24 h, differences in the protease efficiency were observed at earlier time points.

Considering these observations, we now used the Ni-NTA agarose resin in the first step to purify the entire fusion protein and then performed an overnight digestion with His$_6$-enterokinase at room temperature. The removal of MBP-His$_6$ and His$_6$-enterokinase from digestion mixture was accomplished in the second step purification using a mix of amylase (to bind MBP-His$_6$ tag) and Ni-NTA agarose (to bind His$_6$-enterokinase) in different ratio combinations, 2:1, 5:1 and 10:1, respectively. We found that the most optimal ratio amylase:Ni-NTA agarose was 10:1 (Figure 4). The expressed AMPs, LcnA (Figure 4a, lane 5) and hBD (Figure 4b, lane 5), were isolated in over 95% purity when compared to the samples obtained after the cleavage with His$_6$-enterokinase (Figure 4a,b, lane 4).

The production and final yield of LcnA expressed from the pMALc5HisEk_LcnA1 was 2.5 times higher than hBD produced from the pMALp5HisEk_hBD1 (see Table 2). This is understandable because the LcnA AMP is expressed in the cytoplasm while the hBD AMP is expressed in the periplasm. However, during the purification, protein loss levels remained almost the same for both AMPs.

To confirm that the improved pMAL vector that underwent modifications did not lose typical properties related
to induction, purification and solubility of the peptides, we checked the efficiency of purification and proteolytic cleavage efficacy using Xa protease to release the LcnA from MBP fusion protein, and tested the activities of LcnA. The starting material was LcnA cloned into either pMALc5X commercial vector (Malesevic et al., 2021) or pMALc5HisEk_LcnA1. We showed that the levels of induction, solubility (which was higher than 90%) and purification are comparable, suggesting the newly constructed vectors, besides enabling production of clean native peptides, retained all positive properties of the commercial vector.

The functionality/bioactivity of the purified native LcnA (using both, commercial and improved pMAL vectors) and hBD was analysed by spot on the lawn antimicrobial assay using *L. lactis* BGMN-596-sensitive strain.

All three purified native AMPs (200 ng in 5 μl) showed an antimicrobial activity (Figure 5). The activity of LcnA was higher than hBD, while the difference in antimicrobial activity between LcnA produced using commercial and newly constructed pMAL vectors was negligible.

**DISCUSSION**

Many protein expression and purification systems are commercially available to provide a sufficient amount of pure, soluble and active native protein. Adding specific amino acid tags to the N- or C-terminus of the protein increases solubility and facilitates affinity purification of proteins. However, many of the expressed tagged proteins consequently lose functionality, particularly small peptides...
By analysing the expression vectors, we found that only vectors containing Xa cleavage site, for example, pMAL (NewEngland Biolabs) and pQE-30Xa (QIAGEN) provide the precise tag separation from the native protein. This was enabled by the incorporation of a specific restriction sites (XmnI or StuI, respectively) for cloning of DNA fragments carrying genes for proteins expression. On the other hand, unlike Xa, there are His-tagged proteininases such as TEV protease and His6-enterokinase that can be removed after digestion from solution by affinity chromatography along with tag. Expression vectors that carry the enterokinase cleavage site (like pET vectors, with multiple tags, Novogen; pMAL-c5E expression vector, New England Biolabs) have also been designed, but they add a few amino acids to the expressed native protein.

Here we report an efficient procedure for purification and separation of native peptides from the tag(s) using redesigned pMAL expression vector and the two-step affinity chromatography. We constructed novel pMAL-based vectors, pMALc5HisEk and pMALp5HisEk and applied the two-step affinity chromatography protocol, which enables purification of active native AMPs from the cytoplasm and periplasm (see Figure 6).

The key element that enabled the construction of such an efficient vector is the introduction of a BsaBI restriction site for the initial cloning of the in-frame peptides DNA which is also the basis for the formation of a complete enterokinase cleavage site ensuring the expressed peptides have no additional amino acids and are purified in their native and active form following the proteolytic cleavage. This construction strategy can also be applied for the redesign of other expression vectors that have a larger number of tags (e.g., pET32 vectors). That the entire system (containing BsaBI blunt restriction enzyme site) can be transferred to any vector, we confirmed by the construction of additional pQE_Ek expression vector (Figure 1). Based on the latest results in the development of new improved expression vectors, we can conclude that the improvement of expression vectors will most likely go in the direction of reducing the toxicity of expressed AMPs (sandwich tag vector construction) and those contributing to AMP modification to increase stability and functionality (Lamer et al., 2022; Zhu et al., 2021). The improvements we have presented in this study can be combined with other improvements (most are compatible) in the construction of new expression vectors with multiple tags (sandwich tag) and with enzymatic modifications and processing.

It was originally intended to use two tags: MBP in the first step of fusion protein purification, and His6 for the tag and His-tagged enterokinase removal after cleavage, but this order of use of resins did not function. We have overcome this problem by reversing the order of use of tags in purification, where we noticed much greater efficiency of the His6-enterokinase cleavage efficacy after initial purification with the Ni-NTA agarose and not with the amylose. The lack of His6 binding and differences in His6-enterokinase cleavage efficacy are likely presenting the same or similar problem we have in purification of the complex fusion protein.

We fully demonstrated validity of the novel protocol using LcnA and hBD AMPs. The functionality of constructed vectors and two-step purification protocol was confirmed by cloning, expressing and purifying two AMPs, the LcnA and hBD, where the purity of the native peptides, which showed antimicrobial activity in the test, was greater than 95%.

In conclusion, this redesigned pMAL-based expression and purification system is intended for cloning, expression and purification of the heterologous proteins/peptides in E. coli and includes fast and reliable procedure for the isolation of active and native recombinant AMPs.
proteins and peptides (see Figure 7). By applying this expression and purification protocol, thanks to a double tag separation and protease removal the protein purity is greater than 95%. And last but not least, this system is simple and cost-effective, and can be implemented in any laboratory.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

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