

MINIREVIEW

Carrier proteins for fusion expression of antimicrobial peptides in *Escherichia coli*

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Antimicrobial peptides are an essential component of innate immunity and play an important role in host defence against microbial pathogens. They have received increasing attention recently as potential novel pharmaceutical agents. To meet the requirement for necessary basic science studies and clinical trials, large quantities of these peptides are needed. In general, isolation from natural sources and chemical synthesis are not cost-effective. The relatively low cost and easy scale-up of the recombinant approach renders it the most attractive means for large-scale production of antimicrobial peptides. Among the many systems available for protein expression, *Escherichia coli* remains the most widely used host. Antimicrobial peptides produced in *E. coli* are often expressed as fusion proteins, which effectively masks these peptides' potential lethal effect towards the bacterial host and protects the peptides from proteolytic degradation. Although some carriers confer peptide solubility, others promote the formation of inclusion bodies. The present minireview considers the most commonly used carrier proteins for fusion expression of antimicrobial peptides in *E. coli*. The favourable properties of SUMO (small ubiquitin-related modifier) as a novel fusion partner are also discussed.

Recombinant production of antimicrobial peptides

Antimicrobial peptides are gene-encoded peptide antibiotics that exhibit bactericidal activity against diverse microbes. They are present in virtually every life form and are important components of the innate immune system [1]. Because of their broad spectrum of activities and low propensity for resistance development, these peptides have recently received increasing attention as potential new anti-infective therapeutics [2]. To better understand their mechanisms of action and structure–function relationships, extensive biological and structural studies need to be performed, and these require a sufficient amount of highly purified peptides. Moreover, if these peptides can be eventually developed

as alternative antibiotics for widespread clinical use, huge amounts of peptides should be readily available in a cost-effective manner. Preparative isolation from natural sources is typically complicated and time-consuming, and therefore it is not an efficient way for obtaining peptides in large amounts. Besides, it does not apply to designed peptides with altered sequences. While chemical synthesis allows production of both natural and designed peptides, its cost is prohibitive for large-scale manufacture. Generally, recombinant DNA technology provides a means for economical protein production. Indeed, many antimicrobial peptides have been successfully produced through recombinant expression in various heterologous hosts [3]. In addition to cost-effectiveness, the recombinant approach also facilitates the production of peptides with modified sequences and allows the incorporation of isotopes for structural studies. *Escherichia coli* and yeast are the two major systems used to produce recombinant antimicrobial peptides, which together account for over 95% of all reported cases [3,4]. A number of antimicrobial peptides have been produced in yeast with good yields [5–7]. However, several others were expressed in negligible amounts [8,9] or obtained as an inactive form [10] when the yeast system was used. In one case, no recombinant peptide was detected, even though the gene was transcribed [11]. Overall, yeast is used much less commonly than bacteria for recombinant production of antimicrobial peptides [4].

Need for fusion expression in *E. coli*

The popularity of *E. coli* comes from its attractive features (e.g. fast growth rate and low cost) [12] and the simple fact that post-translational modification is not required

Key words: antimicrobial peptides, carrier proteins, *Escherichia coli*, glutathione transferase (GST), small ubiquitin-related modifier (SUMO), thioredoxin. Abbreviations used: CRAMP, cathelicidin-related antimicrobial peptide; GST, glutathione transferase; His-tag, histidine tag; IMAC, immobilized metal-affinity chromatography; LL-37, 37-residue protein whose N-terminal sequence is Leu-Leu (cathelicidin); PaP3.30, encoded by open reading frame 30 of *Pseudomonas aeruginosa* bacteriophage PaP3; PurF, amidophosphoribosyltransferase; SUMO, small ubiquitin-related modifier; TAF12, TATA box binding protein-associated factor; 20 kDa.

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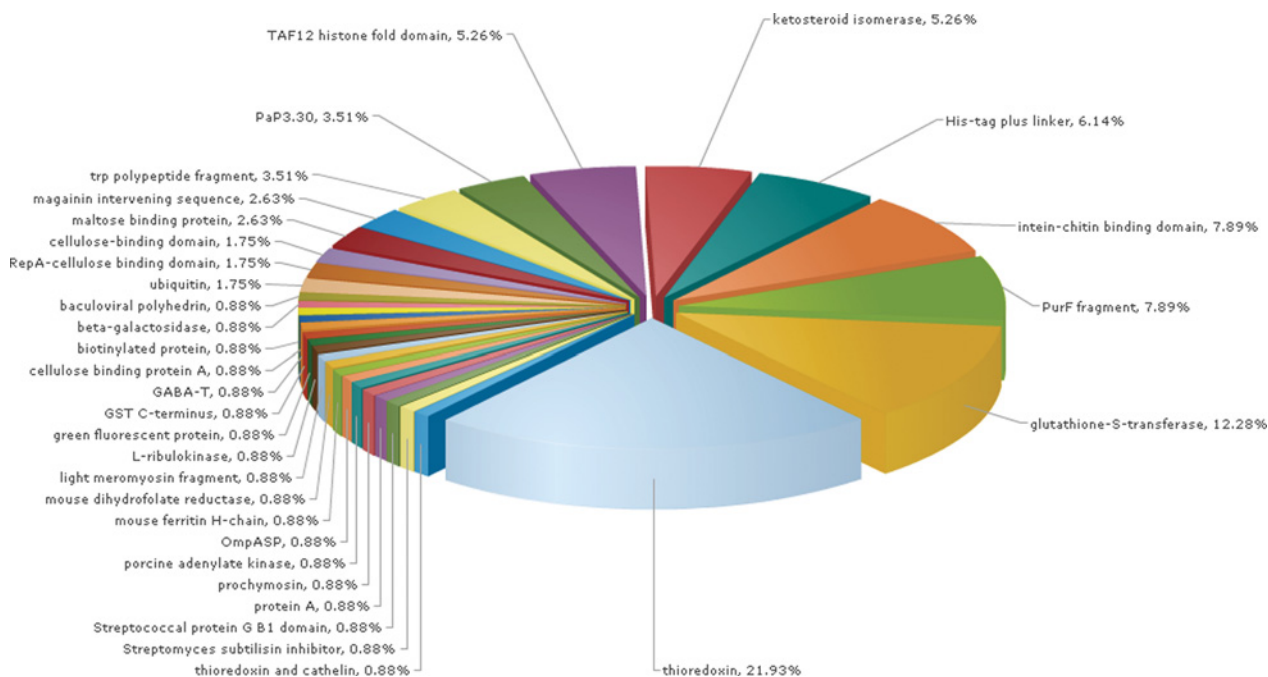


Figure 1 Three-dimensional pie chart showing the relative frequency of proteins being used as carriers for fusion expression of antimicrobial peptides in *E. coli*

The calculation was based on the data collected in the Recombinantly-produced Antimicrobial Peptide Database. Further abbreviations: GABA-T, γ -aminobutyrate: α -oxoglutarate transaminase; OmpASP, outer-membrane-protein-A signal peptide; Trp, tryptophan-operon-encoding.

for the activity of antimicrobial peptides. However, certain properties of antimicrobial peptides pose difficulties for their direct expression in *E. coli* [13]. First, their antibacterial nature makes them potentially fatal to the producing host. Secondly, their small size and high cationic content make them highly susceptible to proteolytic degradation. A strategy found to be effective in overcoming both barriers is to express these peptides as fusions, in which the peptides of interest are genetically attached to carrier proteins [13]. The fusion partners play a similar role as the natural pro-segments in the peptides' inactive precursor forms, which protect both the peptides and their host organisms during peptide biosynthesis [14]. Various proteins with different sizes and properties have been used as carriers (Figure 1). Whereas some of them facilitate soluble expression, others promote the formation of inclusion bodies [3]. In most cases, the carrier/peptide junction usually contains an enzymatic or chemical cleavage site that enables the target peptide to be released by the corresponding method. With very few exceptions, antimicrobial peptides produced in *E. coli* are nearly all expressed as fusion proteins. A closer look at peptides expressed as non-fusions reveals that they are either relatively large in size (i.e. more than 60 residues) and form inclusion bodies upon expression [15], which may alleviate the potential problems of toxicity and instability,

or expressed as tandem multimers [16,17], which can be viewed as a special type of fusion.

Four most commonly used carrier proteins

According to the Recombinantly-produced Antimicrobial Peptides Database (<http://faculty.ist.unomaha.edu/chen/rapd/>; [4]), thioredoxin has been the most frequently used carrier protein, accounting for more than 20% of all reported fusion expressions of antimicrobial peptides (Figure 1). Thioredoxin is a low-molecular-mass protein (~12 kDa) that is highly soluble and possesses chaperone-like activity [18]. Because of these properties, thioredoxin is widely used as a fusion carrier to promote soluble expression of recombinant proteins/peptides in the *E. coli* cytoplasm [18,19]. As summarized in Table 1, most of the thioredoxin-peptide fusions are indeed found in the soluble fraction. For the small percentage of insoluble expressions, some of them could result from unusual designs (e.g. concatamerization or internal fusion) [33,38] or unoptimized parameters [42]. It is noteworthy that low-temperature induction is sometimes needed to obtain soluble thioredoxin fusion [29,32,40]. For peptides

Table 1 Antimicrobial peptides expressed as a thioredoxin fusion

Size is shown as the number of amino acid residues. Further abbreviations used: ABP-CM4, antibacterial peptide CM4, a 35-amino-acid peptide from the silkworm *Bombyx mori*; DCD-1L, human antimicrobial peptide dermcidin 1L; HP signifies antibacterial peptide from *H. pylori*. LFB15-HP is a 28-residue hybrid peptide containing LFB(W4,I10)-HP(4-16): FKCVWR WQWRW KKLGA KVFKR LEKLF SKI; TDEF1, *Trichosanthes kirilowii* (Chinese cucumber) defensin; TvD1, *Tephrosia villosa* (hoary pea) defensin.

Peptide	Size	Fusion solubility	Yield (mg/litre)	Reference(s)
ABP-CM4	35	Soluble	1.2	[20]
Adenoregulin	33	Soluble	3.4 ^a /NA ^b	[21,22]
Arenicin-2	21	Insoluble	5 ^c	[23]
β -Defensin 2	41	Soluble	346	[24]
β -Defensin 3	45	Soluble	90	[25]
β -Defensin 4	50	Soluble	689 ^c	[26]
β -Defensin 5	51	Soluble	140	[27]
β -Defensin 6	45	Soluble	130	[27]
Brevinin-2R	25	Soluble	NA	[28]
Cecropin	36	Soluble	11.2	[29]
DCD-1L	48	Soluble	NA	[30]
Divercin V41	43	Soluble	23	[31]
Hepcidin-25	25	Soluble	0.1 ^a	[32]
Indolicidin	13	Insoluble	0.2	[33]
Latarcin-2a	26	Partially soluble	3.2	[34]
LFB15-HP hybrid	28	Insoluble	11.3	[35]
LL-37	37	Soluble	1.7/2.3	[36,37]
Magainin-2	23	Insoluble	NA	[38]
Pediocin PA-1	44	Soluble	NA	[39]
Perinerin	51	Soluble	1.2	[40]
Piscicolin-126	44	NA	26	[41]
TDEF1	47	Insoluble	NA	[42]
Thanatin analog	20	Soluble	13.2	[43]
TvD1 precursor	75	Soluble	NA	[44]
Viscotoxin-A3	46	Soluble	5.2	[45]

^aFor easy comparison, the original values, which were given in mg/2 (or 50) g of cells, were recalculated on the assumption that the 1-litre bacterial culture yields 4 g of wet cells.

^bNot available.

^cEstimate based on the amount of fusion protein.

containing disulfide bridges, special host strains (e.g. Origami cells from Novagen, Madison, WI, U.S.A.) that are engineered to promote disulfide bond formation greatly increase the chance of obtaining soluble fusions with proper folding [31,32,39]. Glycerol or detergent is frequently added to the cell lysis buffer to increase the yield of soluble protein [21,22,30,36,37,40]. Antimicrobial peptides are known to interact with bacterial membranes, and these agents can help retrieve fusion proteins entrapped by membrane components during cell lysis. In addition to its general properties as a fusion carrier, thioredoxin's small size makes it particularly favourable for peptide production, because it allows the peptide of interest to constitute a relatively large percentage of the fusion. A recent study showed that, among 13 different carrier proteins tested, thioredoxin fusion gave the highest absolute yield of the fused peptide, even though the fusion's overall expression level was not the highest [45]. The actual peptide yield varies from case to case, and extreme values are found at both ends (Table 1).

Table 2 Antimicrobial peptides expressed as GST fusions

Size is shown as the number of amino acid residues. Further abbreviation used: Vv-AMPI, *Vitis vinifera* (common grape vine) antimicrobial peptide 1.

Peptide	Size	Fusion solubility	Yield (mg/litre)	Reference(s)
β -Defensin 2	41	Soluble/insoluble	NA ^a /NA	[47,48]
β -Defensin 3	45	Soluble	NA	[49]
Cecropin	40	Soluble	NA	[50]
CRAMP	34	Soluble	1.5	[51]
Gallinacin-9	42	Insoluble	NA	[52]
Hepcidin-20	20	Insoluble	NA	[53]
Lactoferricin B	25	Soluble	2	[54]
Lactoferrin fragment	54	Partially soluble	NA	[55]
LL-37	37	Soluble	0.3	[56]
Neutrophil defensin I	30	Insoluble	NA	[13]
Puroindoline-A	118	Soluble	1.8	[57]
Puroindoline-B	119	Soluble	0.7	[57]
Sarcotoxin-1A	39	Soluble	NA	[58]
Vv-AMPI	47	Soluble	5	[59]

^aNot available.

For example, the yields of several β -defensins are much higher than that of other peptides. In these cases, the amount of fusion protein is close to, or above, 1 g/litre of culture. Since the wet cell weights were not given, it is difficult to judge whether the high yields resulted from high cell density or from a high expression level. The Novagen pET-32 series has been the most commonly used commercial thioredoxin fusion vector. The presence of a His-tag (histidine tag) in the fusion facilitates protein purification by IMAC (immobilized metal-affinity chromatography). In addition, the pET-32 series contains an enterokinase recognition site upstream of the target peptide, allowing the peptide to be liberated upon enterokinase cleavage. If desired, however, other proteolytic or chemical cleavage sites can be generated at the cloning stage to serve the same purpose. Whereas chemical cleavage has been found to be efficient at releasing the target peptide in all cases where it was applied [20,36,37,41], enzymatic cleavage was found to be poor in several cases [37,39–41].

GST (glutathione transferase) is another commonly used carrier protein for fusion expression of antimicrobial peptides (Figure 1). Like thioredoxin, GST is well established as a carrier for soluble expression of fusion proteins in *E. coli* [46]. GST fusion proteins can be quickly purified from crude lysate by affinity chromatography on immobilized glutathione. Owing to their easy capture and mild elution conditions, GST fusions are routinely used in pull-down assays to confirm or disprove protein–protein interactions. For antimicrobial-peptide expression, GST–peptide fusions are usually found in the soluble portion, as would be expected (Table 2), although sometimes the production of soluble fusions may only be achieved after optimization of several factors, such as host strain and growth temperature [57,59]. For the reason mentioned in the above paragraph, detergent is a common ingredient of the cell lysis buffer to increase

Table 3 Antimicrobial peptides expressed as fusion proteins with a PurF fragment

All the fusions were insoluble. Size is shown as the number of amino acid residues. Further abbreviations used: MSI-344, a magainin analogue [magainins are a family of peptides isolated from the skin of *Xenopus laevis* (South African clawed frog)]; PGQ, 24-amino-acid antimicrobial peptide with N-terminal glycine (G) and C-terminal glutamine (Q) isolated from *Xenopus laevis* stomach. The three values in column 3 for MSI-344 refer to the yield in the corresponding reference in column 4.

Peptide	Size	Yield (mg/litre)	Reference(s)
Bombinin	24	NA ^a	[61]
Bufoforin-2B	22	131	[62]
Histonin	21	167	[63]
Indolicidin	13	NA	[61]
Melittin	26	NA	[61]
MSI-344	22	NA/310/NA	[61,64,65]
PGQ	24	NA	[61]
Pleurocidin	25	9.8	[66]
Tachyplesin-I	17	NA	[61]

^aNot available.

fusion solubility. The commercial GST-fusion plasmids usually contain a specific protease recognition site, cleavage at which releases the desired peptide from the carrier. Chemical cleavage is rarely used for this purpose, probably because non-specific cleavage at the carrier will substantially complicate the subsequent purification. One disadvantage of GST as a carrier for peptide production is its relatively large size (~26 kDa), which decreases the efficiency of the system, as the peptide of interest is usually small and thus comprises only a modest percentage of the expressed fusion. As can be seen in Table 2, the highest yield reported, 5 mg/litre, is lower than the average value given by other systems. GST's large size also makes the fusion highly susceptible to proteolytic degradation [47,56,58]. In one case, fusion degradation could not be stopped by adding protease inhibitors during protein purification, causing a failure to obtain even trace amounts of peptide [58]. In another failed case, uncontrollable degradation was also suspected to be the reason [60].

The protein fragment containing the N-terminal 152 amino acids of PurF (amidophosphoribosyltransferase) is also widely used as a carrier for antimicrobial peptide expression (Table 3). In contrast with thioredoxin and GST, the PurF fragment strongly enhances the formation of inclusion bodies [61]. Compared with soluble fusion, insoluble expression is believed to be able to more completely mask the peptide's intrinsic toxic effects and better protect the peptide from proteolytic activity [61,67]. The inclusion bodies, which contain the PurF fusions, can be collected from the cell lysate by centrifugation. Further purification of the inclusion bodies other than simple washing is not required, since the fusion proteins usually represent the

majority of the insoluble components. Except for one case in which the peptide was released by furin-mediated cleavage [63], all peptides attached to the PurF fragment were liberated by hydroxylamine treatment after the fusions were solubilized in denaturing buffers [61,62,64–66]. The reason for the predominant use of chemical cleavage is probably due to the denaturing conditions required for fusion purification, which make direct enzymatic cleavage impractical. Nearly all antimicrobial peptides expressed as PurF fusions do not contain disulfide bonds and thus refolding is generally not required to restore their activity. For three of the cases listed in Table 4, the peptide yields are relatively high. In two of them protein expression was induced after the attenuation (D_{660}) was above 50 [62,63], suggesting that the high yield probably resulted from high cell density. In another case the total protein in inclusion bodies reached 11.2 g/litre of cell culture [64], further confirming that high cell density cultivation was performed, since the same volume of culture typically yields just 4–5 g of wet cells. Other fusion partners that have been specially designed to target the peptides to inclusion bodies include oxosteroid ('ketosteroid') isomerase [68], the TAF12 (TATA box binding protein-associated factor, 20 kDa) histone fold domain [67], PaP3.30 (encoded by open reading frame 30 of *Pseudomonas aeruginosa* bacteriophage PaP3) [69] and baculoviral polyhedron [70].

Nine peptides collected in the Recombinantly-produced Antimicrobial Peptides Database were produced using the intein-mediated system, making it the third most commonly used carrier along with the PurF fragment. Inteins are the protein counterpart of introns that can excise themselves from a precursor protein and rejoin the flanking regions [71,72]. Intein-mediated protein splicing is an autocatalytic event and requires no auxiliary enzymes. Combined with affinity tags, intein allows efficient protein purification by utilizing its inducible self-cleaving capability. For example, with one terminus fused to a chitin-binding domain and the other fused to the protein of interest, intein fusion can be captured on a chitin column. Upon induction with thiol reagents or pH and temperature shift, the target protein is released by intein-mediated self-cleavage [73–76]. The intein system eliminates the need for exogenous proteases or chemicals required by other fusion systems to remove the carrier, and allows the target protein to be obtained at high purity in a one-step purification employing a single column. Thus this technology lowers production costs and simplifies protein recovery. Commercial intein systems are available from New England Biolabs (Beverly, MA, U.S.A.). In addition to the initially developed system using an engineered *Scv* VMA (*Saccharomyces cerevisiae* vacuolar ATPase subunit) intein (69 kDa), a carrier based on *Ssp* (*Synechocystis* sp.) DnaB mini-intein (18 kDa) is also now available. Both systems yield proteins with the native sequence. Various biologically important peptides, including antimicrobial peptides,

Table 4 Antimicrobial and other bioactive peptides produced by the intein-mediated system

The peptides labelled with an asterisk (*) are antimicrobial peptides. Size is shown as the number of amino acid residues. Further abbreviations used: ABP-CM4, antibacterial peptide CM4 (see Table 1); Bin-1b, an antimicrobial-like protein also known as sperm-associated antigen 11 precursor; hBNP, human brain natriuretic peptide; hEGF, human epidermal growth factor; M65, a deletion mutant of Maxadilan, a vasodilator peptide isolated from sandfly salivary glands; rhPACAP, recombinant human pituitary adenylate cyclase-activating polypeptide; rMBAY, a peptide designed to imitate BAY 55-9837, a VPAC2 (vasoactive intestinal peptide receptor 2)-selective agonist developed by Bayer Corporation; rMROM, a 32-amino-acid VPAC2 agonist; SMAP-29, sheep myeloid antimicrobial peptide 29.

Peptide	Size	Fusion solubility	Yield (mg/litre)	Reference
ABP-CM4*	35	Soluble	4.2	[60]
Aldolase A fragment	26	Soluble	1.4	[77]
β -Defensin 1*	37	Partially soluble	4.5	[78]
β -Defensin 2*	41	Partially soluble	4.1	[78]
Bin-1b*	49	Partially soluble	3.2	[78]
Divercin V41*	43	Soluble	NA ^a	[79]
Enterocin P*	44	Soluble	NA	[79]
Glucagon	29	Partially soluble	11.9	[80]
hBNP	32	Insoluble	2.4	[81]
hEGF	53	Insoluble	17–18	[82]
Huwentoxin-I	33	Soluble	7.8	[83]
Maxadilan	67	Soluble	5.0	[84]
M65	50	Soluble	5.3	[84]
Pediocin PA-1*	44	Soluble	NA	[79]
Piscicolin 126*	44	Soluble	1.1	[79]
rhPACAP	39	Soluble	22	[85]
rMBAY	32	Soluble	53	[86]
rMROM	32	Soluble	6.8	[87]
SMAP-29*	29	Soluble	0.1–0.2	[88]

^aNot available.

have been produced at the laboratory scale using this system (Table 4). In order for intein-mediated cleavage to be effective, the fusion protein should be in a soluble, correctly folded, form. In most cases soluble expression can be achieved at a relatively low growth temperature (e.g. 30 °C) [84–87]. If the fusion goes to the inclusion body, refolding is required prior to purification [81,82]. The highest peptide yield in Table 4, namely 53 mg/litre, is a result of high-density culture (30 g of cells from 1 litre of culture) [86]. However, even without this case, the average yield of non-antimicrobial peptides is still much higher than that of antimicrobial peptides. The lower yield of antimicrobial peptides could be due to uncontrolled autocleavage, which releases a small amount of peptide that negatively influences the cell's protein-making machinery. Premature cleavage of the fusion protein during expression is an intrinsic problem associated with the intein system, which causes loss in the yield of target protein [89]. For proteins other than antimicrobial peptides, the loss due to *in vivo* cleavage can probably be offset by the gain from the relatively simple purification of this approach as long as the uncontrolled cleavage is kept to a low level, and allows

Table 5 Bioactive peptides expressed as SUMO fusions

Size is shown as the number of amino acid residues. Further abbreviations: GLP-1(7–37), glucagon-like peptide-1 7–37-peptide; hEGF, human epidermal growth factor; PTH(1–34), parathyroid hormone 1–34-peptide.

Name	Size	Fusion solubility	Yield (mg/litre)	Reference
Aprotinin	58	Insoluble	9.0	[95]
Brazzein	53	Soluble	20 ^a	[96]
Exendin-4	49	Soluble	1.5	[97]
Exendin(9–39)	31	Soluble	1	[97]
GLP-1(7–37)	31	Soluble	1.5	[97]
hEGF	53	Soluble	16.7	[98]
PTH(1–34)	34	Soluble	3–4	[97]
Urodilatin	32	Partially soluble	5.6	[99]

^aFor easy comparison, the original figure which was given in mg per 10 g of cells was recalculated on the assumption that a 1-litre bacterial culture yields 4 g of wet cells.

this method to still give a better yield than other fusion systems. However, for antimicrobial peptides this could be a serious problem. Certain antimicrobial peptides are known to inhibit cellular function through direct interaction with nucleic acids [90,91]. Therefore even trace amounts of released peptide can be fatal to the host.

SUMO (small ubiquitin-related modifier) as a novel fusion carrier

Recently, SUMO has been used as a novel fusion carrier for the production of recombinant proteins [92,93]. SUMO is a small protein (consisting of about 100 amino acids) that is structurally related to but functionally different from ubiquitin. The protein has a hydrophobic core and a hydrophilic surface, and therefore is highly soluble. As a fusion partner, SUMO was found to improve the folding and solubility of the target protein [92,93]. In addition, the existence of SUMO protease I offers a unique advantage to this system. Unlike other commonly used proteases, which recognize short sequence motifs, SUMO protease I only recognizes SUMO's tertiary structure. As a result, the enzyme never cleaves within the protein of interest and allows the production of target protein with native N-terminus when the fusion is properly designed [92]. Several difficult-to-express proteins, including matrix metalloprotease and SARS-CoV (severe-acute-respiratory-syndrome coronavirus) proteins, have been successfully expressed in *E. coli* using a SUMO fusion strategy [92,94]. This system has also been applied to the production of various bioactive peptides (Table 5). SUMO's small size (~11 kDa), like that of thioredoxin, allows a relatively high peptide-to-carrier ratio, which favours peptide yield. The yields in Table 5 lie within a range of 1–20 mg/litre of culture, which is reasonable

for small peptides and is higher on average than that of the GST fusion. Although application of the SUMO fusion strategy to antimicrobial peptides has not been widely reported, a research group in Emory University, Atlanta, GA, U.S.A. has recently been granted a patent on antimicrobial peptide production using this system. The authors of the patent claimed that various antimicrobial peptides, including CRAMP (cathelicidin-related antimicrobial peptide) and LL-37 (37-residue protein whose N-terminal sequence is Leu-Leu; cathelicidin) can be efficiently produced [100]. Commercial SUMO fusion vectors can be obtained from LifeSensors (Malvern, PA, U.S.A.) and Invitrogen (Carlsbad, CA, U.S.A.). In these vectors, the presence of a His-tag at the N-terminus of the SUMO protein sequence allows for rapid purification of fusions by IMAC.

Conclusions

Fusion expression is a necessary and effective strategy for antimicrobial-peptide production in *E. coli*. Although most commonly used carrier proteins promote proper folding and enhance solubility of the fused peptides, certain carriers, such as the PurF fragment, are specially designed to promote inclusion-body formation. Since many antimicrobial peptides have been successfully expressed as soluble fusions, forcing fusion proteins into inclusion bodies is not necessary to protect the bacterial host. For short peptides without disulfide linkages, refolding is generally not required to restore activity, and insoluble expression has the advantage of easy purification. However, for disulfide-bond-containing peptides, which need to be correctly folded in order to show activity, soluble expression is preferred, because refolding can be time-consuming and may not guarantee high recovery of activity. Thioredoxin and GST are two well established carriers that promote soluble expression of the fused proteins [18,19,46]. Although both carriers are highly efficient at improving solubility, growth temperature and cell-line optimization are sometimes needed to obtain the best results. The size of GST, which is more than twice as big as that of thioredoxin, is a drawback for yield, as it causes an unfavourable ratio of peptide to carrier. For both thioredoxin and GST fusions, enterokinase, Factor Xa and thrombin are routinely used to liberate the peptide. However, cleavage with these enzymes has sometimes been found to be inefficient [37,39–41,54,56]. Although enzymatic cleavage is normally efficient at releasing target protein from its fusion carrier, in the case of antimicrobial peptides it is found to be less efficient than chemical cleavage [13]. A possible reason for this is that certain antimicrobial peptides tend to form oligomers [37,101–104], which block the cleavage sites. The intein system, which eliminates the need for proteases or chemical reagents for carrier

removal, offers a great benefit to target protein purification. However, its uninduced self-cleavage activity may limit its value for antimicrobial peptide production.

SUMO is a relatively new fusion carrier and this system has several advantages. First, like thioredoxin and GST, SUMO promotes soluble expression of the fused protein. Secondly, SUMO's small size results in a relatively high mass ratio of the target protein. Thirdly, SUMO protease is highly specific and efficient at removing the carrier, and generates target protein with native N-terminus. While these properties benefit the production of any type of protein, the second and the third advantages are especially relevant to antimicrobial peptides. The size of antimicrobial peptides is small and therefore a higher stoichiometric ratio will dramatically increase the net yield. As mentioned above, the commonly used proteases are sometimes inefficient at releasing the target peptide. For example, when expressed as thioredoxin and GST fusions, the human antimicrobial peptide LL-37 cannot be efficiently released by thrombin or factor Xa cleavage [37,56]. Recently Bommarius *et al.* reported the production of LL-37 using the SUMO system [100]. However, detailed information about cleavage efficiency and peptide yield was not provided. It would be interesting to know whether SUMO protease's unique property will allow it to overcome the steric hindrance imposed by antimicrobial peptide oligomerization. Although the SUMO fusion system has not been widely used for recombinant production of antimicrobial peptides, it certainly offers an attractive alternative.

Besides the toxicity issue and other technical challenges, the high cost of manufacturing peptides is another obstacle to the wide application of antimicrobial peptides. The typical cost of peptides ranges between \$100 and \$600 per gram, which is much higher than that of conventional antibiotics [2]. Fusion expression in bacteria is so far the most successful approach among many others that have been made to reduce the cost. However, the current cost, though lower than that of chemical synthesis, remains too high to be commercially acceptable. One factor that limits the success of fusion expression might be the lack of a carrier specifically designed for peptides. The current systems, as reviewed in this article, are all for the general purpose of recombinant protein production. In this regard, tandem repeats, a special type of fusion, is an exception. However, protein expression levels in tandem design are usually not proportional to the degree of multimerization and it does not necessarily guarantee a better peptide yield than normal fusions [105,106]. Vassilevski *et al.* recently suggested that many short modules with helper function await their discovery [14]. Thus, novel systems based on these modules that are specialized for peptides, combined with optimized expression and/or cultivation (e.g. new cell lines and high cell density cultivation), may move the yield to the next level in the future.

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