Intein-mediated expression of cecropin in *Escherichia coli*

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Abstract Different strategies have been used to overcome the difficulties to produce antimicrobial peptides. Here we used Intein Mediated Purification with an Affinity Chitin-binding Tag (IMPACT-System, New England Biolabs) for the expression of the antimicrobial peptide cecropin to reduce its sensitivity to intracellular proteases and use its inducible self-cleaving capability to remove the carrier. Cecropin was cloned into suitable expression vector pTYB11, and expression induced by IPTG in *Escherichia coli* ER2566. The use of 22ºC induction allowed the expression of cecropin with its intein carrier in soluble form. Cell extracts were purified by chitin affinity chromatography and intein-mediated splicing of the target protein was achieved by thiol addition, obtaining a final yield of 2.5 mg cecropin/l. Cecropin cleaved from the intein had its proper biologically active form, showing a micromolar antimicrobial activity against *Vibrio ordalii*, *Vibrio alginolyticus* and *Escherichia coli.*

Keywords: antimicrobial, cecropin, fusion, intein, peptide, soluble

INTRODUCTION

Antimicrobial peptides (AMPs) are an essential component of innate immunity and play an important role in host defence against microbial pathogens (Zasloff, 2002). They have received increasing attention to become an alternative to antibiotics. Cecropin A from *Drosophila melanogaster* is a polycationic peptide that allows the interaction with the microbial membrane, and a large hydrophobic tail that induces lysis by altering membrane permeability (Silvestro et al. 1997). This peptide presents a wide range of activity against both gram positive and negative bacteria at micromolar concentrations (Andreu et al. 1983; Boman, 1998; Zasloff, 2002). Chemical synthesis of antimicrobial peptides is an alternative but its high cost makes it prohibitive (Ingham et al. 2005). The biosynthesis of recombinant antimicrobial peptides appears to be the most viable form of preparing polypeptides. However a common problem is the toxicity and proteolytic degradation of an expressed antimicrobial peptide. There are numerous reports of expression of antimicrobial peptides in *E. coli* with different success (Kim et al. 2006; Wang and Cai, 2007; Xu et al. 2007; Zhang et al. 2009; Shen et al. 2010). The production of big quantities of protein should be possible if the host is not sensible to the effects of the expressed peptide. The expression of foreign genes in *E. coli* often leads to densely packed denatured peptide molecules in the form of inclusion bodies (Tsumoto et al. 2003). To manage the expression in bacteria it's generally necessary to express antimicrobial peptides in the form of a peptide fusion to block the antimicrobial activity of the peptide (Morassutti et al. 2005; Li, 2009). This is important so the recombinant peptide is not toxic to the host, something that affects its viability, expression and the degradation of the protein (Li, 2011). Different fusion partners have been used to improve yield of antimicrobial peptides. Anionic peptide increased expression and efficiently masked lactoferricin's

toxicity by neutralizing its positive charge (Kim et al. 2006). Thioredoxin (Li et al. 2006), glutathione transferase (GST) (Liang et al. 2006) and Small Ubiquitin-related Modifier (SUMO) (Bommarius et al. 2010) improved expression and increased the solubility of fusion proteins. Despite its success in many applications, GST fusions were found highly susceptible to proteolytic degradation in several cases, causing inefficient or failed peptide production (Skosyrev et al. 2003). Thioredoxin is also more favourable than GST for peptide production as its small size allows the target to constitute a relatively large percentage of the fusion, but is not an affinity tag as GST (Waugh, 2005). The existence of highly specific SUMO protease facilitates efficient release of the passenger protein/peptide, offering a unique advantage to this system (Li, 2011). Cellulose binding domain allowed the expression and purification of LL37 (Ramos et al. 2010) and other AMPs (Guerreiro et al. 2008). Cellulose is cheap and available in many forms. Therefore, the expression system using the cellulose binding domain as the expression and purification module is very attractive. The problem with fusion partners is to separate them from the peptide to recover activity, a process that generally needs chemical cleavage or enzymatic cleavage adding to the expense and complexity of the system (Diao et al. 2007). In this sense inteins have become an attractive alternative. Inteins are protein splicing elements with the ability to induce their own excision from the precursor polypeptide and rejoin the remaining portions (exteins) with a peptide bond (Perler et al. 1994). Complete splicing of the intein can be inhibited by mutation of conserved residues at the splice junction fused to the affinity tag. This results in site-specific cleavage only at the intein-target protein border (Elleuche and Pöggeler, 2010). The inteins used for bioseparations are modified versions that can selectively undergo N- or C terminal cleavage when induced by thiol reagents like DTT or pH and/or temperature shift (Xu and Evans, 2003). The use of inteins eliminates the need for exogenous proteases or chemicals that are usually required for tag removal. Inteins are usually used in combination with chitin-binding domain (CBD) affinity tag, which allows the fusion proteins to be isolated by chitin resin through a relatively simple procedure (Li, 2011). Recently intein-mediated expression of antimicrobial peptides like bacteriocins (Ingham et al. 2005), β-defensins (Diao et al. 2007) and moricin CM4 (Shen et al. 2010) has been successfully used.

Based on these observations the aim of this study was to develop a system for cloning and producing active recombinant cecropin with a good yield without the need of exogenous enzymes or refolding using a self-cleaving carrier Intein based on the mediated Purification with an Affinity Chitin-binding Tag (IMPACT-System, NEB).

MATERIALS AND METHODS

Bacterial strains and growth conditions

Escherichia coli TOP10 (Invitrogen) was routinely used as the host for propagation and maintenance. *E. coli* ER2566 (New England BioLabs) was used as the host strain for protein expression. *E. coli* TOP10 and ER2566 were grown at 37ºC in Luria-Bertani (LB) broth or agar, and 100 µg ampicillin/ml was added to the media when TOP10 and ER2566 were transformed with the expression plasmids. *Vibrio ordalii* (ATCC 33509), *Vibrio alginolyticus* (ATCC 19108) and *E. coli* (ATCC 11303) were grown in Trypticase Soy Broth (TSB). Vibrio was grown at 26ºC and *E. coli* at 37ºC.

Cloning and transformation

The mature coding sequence of cecropin A1, from GenBank accession number X16972.1 (a kind gift from Dr. Dan Hultmark) cloned in pET27 (Novagen) was used as template for PCR and subcloning in the expression vector pTYB11 (New England Biolabs IMPACT-System). In this plasmid the transcription of the cloned genes is controlled by the T7/lac promoter. The use of pTYB11 allowed the coding sequence of cecropin to be put in frame with the coding sequence of the intein Sce VMA (New England Biolabs IMPACT-System). The PCR was done using a platinum Taq polymerase (Invitrogen) using the following conditions: Incubation at 94ºC for 10 min, 30 cycles at 94ºC for 30 sec, 65ºC for 30 sec, 72ºC for 45 sec and a final incubation at 72ºC for 10 min, using the primers 5' (5'-GGTGGTGCTCTTCCAACGGGTGGCTGAAGAAAA-3') with a SapI restriction site and the primer 3' (5'-GGTGGTGAATTCTCATCAACCTCGGGCAGTTGCG-3') with a EcoRI restriction site. The amplicon was analyzed by electrophoresis in agarose 2% and precipitated by standard methods, digested with the restriction enzymes SapI and EcoRI and was ligated to pTYB11 previously digested with SapI and EcoRI. Ligation mixture was used to transform competent TOP10 cells (Invitrogen). Plasmid DNA was isolated by miniprep kit (Qiagen). Plasmid was sequenced to confirm the identity of the constructs.

Expression plasmid was later used to transform chemical competent ER2566 cells (New England BioLabs).
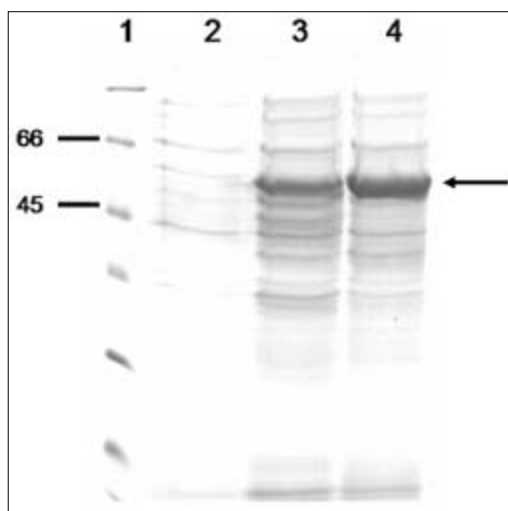
Fig. 1 Expression profile of pTYB11-cecropin in ER2566 cells. SDS-PAGE stained with coomassie blue. Lane 1: Molecular weight marker (Bio-Rad) (kDa); Lane 2: 3 hrs culture before IPTG induction; Lane 3: Culture without IPTG after additional 16 hrs at 22ºC; Lane 4: Culture with 0.3 mM IPTG for 16 hrs at 22ºC. The cecropin-intein carrier in shown by an arrow.

Expression and recovery of peptide

For the expression *E. coli* ER2566 pTYB11/cecropin a transformation colony was used to inoculate 4 ml LB Broth with 100 µg ampicillin/ml at 37ºC for 16 hrs. This culture was used to inoculate 400 ml LB Broth with antibiotic and incubated for 3-4 hrs until an OD_{600} 0.5. The induction of the expression was done with 0.3 mM IPTG final concentration and incubated at 22ºC for 16 hrs until sampling. The bacterial culture was subjected to centrifugation at 5000 g x 10 min at 4ºC and separated into cell pellet and media. Cell pellet was treated with 25 ml lysis buffer (20 mM Tris-HCl, 500 mM NaCl, 0.1%, Triton X-100, 20 µM protease inhibitor PMSF and 20 µg lysozyme/ml, pH 8), and incubated at 4ºC for 1 hr and then subject to centrifugation at 11000 g x 30 min at 4ºC, recovering the supernatant or soluble fraction. Samples of bacterial cultures and recovered soluble fraction were subjected to 12% and 18% acrylamide SDS-PAGE respectively to verify expression and recovery of the peptide.

Cleavage and purification of peptides by affinity chromatography

Supernatant fraction was purified trough 6 ml of chitin columns (New England BioLabs) previously equilibrated with column buffer (20 mM Tris-HCl, 500 mM NaCl, 1 mM EDTA, pH 8). Column was charged with the clarified extract with a 0.5 ml/min flow and then washed with 60 ml column buffer with a 1-2 ml/ min flow. A sample of the chitin column before cleavage was stored for western blot. For the intein cleavage a quick flow of 18 ml cleavage buffer (20 mM Tris-HCl, 500 mM NaCl, 1 mM EDTA, 30 mM DTT, pH 9) was followed by incubation at 18ºC for 40 hrs. A sample of the chitin column after cleavage was subjected to 18% acrylamide SDS-PAGE and stained by coomassie blue R-250 to confirm the identity of the peptide and to verify cleavage efficiency. The peptide was eluted with 18 ml column buffer and fractions recollected for further purification by hydrophobic interaction chromatography and reversed-phase HPLC.

Western blot of cecropin-intein carrier

To confirm the nature of cecropin-intein carrier Sce VMA a sample of the chitin column before cleavage was separated by SDS-PAGE and transferred to a membrane of poli-vinylidene fluoride (PVDF) (Inmobilon P, Millipore), previously treated with methanol, through semi-dry blotting. This membrane

was blocked with 3% bovine serum albumin (BSA) for 1 hr and then washed with PBS. The membrane was subject to hybridization with a rabbit primary antibody specific to the chitin binding domain (New England Biolabs) and washed 3 x with 0.05% tween 20 in PBS to eliminate the antibody not bound to antigen. The membrane was subject to a mouse anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP) (Pierce) that has affinity for the primary antibody and washed 3 x with tween 20 0.05% in PBS. For the visual detection 3,3' diaminobenzidine (DAB) (Pierce) was used. To exclude the possibility that the expressed protein was part of *E. coli* ER2566 and not by the intein-mediated strategy a rabbit primary antibody was produced by immunization of rabbits with *E. coli* expression strain ER2566 whole cell lysate without pTYB11/cecropin. A sample of the chitin column was subjected to immunoblotting as previously described for intein Sce VMA.
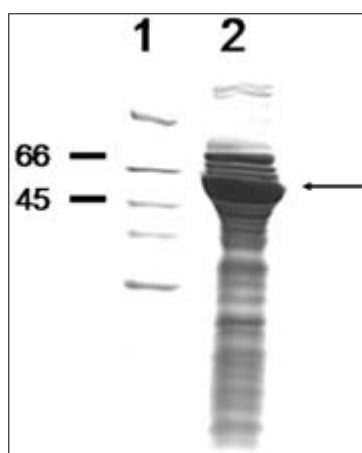

Fig. 2 Purification and recovery of peptides from cellular extract. SDS-PAGE stained with coomassie blue. Lane 1: Molecular weight marker (Bio-Rad) (kDa); Lane 2: Soluble fraction of crude protein extract of IPTG induced pTYB11-cecropin ER2566 cells. The cecropin-intein carrier in shown by an arrow.

**Purification of peptides by reversed-phase HPLC**

Fractions recollected from affinity chromatography were purified further by hydrophobic interaction chromatography in Sep-Pak C-18 columns previously equilibrated with acidified water (0.05% trifluoroacetic acid or TFA in ultrapure water or UPW), washed with acidified water and eluted with 5, 40 and 80% acetonitrile (ACN). Eluted fractions were lyophilized and reconstituted in UPW. Additional purification was done through reversed-phase HPLC (RP-PHPLC) model LaChrom D-7000. The 40 and 80% Sep-Pak eluted fractions were loaded onto a sephasil C-18 (250 x 4.1 mm) (LiChroCART). The peptide was eluted with a linear gradient of ACN (5-60%) with acidified water 0.1% TFA for 90 min with a 0.6 ml/min flow. Fractions were recollected, lyophilized and reconstituted in UPW and stored at -20ºC until antimicrobial assay. A sample of eluate from chromatogram peak was resolved through Tris-tricine/Urea (18%) gel electrophoresis to confirm recovery. Total proteins were determined trough the bicinchoninic acid assay (BCA).

Antibacterial activity tests

Antimicrobial activity of the peptides was monitored by liquid growth inhibition assay (Mitta et al. 1999). Ten microliters of serial dilutions of the peptide (0.17-2.16 µM) was mixed with 100 µl of an exponential phase culture of bacteria (OD 0.2-0.3 with 95% of viable cells). The test was performed at a starting OD 0.001 at 620 nm in the specific broth. After 24 hrs of incubation, absorbance values were measured and percentage growth inhibition determined IC_{50} values. For antibacterial tests the following strains were used: *V. ordalii* (ATCC 33509), *V. alginolyticus* (ATCC 19108) and *E. coli* (ATCC 11303).
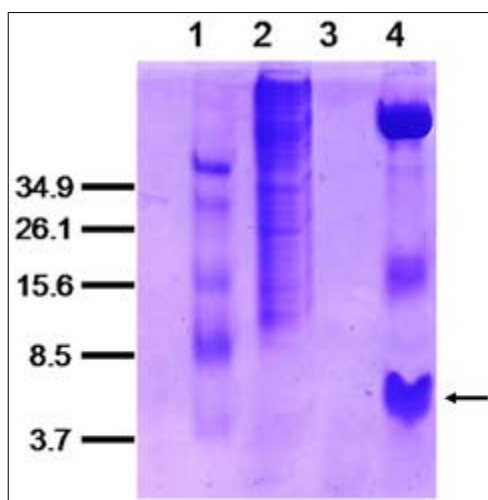
Fig. 3 Purification of cecropin from chitin beds. SDS-PAGE stained with coomassie blue. Lane 1: Molecular weight marker (Bio-Rad) (kDa); Lane 2: First flow through wash of chitin column; Lane 3: Fifth flow through wash of chitin column; Lane 4: Chitin beads after cleavage of cecropin from intein with 30 mM DTT. Released cecropin in shown by an arrow.
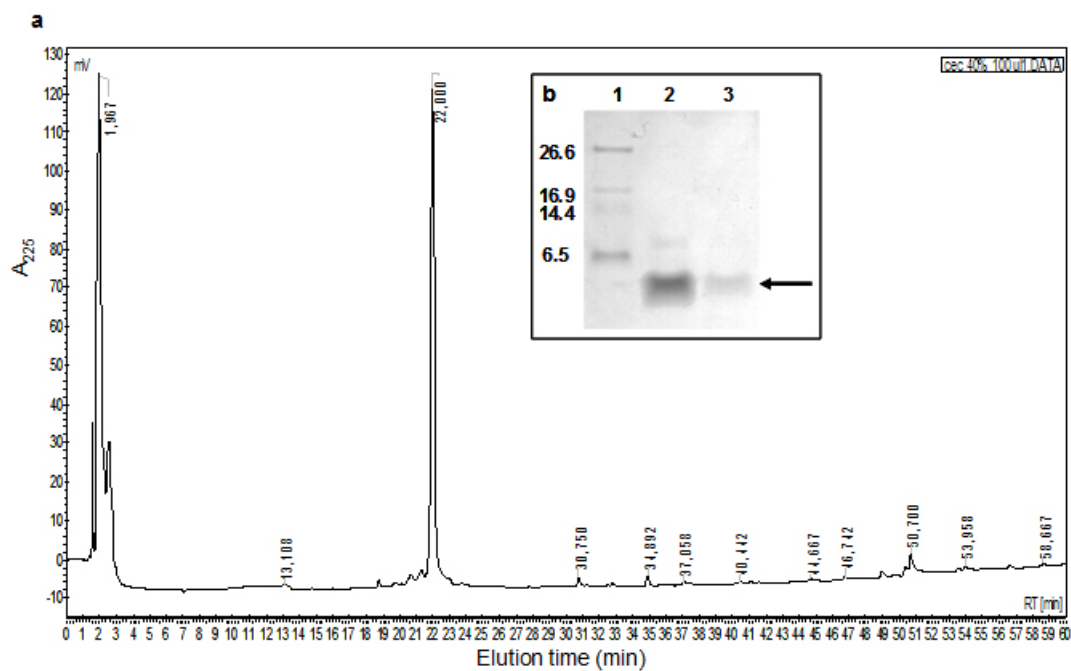
Fig. 4 Purification of cecropin by RP-HPLC. (a) Elution profile by RP-HPLC of the 40% ACN post-Sep-Pak C-18 using a linear gradient of 5-60% CAN; (b) Protein profile resolved through Tris-tricine/Urea (18%) gel electrophoresis is shown. Lane 1: Molecular Weight Standards (Bio-Rad) (kDa); Lane 2: Eluate from Sep-Pak C-18 solid phase chromatography; Lane 3: Eluate from chromatogram peak with a retention time of 22.00 min corresponding to cecropin (shown by an arrow).
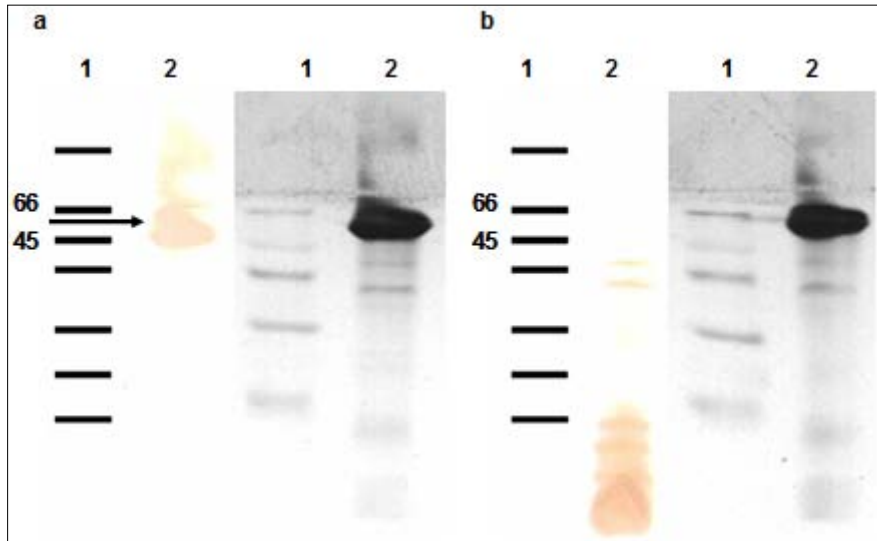
Fig. 5 Western blot of cecropin-intein carrier. (a) Immunoblotting with anti-CBD antibody; (b) Immunoblotting with anti-*E. coli* ER2566 cell lysate antibody. Lane 1: Molecular weight marker (Bio-Rad) (kDa); Lane 2: Chitin beds with cecropin-intein carrier before cleavage with DTT. Membrane was revealed with diaminobenzidine. SDS-PAGE used for immunoblotting in sections a and b are shown as reference to the right of revealed membranes. The cecropin-intein carrier detection with anti-CBD antibody in shown by an arrow in section a.

RESULTS AND DISCUSSION

In this work the expression and recovery of active cecropin without the need of enzymatic cleavage from its carrier was achieved for first time using the intein based system. For industrial applications, the removal of the affinity tag by endoproteases is the most costly step in protein production, and can interfere with the biological activity of the purified component (Wood et al. 2005). Restriction endonuclease digestion and DNA sequencing confirmed that the plasmid pTYB11/cecropin was successfully constructed. The plasmid pTYB11/cecropin was transformed into *E. coli* ER2566 and PCR positive clones were used to express the cecropin-intein carrier. After 16 hrs of induction with 0.3 mM IPTG at 22ºC, recombinant proteins were analyzed by SDS-PAGE (Figure 1). A prominent protein band at 59.2 kDa compatible with the 55 kDa from intein-CBD plus 4.2 kDa from cecropin was observed after induction with IPTG, although some leaky expression was obtained even without the inductor (Figure 1). After the cell lyses and recovery of the supernatant fraction it was shown that cecropin-intein carrier was soluble (Figure 2). The intein was designed to block the antimicrobial activity of cecropin against host cells producing it. This feature could be important to express the peptide in the soluble fraction, compared to results obtained with analogs of cecropin without fusion protein obtained in inclusion bodies (Schmitt et al. 2008). The induction of expression of cecropin in the soluble fraction of *E. coli* was carried out at 22ºC, because at 37ºC the peptide accumulated in the insoluble fraction (data not shown). Evidence has shown that growth at 37ºC can cause the accumulation of certain proteins to inclusion bodies (IB), while the decrease in temperature can bring them to its soluble and active form (Xu et al. 2006). IB proteins are devoid of biological activity, so obtaining the proteins soluble avoids the solubilization and refolding procedures needed to recover a functionally active product (Vallejo and Rinas, 2004). The cecropin-intein carrier was purified by affinity chromatography. Protein extracts from the soluble fraction was applied to chitin columns, where most of the protein was bound to the chitin beads. The data, presented in Figure 3, demonstrate that cecropin-intein carrier was purified by chitin beads from most *E. coli* contaminants. In the chitin beads the intein-mediated self splicing reaction was induced by adding 30 mM DTT as nucleophilic agent, at 18ºC for 40 hrs. No exogenous proteolitic cleavage was needed. A small peptide migrated somewhat slower than expected from the calculated molecular weight of cecropin (4.2 kDa). Small peptides often fail to obey the standard relationship between mass and mobility in SDS gels (Huang and Mathews, 1990). This peptide was released and eluted from the column while the intein-chitin binding domain (CBD) remained bond to the chitin beads. The cleavage of the cecropin-intein carrier was not completely efficient, but never the less allowed obtaining an appreciable amount of cecropin free from

the intein carrier (Figure 3). The small difference in the size of the intein carrier before and after cleavage of cecropin makes the estimation of cleavage efficiency more difficult. After the purification using chitin beads cecropin was desalted and DTT was eliminated by Sep-Pak solid phase chromatography column and recovered in the 40% ACN fraction (Figure 4). Total protein was quantified by bicinchoninic acid assay obtaining a recovery of 2.5 mg cecropin/l (1.0 mg from 400 ml culture). This is in range with other studies using the intein mediated system like 4.2 mg ABP-CM4 (Chen et al. 2008), 1.1 mg piscicolin-126/l (Ingham et al. 2005) and 200 µg SMAP-29/l (Morassutti et al. 2005). Higher yields of cecropin have been accomplished using fusion carriers, but these involve the use of expensive enzymes, making them economically unfeasible. *Musca domestica* cecropin yield of 11.2 mg/l was obtained with thioredoxin in *E. coli* (Xu et al. 2007) and 30.6 mg/l cecropinAD was obtained with SUMO in *Bacillus subtilis* (Chen et al. 2009). Earlier reports involving expression of cecropin with GST and obelin as carriers on *E. coli* showed significant degradation, but success with green fluorescent protein (GFP) that accumulated at 0.5 mg/l in inclusion bodies (Skosyrev et al. 2003). Proteolisis occurred in obelin-cecropin fusion because only the N-terminal region of obelin remain to be used for fusion, leaving the N-terminal part of sarcotoxin exposed (Skosyrev et al. 2003). Additional studies with GST (Liang et al. 2006), lysozyme (Lu et al. 2010), GFP (Yu et al. 2010) and thioredoxin (Kang et al. 2008) have been reported.

The intein carrier was much larger (55.0 kDa) than our target peptide (4.2 kDa) decreasing the

Table 1. Antimicrobial profile of cecropin for different bacteria.

Bacterial species	MIC_{50} (µM)[a]
Vibrio ordalii	1.08
Vibrio alginolyticus	1.08
Escherichia coli	1.08

[a] Aliquots of 100 µl of each overnight culture of the strains diluted in TBS medium to OD_{620} of 0.001 were transferred into the wells of the microplate. Serial dilutions of purified cecropin (0.17-2.16 µM) were added to each well to a final volume of 110 µl. Plates were incubated at 26ºC for *Vibrio* and 37ºC for *E. coli* for 24 hrs and the absorbance at OD_{620} for each well was recorded. Minimum concentrations to inhibit 50% of culture growth (MIC_{50}) are shown for each species of bacteria, and represent the lowest concentration that causes 50% decrease in optical density of microorganism suspension.

cecropin mass percentage in the cecropin-intein carrier. One of the drawbacks for peptide yield is the large size of the Sce VMA intein/chitin domain, although recently smaller intein carriers like the Ssp dnaB derived mini-intein are beginning to be used (Esipov et al. 2008). The heterologous nature of cecropin-intein carrier was confirmed by its affinity against antibody specific for the CBD of the intein, while the rest of the proteins of the bacterial extract reacted with antibodies directed against *E. coli* ER2566 whole cell lysate, but not against the cecropin-intein carrier. In this way the possibility that the expressed protein visualized in Figure 1 was part of *E. coli* ER2566 and not by heterologos expression was excluded (Figure 5). A second purification step through RP-HPLC was performed to assure fine resolution, obtaining a single symmetrical peak with a retention time of 22.00 min (Figure 4). The recovery of the peptide was confirmed by the analysis of the eluate from chromatogram peak that showed a clean band similar in size to cecropin (Figure 4). The recovered cecropin was in its active form as shown by its antimicrobial activity against different gram negative bacteria in the micromolar range (Table 1). This way the activity of 1.08 µM against *E. coli, Vibrio ordalii* and *Vibrio alginolyticus* was comparable to the activity obtained by other studies involving cecropin. Reports of antimicrobial activity against *E. coli* range from 0.5-0.9 µM for cecropin AD (Chen et al. 2009) and cecropin A (Silvestro et al. 2000). Activity for cecropin A against *Vibrio ordalii* and *Vibrio alginolyticus* was 2.06 µM (Díaz et al. 2008; Schmitt et al. 2008) and 0.98-7.81 µM for cecropin against Vibrio (Ho et al. 2002). The choice of pTYB11 for cloning and expression allowed the intein fusion to the N-terminus of cecropin. This feature allows the protection of positive charged amino acids located in this section of cecropin that are vital for its antimicrobial activity and also more prone to degradation (Okemoto et al. 2002; Skosyrev et al. 2003). In this way the peptide could be protected in a similar way to what occurs with the pro peptide from cecropin *in vivo* (Zasloff, 2002). One of the drawbacks is that uncontrolled autocleavage of the intein fusions could cause problem as the released antimicrobial peptides can cause fatal effects through direct interaction with their intracellular targets (Nicolas, 2009). An advantage of the intein-mediated system is that target proteins with native N-terminus can be generated upon cleavage of intein, feature that can be important for the activity of the recovered peptide. This compared with other expression systems where sometimes this is not possible, and

additional amino acids must be added. Inefficient cleavage to free target peptide from fusion carriers like thioredoxin and GST, by endoproteases like factor Xa or enterokinase, has been observed in several studies (Beaulieu et al. 2007; Lee et al. 2008). Among the causes of inefficient cleavage by these enzymes are sensibility to pH and chaotropes (Li, 2011). The removal of the affinity tag by endoproteases is the most costly step in protein production. Therefore intein-mediated bioseparation has become an excellent vehicle for affinity-tag-based protein purification techniques, and is an alternative to conventional cleavage by site-specific endoproteases (Elleuche and Pöggeler, 2010). Our results show that the intein carrier can be used successfully to express cecropin *in E. coli*. The use of this technology using a self-splicing carrier of low production costs and simplified protein purification allowed us to recover the peptide with intact antimicrobial activity from chitin beads. This study gives additional evidence that the IMPACT System could be considered a promising strategy for the production of different antimicrobial peptides in *E. coli*.

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