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Cloning and expression of synthetic genes encoding angiotensin-I converting enzyme (ACE)-inhibitory bioactive peptides in *Bifidobacterium pseudocatenulatum*

Luca Losurdo[1,2,4], Laura Quintieri[3], Leonardo Caputo[3], Raffaele Gallerani[1], Baltasar Mayo[2] & Francesca De Leo[4]

[1]Dipartimento di Bioscienze, Biotecnologie e Scienze Farmacologiche, Università degli Studi di Bari 'Aldo Moro', Bari, Italy; [2]Departamento de Microbiolog´a y Bioqu´mica, Instituto de Productos Lácteos (IPLA-CSIC), Villaviciosa, Asturias, Spain; [3]Istituto di Scienze delle Produzioni Alimentari (ISPA), Consiglio Nazionale delle Ricerche (CNR), Bari, Italy; and [4]Istituto di Biomembrane e Bioenergetica (IBBE), Consiglio Nazionale delle Ricerche (CNR), Bari, Italy

Correspondence: Francesca De Leo, Istituto di Biomembrane e Bioenergetica-CNR c/o Dipartimento di Bioscienze, Biotecnologie e Scienze Farmacologiche Campus "Ernesto Quagliariello" Via Amendola 165/A 70126 Bari, Italy. Tel.:+39 080 5443311; fax: +39 080 5443317; e-mail: f.deleo@ibbe.cnr.it

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Abstract

A wide range of biopeptides potentially able to lower blood pressure through inhibition of the angiotensin-I converting enzyme (ACE) is produced in fermented foods by proteolytic starter cultures. This work applies a procedure based on recombinant DNA technologies for the synthesis and expression of three ACE-inhibitory peptides using a probiotic cell factory. ACE-inhibitory genes and their pro-active precursors were designed, synthesized by PCR, and cloned in *Escherichia coli*; after which, they were cloned into the pAM1 *E. coli*-bifidobacteria shuttle vector. After *E. coli* transformation, constructs carrying the six recombinant clones were electrotransferred into the *Bifidobacterium pseudocatenulatum* M115 probiotic strain. Interestingly, five of the six constructs proved to be stable. Their expression was confirmed by reverse transcription PCR. Furthermore, transformed strains displayed ACE-inhibitory activity linearly correlated to increasing amounts of cell-free cellular lysates. In particular, 50 μg of lysates from constructs pAM1-Pro-BP3 and pAM1-BP2 showed a 50% higher ACE-inhibitory activity than that of the controls. As a comparison, addition of 50 ng of Pro-BP1 and Pro-BP3 synthetic peptides to 50 μg of cell-free extracts of *B. pseudocatenulatum* M115 wild-type strain showed an average of 67% of ACE inhibition; this allowed estimating the amount of the peptides produced by the transformants. Engineering of bifidobacteria for the production of biopeptides is envisioned as a promising cell factory model system.

Introduction

In addition to a role in nutrition, proteins may have further health benefits due to the release by proteolysis of encrypted bioactive peptides (BPs). It has been widely demonstrated that BPs are intervention agents against chronic human diseases such as hypertension and for maintaining general well-being (Hartmann & Meisel, 2007; De Leo *et al.*, 2009; Udenigwe & Aluko, 2012). The use of BPs allows for a number of advantages in comparison with chemical drugs, as they come from natural sources and are therefore safer than synthetic molecules. Although proteins of animal and plant origins, such as milk, fish, cereals, eggs, muscles, and peas, have been exploited as relevant sources for BPs production (Roy *et al.*, 2010; Gomez-Guillen *et al.*, 2011; Holdt & Kraan, 2011; Ryan *et al.*, 2011; Urista *et al.*, 2011; Lico *et al.*, 2012), since extraction of BPs from proteins of plant and animal origin is difficult and costly, companies should be encouraged to invest in new methods that lead to cheaper recovery. BPs are considered to be key ingredients in functional foods, which can be used for struggling against chronic diseases (Korhonen, 2009). These are classified according to their physiological activity as antihypertensive

(Jiang *et al.*, 2010), antimicrobial (Singh & Singh, 2011; Espitia *et al.*, 2012), antioxidative (Sarmadi & Ismail, 2010; Coda *et al.*, 2012), antitumor (Rizzello *et al.*, 2012), mineral-binding (Berrocal *et al.*, 1989), and opiate-like compounds (Varamini *et al.*, 2012), among others. The most widely studied peptides are those belonging to the antihypertensive group, which are capable of inhibiting the angiotensin-I converting enzyme (ACE, EC 3.4.15.1), a type-I membrane anchored dipeptidyl carboxypeptidase that is essential for blood pressure regulation and electrolyte homeostasis through the renin-angiotensin system (Hernandez-Ledesma *et al.*, 2011; Phelan & Kerins, 2011). Three ACE-inhibitory BPs arising from bovine β-casein have been characterized (Gobbetti *et al.*, 2000; Minervini *et al.*, 2003). These compounds, named BP1, BP2, and BP3, correspond, to fragments 57–66, 73–82, and 47–52 of bovine β-casein (A^2 allelic variant), respectively. The pro-active precursors of these BPs (termed as Pro-BP1, Pro-BP2, and Pro-BP3) were previously expressed as fusion proteins in *Escherichia coli* cells, using the GST expression system. The pro-active precursors were generated by the addition of two fragments consisting of five amino acids, at N- and C-term of each BP. After expression in *E. coli* as fusion proteins with the GST protein, BP1, BP2, and BP3 were released from Pro-BPs using partially purified membrane proteinases isolated from *Lactobacillus helveticus* PR4 (Losacco *et al.*, 2007).

In this work, synthetic genes encoding BPs and related Pro-BPs pro-active precursors were designed, cloned and expressed using pAM1, an *E. coli-Bifidobacterium* shuttle vector. The recombinant plasmids isolated from *E. coli* clones were then electrotransferred into the probiotic strain *Bifidobacterium pseudocatenulatum* M115. Expression of the cloned synthetic sequences was assessed by reverse transcription PCR (RT-PCR) analysis, while correct activation of the BPs released in cell-free cellular lysates of the transformed strains was evaluated by the analysis of their ACE-inhibitory activity.

Materials and methods

Bacterial strains and incubation conditions

Table 1 reports the bacterial strains used in this work. Bifidobacteria were grown in anaerobiosis at 37 °C in either MRS (Merck, Darmstad, Germany) or RCM broth (Merck), both media supplemented with 0.25% (w/v)

Table 1. Bacterial strains, plasmids, and oligonucleotide primers utilized in this work

Item	Genotype, phenotype or sequence	Source or reference
Strains		
Escherichia coli DH10β	*mcrA, Δ(mrr-hsdRMS-mcrBC), Δ(lac-proAB), Δ(rec1398), deoR, rpsL, srl-thi-/F'proAB+ lacIqZΔ*M15	Invitrogen
E. coli JM109	*endA1, glnV44, thi-1, relA1, gyrA96, recA1, mcrB+, Δ(lac-proAB), e14-[F' traD36, proAB+, lacIq, lacZΔM15], hsdR17, (rK-mK+)*	New England Biolabs
Bifidobacterium pseudocatenulatum M115	Human intestinal isolate, plasmid-free	IPLA Laboratory Collection
Plasmids		
pAM1	*E. coli-Bifidobacterium* shuttle-vector; Ap^r, Em^r; 6100 bp.	Alvarez-Martin *et al.* (2008)
Oligonucleotides	Sequence 5′-3′, restriction sites are underlined, RBS are italicized	
BP1 F	GATTACGCCAAGCTT*AGGAG*ATGTCTCTGGTGTATCCGTTCCCG	This work
BP1 R	TGGAGATCCTCTAGATTAAATCGGGCCCGGGAACGGATACAC	This work
BP2 F	GATTACGCCAAGCTT*AGGAG*ATGAATATTCCGCCGCTGACGCAGACG	This work
BP2 R	TGGAGATCCTCTAGATTAAATCGGCGTCTGCGTGAGCGG	This work
BP3 F	GATTACGCCAAGCTT*AGGAG*ATGGATAAGATTCATCCG	This work
BP3 R	TGGAGATCCTCTAGATTAGAACGGATGAATCTTATC	This work
Pro-BP1F	GATTACGCCAAGCTT*AGGAG*ATGTTCGCTCAGACGCAGTCTCTGGTGTATCCGTTCCCGGC	This work
Pro-BP1-R	TGGAGATCCTCTAGATTACGGCAGAGAATTCGGAATCGGGCCGAACGGATACACC	This work
Pro-BP2-F	GATTACGCCAAGCTT*AGGAG*ATGAATTCTCTGCCGCAGAATATTCCGCCGCTGACGCAG	This work
Pro-BP2-R	TGGAGATCCTCTAGATTAGAACGGCGGCAGCAGCAGCGGTGTCTGCGTGACCGGCGG	This work
Pro-BP3-F	GATTACGCCAAGCTT*AGGAG*ATGGAAGATGAACTGCAGGATAAGATTCATCCGTTC	This work
Pro-BP3-R	TGGAGATCCTCTAGATTAAGACTGCGTCTGAGCGAACGGATGAATCTT	This work
Polylinker External F	CATGATTACGCCAAGCTT	This work
Polylinker External R	TTTTGGAGATCCTCTAGA	This work
pAM1-R-seq	CCACTTGTGTGACCGTGGTGT	This work

L-cysteine (Sigma-Aldrich, St. Louis, MO). *Escherichia coli* was grown with shaking at 37 °C in Luria Bertani (LB) broth (Sambrook & Russell, 2006). Agar plates were prepared by adding 1.5% w/v agar (Oxoid, Basingstoke, UK) to the same broth media. Antibiotics were added when required at the following concentrations: ampicillin 100 μg mL^{-1} for *E. coli*, and erythromycin 5 μg mL^{-1} for bifidobacteria. Isopropyl-β-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-thiogalactopyranoside (X-gal) (both from Sigma-Aldrich) were incorporated to LB plates at respective concentrations of 75 and 60 μg mL^{-1}.

Substrates and chemicals

Unless otherwise indicated, hippuryl-L-histidil-L-leucine (HHL), ACE (from rabbit lung, *c.* 3 U mg^{-1} of protein), bovine serum albumin (BSA), tryptone and other chemicals were all from Sigma-Aldrich. Pro-BP1 (NH_2-FAQTQSLVYPFPGPIPNSLP-COOH) and Pro-BP3 (NH_2-EDELQDKIHPFAQTQS-COOH) synthetic peptides were purchased from Primm Italia Srl (Milan, Italy) and used as standard references in RP-HPLC and ACE inhibition analyses.

Molecular DNA techniques

Cloning, overlapping PCR, colony PCR and DNA manipulation were carried out following standard procedures (Sambrook & Russell, 2006). Fragments amplified by overlapping PCR (Delidow, 1997) were cloned into the T-A cloning vector pGEM-T® (Promega, Madison, WI). DNA amplicons were then purified from agarose gel using the Montage DNA Gel Extraction Kit® (Millipore, Billerica, MA). The NucleoSpin® Plasmid Kit (Macherey-Nagel, Düren, Germany) was used for purification of plasmid DNA from *E. coli* with reverse transcription PCR (RT-PCR) performed with the SuperScript™ First-Strand Synthesis System (Invitrogen, Carlsbad, CA) using random primers.

Plasmid DNA from bifidobacteria was purified following the (O'Sullivan & Klaenhammer, 1998) procedure with minor modifications. In brief, overnight cultures (5–10 mL) were harvested by centrifugation for 1 min at maximum speed. The pellet was suspended in 200 μL of a 25% sucrose solution containing lysozyme (30 mg mL^{-1}), mutanolysin (100 units mL^{-1}), and RNase A (200 μg mL^{-1}). The mixture was incubated at 37 °C for 30 min. DNA was precipitated with isopropanol and the pellet suspended in 320 μL of sterile water. An aliquot of 350 μL of a phenol:chloroform:isopropanol mixture (24 : 24 : 1) and 200 μL of 7.5 M ammonium acetate were then added to the solution. After extractions, plasmid DNA was precipitated with ethanol, the pellet washed with ethanol at 70%, dried under vacuum and suspended in sterile water or TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0).

Extraction of total RNA from bifidobacteria was performed using the RNeasy Mini Kit® (Qiagen, Hilden, Germany) with some modifications in the lysis step. Briefly, cells, grown at an $OD_{600\ nm}$ of 0.7–0.8, were harvested by centrifugation, the pellet mashed with a pestle in the presence of liquid nitrogen until a fine powder was obtained. Samples were then incubated for 1 h at 37 °C with 200 μL of a TE solution containing lysozyme (30 mg mL^{-1}) and mutanolysin (100 units mL^{-1}), later following the recommendations of the kit protocol.

Amplification and cloning of synthetic genes encoding BPs and Pro-BPs

Table 1 reports the oligonucleotides used in this work. Complementary primer pairs were synthesized and used in overlapping PCR (Delidow, 1997), following amplification conditions reported in Table 2. For each construct, 30 PCR cycles were performed. To increase specificity, progressive longer annealing steps were utilized (Table 2). Amplifications were performed using High Fidelity Taq polymerase (Fermentas, Glen Burnie, MD) following conditions recommended by the supplier. Amplified fragments were cloned in pGEM-T®. Recombinant clones were screened by colony PCR and sequenced. Positive clones were cloned into the *E. coli*-bifidobacteria shuttle vector pAM1 (Alvarez-Martin *et al.*, 2008). Subsequent to

Table 2. Amplification scheme to produce the synthetic DNA sequences encoding BPs and Pro-BPs

	Gene encoding the peptide					
Step	BP1	BP2	BP3	Pro-BP1	Pro-BP2	Pro-BP3
Denaturation	94 °C, 30 s	94 °C, 30 s	94 °C, 30 s	94 °C, 30 s	94 °C, 30 s	94 °C, 30 s
Annealing	35 °C, 30 s	37 °C, 30 s	39 °C, 30 s	37 °C, 30 s	37 °C, 30 s	59 °C, 30 s
	40 °C, 5 s	42 °C, 5 s	44 °C, 5 s	42 °C, 5 s	42 °C, 5 s	62 °C, 5 s
	45 °C, 5 s	47 °C, 5 s	50 °C, 5 s	50 °C, 5 s	47 °C, 5 s	66 °C, 5 s
	50 °C, 10 s	52 °C, 10 s	55 °C, 10 s	60 °C, 10 s	52 °C, 10 s	70 °C, 10 s
Elongation	72 °C, 10 s	72 °C, 10 s	72 °C, 10 s	72 °C, 10 s	72 °C, 10 s	72 °C, 10 s

E. coli transformation pAM1 clones, carrying bioactive peptides and their precursor forms, picked up from LB plates amended with 100 μg mL^{-1} ampicillin, were verified for their in-frame position by sequencing. Positive recombinant plasmids carrying in-frame genes were then electrotransformed into *B. pseudocatenulatum* M115 as previously reported (Alvarez-Martin *et al.*, 2008).

Production of water-soluble protein extracts

Cell-free water-soluble protein extracts (CPE) from recombinant *B. pseudocatenulatum* and *E. coli* strains were obtained by sonication and glass beads treatments. Briefly, bacterial pellets from overnight cultures were suspended in 4.5 mL of sterile milli-Q water supplemented with 500 μL of 1 mM phenyl-methyl-sulfonyl fluoride (PMSF) and 1 g of sterile acid-washed glass beads (106 μm of diameter; Sigma-Aldrich). Samples were sonicated three times (5 min at maximum amplitude, followed by 1 min on ice) by a Vibra-Cell VC130 (Sonics & Materials, Newtown, CT). Protein content was measured using the Quick Start™ Bradford Protein Assay (BioRad, Hercules, CA) following the protocol provided by the manufacturer.

ACE inhibition assay

CPE were used in ACE inhibition assays, following the method of Nakamura *et al.* (1995) with minor modifications. Briefly, increasing volumes of CPE (containing 10–25–50 μg of total protein content) were added to a 5 mM Hip-His-Leu solution and 2 mU of ACE and incubated for 1 h at 37 °C. The hippuric acid liberated was then extracted with 800 μL of ethyl-acetate, dried in an oven at 100 °C for 1 h to remove the solvent, and suspended in Milli-Q water (Millipore). Absorbance of the solutions was then read at 228 nm in an Ultrospec 3100 pro-UV/Visible Spectrophotometer (Amersham-GE Healthcare, Pittsburgh, PA). Reactions were performed in triplicate. For all measurements, a control without addition of ACE inhibitors was included.

RP-HPLC analysis of Pro-BP1 and Pro-BP3 in bacterial lysates

RP-HPLC was carried out on the CPE obtained from cultures of wild-type and recombinat strains of *B. pseudocatenulatum* M115. In particular, 1 mL of each bacterial lysate was precipitated by the addition of 4 mL of cold methanol following the Janini procedure (Janini *et al.*, 2004). Samples were then centrifuged (17 500 ***g***) for 15 min at 4 °C, and the supernatants were collected and dried in a SpeedVac concentrator (Thermo-Savant, Holbrook, NY). Subsequently, all samples were suspended in 1 mL of Milli-Q water (Millipore), filtered through a 0.22-μm membrane (Millipore) and injected (10 μL) on a RP-HPLC Zorbax column (SB300 C18;4.6 × 150 mm, 5 μm; Agilent, Waldbronn, Germany) mounted on an Agilent 1100 apparatus equipped with G1312A binary pump, G1313 Autosampler, G1316A, G1315B UV-visible DAD and Agilent Chemstation G2170AA Windows 2000 operating system. The column was equilibrated in water-acetonitrile (95:5, v/v; solvent A) with 0.1% trifluoro acetic acid (TFA). Peptides were eluted at 1 mL min^{-1} using a 20-min linear gradient from the solvent A to water-acetonitrile (35:65, v/v; solvent B) containing 0.1% TFA, with elution monitored by measuring the absorbance at 220 nm. Linear regression analysis (95% confidence limit), using the least square method, was applied to relate RP-HPLC peak areas of the Pro-Bp1 and Pro-BP3 peptides to their respective concentration.

CPE extracts were also spiked with Pro-BP1 or Pro-BP3 synthetic peptides at different concentrations (0.025 and 0.050 mg mL^{-1}) and processed as above.

In addition, following RP-HLPC analyses, ACE activity was tested by adding 50 ng of synthetic Pro-BP1 and Pro-BP3 to 300 μL (1 mg mL^{-1}) of CPE obtained from *B. pseudocatenulatum* M115 wild-type strain and incubated for 2 h at 37 °C.

Results

Design of DNA sequences encoding BPs and Pro-BPs

The three bovine β-casein (A^2 allelic variant) derived BPs (named respectively BP1, BP2, and BP3) and their pro-active precursors (Pro-BP1, Pro-BP2, and Pro-BP3; Losacco *et al.*, 2007) were selected to be expressed in a probiotic strain. Figure 1 shows amino acid sequences corresponding to BP1, BP2, and BP3 and their precursor forms, including the corresponding primers. Synthetic genes expressing BPs and their precursor forms were produced by overlapping PCR. Primer design took into account the codon usage of *B. pseudocatenulatum.*

To ensure optimal expression of BPs and Pro-BPs, a Shine-Dalgarno ribosome binding site (RBS) consensus sequence for *B. pseudocatenulatum* was placed at the 5′ end of all synthetic genes. This sequence was chosen after the analysis of contigs resulting from the whole genome shotgun sequencing project of *B. pseudocatenulatum* DSM 20438 (NCBI: NZ_ABXX00000000.2) using the Biopython software (Cock *et al.*, 2009). Nucleotide sequences corresponding to positions −100 through +1 of the putative starting codon of 2032 genes were aligned by PatSearch software (Grillo *et al.*, 2003). Among the four RBS

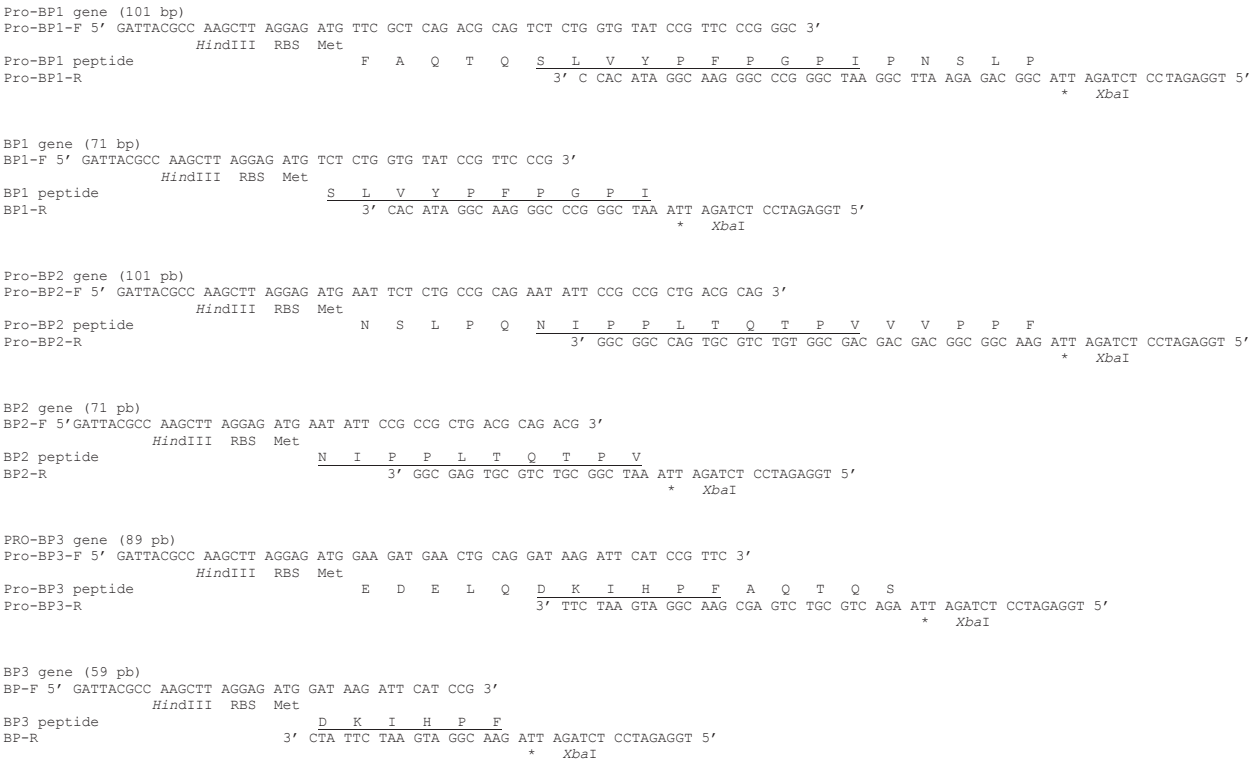

Fig. 1. Nucleotide sequences of the corresponding synthetic BP and Pro-BP genes including primers, RBS, enzyme restriction sites, and start and stop codons. Figure also includes amino acid sequences of Pro-BPs and BPs.

sequences already identified in previous reports (AGGAG, AGGAA, AGAAA, and AGAAG; Nunoura *et al.*, 1997a, b; MacConaill *et al.*, 2003; Park *et al.*, 2008; Table 3), the first one showed the highest frequency in the *B. pseudocatenulatum* genome (21%) and was thus selected for inclusion before the synthetic genes. In addition, HindIII and XbaI restriction enzyme sites were placed at 5′ and 3′ terminal ends of the coding sequences to facilitate directional cloning of synthetic genes into the pAM1 vector (Figs 1 and 2).

Table 3. PatSearch analysis of putative RBS sequences in *Bifidobacterium pseudocatenulatum* genes

Sequence	Number of entries	Frequency (%)
AGGAG	253	21
AGGAA	202	16
AGAAA	172	14
AGAAG	101	8
Others	502	41
Total	1230	100

Cloning of synthetic genes in *E. coli* and bifidobacteria

The cloning procedure, as described in Fig. 2, was performed in two steps. The procedure was necessary to obtain adequate amount of DNA encoding the synthetic genes for cloning into bifidobacteria. The first step involved the cloning of amplified fragments into pGEM-T® Easy Vector with *E. coli* as host. Recombinant clones were analyzed by colony PCR and sequencing using Polylinker External F and R primer pairs (Table 1). Correct inserts from the clones were rescued by double digestion with HindIII and EcoRI and cloned into pAM1 plasmid vector digested with the same enzymes. The ligation mixture was transferred into M115 *B. pseudocatenulatum*. As a control, the same constructs were also transferred to *E. coli* JM109 and DH10β strains. In this way, five of the six constructs could be recovered in *B. pseudocatenulatum* M115, where they proved to be stably maintained. The exception was pAM1-BP1, as no viable colonies were obtained by transformation of bifidobacteria with the pAM1 plasmid vector carrying the BP1-encoding gene. This suggested that the BP1 peptide might have an antimicrobial activity against bifidobacteria.

To demonstrate the stability of recombinant plasmids in *B. pseudocatenulatum* cells, plasmid DNA isolated from recombinant strains was used to transform once again two different *E. coli* strains (XL1-Blue and

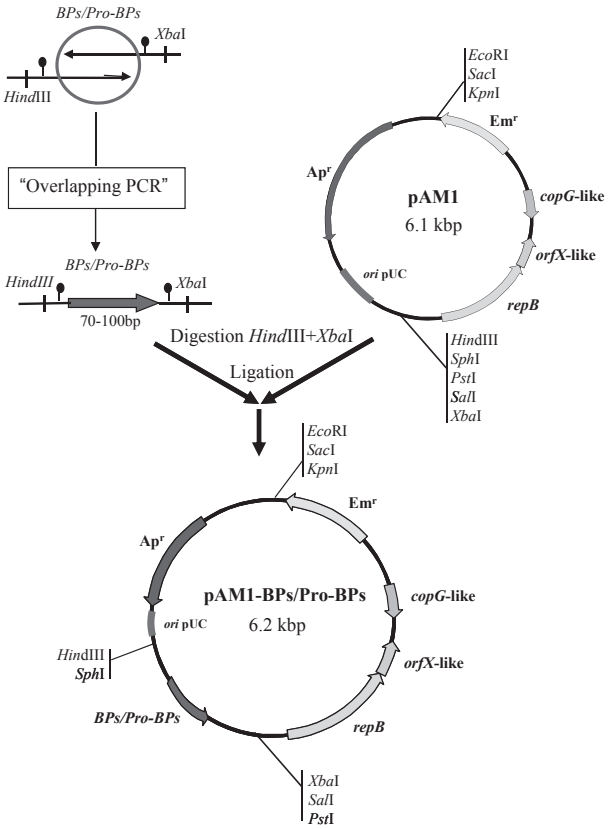

Fig. 2. General procedure of amplification of Pro-BPs and BPs genes by overlapping PCR and cloning of the synthetic genes in *Escherichia coli* using pGEM®-T Easy Vector (Promega) and in *Bifidobacterium pseudocatenulatum* M115 using pAM1 cloning vector.

DH10β). Analysis of the recombinant plasmids further evaluated the integrity of the constructs and the absence of modifications.

BP expression in bifidobacteria

To confirm the expression of the synthetic genes encoding BP2, BP3, Pro-BP1, Pro-BP2, and Pro-BP3 in bifidobacteria, a transcription analysis was carried out. Total RNA was extracted from recombinant clones and used as a template in RT-PCRs with random primers. To achieve specific cDNAs for BP and Pro-BP genes, amplifications were carried out using the External Polylinker F and R primer pairs (data not shown). Sequencing of the amplicons obtained demonstrated the effective and reproducible transcription of the synthetic genes in the bifidobacteria host strain.

Finally, ACE-inhibitory activity was carried out on protein extracts isolated from transformed strains carrying BP or Pro-BP gene-encoding sequences. As a control, a bifidobacteria strain transformed with pAM1 wild-type plasmid was used. As can be seen in Table 4, the increase in ACE-inhibitory activity of CPE belonging to recombinant bifodobacteria expressing the constructs as compared to the controls further confirmed expression of the synthetic genes.

A further attempt to demonstrate gene expression was carried out by reverse phase (RP)-HPLC analyses of CPE extracts of wild and recombinant strains of *B. pseudocatenulatum* M115 carrying Pro-BP1 and Pro-BP3 genes. Even though the lysates were previously extracted with methanol, peaks corresponding to the precursor forms of the bioactive peptides were not observed. By contrast, the same peptides, synthesized at an HPLC grade and spiked at two different concentrations (0.025 and 0.050 mg mL^{-1}) in the bacterial lysates, were quantitatively recovered (about 97%). The failure to retrieve Pro-BP1 and Pro-BP3 peptides by (RP)-HPLC analysis could be probably due to their hydrolysis by intracellular endopeptidases of the host strain (particularly aminopeptidases and iminopeptidases), which may be released during the cell lysis treatment, as previously reported by (Elsoda *et al.*, 1992). As the precursor peptides were not recovered, isolation of BPs was not attempted.

As reported in Table 4, the presence of BPs and their precursor forms in the cell-free extracts was finally demonstrated by the increasing ACE-inhibitory activity of increasing amounts of CPE extracts from the recombinant clones

Table 4. ACE inhibitory activity in cell-free lysates isolated from *Bifidobacterium pseudocatenulatum*. (A) ACE inhibitory activity in cell-free cellular lysates obtained from *B. pseudocatenulatum* transformed with pAM1 wild-type and pAM1-BPs and pAM1-Pro-BPs recombinant plasmids*. (B) ACE-inhibitory activity of 50 ng of synthetic Pro-BP1 and Pro-BP3 with or without CPE (1 mg mL^{-1}) of bifidobacteria carrying pAM1-Pro-BP1 and Pro-BP3, respectively*

	Cellular protein extract amounts (μg)		
	10	25	50
Recombinant strain	Activity (%)		
(A)			
pAM1 wt	0	26 ± 1.5	29 ± 1.5
pAM1-BP2	14 ± 0.7	36 ± 2	54 ± 3
pAM1-BP3	18 ± 1	26 ± 1.5	41 ± 2
pAM1-Pro-BP1	25 ± 1.40	37 ± 2	48 ± 2.5
pAM1-Pro-BP2	5 ± 0.3	14 ± 1	43 ± 2.2
pAM1-Pro-BP3	19 ± 1	32 ± 1.9	58 ± 3

	ACE-inhibitory activity (%)	
Synthetic peptide	Pro-BPs	Synthetic Pro-BPs plus CPE
(B)		
Pro-BP1	64 ± 3	70 ± 3.5
Pro-BP3	50 ± 2.5	64 ± 3

*Data are the average of triplicate-independent assays, and the confidence interval was computed at 5%.

(Table 4). These results confirmed the production and the expression of bioactive peptides and their precursor forms even though they were not detectable by RP-HPLC.

To demonstrate the correct intracellular activation in the biological hypotensive form through the proteolytic endogenous machinery of *B. pseudocatenulatum* M115, synthetic Pro-BP1 and Pro-BP3 were added to water-soluble protein extracts from *B. pseudocatenulatum* M115 and incubated for 2 h at 37 °C, after which an ACE inhibition assay was carried out. The results are also summarized in Table 4, and, as compared to the control, bifidobacteria transformed with the BPs genes showed a significant increase in their ACE-inhibitory capability. These results strongly suggest that the increase in ACE inhibition arose from the activation of the Pro-BPs by the proteolytic machinery of the host, producing the fully active ACE-inhibitory BPs.

Discussion

In this study, we established a procedure to obtain ACE-inhibitory peptides in a probiotic strain by recombinant DNA techniques. Even though the results are preliminary, they are relevant because the main advantage of this approach consists in the engineering of bifidobacteria for the production of active biopeptides. These could be used, for example, as supplements in functional foods, bypassing the purification of complex mixtures of peptides from food matrices or protein hydrolysates. Furthermore, DNA recombinant technologies allow the modification of single nucleotides to produce from scratch new highly efficient and selective BPs. *B. pseudocatenulatum* M115 has been proposed over the past decades as a probiotic candidate along with certain strains belonging to other species of the human gut (Delgado *et al.*, 2008). In addition, M115 strain had been already selected as an optimal cell factory, demonstrating effectiveness for the heterologous expression of interesting enzymes (Alvarez-Martin *et al.*, 2008).

A number of antihypertensive molecules, including BPs isolated from natural sources have been already incorporated into several food and drink products (Hernandez-Ledesma *et al.*, 2011). Despite their proven usefulness, purification of BPs from lysates, gastrointestinal digests, or food stuffs is labor-intensive and costly, prompting us in this study to explore a new procedure for the production of BPs using a recombinant DNA technology. Indeed, even though several BPs have been successfully transferred and expressed by recombinant DNA technologies in bacteria (Renye & Somkuti, 2008), plants (Yang *et al.*, 2008; Wang *et al.*, 2009; Lico *et al.*, 2012), and yeasts (Wang *et al.*, 2009), their isolation and industrial exploitation is rather difficult.

To the best of our knowledge, this is the first work that reports gene expression of synthetic genes encoding BP2, BP3, Pro-BP1, Pro-BP2, and Pro-BP3 in bifidobacteria. Thanks to their ability to colonize the human gut, these microorganisms could release ACE-inhibitory bioactive peptides in the intestinal mucosa. Nevertheless, the actual extent of their assimilation in the bloodstream and their physiological effects on human health should be evaluated by appropriate clinical trials. In this regard, further studies have to be addressed to search for novel food-grade plasmids that not bear antibiotic resistances. Furthermore, the over-expression of the synthetic BP-encoding genes under the control of strong promoters based on constitutive and inducible bifidobacterial genes could enhance BP production. The capability of bifidobacteria to express and activate BPs is a fascinating feature for the formulation of innovative functional foods (as for instance, fermented milks) that could prove useful in the treatment of mild forms of hypertension.

Using the approach described here, the biopeptides were expressed and efficiently activated, making it possible to determine the ACE-inhibitory activity of each individual BP. For the detection of these small BPs, more sensitive methods of analysis should be applied. This study is not exhaustive and other data should be collected to assess the bioavailability of BPs during digestion. Although our results leave open many questions, the data reported in this study are encouraging in the use of *B. pseudocatenulatum* M115 as suitable host strain for the expression of bioactive molecules. Then, the M115 strain could be converted in a suitable cell factory model system.

The novelty of this study consists not only in the procedure used to express the three BPs (BP1, BP2, and BP3) and their precursor forms, but also in the evidence of toxicity of the sequence (BP1) whose antimicrobial activity has never been reported (Losacco *et al.*, 2007). Further analysis should be aimed at a definitive demonstration of cytotoxic activity for BP1.

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