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Production of Cell-Penetrating Peptides in *Escherichia coli* Using an Intein-Mediated System

Vida Rodríguez · Jorge Lascani · Juan A. Asenjo · Barbara A. Andrews

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Abstract Cell-penetrating peptides are molecules with the ability to cross membranes and enter cells. Attention has been put on these peptides as a tool for drug delivery research, as they are able to serve as delivery vectors for large molecules. Intracellular delivery of bioactive peptides is a very promising research area for clinical applications, since peptides are able to simulate protein regions and thus modulate key intracellular protein-protein interactions. Therefore, evaluation of different strategies for production of these peptides is necessary. In this work, an intein-mediated system was used to evaluate *Escherichia coli* recombinant production of p53pAnt and PNC27 anticancer cell-penetrating peptides. It was demonstrated that the pTXB1 and the pTYB11 vector systems are suitable for production of this kind of peptides. The production process involves a low-temperature induction process and an efficient on-column intein-mediated cleavage, which allowed an effective peptide recovery using a single chromatographic step.

Keywords Cell-penetrating peptides · Intein cleavage · Recombinant peptide production · *E. coli*

Abbreviations

CPP	Cell-penetrating peptide
Ni-NTA	Nickel-nitrilotriacetic acid
CBD	Chitin-binding domain
MALDI-TOF	Matrix-assisted laser desorption ionization time-of-flight

Introduction

Cell-penetrating peptides (CPPs) are amino acid chains of 10 to 35 residues in length that have the ability to enter cells [1, 2]. They are characterized by having a high content of lysine and arginine amino acids, a positive net charge at physiological pH, and an alpha helix structure [2]. Their cellular internalization mechanism is under debate [1], but they have been shown to lack cellular specificity, and the main cellular uptake mechanisms are endocytosis and direct translocation across the plasma membrane [1]. It has been demonstrated that diverse molecules

V. Rodríguez (✉) · J. Lascani · J. A. Asenjo · B. A. Andrews
Centre for Biotechnology and Bioengineering (CeBiB), Department of Chemical Engineering and Biotechnology, University of Chile, Beauchef 851, Santiago, Chile
e-mail: vidrodri@ing.uchile.cl

covalently bound to CPPs are able to enter the cell [3, 4] and that CPPs are capable of transporting biologically active peptides to their intracellular target, with positive results in vitro [5–10] and in vivo [7, 8]. Thereby, these peptides have an attractive potential for biological research and pharmacological applications.

Penetratin is a CPP corresponding to the third helix of the DNA-binding domain of Antennapedia, a *Drosophila melanogaster* transcription factor [11], and is one of the most studied CPPs [1]. Interesting research related to peptide sequences derived from regions of the p53 tumor suppressor protein, fused to Penetratin, has led to the peptides called p53pAnt and PNC27 which have shown selective antitumor activity in cancer cells.

The p53pAnt peptide (GSRAHSSHLKSKKGQSTSRH*KKWKMRRNQFWVKVQRG*) comprises the regulatory region of the p53 protein, corresponding to residues 361–382, fused by its carboxyl end to the CPP Penetratin [12]. This peptide operates intracellularly and selectively induces apoptosis in tumor cells containing mutated or overexpressed p53, through a p53-dependent process that does not occur in tumor cell lines with no p53 [12, 13]. It was successfully tested in vitro in several cell lines from different tissues, such as the colon [12, 14], breast [13, 14], lung [14], and lymphoma [12], and in vivo in a glioma rat model [15]. Moreover, it is known that cancer cells eventually are able to activate specific mechanisms of resistance to therapeutic agents that induce apoptosis, but studies have shown that p53pAnt induces targeted necrosis on chemotherapy-resistant prostate cancer cells [16].

The PNC27 (PPLSQETFSDLWKLL*KKWKMRRNQFWVKVQRG*) peptide consists of the mdm-2-binding domain of the p53 protein (residues 12–26), fused by its carboxyl end to Penetratin as carrier [17]. Although this peptide was designed to bind mdm-2 inside the cell, and thus reduce p53 degradation [17], in vitro experiments showed a different mechanism of action for PNC27, since it induces selective necrosis in various carcinogenic cell types [18–20]. This peptide has an amphipatic structure [21, 22] which allows it to integrate into the cell membrane forming pores, producing membrane disruption and cell death by a p53-independent mechanism [18, 21, 23]. The selectivity on tumor cells would be given by the presence of mdm-2 on the plasma membrane of these cells [24, 25], which is related to the role of mdm-2 in E-cadherin ubiquitination and degradation [25]. Thus, in nontumor cells, PNC27 enters the cell, but in cancer cells, it is retained in the membrane due to its affinity for mdm-2 and forms pores [24].

Efficient production of CPPs with therapeutic activity could promote research and development of these potentially new drugs. These peptides usually contain two domains (one carrier and one bioactive) which make them exceed 30 amino acids in length. So, traditional production through chemical reactions is difficult [26], and recombinant production appears to be an attractive process as it presents good prospects of cost and scalability [27, 28].

Recombinant production of proteins is a standard procedure comprising genetic engineering, fermentation, recovery, and purification. However, it is an empirical process that depends on the particular properties of the protein of interest. To resolve this point, numerous fusion tags have been developed for recombinant peptide/protein production in order to direct the target sequence properties and improve production yields. In general, these tags modify the protein solubility and facilitate purification through affinity chromatography, so that a single step can isolate and purify the protein of interest.

However, subsequent removal of the fusion tag can be necessary as it could affect the structure or the biological function of the target molecule. To recover the native target protein, site-specific proteases are usually used, after which the protein of interest must be separated from the affinity tag and the protease through an additional purification step.

As there is not a single strategy which guarantees success in production for every peptide or protein, evaluation of different recombinant protein production systems is necessary, thus exploring diverse approaches to obtain an efficient peptide production.

The study of protein splicing elements termed inteins has lead to improvements in the control protein splicing process [29, 30] and thus, its application in development of self-cleavage fusion tag methods. In this context, some inteins have been modified in order to prevent in vivo cleavage and to release the fused target protein in response to a chemical or physical stimulus [31, 32].

The use of self-cleaving fusion tags for the purification of recombinant proteins eliminates the need for protease treatment. This, combined with purification tags, allows elimination subsequent removal of the cleaved tag, minimizing the chromatographic steps.

The aim of this work was to evaluate the IMPACT® system as a method for recombinant production of CPPs in *Escherichia coli*. This system allows expression of recombinant proteins as a fusion with a self-cleaving intein tag and a chitin-binding domain (CBD) for affinity chromatography purification. The fusion protein is purified by adsorption onto a chitin column and while immobilized undergoes an inducible self-cleavage that release the target protein while the fused tags remain bound to the column.

Unlike other recombinant protein production strategies, this commercially available system allows production of recombinant peptides with no or minor modifications in the native sequence, without addition of proteases and minimizing the chromatographic steps.

The IMPACT® system provides two expression vectors: pTXB1 and pTYB11, which enable fusion of the recombinant protein either to the amino terminal extreme of the *intein-CBD* fusion protein or to the carboxyl terminal extreme of the *CBD-intein* tag, respectively. Since inteins exhibit different preferences for the amino acid at the cleavage site, attention should be placed on the first and last amino acid of the target sequence. Moreover, when sequences are cloned into pTXB1, an N-terminal methionine is added to the target sequence from the translational start codon.

This paper presents the production of the CPPs p53pAnt and PNC27 using both pTXB1 and pTYB11 vector systems.

Materials and Methods

Strains, Vectors, and Enzymes

E. coli strains DH5α and BL21(DE3) were used as hosts for cloning and expression, respectively. The pGEM-T Easy vector (Promega) was used for cloning oligonucleotide sequences of the peptides. For recombinant expression of the peptides, pTXB1 and pTXB11 vectors (IMPACT® system, NEB) were used. Restriction enzymes *EcoRI*, *SapI*, *NdeI*, *PstI*, and *DpnI* (New England Biolabs) were used according to the recommendation of the supplier. Oligonucleotide primers were synthesized by Integrated DNA Technologies (USA). Elongase enzyme (Invitrogen) was used for the PCR amplification of sequences, and T4 DNA Ligase (Invitrogen) was used for DNA ligation. Prestained protein molecular weight marker (Thermo scientific) and Ultra low molecular weight marker (Sigma) were used for SDS-PAGE protein and peptide analysis, respectively.

Cloning of the DNA Sequences Encoding the Peptides p53pAnt and PNC27

Codon-optimized DNA sequences encoding the peptides were available in our laboratory on pGEM-T Easy cloning vectors on the pGEM-T-p53pAnt and pGEM-T-PNC27 constructs [33]. The oligonucleotide sequence that encodes p53pAnt contains a *SapI* restriction enzyme recognition site which was eliminated by a silent site-directed mutagenesis on the tenth codon

AAG, which was changed to AAA. Site-directed mutagenesis was done on the complete pGEM-T-p53pAnt plasmid, through a method based on the Stratagene QuickChange®protocol using Mutp53ps and Mutp53pr primers (Table 1) and KOD Hot Start DNA polymerase (Novagen) for the PCR reaction. *DpnI* was used for digestion of the amplification product.

Peptide-encoding DNA sequences were released from the vectors pGEM-T-p53pAntMut and pGEM-T-PNC27 by *EcoRI* (NEB) digestion and used as templates for PCR reactions with Elongase enzyme (Invitrogen). For cloning the peptides into the pTXB1 vector, sequences were flanked by *NdeI* and *SapI* restriction enzyme sites using primer pairs pTXp53ps/pTXr and pTXPNCs/pTXr for amplification of p53pAnt and PNC27, respectively. Likewise, for cloning the peptide sequences into the pTYB11 vector, *SapI* and *PstI* restriction enzyme sites were added using primer pairs pTYp53ps/pTYr1 for amplification of the p53pAnt sequence and pTYPNCs/pTYr2 for the PNC27 sequence. The latter reaction also incorporates a glycine amino terminal amino acid into the PNC27 peptide sequence, in order to improve the cleavage efficiency at that site. Sequences of the primers used are shown in Table 1.

Amplification products were cloned into the pGEM-T Easy cloning vector, and the resulting constructs were used to transform *E. coli* DH5α electrocompetent cells. Colony PCR was done on the colonies obtained; DNA from the positive colonies was prepared by the miniprep procedure (QIAprep Spin Miniprep, QIAGEN) and sequenced by Macrogen (Korea).

The final constructs were built from the confirmed pGEM-T constructs and the pTXB1 and pTYB11 expression vectors. The pGEM-T constructs and the pTXB1 vector were double digested with *NdeI* and *SapI*, according to the recommendations of the supplier, and then, resulting fragments were ligated using T4 DNA Ligase, resulting in pTXB1-p53pAnt and pTXB1-PNC27. In the case of pTYB11-p53pAnt and pTYB11-PNC27 vector assembly, the pGEM-T constructs and the pTYB11 expression vector were double digested with *SapI* and *PstI*, before fragment ligation.

All the resulting plasmids were sequenced by Macrogen (Korea) and transformed into *E. coli* BL21(DE3) electrocompetent cells.

Production of Recombinant-Fused Peptides

E. coli BL21(DE3) harboring the expression vectors was cultivated in LB medium containing 100 μg/ml ampicillin, at 37 °C with shaking. The overnight culture was used to

Table 1 Primer sequences for cloning and mutagenesis of peptide-encoding DNA sequences

Primer name	T_m (°C)	Oligonucleotide sequence
pTXp53ps	60.2	5′CATATGGGCAGCCGCGCCCAT 3′
pTXPNCs	60.2	5′CATATGCCGCCGCTGAGCCAG 3′
pTXr	60.6	5′GCTCTTCCGCAACCGCGTTGAAC 3′
pTYp53ps	60.0	5′GCTCTTCCAACGGCAGCCGC 3′
pTYPNCs	67.4	5′GCTCTTCCAACGGTCCGCCGCTGAGC 3′
pTYr1	59.7	5′CTGCAGTTAACCGCGTTGAACTTTCAC 3′
pTYr2	67.0	5′CGCGGACGCTGCAGTTAACCGCGTTGAACTTT 3′
Mutp53ps	63.2	5′GCCCATAGCAGTCATCTGAAAAGCAAAAAGGGT 3′
Mutp53pr	63.2	5′ACCCTTTTTGCTTTTCAGATGACTGCTATGGGC 3′

inoculate 100-ml LB media (with 100 μg/ml ampicillin) to an initial OD_{600}=0.05. Different culture conditions were analyzed in order to obtain the greatest amount of soluble recombinant protein. Cultures were grown at 30 or 37 °C with shaking. When the OD_{600} of the cultures reached 2.0, IPTG was added to final concentrations of 0.4, 0.2, 0.1, or 0.05 mM and induced at different conditions of temperature and time: 37 °C for 6 h, 25 °C for 10 h, 18 °C for 16 h, and 12 °C for 20 h. Cells were harvested by centrifugation (5000×*g*, 10 min, 4 °C), resuspended in column buffer (20 mM Tris-HCl, 500 mM NaCl, 1 mM EDTA, pH 8.5), and lysed by sonication in an ice water bath. Samples of suspensions were collected for analysis of total intracellular protein. The suspensions were centrifugated (12,000×*g*, 10 min, 4 °C) to pellet the insoluble matter. The supernatants containing the soluble proteins were recovered. The pellets were resuspended in denaturing column buffer (20 mM Tris-HCl, 500 mM NaCl, 1 mM EDTA, 8 M urea, pH 8.5) and centrifuged again. Samples of the supernatants were collected for insoluble protein analysis. SDS-PAGE was used to visualize and analyze the different samples. The Bradford assay was used for protein quantification using bovine serum albumin as standard.

Purification of Recombinant Fused Peptides and Self-Cleavage Induction

A chitin affinity matrix (IMPACT ®Kit, NEB) was used to purify the recombinant proteins at a ratio of 1 to 1.4 mL per mg of fusion protein. Samples containing soluble recombinant proteins were incubated on a rotary shaker with chitin beads, previously equilibrated in column buffer, at 4 °C for 1 h. Then, the columns were packed and washed with washing buffer (20 mM Tris-HCl, 1.5 M NaCl, 0,3 % *v*/*v* Triton X-100, pH 8.5) for reduction of unspecific binding of protein, retaining the recombinant proteins bound to the matrix. Samples of every fraction and of loaded chitin beads were collected and loaded on to SDS polyacrylamide gels for visualization. Chitin-binding efficiency factor (CBF) for each recombinant protein was estimated by densitometry analysis (ImageJ software).

On-column cleavage was induced by adding cleavage buffer (20 mM Tris-HCl, 500 mM NaCl, 50 mM DTT, 1 mM EDTA, pH 8.5) incubating the reaction on a rotary shaker at 23 °C for 40 h.

Samples of chitin beads were collected before and after cleavage induction and loaded on SDS-polyacrylamide gels for estimation of cleavage efficiency factors (CEFs) by densitometry analysis.

Recovery and Analysis of Recombinant Peptides

The supernatants of the on-column cleavage reaction products were collected and used to wash the columns three times. The columns were then washed with column buffer for recovery of the remainder peptides.

Protein concentration was determined by the Bradford assay, using bovine serum albumin as standard. Elution efficiency factor (EEF) was estimated as the amount of peptide recovered in the column supernatant, with respect to total peptide available which includes the peptide in the washing fraction.

Analysis of the peptide samples was done on tricine-SDS-polyacrylamide gels and visualized with Coomasie staining. The resultant peptides were also analyzed by MALDI-TOF mass spectrometry. Samples were solubilized in acetonitrile 50 % and phosphoric acid 1 % and mixed with α-cyano-4-hydroxycinnamic acid (CHCA/PA matrix). The spectra were obtained in a Microflex equipment (Bruker Daltonics INC., USA) in positive linear mode.

Results and Discussion

Cloning of the DNA Sequences Encoding the Peptides p53pAnt and PNC27

Site-directed mutagenesis was done on the p53pAnt DNA sequence in order to eliminate its *SapI* restriction enzyme site, since this enzyme is used for cloning into the expression vectors pTXB1 and pTYB11. The mutation was made directly in the construction pGEM-T-p53pAnt available in our laboratory. The resulting plasmid, pGEM-T-p53pAntMut, contains a different oligonucleotide sequence which encodes the p53pAnt amino acid sequence without any modification.

Template sequences of p53pAnt and PNC27 were obtained from *EcoRI* digestion of pGEM-T-p53pAntMut and pGEM-T-PNC27 and used for PCR reactions which incorporated the restriction enzyme sites required for cloning into the expression vectors. The PCR reaction on the PNC27 encoding sequence by using pTYPNCs/pTYr2 primer pair was designed to also incorporate a mutation which adds an N-terminal glycine, as PNC27 starts with proline, an unfavorable amino acid for cleavage, which would be encoded adjacent to the intein if the native sequence were cloned into the pTYB11 expression vector.

PCR products were cloned into the pGEM-T Easy vector. Screening of plasmid inserts directly from transformant colonies by PCR reaction showed amplification of target sequences in white colonies, as expected, and also in some light blue colonies and even in blue colonies. Subsequent sequencing of the cloning site indicates that the peptide-encoding sequences were present within the gene-encoding β-galactosidase of constructs obtained from white, light blue, and blue colonies. This result indicates that some positive clones retained β-galactosidase activity, despite interruption of the *lacZ* gene, probably due to the minor size of the inserted peptide with respect to the complete enzyme.

Sequencing of the constructs confirmed that they contain restriction enzyme sites added at both sides of the target sequences and also showed that specific mutations were incorporated correctly. These modifications made to the DNA sequences encoding p53pAnt and PNC27 allowed cloning into the expression vectors pTXB1 and pTYB11. The constructs pTXB1-p53pAnt and pTXB1-PNC27 were built from the sequences of p53pAnt and PNC27 cloned into the pGEM-T Easy vector and the pTXB1 expression vector, through the *NdeI* and *SapI* restriction enzyme sites. In the case of pTYB11-p53pAnt and pTYB11-PNC27 constructs, they were built by cloning p53pAnt and PNC27 from the pGEM-T Easy vector into the pTYB11 expression vector, through the *SapI* and the *PstI* restriction enzyme sites. The resulting constructs were confirmed by sequencing and transformed into *E. coli* BL21(DE3) electrocompetent cells.

Expression and Purification of Recombinant Fused Peptides

Recombinant protein expression was carried out at different conditions for *E. coli* BL21(DE3) harboring the expression vectors pTXB1-p53pAnt, pTXB1-PNC27, pTYB11-p53pAnt, or pTYB11-PNC27. Induction conditions were studied by SDS-PAGE analysis of the different intracellular protein fractions for every sample.

It was determined that cells carrying the pTYB11-p53pAnt vector produce soluble recombinant protein only when the culture is grown at 30 °C before induction. Analysis of different induction conditions showed that experimental conditions which maximized recombinant protein production in a soluble form were induction at 12 °C for 20 h and 0.1 mM final inducer concentration. This result is not surprising since it is known that decreasing induction temperature may improve protein folding by reduction of the rate at which the recombinant

protein is formed and proteolytic activity is reduced [34]. In all cases, a fraction of the recombinant protein was expressed in insoluble form (Fig. 1, lanes 1, 5, and 9). Moreover, for cells harboring the pTXB1-p53pAnt vector, expression of recombinant protein was always found in the insoluble fraction, and soluble recombinant protein was not detected under any culture conditions (Fig. 2). The corresponding plasmid sequence was revised and showed no mutation in the encoding region nor in transduction and translation-related areas. mRNA analysis for self-complementarity and secondary structures, and plasmid stability studies were done on the pTXB1-p53pAnt construct without finding any irregularities. It is believed that the presence of the peptide p53pAnt on the amino terminal extreme of the recombinant protein somehow affects correct protein folding and leads to misfolded protein aggregation, since a control experiment on BL21 cells transformed with pTXB1 showed soluble recombinant protein production.

From the Bradford quantification of intracellular protein fractions, together with densitometry analysis of SDS-polyacrylamide gels, production levels of soluble recombinant protein per liter of culture were estimated as 8 mg for pTXB1-PNC27, 20 mg for pTYB11-p53pAnt, and 8.5 mg for pTYB11-PNC27.

Samples containing soluble recombinant proteins were loaded onto a chitin affinity matrix and incubated with rotation for 1 h. The matrix was packed into a column eliminating the flow through. The column was washed, and samples of chitin beads were collected. Proteins bound to the beads were recovered and loaded on SDS-polyacrylamide gels. As expected, no protein was detected in pTXB1-p53pAnt samples (Fig. 2, lane 4). Densitometry analysis allowed estimation of a chitin-binding efficiency factor (CBF) for each recombinant protein as 0.93 for pTYB11-p53pAnt, 0.94 for pTXB1-PNC27, and 0.88 for pTYB11-PNC27.

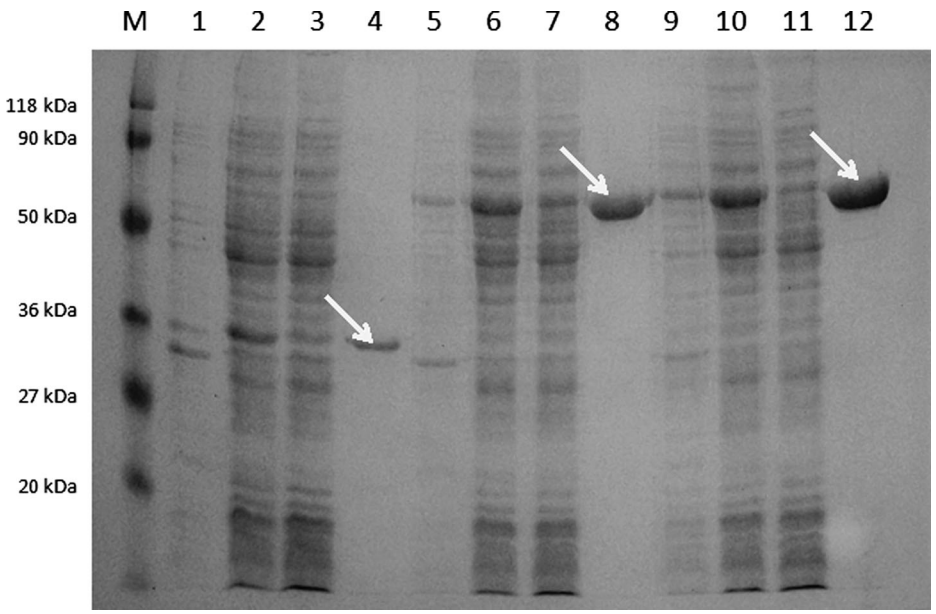

Fig. 1 SDS-PAGE analysis of expression and purification fractions. *1–4* pTXB1-PNC27; *1* insoluble protein, *2* soluble protein, *3* protein in flow through, *4* protein bound to matrix. *5–8* pTYB11-PNC27; *5* insoluble protein, *6* soluble protein, *7* protein in flow through, *8* protein bound to matrix. *9-12* pTYB11-p53pAnt; *9* insoluble protein, *10* soluble protein, *11* protein in flow through, *12* protein bound to matrix

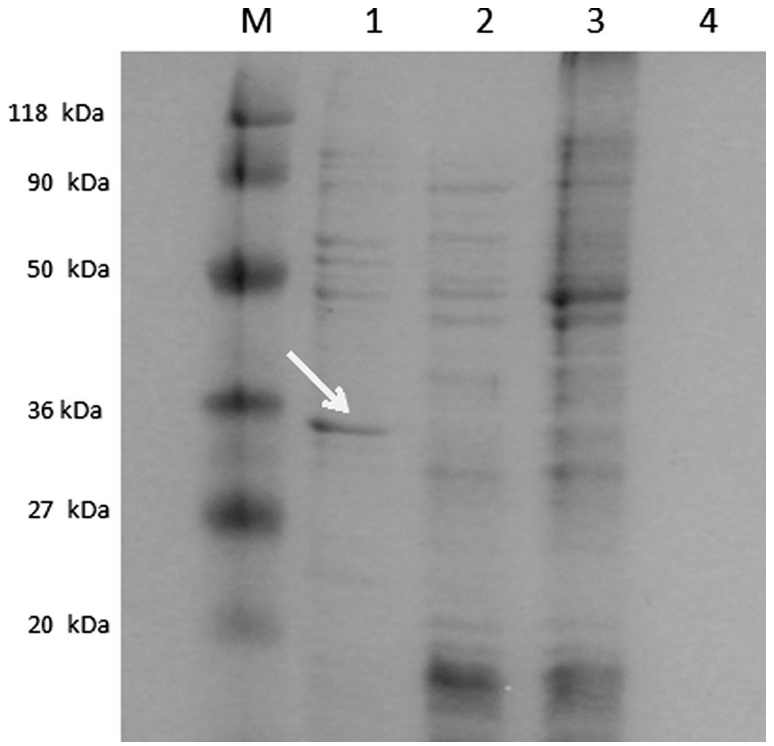

Fig. 2 SDS-PAGE analysis of expression and purification fractions of pTXB1-p53pAnt. *1* Insoluble protein, *2* soluble protein, *3* total intracellular protein, *4* protein bound to matrix

Self-Cleavage Induction and Recovery of Recombinant Peptides

DTT solution was added for self-cleavage induction of recombinant proteins bound to chitin columns, and the reaction was incubated for 40 h. The collected flow through was subjected to SDS-PAGE analysis. Cleavage efficiency factors (CEF), shown in Table 2, were determined from SDS-PAGE analysis of chitin beads before and after cleavage induction. The values are similar or better than the values from the efficiency guide provided by the IMPACT® system supplier (Table 2, column 2). These values are only a guide, as cleavage efficiency depends not only on the residue adjacent to the intein and incubation temperature and time, but also on the entire fusion protein folding, due to the different degrees of steric hindrance that the structure

Table 2 Efficiency factors for different steps of peptide production process

	CBF	CEF[a]	CEF	EEF
pTXB1-PNC27	0.94	0.6	0.73	0.93
pTYB11-p53pAnt	0.93	>0.9	0.95	0.96
pTYB11-PNC27	0.88	>0.9	0.90	0.90

CBF chitin-binding efficiency factor, *CEF* cleavage efficiency factor, *EEF* elution efficiency factor

[a] Values from IMPACT® system guide

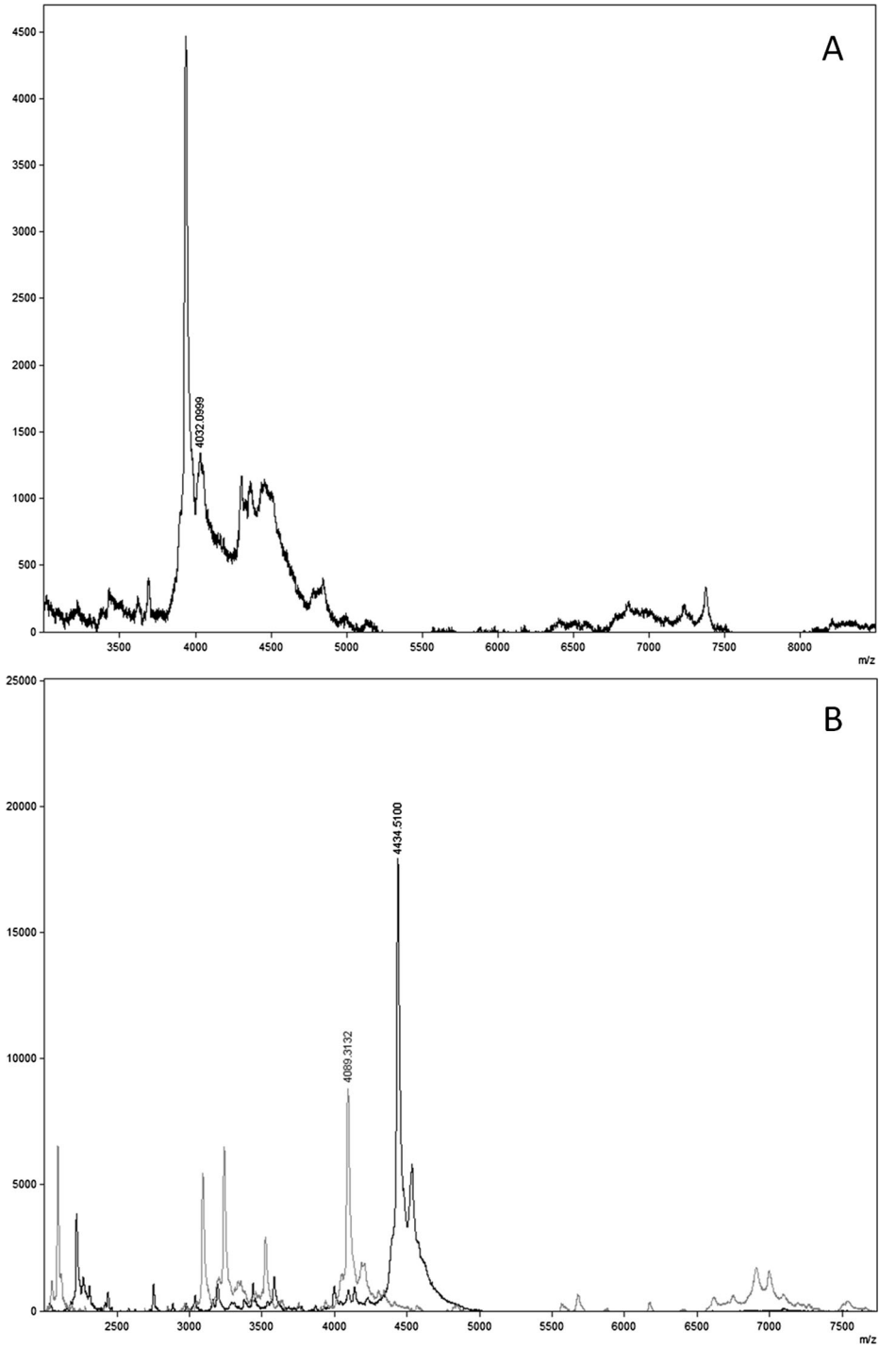

Fig. 3 **a** MALDI-TOF mass spectrum of PNC27 peptide produced by the pTXB1 system. **b** MALDI-TOF mass spectra of peptides produced by the pTYB11 system. *Black* p53pAnt; *gray* PNC27

could impose on the separation. It is worth noting that addition of a single amino terminal glycine to PNC27 sequence allowed effective cleavage even though the native sequence was unfavorable for the self cleavage reaction when using pTYB11 expression vector.

An Elution Efficiency Factor (EEF) was obtained from peptide recovery on collected flow through and total peptide available, determined by quantification of peptide in fractions from subsequent washing of the column. The resulting values are shown in Table 2.

Samples of PNC27 produced by pTXB1 system and p53pAnt and PNC27 peptides produced by the pTYB11 system were subjected to MALDI-TOF analysis. The obtained spectra are shown in Fig. 3.

Analysis of PNC27 produced by pTXB1 system showed a m/z average ratio of 4032 corresponding to the protonated form of the native PNC27 peptide sequence without the amino-terminal methionine (Fig. 3a). This result shows the removal of the translation initiator methionine from the recombinant protein allowing obtention of the native peptide sequence. Protein N-terminal methionine excision is highly probable if the next amino acid of the peptide chain is proline [35], which is the case.

The expected m/z average ratio of the p53pAnt peptide and the N-terminal glycine-PNC27 peptide produced by pTYB11 system (4431 and 4089, respectively) were identified in the obtained spectra (Fig. 3b), confirming the peptide presence.

The amount of peptide produced was calculated from the quantity of soluble recombinant protein, the relative ratio of the peptide size compared to the sequence of the complete recombinant protein, and the different efficiency factors for every process step (see Table 2). The results showed that the productivity showed some differences for every vector-peptide system (Table 3, column 3).

Peptide samples were quantified by the Bradford assay (Table 3, column 4). Some of the values obtained for protein quantification were somewhat larger than those estimated by the calculation. SDS-PAGE analysis of the peptide samples produced by the pTYB11 system presented a single protein band corresponding to the generated peptides. The analysis of PNC27 peptide produced by the pTXB1 system showed a high molecular weight contaminant protein. This indicates that in the case of peptides produced by pTYB11 vector system, each protein different of the target peptides would be present at a concentration lower than 0.1 μg (detection limit of Coomasie staining for a single protein band [36]).

Peptide yields for the expression systems per gram of dry cell weight are shown in Table 3, column 5. From these production levels, we proposed that by using high cell density cultures which have been shown to be applicable to the IMPACT® system up to 35 g of cells per liter of culture [37], more than 20 mg of peptide per liter of culture could be obtained, exceeding production levels reported for other peptides produced by a similar system [38, 39].

Table 3 Production level and peptide purity of recombinant peptide production process

	Soluble recombinant protein [mg][a]	Peptide/ recombinant protein ratio	Produced peptide [mg][a]	Peptide [mg][a] (Quantification)	Peptide yield [mg/g dry cell weight]
pTXB1-PNC27	8.0	0.13	0.65	0.82	0.9
pTYB11-p53pAnt	20.0	0.07	1.18	1.26	0.7
pTYB11-PNC27	8.5	0.07	0.40	0.41	0.6

[a] Data per liter of cell culture

The experimental data shows that main drawbacks on final peptide yield were the soluble recombinant protein yield and also the cleavage process efficiency which is related, in principle, to peptide sequence. The CBF could be improved, since handling and successive regeneration of the chitin resin could have affected its performance. On the other hand, to increase EEF could be difficult, but the peptide in the fraction corresponding to the later wash of the column could be recovered and mixed with the flow through, thus collecting most peptide available.

Evaluation of the IMPACT® system for recombinant production of CPPs indicates that the system is suitable for production of this kind of peptides. Integration of this biological synthesis strategy to reversed-phase HPLC method for peptide purification will permit to polish the product to obtain the final pure peptide.

Conclusions

Recombinant CPPs were produced fused to an intein protein with self-cleavage activity and to a CBD for chitin affinity purification, using pTXB1 and pTYB11 expression vectors. DNA sequences encoding p53pAnt and PNC27 peptides were cloned into both expression vectors. It was possible to induce production of recombinant proteins in all cases; nevertheless, in the case of induction of cells harboring the pTXB1-p53pAnt vector, recombinant protein was obtained in an insoluble form. The other three constructs led to soluble recombinant protein production by a low-temperature induction process. Soluble recombinant proteins were purified by chitin-binding affinity chromatography and subjected to self-cleavage induction to liberate the fused peptide.

Efficiency factors of the main steps of the process were estimated. It was determined that peptide yields were affected mainly by soluble recombinant protein productivity which could be improved by inducing protein expression at low temperature and by self-cleavage efficiency, which depends on peptide sequence. It was observed that addition of an amino terminal glycine to PNC27 sequence allowed effective cleavage although the native sequence was unfavorable for this reaction when using pTYB11expresion vector.

The PNC27 peptide produced by the pTXB1 vector system was obtained as the native sequence at 0.9 mg per gram of dry cell weight. Peptide production levels for the pTYB11 expression system were 0.7 mg for p53pAnt and 0.6 mg for N-terminal-glycine-PNC27, from 1 g of dry cell weight.

Thus, it was determined that the IMPACT® system allows production of recombinant CPPs in *E. coli*, through a process comprising a single chromatographic step.

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