Protein Expression and Purification 71 (2010) 1–7

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Escherichia coli expression and purification of LL37 fused to a family III carbohydrate-binding module from *Clostridium thermocellum*

Reinaldo Ramos, Lucília Domingues, Miguel Gama *

IBB – Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal

ARTICLE INFO

Article history:
Received 8 September 2009
and in revised form 20 October 2009
Available online 31 October 2009

Keywords:
Antimicrobial peptide
LL37
CBM3
Cellulose
Formic acid

ABSTRACT

The cathelicidin derived human peptide LL37 has a broad spectrum of antimicrobial and immunomodulatory activities. The large variety of biological activities makes LL37 a very promising candidate for clinical applications. The production of biologically active LL37 in large amounts with reduced costs can only be achieved using recombinant techniques. In this work, LL37 has been cloned to the N- and C-termini of a family III carbohydrate-binding module fused to the linker sequence (LK-CBM3) from *Clostridium thermocellum;* both constructions (LL37-LK-CBM3 and LK-CBM3-LL37) were cloned into the pET-21a vector. A formic acid recognition site was introduced between the two modules, allowing the isolation of LL37 after chemical cleavage.

The recombinant proteins were expressed in *Escherichia coli* BL21 (DE3) and solubilized with Triton X-100. The purification was achieved using cellulose CF11 fibers, taking advantage of the CBM3 specific affinity for cellulose; after hydrolysis with formic acid, LL37 was further purified by reverse-phase HPLC, as confirmed by MALDI-TOF mass spectrometry. The production and purification methodology developed in this work compares advantageously to other protocols previously described, having fewer purification steps. Only the recombinant LL37 obtained from the C-terminally fused protein (LK-CBM3-LL37) showed antibacterial activity against *E. coli* K12, with a MIC of 180 μg/ml.

Introduction

The increasing resistance of microorganisms against common antibiotics has become a growing threat for the public health. Antimicrobial peptides (AMPs)[1] are part of the innate immune system and have a large spectrum of antimicrobial activities against Gram-positive and Gram-negative bacteria, fungi [1] and viruses [2]. AMPs are generally defined as cationic, amphipathic peptides, with less than 50 amino acids, including multiple arginine and lysine residues [3] and show inhibitory concentrations (MIC) as low as 0.25–4 μg/ml [2]. They are widely distributed in nature, from insects to plants and animal species; over 1440 AMPs have been identified so far (http://aps.unmc.edu/AP/main.php; July 2009).

In mammals, defensins and cathelicidins represent the two major types of AMPs. Cathelicidins share a highly conserved N-terminal cathelin domain, flanked by a rather variable antimicrobial peptide on the C-terminus [4]. The hCAP-18/LL37 is the only human cathelicidin. The antimicrobial peptide is referred to as LL37, since it has a 37 amino acids sequence starting with two leucines. It is a 4.5 kDa, cationic (+6), amphipathic α-helical peptide, with a broad spectrum of antimicrobial activity. LL37 and the precursor protein, hCAP-18, can be found at different concentrations in many different cells, tissues and body fluids. Durr et al. [5] summarized the various tissues where LL37 expression has been detected. Besides its protective effect against infections, a variety of other biological activities have been described. In fact, LL37 induces chemotaxis of mast cells [6], monocytes, T lymphocytes and neutrophils [7], promotes wound healing [8], angiogenesis and arteriogenesis [9].

Many bacterial and fungal enzymes that hydrolyse insoluble carbohydrates share a common structure composed of a catalytic domain linked to a carbohydrate-binding module (CBM). CBMs that are specific for insoluble cellulose (cellulose binding domain – CBD) represent the predominant category. The CBMs can be grouped into distinctive families on the basis of amino acid sequence similarities [10]; 53 families of CBMs have been recognized so far (http://www.cazy.org/fam/acc_CBM.html; July 2009).

Family III CBDs normally comprise ~150 amino acids residues, have been identified in many different bacterial enzymes, and also in non-hydrolytic proteins [11]. *Clostridium thermocellum* produces a multi-enzyme complex of cellulases and hemicellulases, termed the cellulosome, which is assembled by the scaffoldin protein CipA.

* Corresponding author. Fax: +351 253678986.
E-mail address: fmgama@deb.uminho.pt (M. Gama).

[1] *Abbreviations used:* AMPs, antimicrobial peptides; CBM, carbohydrate-binding module; CBD, cellulose binding domain; PCR, polymerase chain reaction; TFA, trifluoroacetic acid; MIC, minimal inhibitory concentration.

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doi:10.1016/j.pep.2009.10.016

Binding of the cellulosome to the plant cell wall is driven by the action of the CipA family 3 CBM (CBM3), which presents high affinity for crystalline cellulose [12]. CBM3 belongs to the all-β family of proteins and is arranged in two antiparallel β sheets that form a β sandwich with jellyroll topology [11].

In this work, we describe the successful cloning, expression and purification of LL37 using the CBM3 from *C. thermocellum* as fusion partner. The CBM3 is overexpressed in *Escherichia coli* and it is possible to take advantage of its affinity properties to purify recombinant proteins on cellulose fibers, reducing significantly the costs of purification.

Materials and methods

Construction of expression vectors

The gene encoding CBM3 fused to the endogenous CipA N-terminal linker sequence (LK) has previously been cloned in the expression vector pET21-a (Novagen), in our laboratory [12]. Here, two expression vectors were constructed with LL37, either at the N-terminus (LL37-LK-CBM3) or C-terminus (LK-CBM3-LL37) of the pET21-LK-CBM3 vector (Fig. 1).

The DNA fragment encoding LL37 was amplified by polymerase chain reaction (PCR) from hCAP-18/pET15b generously provided by Dr. Ole Sorensen, Lund University, Lund, Sweden. For the LL37-LK-CBM3 construction, the following primers were used: 5′-GGA ATTC **CAT ATG** CTG CTG GGT GAT TTC TTC-3′ (forward primer) and 5′-CTA **GCT AGC** *CGG ATC* GGA CTC TGT CCT GGG TAC-3′ (reverse primer). For the LK-CBM3-LL37 construction, the primers were: 5′-CCG **CTC GAG** *GAT CCG* CTG CTG GGT GAT TTC TTC-3′ (forward primer) and 5′-CCG **CTC GAG** TTA GGA CTC TGT CCT GGG TAC-3′ (reverse primer). *NdeI*, *NheI* (LL37-LK-CBM3) and *XhoI* (LK-CBM3-LL37) recognition sites are shown in bold. In order to allow the chemical cleavage with formic acid, proline and aspartate residues were introduced (in italic). PCRs were performed using the DNA Polymerase VENT (Stratagene). The PCRs were performed as follows: preheating at 95 °C for 2 min, 30 cycles at 95 °C for 30 s, 30 s at 52 °C and 30 s at 72 °C, followed by a final elongation stage at 72 °C for 10 min. The amplified products were recovered from 1% agarose gel, digested with *NdeI* and *NheI* or *XhoI* and ligated into pET21-LK-CBM3 plasmid and sequenced to ensure that no mutations had occurred during the PCR. Fig. 1 represents LL37-LK-CBM3 and LK-CBM3-LL37 constructions. The construction LL37-LK-CBM3 kept the C-terminal His6-tag even though this tag was not used for purification.

Expression and purification of fusion proteins

Fig. 2 summarizes the strategy followed for the production and purification of recombinant LL37. The recombinant plasmids were transformed into the *E. coli* strain BL21 (DE3) (Novagen) for protein expression. The bacterial strains were grown in Luria–Bertani broth (LB) containing 100 μg/ml of ampicillin at 37 °C to mid-exponential phase (OD_{595} = 0.6). Expression of the fusion proteins was initiated by adding isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM and the culture was incubated overnight at 37 °C. The cells were centrifuged at 6000 rpm for 15 min at 4 °C, resuspended in Tris–HCl pH 7.0, 20 mM NaCl, 5 mM $CaCl_2$(2-H_2O) buffer (CBM buffer) and sonicated in ice for 6 min. After centrifugation at 12,000 rpm, for 30 min at 4 °C, the cells were resuspended in CBM buffer with 1% Triton X-100. The cells were centrifuged again at 12,000 rpm, for 30 min at 4 °C and the soluble fraction was collected.

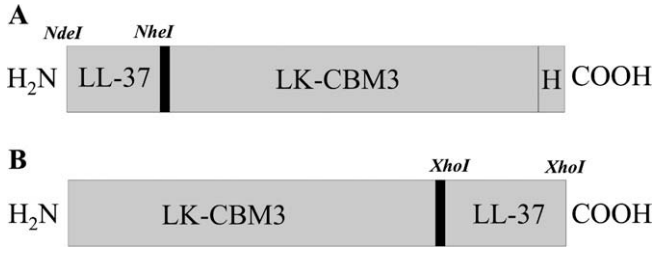

Fig. 1. Schematic representation of LL37-LK-CBM3 (A) and LK-CBM3-LL37 (B) constructions. The AspPro site for chemical cleavage with formic acid is shown in black; H, His-tag.

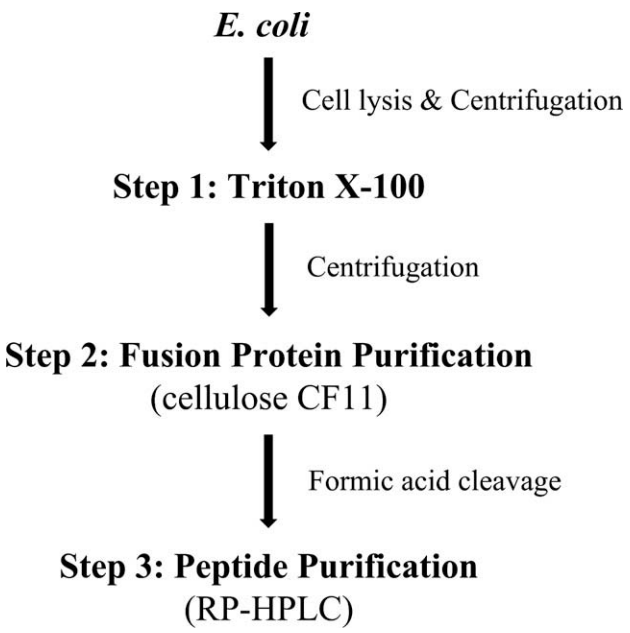

Fig. 2. Flow chart for the expression and purification of LL37 from *E. coli*.

The recombinant proteins were purified on cellulose CF11 (Sigma), exploiting the CBM3 cellulose-binding properties, as follows: 20 ml of cell-free extracts were mixed with 2 g of cellulose and incubated, with agitation, for 1 h at 4 °C. The cellulose with bound proteins was then centrifuged and washed five times with CBM buffer.

Formic acid cleavage and LL37 purification

In order to cleave LL37 of the recombinant protein, a 50% formic acid solution (20 ml of distilled water and 26 ml 88% formic acid) was applied directly to cellulose with the adsorbed fusion proteins. The mixture was then incubated for 24 h at 50 °C. The supernatant was separated after centrifugation at 12,000 rpm, for 30 min, and lyophilized to remove formic acid. The cleavage was confirmed by SDS–PAGE using a 16.5% Tris–Tricine gel.

The purification of LL37 was achieved by reverse-phase HPLC, using an YMC C18 preparative column (250 × 30 mm) equilibrated with aqueous acetonitrile (5%)/0.1% trifluoroacetic acid (TFA). The peptide was eluted using a linear gradient of acetonitrile from 5% to 90% at a flow rate of 5 ml/min. The elution was monitored at 215 nm. Several peaks were collected and analyzed by SDS–PAGE using a 16% Tris–Tricine gel. The fraction corresponding to pure LL37 was lyophilized. The concentration of the recombinant peptides was quantified using Waddell's method [13].

MALDI-TOF mass spectrometry

The purity of LL37 was confirmed by mass spectrometry at the Institute of Molecular Pathology and Immunology of the University

of Porto (IPATIMUP). Samples were desalted and concentrated using microC18 ZipTips (Millipore, USA) accordingly to the manufacturer's protocol. The samples were eluted with the matrix α-cyano-4-hydroxycinnamic acid (5 mg/mL) in 50% acetonitrile/0.1% TFA and spotted onto a stainless steel 192-well MALDI plate. After sample crystallization, mass spectrum acquisition was performed using a 4700 Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystems, USA). Mass spectra were acquired in positive linear mode for 1–15 kDa (m/z) mass window and in positive reflector mode for the ranges 700–7000, 4000–5000 and 4585–4600 Da (m/z). External calibration of the mass spectrum was performed using Applied Biosystems Calibration Mix 3 standards.

Antibacterial activity

The antibacterial activity of the pure peptides was quantified in 96-well polypropylene microtiter plates, as described by Vogt and Bechinger [14], with some modifications. *E. coli* strain K12 was grown at 37 °C in LB medium until mid-logarithmic phase (OD_{600} = 0.5). The cells were then resuspended in LB at 1×10^6 and 1×10^5 cells/ml; 100 μl of peptides, at different concentrations, were inoculated with 50 μl of bacterial suspension, incubated overnight at 37 °C and the bacterial growth was assessed by the measurement of optical density at 620 nm. The results were confirmed by plating 25 μl aliquots of the mixture from each well in LB plates and incubating for 16 h at 37 °C, followed by colony counting. Each assay was repeated three times and controls were made by adding sterile deionized water, instead of the peptide solutions. The minimal inhibitory concentration (MIC) was determined as the lowest peptide concentration that inhibited bacterial growth.

Results and discussion

Expression and purification of recombinant proteins

The protein LL37 was successfully cloned at both the N- and C-terminus of the LK-CBM3 in the expression vector pET21-a. After transformation of the recombinant plasmids into *E. coli* BL21 (DE3), several fermentation conditions were tested, attempting to optimize the production of the recombinant proteins in the soluble form. The following operational parameters and *E. coli* strains were varied or tested: concentration of IPTG, induction temperature and time, fermentation in M9 minimal medium, transformation into *E. coli* Tuner (DE3) and Origami (DE3) and co-expression with chaperones GroEL–GroES and *E. coli* trigger factor (TF) [15]. Only using the nonionic detergent, Triton X-100, the production of the proteins (~27 kDa) in the soluble form was achieved (Fig. 3).

In previous work, Guerreiro et al. [12] reported the expression of the same CBM3 from *C. thermocellum* used in this work. The CBM, fused or not with small antimicrobial peptides, was overexpressed in the soluble form. Only the protein LKLLKKLLKLLKKLGGGK-LK-CBM3 was found to be insoluble when expressed by different *E. coli* strains, under a range of induction conditions. In this work, the CBM3 fused to LL37 could only be solubilized with detergents. The use of CBM3 as fusion partner had two major goals: (1) to express and purify the peptide LL37 by a novel, fast and inexpensive method, using cellulose for purification and (2) to modify the surface of cotton fabrics with LL37, aiming at obtaining textiles with antibacterial and bioactive properties. As already stated, LL37 is an antimicrobial peptide but it also has a broad spectrum of biological activities. Thus, one main objective was to modify cellulose for biomedical applications. However, in this work, we focus on the production of the isolated LL37. For this purpose, the CBM is still useful,

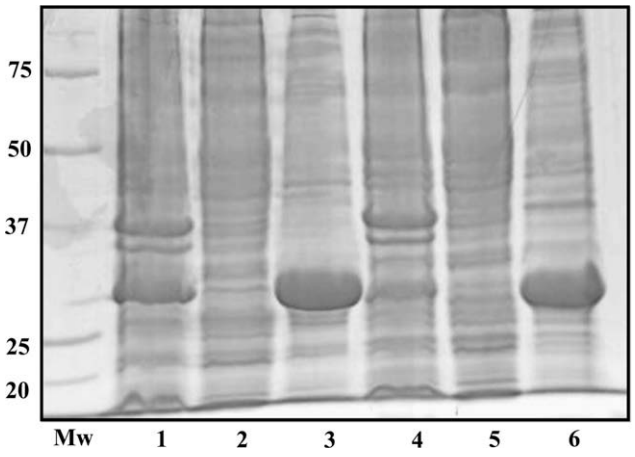

Fig. 3. Expression of recombinant proteins LL37-LK-CBM3 and LK-CBM3-LL37. M_w, protein molecular weight marker; 1, LL37-LK-CBM3 insoluble fraction; 2, LL37-LK-CBM3 soluble fraction; 3, LL37-LK-CBM3 Triton X-100 solubilized fraction; 4, LK-CBM3-LL37 insoluble fraction; 5, LK-CBM3-LL37 soluble fraction; 6, LK-CBM3-LL37 Triton X-100 solubilized fraction.

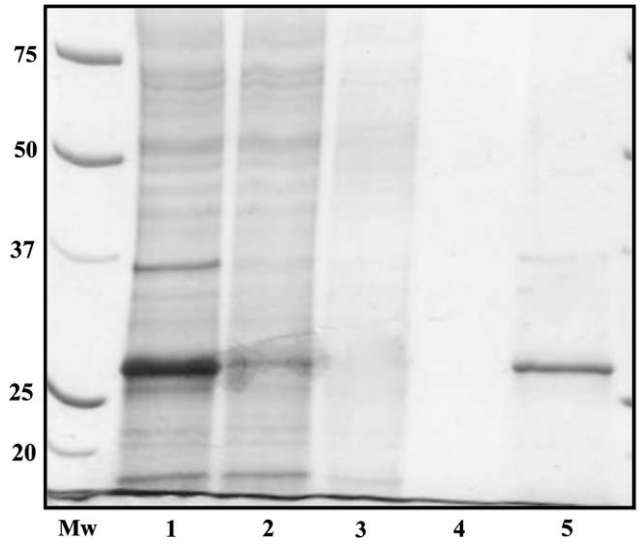

Fig. 4. Binding assay of LK-CBM3-LL37 on CF11 fibers. M_w, protein molecular weight marker; 1, Triton solubilized supernatant; 2, unbound protein; 3, first cellulose wash; 4, fifth cellulose wash; 5, bound protein eluted with 1% SDS.

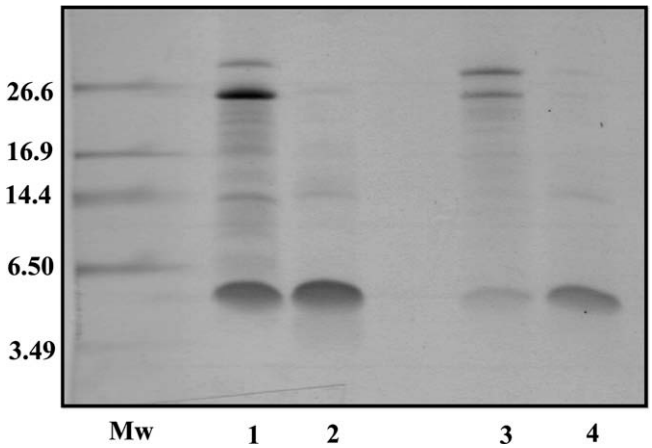

Fig. 5. Formic acid cleavage and release of LL37. M_w, protein molecular weight marker; 1, insoluble fraction of cleaved LL37-LK-CBM3; 2, supernatant of cleaved LL37-LK-CBM3; 3, insoluble fraction of cleaved LK-CBM3-LL37; 4, supernatant of cleaved LK-CBM3-LL37.

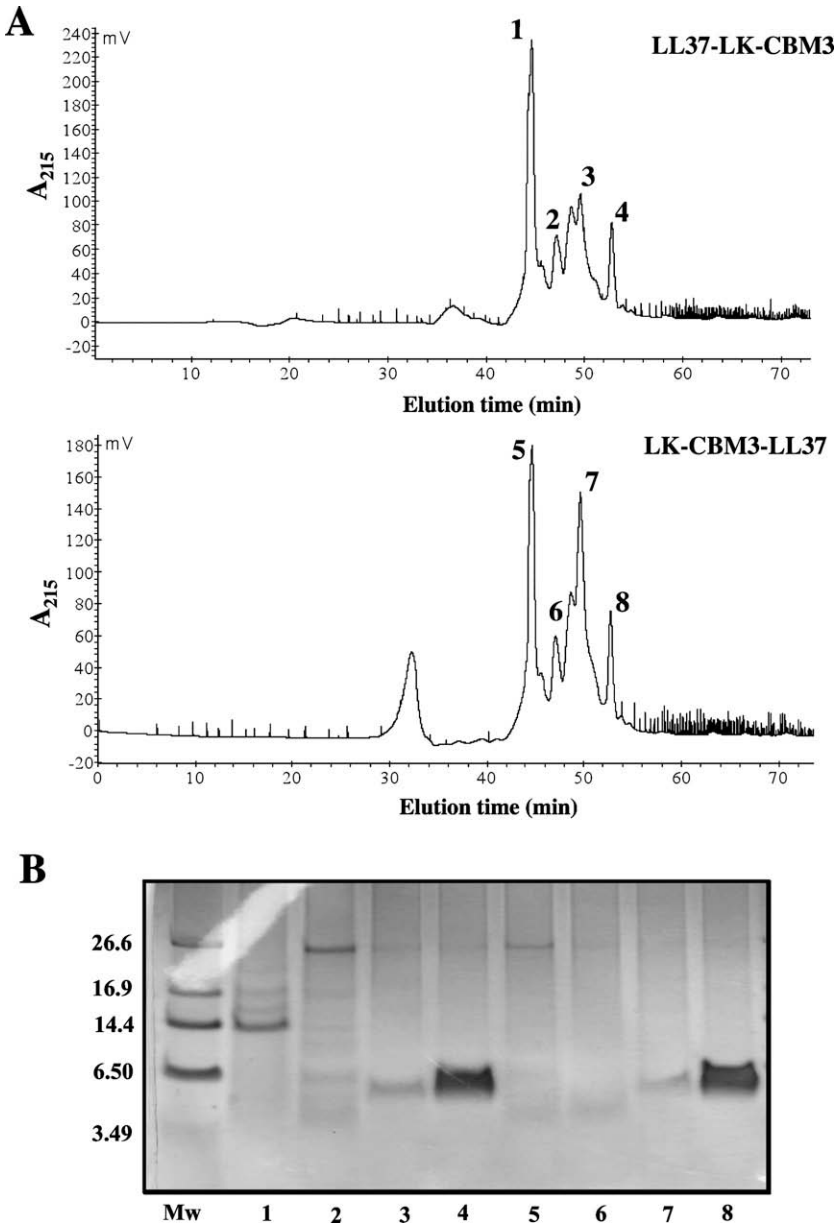

Fig. 6. (A) Reverse-phase HPLC chromatogram of fusion proteins LL37-LK-CBM3 and LK-CBM3-LL37. (B) RP-HPLC peak fractions. M_w, protein molecular weight marker; lanes 1–4, peak fractions of cleaved LL37-LK-CBM3; lanes 5–8, peak fractions of cleaved LK-CBM3-LL37.

allowing the separation of the recombinant protein through inexpensive affinity-based separation, using cellulose fibers.

The purification of the LL37-LK-CBM3 and LK-CBM3-LL37 recombinant proteins was performed on cellulose CF11 fibers. The family III CBM from *C. thermocellum* is well studied. This CBM adsorbs to cellulose with high affinity constants [16]. The fusion with LL37 and the presence of residual detergent did not affect the binding of the CBM3 on CF11 cellulose. Fig. 4 shows a binding assay of the LK-CBM3-LL37 recombinant protein, performed as described previously [12]. The recombinant proteins bind very efficiently to cellulose and only desorb using SDS.

To date, few groups have reported the recombinant expression of LL37 either in the *E. coli* expression system [17–19] or in the *Pichia pastoris* system [20]. In these publications, the purification of the recombinant proteins is always performed through affinity chromatography techniques, using expensive matrixes (sepharose, cobalt). Although very effective, chromatography is a slow process with high costs. LL37 is a peptide with a large potential for biomedical applications. Thus, the production of large amounts of LL37 at low costs is of great importance, purification being generally the most critical step in this regard. Cellulose is cheap and available in many forms. Therefore, the expression system using the CBM3 as the expression and purification module is very attractive. As shown previously [12] and in this work, the CBM3, when fused to antimicrobial peptides, is overexpressed in *E. coli* conserving its binding properties. The adsorption of CBM3 on cellulose is quite fast, allowing the recombinant proteins to be purified in a quick, inexpensive method. Moreover, no columns and chromatography units are required. Compared with previous publications, this method is clearly the best for large-scale purification of LL37.

Chemical cleavage and purification of LL37

Several methods, both enzymatic (factor Xa and thrombin proteases) and chemical (CNBr and formic acid) are widely used to cleave proteins from fusion partners. Formic acid is a good option

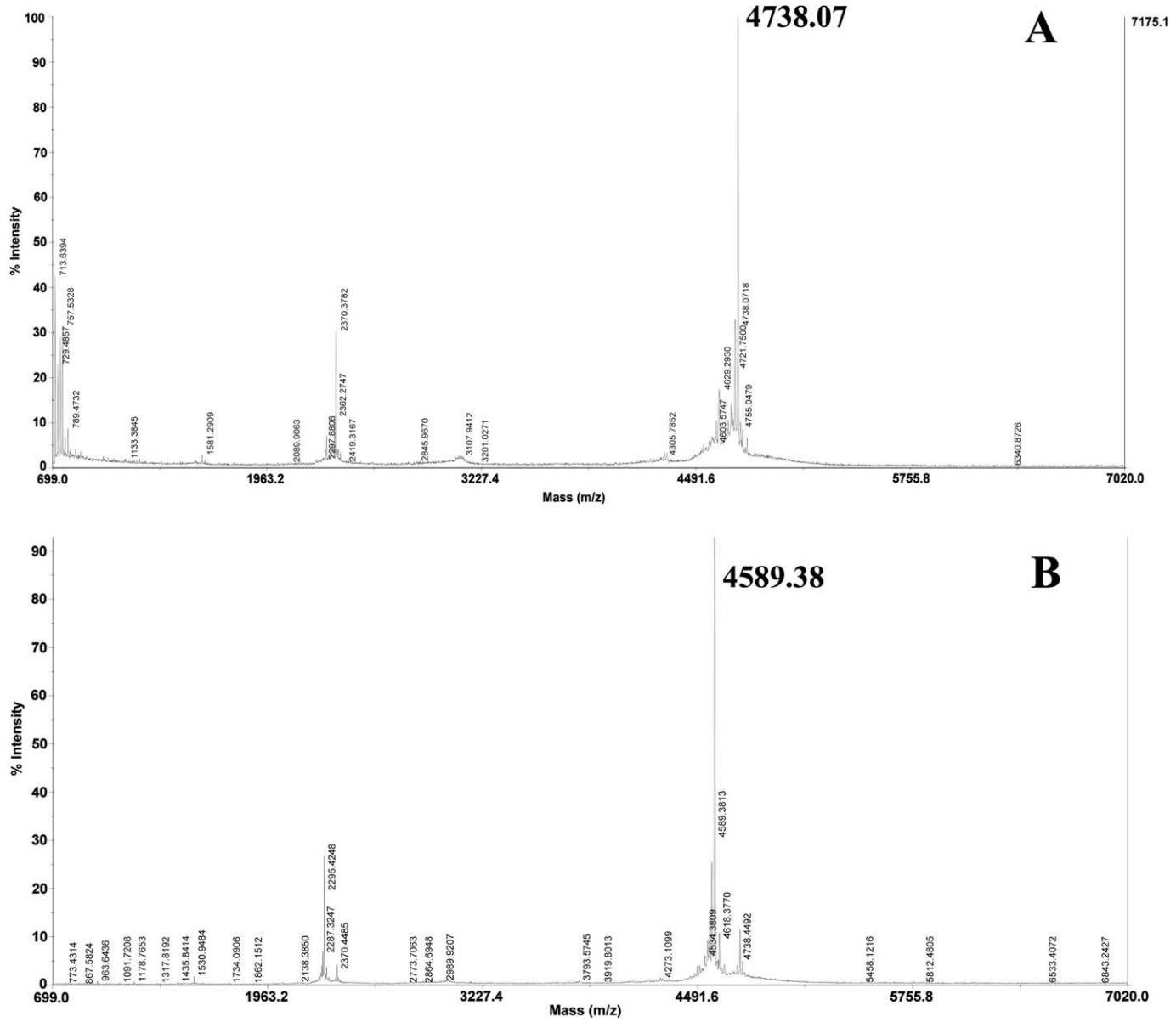

Fig. 7. MALDI-TOF MS analysis of purified LL37. (A) M-LL37-D. (B) P-LL37. The measured molecular weights are very similar to the theoretical values (4739.6 and 4590.4 Da).

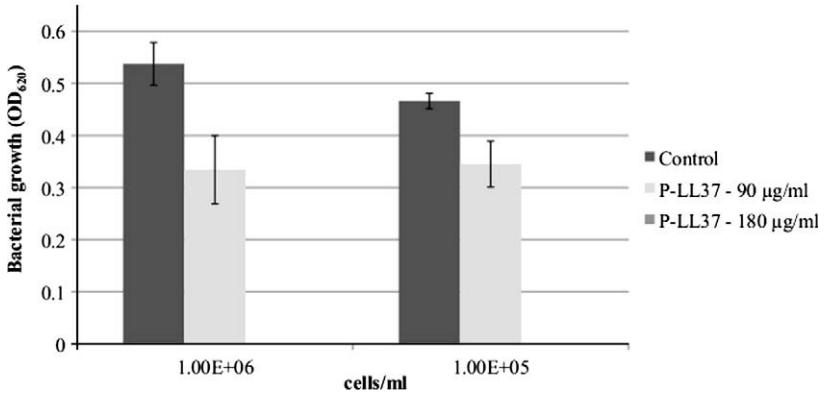

Fig. 8. Antibacterial activity of P-LL37 against *E. coli* K12. Bacterial growth was inhibited at a concentration of 180 µg/ml of P-LL37.

for large-scale production, due to its low cost. The chemical cleavage of the recombinant proteins was performed, in this work, with 50% formic acid, at 50 °C for 24 h. LL37-LK-CBM3 and LK-CBM3-LL37 proteins were first purified on cellulose. Then, formic acid was applied directly on the CF11 fibers with the bound protein. After centrifugation, the supernatants were lyophilized to

completely remove the acid and analyzed by 16.5% Tris–Tricine gel (Fig. 5). After acid hydrolysis and lyophilization, part of the protein (mostly CBM) precipitates, probably degraded by the formic acid. On the other hand, most of the peptide (~4.5 kDa) remains soluble, as shown in Fig. 5 (lanes 2 and 4). The precipitation of the CBM3 facilitates the purification. In fact, LL37 is separated from most of the CBM and other contaminating proteins by centrifugation. The soluble supernatants, with a large proportion of LL37, were then subjected to RP-HPLC to achieve higher purity (Fig. 6a). In this step, the fractions corresponding to the major peaks were collected and the purity of the peptides was checked on 16.5% Tris–Tricine gel and confirmed by MALDI-TOF. Fig. 6b presents the peak fractions from RP-HPLC for the two fusion proteins. Lanes 4 and 8 demonstrate the purity of the two peptides.

The two different proteins, both including the LL37, were obtained after the formic acid cleavage of the CBM constructs. The first peptide, obtained by the cleavage of the LL37-LK-CBM3 protein, has 39 amino acids with a theoretical molecular weight of 4739.6 Da. It is flanked with the N-terminal methionine, remaining from the translation process and a C-terminal aspartic acid, left by the chemical cleavage (M-LL37-D). Although 55–70% of mature proteins are subjected to N-terminal Met excision [21], M-LL37-D conserved the initial amino acid. Indeed, as demonstrated by Sherman et al. [22] most proteins retain the initial Met when the N-terminus residue is leucine. The second peptide, cleaved from the LK-CBM3-LL37 protein, is 38 amino acids long, with a theoretical molecular weight of 4590.4 Da, having a proline residue at the N-terminus (P-LL37). Mass spectrometry (Fig. 7) confirmed the purity of LL37 and the predicted molecular weights.

The production and purification of LL37 using CBM3 as the fusion partner only requires one chromatographic step. All previous publications refer to at least two, and use enzymes to cleave LL37. Li et al. [17,23] also use formic acid for chemical release of LL37, however the purification scheme used is more complex. In this work, it was possible to obtain 1 mg of pure P-LL37 and 2 mg of pure M-LL37-D from 1 L of bacterial culture. This yield is comparable to the ones obtained in previous studies [17–19]. Li et al. [23] also described a novel method to purify LL37 utilizing its property of aggregation. The authors were able to produce 2.6 mg of recombinant LL37 from 1 L of culture. However, this method required two cleavage steps with thrombin and formic acid and three purification steps.

Antibacterial activity

M-LL37-D and P-LL37 antibacterial activities were tested against *E. coli* K12. The concentration of the peptides was quantified by UV spectroscopy using the Waddell's method [13]. Fig. 8 demonstrates that P-LL37 has antimicrobial activity, with a MIC of about 180 μg/ml (40 μM), similar to the values found in the literature [5,17]. At lower concentration, P-LL37 inhibited bacterial growth only partially. The peptide (M-LL37-D) obtained from the N-terminal fusion construct (LL37-LK-CBM3) showed no antibacterial activity. According to Nagaoka et al. [24], the N-terminus and C-terminus of LL37 do not contribute to the formation of α-helical structures, being less important for its antimicrobial activity. However, the C-terminal aspartic acid reduces the global charge of the peptide. Furthermore, Giglione et al. [21] suggest that an N-terminal Met may act as a destabilization signal. This peptide has a total charge of +5, so the second hypothesis seems to be more likely. In fact, other AMPs with lower net charge still exhibit antibacterial activity. As already stated, recombinant LL37 has already been expressed and purified and *E. coli* but only two groups tested the antibacterial activity of the pure peptides. Yang et al. [18] produced the peptide GSLL-39 with a MIC of 12.5 μg/ml against *E. coli* DH5α and 25 μg/ml against *S. aureus;* Li et al. [17] obtained the peptide P-LL37 with a MIC of 180 μg/ml (40 μM) against *E. coli* K12. Durr et al. [5] report that the MIC of LL37 against *E. coli* and *S. aureus* is higher than 144 μg/ml (32 μM), similar to the P-LL37 expressed previously and in this work (Fig. 8).

Conclusions

LL37 has been extensively studied in recent years. The variety of immunological and antimicrobial activities makes LL37 a very promising candidate for clinical applications. For these purposes, high amounts of affordable LL37 can only be obtained using recombinant techniques. Major advances in solid-phase chemical synthesis of peptides, over the last five years have rendered the large-scale synthesis of long chain-length bioactive peptides possible, but this technique is too expensive for the production of large quantities of pure peptides with more than 30 amino acids. In this work, the family III CBM from *C. thermocellum* was used as fusion partner, taking advantage of its cellulose-binding properties for purification. The antibacterial assays showed that only P-LL37 was active. The methodology described in this work appears to be very effective for production and purification of AMPs. The use of cellulose for purification and formic acid for chemical cleavage make this method the more cost effective when comparing to previous publications.

Acknowledgments

This work was supported by the individual Grant SFRH/BD/27404/2006 from Fundação para a Ciência e a Tecnologia (Portugal). We are grateful to Prof. Ole Sorensen at the Lund University, Sweden. The authors acknowledge Dr. Hugo Osório and the IPATIMUP Proteomics Unit at the Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP) for the MALDI-TOF spectrometry analysis of the peptides.

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