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Improvement of cyclic decapeptides against plant pathogenic bacteria using a combinatorial chemistry approach

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ABSTRACT

Cyclic decapeptides were developed based on the previously reported peptide c(LysLeuLysLeuLysPheLysLeuLysGln). These compounds were active against the economically important plant pathogenic bacteria *Erwinia amylovora*, *Pseudomonas syringae* and *Xanthomonas vesicatoria*. A library of 56 cyclic decapeptides was prepared and screened for antibacterial activity and eukaryotic cytotoxicity, and led to the identification of peptides with improved minimum inhibitory concentration (MIC) against *P. syringae* (3.1–6.2 μM) and *X. vesicatoria* (1.6–3.1 μM). Notably, peptides active against *E. amylovora* (MIC of 12.5–25 μM) were found, constituting the first report of cyclic peptides with activity towards this bacteria. A second library based on the structure c($X^1X^2X^3X^4$LysPheLysLysLeuGln) with X being Lys or Leu yielded peptides with optimized activity profiles. The activity against *E. amylovora* was further improved (MIC of 6.2–12.5 μM) and the best peptides displayed a low eukaryotic cytotoxicity at concentrations 30–120 times higher than the MIC values. A design of experiments permitted to define rules for high antibacterial activity and low cytotoxicity, being the main rule $X^2 \neq X^3$, and the secondary rule X^4 = Lys. The best analogs can be considered as good candidates for the development of effective antibacterial agents for use in plant protection.

1. Introduction

Bacterial diseases of plants create challenging problems in commercial agriculture because they are difficult to control, and often result in sudden, devastating economic losses [1,42]. Plant protection to bacteria is mainly based on copper derivatives and antibiotics. However, environmental concerns and the emergence of antibiotic-resistant strains are limiting the value of these compounds in crop protection [28,38].

In recent years, a great deal of research has been undertaken in order to discover antibiotics with unexploited mechanisms of action to counteract the bacterial resistance. Antimicrobial peptides represent potential candidates of such a new class of antibiotics [19,20,44]. They show a broad spectrum of activity, a remarkable level of antibacterial selectivity, low eukaryotic cytotoxicity, and a mode of action and cellular targets different from those of the traditional antibiotics [7,10,18,37,43]. Although a few antimicrobial peptides may interact with intracellular components including mitochondria and nucleic

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acids, they work primarily by compromising the membrane of the target organism [18]. Since their mechanism does not demand a specific organization of their sequence and structure, several diverse architectures have evolved, including different types of linear and cyclic peptides [44]. These sequences meet two common and functionally important requirements; they bear a net positive charge that attracts them to the anionic microbial surface and they have the ability to assume an amphipathic structure that favors their insertion into microbial membranes. Membrane permeation occurs either by a detergent-like disruption of the bacterial cell membrane into peptide coated vesicles or by formation of transient transmembrane pores [7,10,37]. Antimicrobial peptides are unlikely to cause rapid emergence of resistance because it would require significant alteration of phospholipid membrane composition, which is difficult to occur [43].

Practical use of available linear antimicrobial peptides has not been completely satisfactory due to the conformational flexibility of their structure which is often associated to low target selectivity, poor bioavailability, and low stability towards protease degradation. In spite of these drawbacks, linear peptides with significant biological activities have been developed [15,17,25]. However, cyclic peptides exhibit better metabolic stability, selectivity and bioavailability [12–14,26,35,41]. For instance, potent cyclic antimicrobial peptides have been successfully developed and cyclization of cytolytic amphipathic α-helical peptides has been shown to increase the selectivity for bacteria by substantially reducing the hemolytic activity [13,16,24,33].

Despite the advantageous properties of cyclic peptides, few examples of such compounds with antibacterial activity against plant pathogens have been described and are mainly limited to peptides isolated from natural sources [23]. Our current research is oriented toward developing new control methods against economically important plant pathogenic bacteria, such as *Erwinia amylovora*, *Xanthomonas* sp., and *Pseudomonas syringae* for which the available methods are not sufficiently effective [11,32,34]. In particular, a great deal of our research is focused on the identification of small cyclic peptides with specific activity against these bacteria. We have synthesized cyclic peptides of 4–10 residues consisting of alternating cationic (Lys) and hydrophobic (Leu and Phe) amino acids. The cyclic decapeptide c(LysLeuLysLeuLysPheLysLeuLysGln), coded as BPC10L, was active against *P. syringae* and *Xanthomonas vesicatoria*, but it was not active against *E. amylovora* and displayed a high hemolytic activity [4,30]. This peptide constitutes a good lead for the discovery of new cyclic decapeptides with improved biological properties.

Combinatorial chemistry provides a powerful tool to rapidly identify peptides with potentially improved or new properties [8,29,36,39]. Synthesis of libraries of small cyclic peptides by combinatorial chemistry may be considered a suitable strategy for the rapid finding of effective compounds to combat plant diseases. We have recently devised a methodology towards the synthesis of small cyclic peptides (4–10 residues) which can be applied to the preparation of combinatorial libraries [31]. However, a limitation of the combinatorial chemistry approach to optimize molecular properties is the difficulty in determining cooperative effects among the molecular substitutions. Design of experiments (DOE) constitutes a well-known general statistical methodology able to grasp simultaneous, synergic and non-linear effects among experimental factors and to elucidate inner rules governing the system's behavior in order to assist an investigation course [9]. This methodology has been successfully applied in the peptide design and activity prediction [6].

In the present study we report the identification of cyclic decapeptides with higher antibacterial activity and lower eukaryotic cell cytotoxicity than BPC10L through combinatorial chemistry. Two cyclic decapeptide combinatorial libraries were synthesized and screened for in vitro growth inhibition of *E. amylovora*, *P. syringae*, and *X. vesicatoria*. Their cytotoxic effects on eukaryotic cells were determined by evaluating the hemolytic activity. Analysis of internal structural features in conjunction with a DOE led to the identification of cyclic peptides with significant biological properties, and permitted to define general rules that describe a peptide sequence pattern associated with high antibacterial activity and low cytotoxicity.

2. Material and methods

2.1. Materials

All the 9-fluorenylmethoxycarbonyl (Fmoc) acid derivatives, reagents, and solvents used in the peptide synthesis were obtained from Senn Chemicals International (Gentilly, France). D-sized polystyrene Rink amide SynPhase^{TM} lanterns with a 35 μm loading, cogs and spindles were purchased from Mimotopes, Pty Ltd. (Clayton, Australia). Trifluoroacetic acid (TFA), *N*-methyl-2-pyrrolidinone (NMP), $Pd(PPh_3)_4$, sodium *N*,*N*-diethyldithiocarbamate, triisopropylsilane (TIS), and $CHCl_3$ were from Sigma–Aldrich Corporation (Madrid, Spain). Piperidine, *N*-methylmorpholine (NMM), and *N*,*N*-diisopropylethylamine (DIEA) were purchased from Fluka (Buchs, Switzerland). Acetic acid was from Panreac (Castellar del Vallès, Spain). Solvents for reverse-phase high-performance liquid chromatography (RP-HPLC) were obtained from J.T. Baker (Deventer, Holland).

2.2. Synthesis of peptide libraries

Libraries of cyclic decapeptides were synthesized from commercially available D-sized polystyrene Rink amide SynPhase^{TM} lanterns by carrying out solid-phase synthesis of linear sequences, followed by on-lantern cyclization as previously described [31]. A three-dimensional orthogonal Fmoc/tButyl/Allyl strategy was used [22]. Side-chain protection for Lys was as *tert*-butyl carbamate (Boc). A Fmoc-Glu-OAl residue was introduced as trifunctional amino acid to allow peptide anchoring onto the lantern, which resulted in a Gln after peptide cleavage from the solid support.

Libraries were prepared using a split/pool approach to perform washings and common steps. Lanterns were tagged by colored spindles and cogs. Lanterns were placed into a single flask and swelled into CH_2Cl_2 (5 min) to carry out Fmoc protecting group removal by treatment with piperidine–CH_2Cl_2–NMP (1:2:2, 2× 45 min). Washings were performed by dipping lanterns in NMP (3× 5 min) and CH_2Cl_2 (2× 5 min) using plastic syringes fitted with polypropylene frits. Lanterns were

then placed into a capped flask containing a solution of Fmoc-Glu-OAl (120 mM), *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU) (120 mM) and DIEA (240 mM) in NMP. After 24 h, lanterns were washed with NMP (3× 5 min) and CH_2Cl_2 (2× 5 min). Next coupling steps were accomplished by repeated cycles of Fmoc group removal, coupling and washings as described above. For each Fmoc-Lys(Boc)-OH and Fmoc-Leu-OH coupling step, lanterns were sorted and split into two pools, and then combined into a single plastic syringe to be washed. Then they were split again into two groups for the subsequent Fmoc-Lys(Boc)-OH and Fmoc-Leu-OH coupling step or placed into a single flask for the Fmoc-Phe-OH coupling. After chain assembly was completed, the C-terminal allyl ester was cleaved by treatment with $Pd(PPh_3)_4$ (75 mM) in $CHCl_3$–acetic acid–NMM (37:2:1) under nitrogen for 6 h, and lanterns were washed with $CHCl_3$–acetic acid–NMM (37:2:1, 3× 2 min), DIEA–CH_2Cl_2 (1:19, 3× 5 min), sodium *N*,*N*-diethyldithiocarbamate (0.03 M in NMP, 3× 15 min), NMP (5× 5 min) and CH_2Cl_2 (5× 5 min). Linear peptides were obtained by final Fmoc removal. Cyclization was carried out separately into individual flasks by treatment of five stacked disks of each lantern with benzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP) (16 equiv.), 1-hydroxybenzotriazole (HOBt) (16 equiv.), and DIEA (32 equiv.) in 1 ml of NMP at 25 °C for 24 h. Following washes with NMP (3× 5 min) and CH_2Cl_2 (3× 5 min), cyclodecapeptides were cleaved from lanterns by treatment with TFA–H_2O–TIS (95:2.5:2.5) for 1 h in individual polypropylene tubes of a 96-well 1.5 ml plate. Cleavage cocktail was then removed using a Thermo Savant SPD121P SpeedVac concentrator. After Et_2O extraction, cyclic peptides were dissolved in H_2O, lyophilized, and analyzed by analytical RP-HPLC performed at 1.0 ml/min using a Kromasil (4.6 mm × 40 mm; 3.5 μm particle size) C_{18} reversed-phase column. Linear gradients of 0.1% aqueous TFA and 0.1% TFA in CH_3CN were run from 0.98:0.02 to 0:1 over 7 min with UV detection at 220 nm. Final products were confirmed by electrospray ionization mass spectrometry (ESI-MS).

2.3. Bacterial strains and media

The following plant pathogenic bacterial strains were used: *E. amylovora* PMV6076 (Institut National de la Recherche Agronomique, Angers, France), *P. syringae* pv. *syringae* EPS94 (Institut de Tecnologia Agroalimentària, Universitat de Girona, Spain) and *X. vesicatoria* 2133-2 (Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain). All bacteria were stored in liquid Luria Bertani (LB) medium supplemented with glycerol (20%) and maintained at −80 °C. *E. amylovora* PMV6076 and *P. syringae* pv. *syringae* EPS94 were scrapped from LB agar after growing 24 h and *X. vesicatoria* 2133-2 after 48 h at 25 °C. The cell material was suspended in sterile water to obtain a suspension of 10^8 CFU ml^{-1}.

2.4. Antibacterial activity

Lyophilized peptides were solubilized in sterile milli-Q water to a final concentration of 1000 μM and sterilized through a 0.22-μm pore filter. For MIC assessment, dilutions of the synthetic peptides were made to obtain a final concentration of 750, 500, 250, 125, 62.5, 31.2, and 15.6 μM. Twenty microliters of each dilution were mixed in a microtiter plate well with 20 μl of the corresponding suspension of the bacterial indicator and 160 μl of Trypticase Soy Broth (TSB) (BioMèrieux, France) to a total volume of 200 μl. Three replicates for each strain, peptide and concentration were used. Positive controls contained water instead of peptide and negative controls contained peptides without bacterial suspension. Bacterial growth was automatically determined by optical density measurement at 600 nm (Bioscreen C, Labsystem, Helsinki, Finland). Microplates were incubated at 25 °C with 20 s shaking before hourly absorbance measurement for 48 h. Each experiment was repeated twice. The MIC was taken as the lowest peptide concentration with no growth at the end of the experiment. Inhibition of growth (*I*) was calculated as a percentage of the positive control using the equation: $I = 100 \times [(AC - AS)/AC]$, where AC is the area under the curve of the control, and AS is the area under the curve of a given peptide concentration.

2.5. Hemolytic activity

The hemolytic activity of the peptides was evaluated by determining hemoglobin release from erythrocyte suspensions of fresh human blood (5%, v/v). Blood was aseptically collected using a BD vacutainer K2E System with EDTA (Belliver Industrial State, Plymouth, UK) and stored for less than 2 h at 4 °C. Blood was centrifuged at 6000 *g* for 5 min, washed three times with Tris buffer (10 mM Tris, 150 mM NaCl, pH 7.2) and diluted with Tris buffer. Peptides were solubilized in Tris buffer to a final concentration of 750, 500 and 250 μM. Three replicates for each peptide and concentration were used. Sixty-five microliters of human red blood cells were mixed with 65 μl of the peptide solution in MicroAmp® 96-well plates (Applied Biosystems, USA) and incubated under continuous shaking for 1 h at 37 °C. Then, the plates were centrifuged at 3500 *g* for 10 min. Eighty microliter aliquots of the supernatant were transferred to 100-well microplates (Bioscreen) and diluted with 80 μl of milli-Q water. Hemolysis was measured as the absorbance at 540 nm with a Bioscreen plate reader. Complete hemolysis was determined in Tris buffer plus melittin 200 μM (Sigma–Aldrich Corporation, Madrid, Spain) as a positive control. The percentage of hemolysis (*H*) was calculated using the equation: $H = 100 \times [(Op - Ob)/(Om - Ob)]$, where Op was the density for a given peptide concentration, Ob for the buffer, and Om for the melittin positive control.

2.6. Design of experiments (DOE)

DOE was applied over a 16 cyclodecapeptide library of general structure c($X^1X^2X^3X^4$LysPheLysLysLeuGln). Two residues (Leu or Lys) could be present at every molecular position coded as X. According to the nomenclature of DOE methodology, every substitution point constitutes a two-level factor. All the 2^4 possible substitution variations were studied as series, constituting the calculation a two-level full factorial design. In this way, the extraction of information required the comparison of the activity of 16 molecules per numerical experiment. DOE calculations quantitatively assign effects to the distinct factor levels combinations. The magnitude of these effects allows the quantification of the role played in the experimental response (antibacterial or hemolytic activities)

by single, double, triple or quadruple factors interactions. This information was graphically represented in effects plots. The data manipulations have been carried out with the MINITAB program (MINITAB version 14 for Windows. Minitab Inc., State College, PA, 2004).

Among the antibacterial activities, five series of experiments showed a remarkable variability and were selected in order to be statistically analyzed by the DOE methodology. For *X. vesicatoria*, the selected series were the percent of bacterial growth inhibition at peptide concentrations of 3.1 and 6.2 μM. For *E. amylovora*, peptide concentrations were 25 and 50 μM, and for *P. syringae* only the concentration of 6.2 μM was considered. For hemolysis, three series of experiments, corresponding to peptide concentrations of 125, 250, and 375 μM, showed a significant variation and were statistically investigated. All the activity determinations were carried out in triplicate and in a random order.

3. Results

The aim of this study was to improve the biological profile of the previously reported peptide BPC10L c(LysLeuLysLeuLysPheLysLeuLysGln) [4,30]. A library was prepared and screened for antibacterial and hemolytic activities (library I). Results from this library led to a new set of compounds (library II). A DOE based on the biological activities of library II was performed to check for the structural interactions responsible for activity.

3.1. *Library I*

Library I was designed based on the sequence of BPC10L and comprised 56 cyclic peptides. The sequences incorporated a Phe and a Gln residue at positions 6 and 10, respectively. The other positions consisted of all the possible combinations of three Leu and five Lys. Antibacterial activity was tested against *E. amylovora*, *P. syringae* and *X. vesicatoria*. The MIC of bacterial growth of all cyclic peptides is compiled in Table 1. BPC10L was included in the library for comparison purposes.

Most of the peptides exhibited relevant antibacterial activities against at least one pathogen, except for BPC058, BPC068, BPC100, BPC112 and BPC148 which showed low activity against the three bacteria. Interestingly, unlike BPC10L, 21 peptides resulted to be active against *E. amylovora*. Cyclopeptides BPC060, BPC074, BPC084, BPC096, BPC098 and BPC102 displayed the most potent antibacterial activity toward this bacteria with MIC-values 12.5–25 μM. Thirteen peptides showed lower activity (25–50 μM) and two sequences displayed higher MIC values (50–75 μM). The majority of peptides exhibited antibacterial activity against *X. vesicatoria* and *P. syringae*. Five peptides displayed a significantly improved activity toward *X. vesicatoria* compared to BPC10L, in particular, BPC088 and BPC098 (1.6–3.1 μM), and BPC090, BPC092 and BPC096 (3.1–6.2 μM). Twenty-five sequences showed identical MIC-values than BPC10L (6.2–12.5 μM) and 14 peptides displayed moderate activity toward this bacteria (12.5–25 μM). The set of peptides with activity against *P. syringae* included 28 sequences with higher activity than BPC10L. Among them, peptides BPC104 and BPC144 were the most active sequences (3.1–6.2 μM) and 26 peptides showed slightly higher MIC-values (6.2–12.5 μM). Twenty-two compounds of the library resulted as active as BPC10L (12.5–25 μM).

Cyclopeptides were also tested for their hemolytic activity against the highly susceptible human erythrocytes. The percent hemolysis of peptides at 375 μM is shown in Table 1. The results revealed that the hemolytic activity of all peptides was distinctly lower as compared to BPC10L. Thirty-eight peptides exhibited less than 15% hemolysis and only 7 peptides showed a hemolytic activity higher than 35%.

It was performed a frequency analysis of the most occurring residues at each position of peptides of library I displaying the highest antibacterial activity. The data was taken from the peptides with higher values of bacterial growth inhibition (>70%) against *P. syringae* and *X. vesicatoria* at 6.2 μM. For *E. amylovora*, cyclopeptides active at concentrations lower than 100 μM were taken into account. This study revealed that the dominant substitution obeyed to the general structure c($X^1X^2X^3X^4$LysPheLysLysLeuGln). The leftmost residues labeled $X^1X^2X^3X^4$ showed a much more complicated pattern to detect rules for active compounds.

3.2. *Library II*

Library II was designed in order to check for the influence on antibacterial and hemolytic activities of residues at positions 1–4 by using DOE. It comprised 16 cyclopeptides incorporating the substructure $Lys^5PheLysLysLeuGln^{10}$ and all possible combinations of Leu and Lys at positions 1–4. To allow better comparison, compounds BPC088, BPC090, BPC092, BPC094, BPC096, and BPC098 were re-synthesized and included in this library. The data of these compounds are in agreement with those obtained in library I. Antibacterial activity and cytotoxicity of peptides of library II are shown in Table 2.

Peptides BPC184, BPC192, BPC194 and BPC198 showed low MIC values (3.1–6.2 μM) against *X. vesicatoria* (Table 2). Moreover, BPC194 and BPC198 were as active against *P. syringae* as compounds from library I with the highest activity (3.1–6.2 μM). Interestingly, both compounds maintained a low level of hemolysis (around 15%). In addition, BPC194 displayed improved activity toward *E. amylovora* (6.2–12.5 μM). Fig. 1 represents the inhibitory effect on the bacterial growth by peptides BPC194 and BPC198 compared to BPC10L.

3.3. *DOE of library II*

A full two-level factorial design, based on the percent of bacterial growth inhibition and hemolysis obtained for compounds of library II, was performed in order to check the influence of the X^1 to X^4 residues on both biological properties. Results obtained for antibacterial and hemolytic activities are represented by means of effects plots (Figs. 2 and 3, respectively). The effects plots are probabilistic scale graphs commonly used to display the numerical effects obtained from DOE calculations. The points depicted in plots represent calculated effects (increase or decrease of the measured activity) associated to different factor combinations (single, double, triple or quadruple interactions). This magnitude, once expressed in standard deviation units, constitutes the abscissas axis variable. The vertical axis variable is the left queue cumulative

Table 1 – Antibacterial activity (MIC) and cytotoxicity of peptides of library I

Peptide		MIC intervals (μM)			Hemolysis (%)[a]
Code	Sequence	Ps[b]	Xv[c]	Ea[d]	
BPC10L	c(Lys-Leu-Lys-Leu-Lys-Phe-Lys-Leu-Lys-Gln)	12.5–25	6.2–12.5	>100	84 ± 6.9
BPC058	c(Lys-Lys-Lys-Lys-Lys-Phe-Leu-Leu-Leu-Gln)	25–50	25–50	>100	6 ± 0.5
BPC060	c(Lys-Lys-Lys-Lys-Leu-Phe-Lys-Leu-Leu-Gln)	6.2–12.5	6.2–12.5	12.5–25	72 ± 5.5
BPC062	c(Lys-Lys-Lys-Leu-Lys-Phe-Lys-Leu-Leu-Gln)	12.5–25	6.2–12.5	>100	6 ± 0.6
BPC064	c(Lys-Lys-Leu-Lys-Lys-Phe-Lys-Leu-Leu-Gln)	12.5–25	6.2–12.5	25–50	10 ± 2.0
BPC066	c(Lys-Leu-Lys-Lys-Lys-Phe-Lys-Leu-Leu-Gln)	6.2–12.5	6.2–12.5	25–50	22 ± 4.0
BPC068	c(Leu-Lys-Lys-Lys-Lys-Phe-Lys-Leu-Leu-Gln)	25–50	25–50	>100	0 ± 0.5
BPC070	c(Lys-Lys-Lys-Lys-Leu-Phe-Leu-Lys-Leu-Gln)	12.5–25	6.2–12.5	>100	19 ± 0.5
BPC072	c(Lys-Lys-Lys-Leu-Lys-Phe-Leu-Lys-Leu-Gln)	6.2–12.5	6.2–12.5	25–50	7 ± 2.0
BPC074	c(Lys-Lys-Leu-Lys-Lys-Phe-Leu-Lys-Leu-Gln)	6.2–12.5	6.2–12.5	12.5–25	36 ± 1.7
BPC076	c(Lys-Leu-Lys-Lys-Lys-Phe-Leu-Lys-Leu-Gln)	6.2–12.5	6.2–12.5	25–50	13 ± 1.9
BPC078	c(Leu-Lys-Lys-Lys-Lys-Phe-Leu-Lys-Leu-Gln)	12.5–25	6.2–12.5	>100	0 ± 0.4
BPC080	c(Lys-Lys-Lys-Leu-Leu-Phe-Lys-Lys-Leu-Gln)	6.2–12.5	12.5–25	>100	19 ± 0.7
BPC082	c(Lys-Lys-Leu-Lys-Leu-Phe-Lys-Lys-Leu-Gln)	12.5–25	6.2–12.5	25–50	36 ± 2.5
BPC084	c(Lys-Leu-Lys-Lys-Leu-Phe-Lys-Lys-Leu-Gln)	12.5–25	12.5–25	12.5–25	45 ± 3.5
BPC086	c(Leu-Lys-Lys-Lys-Leu-Phe-Lys-Lys-Leu-Gln)	6.2–12.5	25–50	25–50	8 ± 0.9
BPC088	c(Lys-Lys-Leu-Leu-Lys-Phe-Lys-Lys-Leu-Gln)	6.2–12.5	1.6–3.1	25–50	33 ± 3.5
BPC090	c(Lys-Leu-Lys-Leu-Lys-Phe-Lys-Lys-Leu-Gln)	6.2–12.5	3.1–6.2	25–50	35 ± 4.7
BPC092	c(Leu-Lys-Lys-Leu-Lys-Phe-Lys-Lys-Leu-Gln)	6.2–12.5	3.1–6.2	>100	9 ± 0.9
BPC094	c(Lys-Leu-Leu-Lys-Lys-Phe-Lys-Lys-Leu-Gln)	6.2–12.5	6.2–12.5	25–50	73 ± 1.6
BPC096	c(Leu-Lys-Leu-Lys-Lys-Phe-Lys-Lys-Leu-Gln)	6.2–12.5	3.1–6.2	12.5–25	32 ± 7.2
BPC098	c(Leu-Leu-Lys-Lys-Lys-Phe-Lys-Lys-Leu-Gln)	6.2–12.5	1.6–3.1	12.5–25	36 ± 3.7
BPC100	c(Lys-Lys-Lys-Lys-Leu-Phe-Leu-Leu-Lys-Gln)	25–50	25–50	>100	2 ± 0.8
BPC102	c(Lys-Lys-Lys-Leu-Lys-Phe-Leu-Leu-Lys-Gln)	6.2–12.5	25–50	12.5–25	26 ± 3.0
BPC104	c(Lys-Lys-Leu-Lys-Lys-Phe-Leu-Leu-Lys-Gln)	3.1–6.2	6.2–12.5	>100	15 ± 2.2
BPC106	c(Lys-Leu-Lys-Lys-Lys-Phe-Leu-Leu-Lys-Gln)	12.5–25	25–50	>100	0 ± 0.1
BPC108	c(Leu-Lys-Lys-Lys-Lys-Phe-Leu-Leu-Lys-Gln)	6.2–12.5	12.5–25	>100	2 ± 0.2
BPC110	c(Lys-Lys-Lys-Leu-Leu-Phe-Lys-Leu-Lys-Gln)	12.5–25	12.5–25	>100	9 ± 0.4
BPC112	c(Lys-Lys-Leu-Lys-Leu-Phe-Lys-Leu-Lys-Gln)	25–50	25–50	>100	1 ± 0.1
BPC114	c(Lys-Leu-Lys-Lys-Leu-Phe-Lys-Leu-Lys-Gln)	6.2–12.5	6.2–12.5	>100	24 ± 1.8
BPC116	c(Leu-Lys-Lys-Lys-Leu-Phe-Lys-Leu-Lys-Gln)	6.2–12.5	6.2–12.5	25–50	13 ± 0.9
BPC118	c(Lys-Lys-Leu-Leu-Lys-Phe-Lys-Leu-Lys-Gln)	12.5–25	12.5–25	>100	6 ± 0.6
BPC120	c(Leu-Lys-Lys-Leu-Lys-Phe-Lys-Leu-Lys-Gln)	12.5–25	6.2–12.5	>100	8 ± 1.6
BPC122	c(Lys-Leu-Leu-Lys-Lys-Phe-Lys-Leu-Lys-Gln)	6.2–12.5	6.2–12.5	>100	4 ± 1.3
BPC124	c(Leu-Lys-Leu-Lys-Lys-Phe-Lys-Leu-Lys-Gln)	12.5–25	12.5–25	>100	3 ± 0.1
BPC126	c(Leu-Leu-Lys-Lys-Lys-Phe-Lys-Leu-Lys-Gln)	12.5–25	6.2–12.5	>100	4 ± 0.6
BPC128	c(Lys-Lys-Lys-Leu-Leu-Phe-Leu-Lys-Lys-Gln)	12.5–25	25–50	>100	1 ± 0.3
BPC130	c(Lys-Lys-Leu-Lys-Leu-Phe-Leu-Lys-Lys-Gln)	12.5–25	25–50	>100	6 ± 3.0
BPC132	c(Lys-Leu-Lys-Lys-Leu-Phe-Leu-Lys-Lys-Gln)	6.2–12.5	12.5–25	>100	4 ± 0.9
BPC134	c(Leu-Lys-Lys-Lys-Leu-Phe-Leu-Lys-Lys-Gln)	12.5–25	12.5–25	>100	2 ± 0.3
BPC136	c(Lys-Lys-Leu-Leu-Lys-Phe-Leu-Lys-Lys-Gln)	6.2–12.5	6.2–12.5	>100	41 ± 1.8
BPC138	c(Lys-Leu-Lys-Leu-Lys-Phe-Leu-Lys-Lys-Gln)	12.5–25	25–50	>100	5 ± 0.9
BPC140	c(Leu-Lys-Lys-Leu-Lys-Phe-Leu-Lys-Lys-Gln)	6.2–12.5	6.2–12.5	25–50	23 ± 1.1
BPC142	c(Lys-Leu-Leu-Lys-Lys-Phe-Leu-Lys-Lys-Gln)	6.2–12.5	6.2–12.5	25–50	14 ± 1.6
BPC144	c(Leu-Lys-Leu-Lys-Lys-Phe-Leu-Lys-Lys-Gln)	3.1–6.2	6.2–12.5	50–75	9 ± 1.9
BPC146	c(Leu-Leu-Lys-Lys-Lys-Phe-Leu-Lys-Lys-Gln)	6.2–12.5	12.5–25	75–100	3 ± 0.5
BPC148	c(Lys-Lys-Leu-Leu-Leu-Phe-Lys-Lys-Lys-Gln)	25–50	25–50	>100	1 ± 0.2
BPC150	c(Lys-Leu-Lys-Leu-Leu-Phe-Lys-Lys-Lys-Gln)	12.5–25	6.2–12.5	>100	4 ± 0.2
BPC152	c(Leu-Lys-Lys-Leu-Leu-Phe-Lys-Lys-Lys-Gln)	12.5–25	6.2–12.5	>100	2 ± 0.1
BPC154	c(Lys-Leu-Leu-Lys-Leu-Phe-Lys-Lys-Lys-Gln)	6.2–12.5	6.2–12.5	25–50	11 ± 1.7
BPC156	c(Leu-Lys-Leu-Lys-Leu-Phe-Lys-Lys-Lys-Gln)	12.5–25	12.5–25	>100	3 ± 0.4
BPC158	c(Leu-Leu-Lys-Lys-Leu-Phe-Lys-Lys-Lys-Gln)	6.2–12.5	6.2–12.5	50–75	9 ± 0.7
BPC160	c(Lys-Leu-Leu-Leu-Lys-Phe-Lys-Lys-Lys-Gln)	6.2–12.5	12.5–25	>100	3 ± 0.4
BPC162	c(Leu-Lys-Leu-Leu-Lys-Phe-Lys-Lys-Lys-Gln)	12.5–25	12.5–25	>100	2 ± 0.8
BPC164	c(Leu-Leu-Lys-Leu-Lys-Phe-Lys-Lys-Lys-Gln)	12.5–25	12.5–25	>100	4 ± 0.5
BPC166	c(Leu-Leu-Leu-Lys-Lys-Phe-Lys-Lys-Lys-Gln)	12.5–25	12.5–25	>100	0 ± 0.1

[a] Percent hemolysis at 375 μM plus confidence interval ($\alpha = 0.05$).
[b] Ps stands for *P. syringae*.
[c] Xv stands for *X. vesicatoria*.
[d] Ea stands for *E. amylovora*.

Table 2 – Antibacterial activity (MIC) and cytotoxicity of peptides of library II

Peptide		MIC intervals (μM)			Hemolysis (%)[a]
Code	Sequence	Ps[b]	Xv[c]	Ea[d]	
BPC088	c(Lys-Lys-Leu-Leu-Lys-Phe-Lys-Lys-Leu-Gln)	6.2–12.5	1.6–3.1	25–50	33 ± 3.3
BPC090	c(Lys-Leu-Lys-Leu-Lys-Phe-Lys-Lys-Leu-Gln)	6.2–12.5	3.1–6.2	25–50	30 ± 4.1
BPC092	c(Leu-Lys-Lys-Leu-Lys-Phe-Lys-Lys-Leu-Gln)	6.2–12.5	3.1–6.2	>100	7 ± 0.7
BPC094	c(Lys-Leu-Leu-Lys-Lys-Phe-Lys-Lys-Leu-Gln)	6.2–12.5	6.2–12.5	25–50	75 ± 1.6
BPC096	c(Leu-Lys-Leu-Lys-Lys-Phe-Lys-Lys-Leu-Gln)	6.2–12.5	3.1–6.2	12.5–25	24 ± 4.3
BPC098	c(Leu-Leu-Lys-Lys-Lys-Phe-Lys-Lys-Leu-Gln)	6.2–12.5	1.6–3.1	12.5–25	28 ± 2.4
BPC184	c(Lys-Leu-Leu-Leu-Lys-Phe-Lys-Lys-Leu-Gln)	12.5–25	3.1–6.2	50–75	89 ± 5.3
BPC186	c(Lys-Lys-Lys-Leu-Lys-Phe-Lys-Lys-Leu-Gln)	6.2–12.5	6.2–12.5	>100	0 ± 0.4
BPC188	c(Leu-Leu-Leu-Lys-Lys-Phe-Lys-Lys-Leu-Gln)	12.5–25	6.2–12.5	25–50	87 ± 6.5
BPC190	c(Leu-Lys-Lys-Lys-Lys-Phe-Lys-Lys-Leu-Gln)	6.2–12.5	6.2–12.5	>100	0
BPC192	c(Leu-Lys-Leu-Leu-Lys-Phe-Lys-Lys-Leu-Gln)	12.5–25	3.1–6.2	25–50	49 ± 7.7
BPC194	c(Lys-Lys-Leu-Lys-Lys-Phe-Lys-Lys-Leu-Gln)	3.1–6.2	3.1–6.2	6.2–12.5	17 ± 1.7
BPC196	c(Leu-Leu-Lys-Leu-Lys-Phe-Lys-Lys-Leu-Gln)	12.5–25	6.2–12.5	>100	47 ± 7.2
BPC198	c(Lys-Leu-Lys-Lys-Lys-Phe-Lys-Lys-Leu-Gln)	3.1–6.2	3.1–6.2	12.5–25	14 ± 1.4
BPC200	c(Leu-Leu-Leu-Leu-Lys-Phe-Lys-Lys-Leu-Gln)	25–50	12.5–25	>100	71 ± 11.7
BPC202	c(Lys-Lys-Lys-Lys-Lys-Phe-Lys-Lys-Leu-Gln)	12.5–25	6.2–12.5	>100	2 ± 0.2

[a] Percent hemolysis at 375 μM plus confidence interval ($\alpha = 0.05$).
[b] Ps stands for *P. syringae*.
[c] Xv stands for *X. vesicatoria*.
[d] Ea stands for *E. amylovora*.

probability to find a data point in the sequence of points once sorted by its effect magnitude. The straight line in Figs. 2 and 3 shows the ideal fitting line to which the points should approach if a random Gaussian distribution is followed. Points around this line represent effects that are not significant. The points that move far away from the ideal line reveal which kind of interactions are statistically significant.

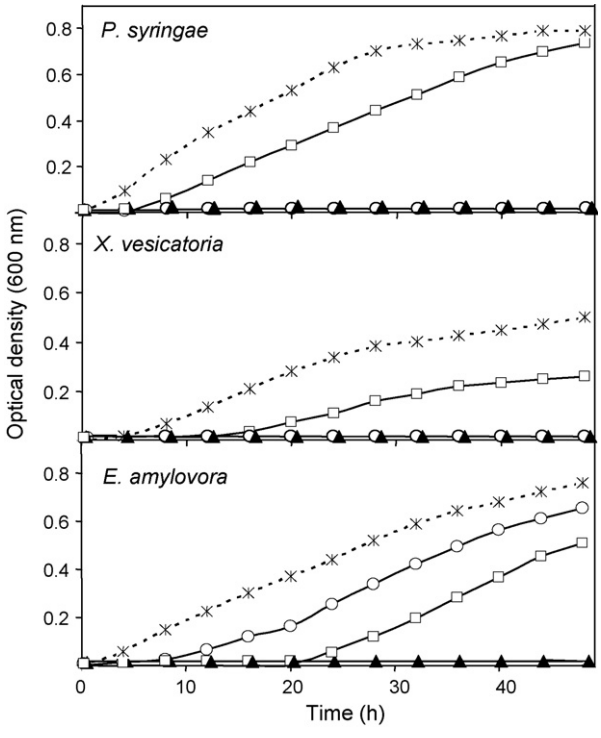

Fig. 1 – Growth of plant pathogenic bacteria in the absence (⁕) and presence of BPC10L (□), BPC194 (▲) and BPC198 (○). The peptide concentration was 6.2 μM for all the peptides in *P. syringae* and *X. vesicatoria*. In *E. amylovora* the concentration was 12.5 μM for BPC194 and BPC198, and 100 μM for BPC10L.

Effects plots were obtained by Minitab from the analysis of the percent of bacterial growth inhibition at peptide concentrations of 3.1 and 6.2 μM for *X. vesicatoria*, 6.2 μM for *P. syringae*, and 25 and 50 μM for *E. amylovora*. Representative data series are shown in Table 3. The numerical analysis revealed that all peptides showed cooperative effects among residues, many of them including statistically significant high-order interactions.

For the three bacteria at all the concentrations studied, almost all the interactions were significant, but the most remarkable and dominant effect was the double interaction involving residues at positions 2 and 3, as it is labeled in Fig. 2 for *X. vesicatoria* at 3.1 μM. The interaction plot represented in the small panel in Fig. 2 revealed that residues X^2 and X^3 had to be distinct. In this interaction plot, the segments delimited by points are attached to a Leu residue at position 2, whereas the segments delimited by squares correspond to a Lys residue at position 2. The left and right parts of this panel specify the Leu (left) or Lys (right) residues at position 3. The vertical axis measures represent compound activities. The overall interpretation is that the cross which can be seen in the panel indicates that when a residue is fixed in one position, a high mean activity is found if the other residue is different.

According to the visual inspection and the numerical data given by Minitab program, other secondary rules contributed to define with more precision the optimal combinations leading to a maximum activity. For *X. vesicatoria* at 3.1 and 6.2 μM, except for BPC196, it was found that the rule to follow is to set $X^2 \neq X^3$ and then preferably set X^2 = Leu and X^4 = Lys. Simple interactions revealed to be also important and compatible with this pattern and consisted into maintaining a Lys at positions 1 and 4. This set of rules selected as the most active peptide the one incorporating the substructure

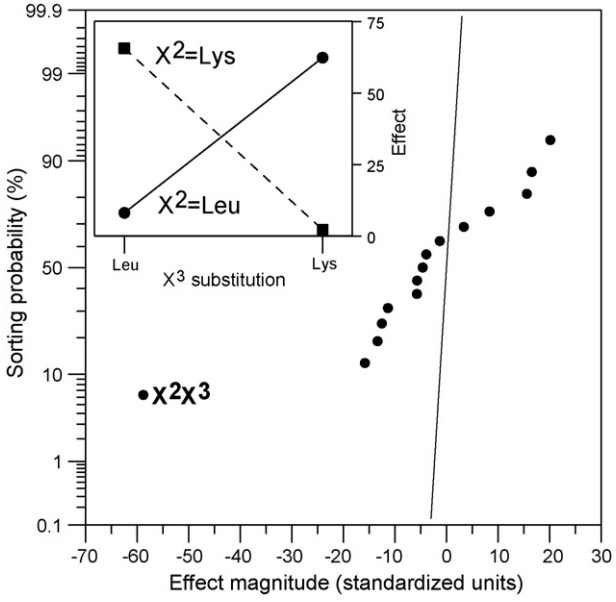

Fig. 2 – Effects plot obtained for *X. vesicatoria* at 3.1 μM. The most statistically significant and dominant factor combination is the labeled double interaction X^2X^3. For this pair of variables, the small panel represents the corresponding interaction plot indicating that substitutions at X^2 and X^3 must be distinct. The same qualitative behavior is found for the other bacteria.

$X^1X^2X^3X^4$ = LysLeuLysLys, that is BPC198. Concerning *P. syringae* at 6.2 μM it was found that the main rule to follow is to keep the $X^2 \neq X^3$ constrain and then maximize the number of Lys. This led to select BPC194 and BPC198 as the most active sequences. For *E. amylovora* at 25 μM it was found that the constraint $X^2 \neq X^3$ must also be preserved and, similarly to *X. vesicatoria*, combined with the secondary rule consisting on maximizing the number of Lys at positions 1 and 4. These rules especially pointed to compounds BPC194 and BPC198. At 50 μM the $X^2 \neq X^3$ constraint was found to be also applicable and has to be combined with some secondary preferences such as to set $X^3 = X^4$ = Lys or X^2X^4 = LeuLys. This set of rules led to fix the attention to compound BPC098 and again to BPC198.

The factorial design analysis of the percent hemolysis at 125, 250 and 375 μM gave similar results. Fig. 3 represents the effects plot analysis at 375 μM (numerical data in Table 3) showing that there are two relevant single effects dominated by residues X^2 and X^3 separately (see labeled points in Fig. 3). The interaction plot (not shown) revealed that simultaneous presence of a Lys at positions 2 and 3 accounted for many of the lowest hemolytic compounds (BPC092, BPC186, BPC190 and BPC202). Although this rule involving single substitution patterns differs from the described for maximizing antibacterial activity, the structures including different residues at positions 2 and 3 showed a low hemolytic level (≤25%), especially the subset with a Lys at position 4 (BPC096, BPC098, BPC194 and BPC198).

4. Discussion

As part of our search for effective methods to control plant pathogenic bacteria of economic importance such as *E. amylovora*, *P. syringae* and *X. vesicatoria*, we have previously identified cationic cyclic peptides with interesting biological properties. These peptides consist of alternating hydrophobic (Leu and Phe) and hydrophilic (Lys) amino acids, adopting an

Table 3 – Data for library II used for DOE analysis (Changes of X^1 to X^4 residues in the cyclic decapeptides c($X^1X^2X^3X^4$LysPheLysLysLeuGln), bacterial growth inhibition and hemolysis at selected peptide concentrations)

Residues				Peptide	Inhibition (%)									Hemolysis (%)[a]		
					Xv(3.1 μM)[b]			Ps (6.2 μM)[c]			Ea(25 μM)[d]					
X^1	X^2	X^3	X^4													
Leu	Leu	Leu	Leu	BPC200	0.0	0.0	0.0	23.4	23.4	22.4	0.0	0.0	0.0	80.8	73.2	60.4
Lys	Leu	Leu	Leu	BPC184	5.5	5.9	5.6	32.7	33.0	35.4	0.0	0.0	0.0	94.9	87.2	86.5
Leu	Lys	Leu	Leu	BPC192	30.5	40.8	43.8	46.9	45.4	51.6	19.1	16.4	17.8	43.1	47.8	56.5
Lys	Lys	Leu	Leu	BPC088	92.0	82.9	92.0	73.4	96.6	79.3	89.2	86.9	76.9	29.9	35.6	34.1
Leu	Leu	Lys	Leu	BPC196	0.0	0.0	0.0	36.2	34.4	37.6	0.0	8.0	0.8	52.5	49.0	40.1
Lys	Leu	Lys	Leu	BPC090	71.2	76.0	69.9	72.0	72.8	75.4	86.9	78.2	85.8	29.9	34.3	27.0
Leu	Lys	Lys	Leu	BPC092	12.8	10.3	3.3	54.1	60.5	60.3	3.5	3.1	1.6	7.7	6.7	6.8
Lys	Lys	Lys	Leu	BPC186	0.0	0.0	0.0	82.9	86.3	86.5	0.0	0.0	0.0	0.0	0.6	0.6
Leu	Leu	Leu	Lys	BPC188	2.5	0.8	0.8	34.0	34.7	35.1	2.1	0.0	0.0	87.4	81.9	93.4
Lys	Leu	Leu	Lys	BPC094	21.3	22.3	32.5	29.7	50.6	50.6	41.8	31.7	40.3	73.4	76.2	75.1
Leu	Lys	Leu	Lys	BPC096	67.6	66.2	66.6	82.1	81.3	83.4	97.0	98.2	99.0	21.3	23.6	28.7
Lys	Lys	Leu	Lys	BPC194	65.9	68.6	70.8	100.0	100.0	100.0	100.0	100.0	100.0	16.9	15.6	18.7
Leu	Leu	Lys	Lys	BPC098	94.2	90.6	100.0	87.3	95.0	95.0	100.0	99.9	100.0	30.7	27.0	27.0
Lys	Leu	Lys	Lys	BPC198	83.8	84.1	78.2	100.0	100.0	100.0	100.0	93.5	83.1	13.1	15.3	15.2
Leu	Lys	Lys	Lys	BPC190	0.0	0.0	0.0	82.7	82.7	82.9	0.0	0.0	0.0	0.0	0.0	0.0
Lys	Lys	Lys	Lys	BPC202	0.0	0.0	0.0	37.1	37.8	37.6	0.0	0.0	0.0	2.1	1.8	1.9

[a] Percent hemolysis at 375 μM.
[b] Xv stands for *X. vesicatoria*.
[c] Ps stands for *P. syringae*.
[d] Ea stands for *E. amylovora*.

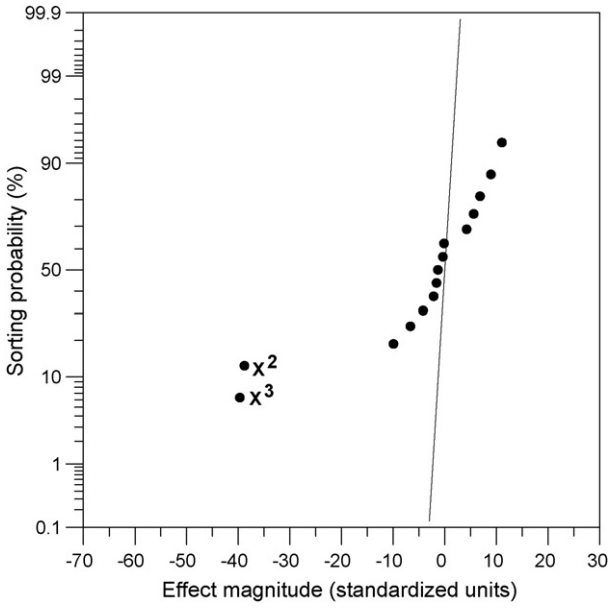

Fig. 3 – Effects plot obtained for the hemolysis experiments. The statistically significant effects are the labeled single interactions X^2 and X^3. The interaction plot (not shown) revealed that a Lys residue must be placed at every substitution point.

amphipathic structure which influences their antibacterial and cytotoxic activities [4,30]. In particular, the cyclodecapeptide BPC10L, consisting of c(LysLeuLysLeuLysPheLysLeuLysGln), was active against *P. syringae* (12.5–25 μM) and *X. vesicatoria* (6.2–12.5 μM), but it was inactive against *E. amylovora* and showed a significant hemolytic activity (84%).

In the present work, library I consisting of 56 peptides allowed for a first optimization of the biological properties of BPC10L. The activity against *X. vesicatoria* and *P. syringae* was four-fold improved as compared to BPC10L. Most peptides were active against *X. vesicatoria*, indicating that this bacteria is highly susceptible toward this family of cyclic peptides, as previously reported with related compounds [30]. Notably, peptides active against *E. amylovora* were identified, constituting the first report of cationic cyclic peptides with activity toward this bacteria. Furthermore, 34 peptides were eight-fold less hemolytic than BPC10L.

Frequency analysis of the most occurring residues at each position in the most active compounds of library I permitted the identification of structural features not easily observed. This approach enables the identification of the relative importance of each amino acid for every position in the peptide, similarly to the positional scanning methodology [27,40]. The analysis suggested that the residues $Lys^5PheLysLysLeuGln^{10}$ constituted a structural requirement that could lead to improved activity. In fact, decapeptides from library I containing this substructure in their sequence exhibited low MIC values against the three bacteria. Among them, BPC088 and BPC098 displayed the best activity toward *X. vesicatoria*, and BPC096 and BPC098 were among the most active peptides against *E. amylovora*.

Library II led to the identification of peptides of general structure $c(X^1X^2X^3X^4LysPheLysLysLeuGln)$ with better antibacterial and hemolytic profiles. BPC194 and BPC198 displayed low MIC values against *P. syringae* and *X. vesicatoria* while maintaining a low hemolytic level. Moreover, BPC194 exhibited the highest activity against *E. amylovora*, implying a two-fold increase as compared to peptides of library I.

Results obtained for library II were numerically analyzed by DOE in order to find a general rule to identify peptides incorporating an optimal amino acid combination at positions 1–4 associated to improved biological properties. DOE has been reported as a useful tool in peptide chemistry [21] to direct the design of libraries and property prediction as well as to extract optimization rules [2,3,5]. In this context, DOE has been successfully performed over dichotomic data [6]. Due to their dichotomic nature, this data can be arbitrarily codified and numerically studied by means of a two-level factorial design, which is especially suitable when a reduced number of molecules are studied. This methodology was applied in the present study since library II consisted of a small set of compounds incorporating only two possible amino acids at each position 1–4. This approach allowed us to simultaneously study all the effects of each residue and also to supervise and discriminate single or multiple interactions between them. These interactions cannot be detected with the experimental methods commonly used in the design of peptide libraries by combinatorial chemistry that are based on varying only one factor at a time.

The simultaneous study of the antibacterial activities allowed us to find a general rule defining a peptide sequence pattern valid for high activity against the three bacteria. The general rule obtained with DOE consisted in setting different residues X^2 and X^3. Although secondary interactions characteristic for each bacteria were also determined, the general rule accounted for compounds exhibiting the highest activity against the three bacteria, in particular, peptides BPC088, BPC090, BPC096, and especially BPC098, BPC194, BPC198. The only exceptions were compounds BPC192 and BPC196 that did not display high activity despite obeying the $X^2 \neq X^3$ constraint.

Notably, the general rule obtained for antibacterial activity was compatible with low eukaryotic cell cytotoxicity. Although this rule did not account for the less hemolytic compounds, the hemolytic values obtained when maximizing the antibacterial activity were acceptable, even more taking into account that hemolysis was evaluated at concentrations 30–120 times higher than the antibacterial activity. Peptides that showed the best biological properties were BPC096, BPC098, BPC194, and BPC198, which fulfill the substitution rule $X^2 \neq X^3$ and include a Lys at position 4.

Therefore, in the present study, DOE was a useful tool to assist and complement the identification of bioactive peptides. The method allowed describing general rules applicable for bacterial growth inhibition and hemolysis at different peptide concentrations, leading to the selection of optimal compounds minimizing at the same time the number of false positives. It is expected that this methodology could be extended to similar studies in searching for new antimicrobial peptides.

In summary, cyclodecapeptides with better biological properties than BPC10L have been identified. The sequence pattern for high antibacterial activity and low hemolysis is

c($X^1X^2X^3X^4$LysPheLysLysLeuGln) where $X^2 \neq X^3$ (main rule) and X^4 = Lys (secondary rule). BPC194 and BPC198 were the peptides with better activity profile. They were active against *E. amylovora*, *P. syringae* and *X. vesicatoria*, and displayed a low hemolytic activity. The peptides reported here are comparable in terms of activity to antibiotics, such as streptomycin, used in agriculture for bacterial disease control with MIC values of 2–9 μM, and operational doses for field treatment of around 100 μM. At field doses similar to those of streptomycin, these peptides are not expected to present cytotoxic effects. Therefore, these peptides might be considered as suitable candidates to develop antimicrobial agents for use in plant protection.

Acknowledgments

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