Expression of cecropin B in *Pichia pastoris* and its bioactivity *in vitro*

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Received April 5, 2011; Accepted May 2, 2011

DOI: 10.3892/etm.2011.262

Abstract. Natural cecropin B (CB), consisting of 35 amino acids, is a member of the cecropin family with the highest antibacterial activity. Here, a novel approach for the generation of recombinant CB in the methylotrophic yeast *Pichia pastoris* was explored. For this purpose, the CB gene was amplified by recursive PCR (rPCR) and cloned into the pPICZαA vector. The *Sac*I-linearized plasmid pPICZαA-CB was transformed into *P. pastoris* SMD1168 by electroporation. The expression of recombinant CB was induced with 1.0% methanol at pH 5.0 for 60 h at 28˚C. Recombinant CB was purified using cationic exchange chromatography; 5.0 mg of pure active CB was obtained from 100 ml of culture broth supernatant. Antimicrobial assays demonstrated that CB has a broad spectrum of antimicrobial activity against both Gram-positive and Gram-negative bacteria. Our results suggest that the *P. pastoris* expression system can be used to produce large quantities of fully functional CB for both research and industrial purposes.

Introduction

Antimicrobial peptides (AMPs) are amphiphilic, positively charged molecules (1-4) that have emerged as novel antimicrobial agents for use in therapeutics, animal drugs and food preservatives. In recent years, many different types of AMPs have been identified in various organisms, including amphibians, mammals, plants, invertebrates and prokaryotes (1,5-7). Currently, the database of the National Center for Biotechnology Information records more than 800 sequences of antibacterial peptides and proteins from both animals and plants.

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Key words: antibacterial activity, cecropin B, expression, *Pichia pastoris*, antimicrobial assays

The cecropin family of antibacterial peptides is found in the immune hemolymph of silkworm pupae (8), insects and mammals (9,10) and plays an important role in the innate immune systems of these animals. The cecropin family is comprised of cecropins A, B, C, D, E and F, all of which are 35 to 39 amino acids in length and share highly homologous sequences (11). These peptides were originally described as having a broad spectrum of antibacterial activity against both Gram-positive and Gram-negative bacteria, but lacking the ability to lyse eukaryotic cells (12). Therefore, cecropins are considered to be valuable peptide antibiotics, particularly against bacteria that have developed resistance to chemical antibiotics. Moreover, previous studies (5) have demonstrated that cecropins lyse cancer cells, opening the possibility for their use in medical applications (13).

Over the past decade, bacterial resistance to antibiotics has risen dramatically. Thus, there is an increasing need to discover and introduce novel effective antibiotic drugs. Cationic antimicrobial peptides are one potential source of novel antibiotics. Antimicrobial peptides are naturally occurring antibiotics (14). As part of the innate immune system of vertebrates, these peptides have direct antimicrobial function (15). However, few peptide antibiotics have been tested in clinical trials, and these few have exhibited mixed results (16-19). ABP-CB is a promising novel antibiotic candidate. However, the yield of ABP-CB extracted from the silkworm is fairly small. The use of recombinant expression methods may allow for the production of a sufficient quantity of ABP-CB in order to meet the demand. We expressed ABP-CB in the methylotrophic yeast *Pichia pastoris*, which has been widely utilized as a heterologous gene expression system. The increasing popularity of this expression system is attributed to three key advantages: i) it possesses a highly regulated alcohol oxidase (AOX1) promoter gene that can be induced by methanol and repressed by glucose and glyceroland; ii) a minimal salt medium yields a high cell density, making this system cost-effective; and iii) *P. pastoris* possesses subcellular organelles, such as an endoplasmic reticulum and Golgi apparatus, allowing for post-translational modifications that include protein folding, disulfide bridge formation and glycosylation (20-24).

Here, the synthesis of the ABP-CB gene, the extracellular expression of ABP-CB protein in *P. pastoris* SMD 1168 and

the characterization of the biological activity of recombinant ABP-CB are described in detail.

Materials and methods

Materials. E. coli DH5α was used for cloning the pPICZα-A-ABP-CB plasmid. *E. coli* K88 and *Staphylococcus aureus* (Cowan I) were used for the antibacterial assay.

The pPICZα-A vector (Invitrogen) was used for the expression of ABP-CB. The *P. pastoris* strain SMD1168 (his^- mut^+) was used for the expression of the ABP-CB protein.

The restriction enzymes *Xho*I, *Xba*I and *Sac*I and T4 DNA ligase were purchased from Takara (Nanjing, China).

Synthesis of cecropin B. Three 60-bp oligomers with 20 bases complementary to their 3' ends were designed based on the CB amino acid sequence. In order to express the native N-terminus of CB, a *Xho*I restriction site was introduced into the α-factor secretion signal of the pPICZ-A expression vector to allow in-frame cloning, and a nucleotide sequence encoding the KEX2 cleavage site was placed upstream of the CB gene. At the C-terminus, an Asn codon, a *Xba*I restriction site and a stop codon were introduced. The sequences of the primers were as follows: F1, cctctcgagaaaagaaagtggaaggtcttcaagaaga tcgaaaagatgggtcgtaac; F2, gatagctggaccagccttgacgataccgttacg gatgttacgacccatcttttc; F3, gctggtccagctatcgctgtcctaggtgaagct aaggctctacaa tga tct aga act.

Gene splicing by overlap extension (SOE) was used for gene synthesis (Fig. 1). An additional pair of primers was designed for identification of the recombinant vector and yeast. The upstream primer targeted the 5' AOX gene region of *P. pastoris*, and the downstream primer targeted the insertion region of CB; the amplification length was 496 bp. The sequences of the primers were as follows: P1: 5'GTCTCC ACATTGTATGCTTC3'; P2, 5'CTGTGCAGGAACTTGAT3'.

All primers were synthesized by the Shanghai Branch Office of Invitrogen.

PCR amplification was performed according to the SOE method using the F1, F2 and F3 elements as templates and primers. In addition, Touchdown (TD) PCR technology was used to optimize the PCR method in order to ensure the specificity of the SOE synthesis. The TD-PCR reaction involved the following solutions: 5 µl 10X Mg^{2+}-free PCR buffer; 3 µl 25 mmol/l $MgCl_2$; 1 µl 10 mmol/l dNTP; 2 µl each 40 pmol/l F1, F2, F3; 0.5 µl Takara ExTaq™; and 38.5 µl sterile ultrapure water. All solutions were mixed uniformly and centrifuged for use in TD-PCR. The reaction was initialized at 94˚C for 2 min and TD-PCR circulation was performed at 94˚C for 30 sec; the annealing temperature was decreased from 65 to 50˚C in 0.5˚C increments/min and maintained at 72˚C for 1 min; the temperature was then maintained at 50˚C after 30 cycles, and then another 15 cycles were performed at an optimization temperature of 52˚C; extension was performed at 72˚C for 6 min.

Construction of recombinant expression plasmids. PCR products and pPICZα-A vectors were digested with *Xho*I and *Xba*I, gel-purified and ligated together. *E. coli* DH5α was chemically transformed with the recombinant vectors and then cultured at 37˚C on low-salt LB supplemented with 25 µg/ml Zeocin for selection of recombinants. The nucleotide sequence of the recombinant pPICZα-A-CB plasmid was verified by restriction analysis and DNA sequencing in order to ensure 100% identity with the expected sequence and in-frame orientation. The positive clones were then grown for further DNA transformation.

Transformation and PCR analysis of P. pastoris. Yeast cells were transformed using the Pichia Expression kit. Briefly, 80 µl of competent *P. pastoris* SMD1168 cells mixed with 5 µg *Sac*I-linearized pPICZα-A-CB were transferred into an ice-cold 0.2-cm electroporation cuvette and incubated in an ice bath for 5 min. Immediately following electroporation at 1.5 kV, 25 µF, 200 Ω, 1 ml of ice-cold 1 M sorbitol was added to the cuvette, which was then incubated for 1 h at 30˚C without shaking. Aliquots of 200 µl were spread on YPDS plates supplemented with 100 µg/ml Zeocin, and the plates were incubated at 30˚C until colonies appeared. As a negative control, linearized pPICZα-A vector alone was transformed into *P. pastoris* SMD1168 cells.

The transformants bearing the genomic integrants of the pPICZα-A-CB plasmids were identified by genomic PCR using primers P1 and P2. The method of 'boiling-freezing-boiling' was used to prepare the Pichia genomic DNA template. PCR amplification was performed with the following conditions: initial denaturation at 94˚C for 5 min, followed by 26 cycles at 94˚C for 1 min, 46˚C for 1 min and 72˚C for 1 min, and elongation at 72˚C for 7 min.

Induction of ABP-CB expression in recombinant P. pastoris using the shake-flask method. A single colony of transformants was grown in 5 ml of buffered glycerol-complex medium [BMGY, 1% (w/v) yeast extract, 2% (w/v) peptone, 1.34% YNB, 1% glycerol, 0.4 µg/ml biotin, buffered with 1/10 volume potassium phosphate buffer pH 6.0] in a 50-ml flask. The culture was grown at 28˚C in a rotating shaker at 230 rpm for 24 h until the culture reached an OD_{600} equal to 5. The culture was harvested after centrifugation at 1,500 g for 5 min at room temperature. To induce ABP-CB expression, the pellet was resuspended in 25 ml of buffered methanol-complex medium [BMMY, 1% (w/v) yeast extract, 2% (w/v) peptone, 1.34% YNB, 0.5% methanol, 0.4 µg/ml biotin, buffered with 1/10 volume potassium phosphate buffer pH 6.0] in a 250-ml flask and grown at 28˚C with shaking. To maintain expression of ABP-CB, methanol was added to the culture at a final concentration of 1%. In addition, aliquots were sampled throughout the incubation time in order to perform activity assays. Several parameters that could influence the level of protein expression were analyzed. Antibacterial activity assays were performed by measuring zones of growth inhibition of *E. coli* K88 in thin agar plates, as described by Cipakova *et al* (25) A total of 100 µl of each condensed expression supernatant was added to the wells.

Determination of ABP-CB expression efficiency and protein concentration. The efficiency of ABP-CB expression was assessed by Tricine-SDS-PAGE. Protein concentration was determined using the Bio-Rad dye agent with bovine serum albumin (BSA) as a standard (23).

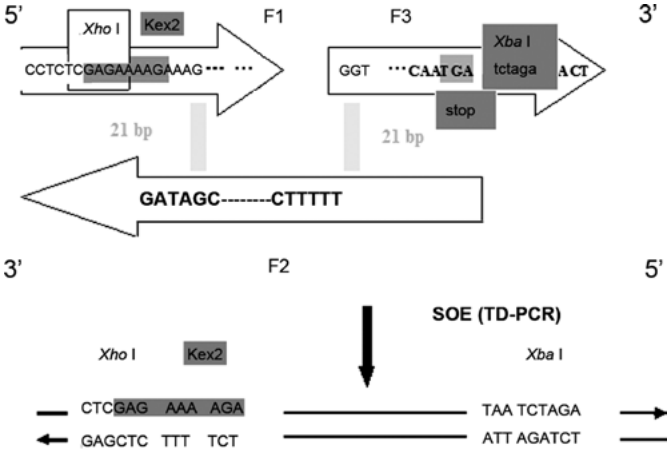

Figure 1. Schematic representation of synthetic cecropin B.

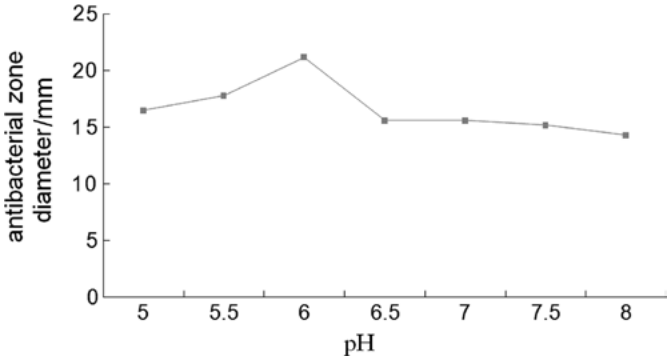

Figure 2. Effect of pH on the production of recombinant cecropin B.

Purification of secreted cecropin B. After 60 h of culture, the culture medium (100 ml) was collected by centrifugation at 12,000 g for 30 min. The supernatant was filtrated using the Amicon ultrafiltration device. The filtrate, which contained proteins that ranged from 3 to 10 kDa in size, was dialyzed overnight in 0.1 M sodium acetate and then applied to a CM-Sepharose CL-6B column (Pharmacia Biosciences, USA) pre-equilibrated with 0.1 M sodium acetate (pH 4.5). The column was washed with 0.1 M acetate buffer, and the proteins were eluted using a linear gradient of 0.1-1.0 M sodium acetate (pH 4.5). The purified proteins were analyzed by Tricine-SDS-PAGE.

Antimicrobial assays. The antimicrobial activity of CB was tested against several Gram-positive and Gram-negative bacteria. The minimal growth inhibition concentration (MIC), which is defined as the lowest concentration of a peptide that can inhibit the growth of a microorganism (26), was determined by performing a liquid growth inhibition assay (27). A stock solution of CB was serially diluted 10-fold in 0.01% acetic acid and 0.2% BSA. Aliquots of each dilution (10 μl) were distributed into the wells of a 96-well polypropylene microtiter plate, and each well was inoculated with 100 μl of a suspension of mid-log bacteria (2-7x10^5 CFU/ml) in LB broth. Cultures were grown for 24 h at 30˚C with vigorous shaking, and the bacterial concentration was evaluated by measuring the absorbance of the cultures at 600 nm using a microplate reader (Bio-Rad, USA).

Acid stability analysis of recombinant CB. Buffers of pH ranging from 2.5 to 8.5 were prepared. Antibacterial CB peptide (20 μl) and of the different buffers were mixed in order to check the acid stability of CB. Buffer alone was used as a negative control.

Heat stability of recombinant CB. Recombinant CB was heated at 60, 80 and 100˚C for 5 min, and then centrifuged at 12,000 rpm for 10 min in order to separate the precipitate. The supernatant was then used in the antibacterial assays.

Results

Construction and transformation of pPICZα-A-CB. Fig. 1 shows a schematic representation of the sequence of the synthetic CB peptide. The amplification of the CB gene was achieved through TD-PCR, resulting in a product of 129 bp in length. After transformation, transformants were screened by restriction enzyme analysis using *Xho*I and *Xba*I and sequencing. The results of restriction enzyme analysis and DNA sequencing revealed that the CB gene was inserted correctly into the expression vector. Transformation with the *Sac*I-linearized version of pPICZα-A-CB favored its insertion into the yeast genome by homologous recombination.

Determination of the optimal conditions for secretion of recombinant CB. The activity of the recombinant CB secreted by *P. pastoris* was analyzed under several different conditions. The supernatant was collected and its antimicrobial activity was monitored by performing bacterial inhibition assays. In addition, several parameters that affect the level of protein expression were assayed. The secretion of proteases into the medium, and possibly their release due to cell lysis, is a significant problem in many high-cell-density cultures because it can lead to proteolytic degradation. The induction temperature is also an important parameter. In *P. pastoris*, incubation at 30, 25, 20 and 15˚C was performed in attempts to minimize extracellular proteolysis. Lower temperatures have been shown to reduce protease levels and to greatly enhance the yield of biologically active proteins in *P. pastoris* (8,28-31). We found that reducing the incubation temperature from 30 to 20˚C during the methanol-feed phase dramatically increased the yield of recombinant protein (data not shown). Culture of *P. pastoris* on BMMY medium at pH 6.0 provided the most optimal conditions for the expression of CB, and a methanol induction time of 60 h was the most suitable (Figs. 2 and 3).

Expression of CB in P. pastoris. The highest CB-producing *P. pastoris* clone was selected for subsequent large-scale protein expression under the most optimal conditions of 28˚C with 1.0% methanol for 60 h. Approximately 50 mg of ABP-CB was secreted into 1 l of medium. The resultant protein appeared as a single homogeneous band on a Coomassie blue-stained Tricine-SDS-PAGE gel (Fig. 4). The size of the recombinant CB was consistent with the expected

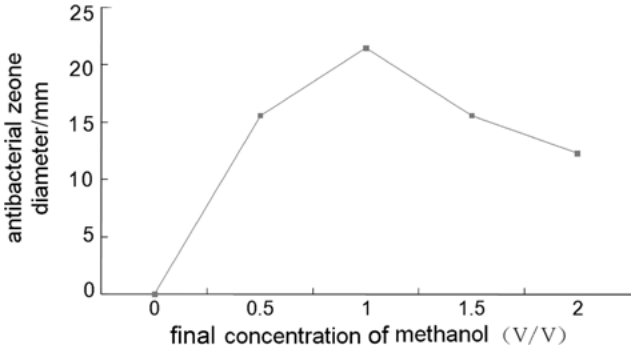

Figure 3. Effect of methanol concentration on the production of recombinant cecropin B.

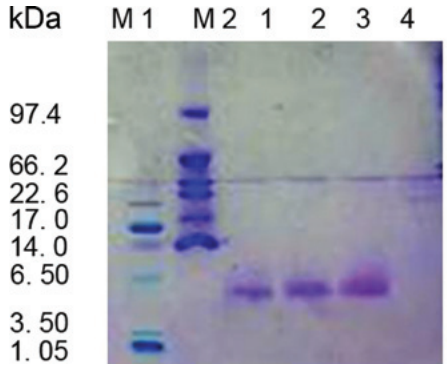

Figure 4. Tricine-SDS-PAGE analysis of cecropin B. M1, ultra-low molecular weight protein marker; M2, low molecular weight protein marker; lanes 1 and 2, culture supernatant of SMD1168/pPICZα-CB; lane 3, purified cecropin B; lane 4, supernatant of SMD1168/pPICZα.

molecular weight of 4.7 kDa calculated from the amino acid sequence.

Antimicrobial assay. The antibacterial activity of recombinant CB was qualitatively determined with a radial diffusion assay using *E. coli* as a representative Gram-negative bacterium. CB was found to be highly effective against *E. coli* (Fig. 5). Similar results were obtained from radial diffusion assays using the Gram-positive bacterium *S. aureus* (Fig. 6, Table I).

Acid stability of recombinant CB. Recombinant CB had the strongest antibacterial activity at pH 4.0. There were no obvious changes observed in the diameters of the antibacterial zone at pH values between 3.5 and 7.5. The diameter of the antibacterial zone decreased gradually at pH values >7.5. Recombinant CB was stable at low (3.5) and high pH (8.5), resulting in suppressed antibacterial activity (Fig. 7).

Heat stability of recombinant CB. Incubation of the CB supernatant at 60˚C for 5 min had no effect on its antibacterial activity. We found that there were many ingredients in the supernatant of the CB peptide, which were removed by treatment at high temperature. Boiling the supernatant for 30 min removed approximately 60% of the mixed proteins found in the supernatant without changing the heat resistance of CB (Fig. 8).

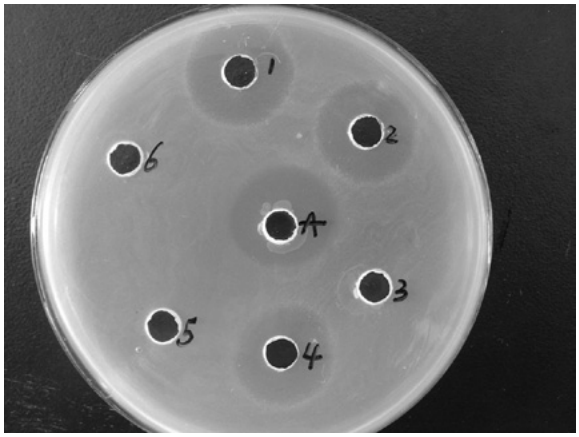

Figure 5. Analysis of the antibacterial activity of cecropin B against *E. coli*. A, Amp control (25 μg/ml); 1, 3 and 4: supernatant of cecropin B; 2, 5 and 6: control supernatant from *P. pastoris* SMD1168/pPICZα-A.

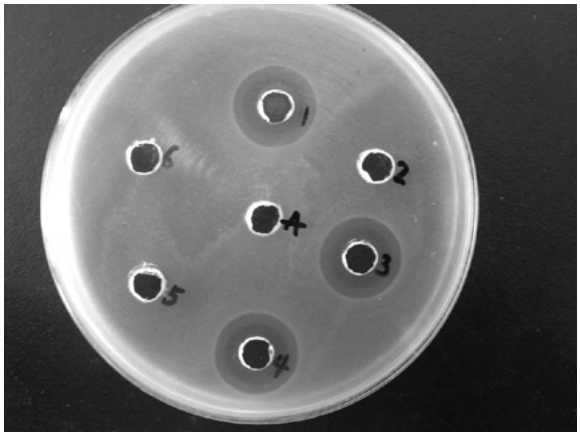

Figure 6. Analysis of the antibacterial activity of cecropin B against *S. aureus*. A, Amp control (25 μg/ml); 1, 2 and 4: supernatant of cecropin B; 3, 5 and 6: control supernatant from *P. pastoris* SMD1168/pPICZα-A.

Table I. Antibacterial activity of cecropin B (MIC) expressed as the final concentration in μM.

Microorganism	MIC (μM)
Gram-positive bacteria	
Staphylococcus aureus	0.89
Bacillus subtilis	0.98
Sporosarcina	0.88
Curtobacterium	0.97
Gram-negative bacteria	
E. coli	0.50
Salmonella pullorum	0.78
Pseudomonas aeruginosa	0.98

Discussion

Cecropins are a family of small basic polypeptides that are mainly present in the hemolymph of insects (30). CB exhibits significant antibacterial activity against Gram-positive and Gram-negative bacteria, and also has potent antitumor activity

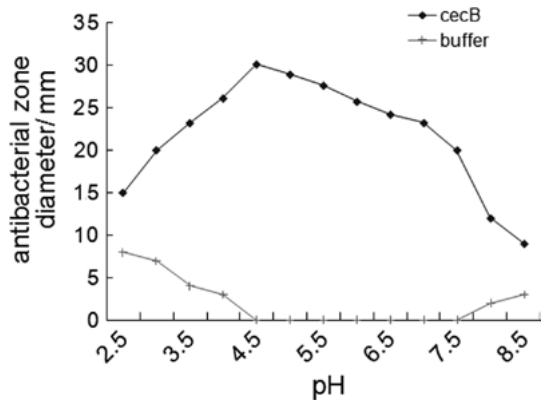

Figure 7. Effect of pH on the antibacterial activity of cecropin B.

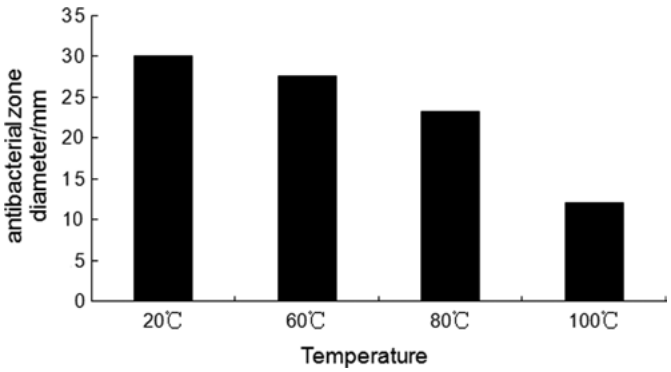

Figure 8. Effect of temperature on the antibacterial activity of cecropin B.

against certain transformed tumor cells, but rather than the hemolytic effect on human erythrocytes. To date, potent CB analogues have been synthesized and have been proven to exhibit effective antibacterial activity; however, heterologous expression of recombinant cecropin has seldom been reported (32,33). Heterologous expression of recombinant proteins results in toxicity for the host cells (27). For a peptide to be used as an antibiotic, the fusion protein partner must be removed from the bacterial expression system; this process is expensive and laborious. Therefore, in the present study, the methylotrophic yeast *P. pastoris* was used as a host for high level expression and secretion of recombinant CB. Recombinant CB was successfully secreted by *P. pastoris* into the culture supernatant, and 5.0 mg of recombinant CB was obtained from 100 ml of crude extract. The large-scale production of antibiotic peptides, such as cecropin, will require further exploration regarding the manufacturing methods.

Previously, it was reported that the recombinant protein Psd1, which originates from the C-terminus of the α-factor secretion signal of *S. cerevisiae* and contains four additional amino acids (EAEA) at the N-terminal region, was produced by *P. pastoris*. In addition, recombinant Psd1 was shown to decrease antifungal activity against *A. niger* by at least 10-fold (22,34). In the present study, we cloned the gene encoding CB, with only the KEX2 cleavage site, by recursive PCR in order to obtain CB protein with its native N-terminus. Tricine-SDS-PAGE revealed that CB was successfully secreted by the *P. pastoris* α-mating factor signal sequence into the culture supernatant. Activity assays demonstrated that CB had low MIC against all Gram-negative and Gram-positive bacteria tested. Additionally, our results showed that the signal peptide was efficiently processed by the KEX2 protease through the *P. pastoris* secretory pathway.

Expression of a foreign gene, in this case CB, in *P. pastoris* and secretion of the active antibacterial peptide suggest that the CB gene could be introduced into animal cells in order to enhance their resistance to bacterial pathogens. Previously, we showed that both CB and cecropin P1 produced by transgene constructs under the control of the cytomegalovirus (CMV) promoter in Chinook salmon embryo cells (CHSE-214) exhibited bactericidal activity against three pathogens (35). In addition, the expression of cecropin in yeast and fish cells demonstrates that the cecropin gene can be introduced into animal cells. Moreover, the expression of CB by transgenic plants has also been reported (36,37). However, further studies are required to address the feasibility of generating cecropin-expressing transgenic animals for the purpose of increasing their resistance to microbial diseases. In this study, we showed that heterologous CB can be expressed in *P. pastoris* under the promoter for the secretion signal of the yeast-mating factor. Recombinant CB was expressed at a concentration of 50 mg/l in culture medium, and it exhibited strong antimicrobial activity. Our results show that *P. pastoris* is a robust system that can be used for the expression of secreted CB, which exhibits antibacterial activity against both Gram-positive and Gram-negative bacteria (7-11).

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