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Heterologous Expression and Functional Characterization of CAP18 from *Oryctolagus cuniculus*

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Abstract

Background: Antimicrobial peptides belong to the innate defence system of creatures. These peptides attach to the bacterial membrane in order to die microorganisms by penetrating them. Hence, biotechnology researchers pay more attention to produce antimicrobial peptides for use in various fields. The studies showed that rabbit tissue with inflammation and skin ulcers would be producing CAP18 peptide, which belongs to the cathelicidin group.

Methods: In this study, the optimized sequence of the *cap18* gene was placed into the pPICZAα plasmid after the alpha-factor signal and transformed into *Pichia pastoris* (X-33 strain). Purification of the recombinant peptide was done based on its histidine tail at C-terminal, and western blotting method was used to demonstrate the purification of rCAP18. The antibacterial activity of the purified and desalted rCAP18 was investigated at different concentrations against pathogenic bacteria.

Results: The maximum expression level of rCAP18 (17.5 kDa) was seen 90 h after induction of alcohol oxidase I (AOX1) promoter with methanol. The concentration of rCAP18 was 33 mg/L after purification with Ni-NTA Sepharose column. The function of rCAP18 (4.3, 5.7, 7 µg/ml) was investigated against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Results showed that %CFU/cm^2 reached 28% after *P. aeruginosa* cells treatment with 7 µg/ml of rCAP18.

Conclusions: This study presented the findings related to heterologous expression of *cap18* gene, and evaluation of rCAP18 antibacterial effects. Our results showed that rCAP18 plays a significant role in inhibiting bacterial growth, especially Gram-negative bacteria.

Keywords: Antibacterial activity, Cathelicidin, Recombinant CAP18, Heterologous expression.

Introduction

Antimicrobial peptides (AMPs) are the most critical host defense molecules in the innate immune system. So far, more than 5,000 various types of these peptides have been synthesized or discovered (1). AMPs are an amphipathic molecule which contains from 5 to more than 100 amino acids. They include hydrophobic and cationic residues (2) and show a broad range of antimicrobial activity (3,4). Furthermore, AMPs can interact with the pathogens' surface and destroy them due to their amphipathic nature and positive residues. These interactions between AMPs and the hydrophobic areas of the membrane led to the formation of channels or pores to bacterial death (5,6). Therefore, bacteria have low resistance relative to AMPs (7). One of the reasons which are interested in researchers about antimicrobial peptides is the bacterial resistance to conventional antibiotics. Antibiotics play an essential role in modern therapeutic systems and act as adjuncts to the innate immune system. However, the advent of antibiotic-resistant strains led to a continuous search for finding different kinds of compounds with high efficiency (8-10). Since

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AMPs' goal is prokaryotic cell membranes, they are classified into the antibiotic group to reduce bacterial resistance risk (11). Some of these peptides are present on epithelial tissue surfaces in mammals and act as barriers to preventing microbial invasion (12). The most critical peptides in mammalian skin are Beta-defensin and cathelicidin induced by skin ulcers or inflammation. One gene has been known for the cathelicidin group in rodents and humans, while pigs, horses, and cows have several genes (12). hCAP18/LL37 is the only member of the human cathelicidin group expressed in neutrophils, skin epithelial cells, the gastrointestinal tract, and the genital tract (13). There are 37 amino acids from the C-terminal of hCAP18 that released and made LL37 peptide, which has high ability than its precursor into antibacterial activity (14).

Several of these peptides have been produced based on recombinant DNA technology due to their importance in the medical and pharmacy fields. Clinical use of antibacterial peptides for the treatment of several types of pathogenic infections has been investigated. Jacobsen et al. (15) used hCAP-18/LL-37 to treat burn wound infections in 2005, and Kougias et al. (16) showed the positive effect of alpha-defensin in preventing vascular diseases. Cathelicidin peptides have unique properties that make them useful as desirable pharmaceutical agents (17-19). Cathelicidins have an immediate effect on bacteria growth inhibition; meanwhile, they quickly create bacteria's morphological changes. These changes are mostly related to the permeability of peptides in the bacterial membrane (18). In a study, the activity of five different cathelicidin types; human (hCAP18), sheep (SMAP29, SMAP34), rabbit (CAP18), rat (rCAMP), and mice (mCAMP) against microorganisms was examined by Travis and his colleagues. Their findings showed that the rabbit CAP18 had the highest antibacterial activity than the other peptides (20). CAP18 relating to rabbit (*Oryctolagus cuniculus*) is a cationic antimicrobial peptide containing 171 amino acids (29 and 142 amino acids belonging to signal and the main chain of the peptide, respectively) with 18 kDa molecular weight. CAP18 is a thermostable peptide without cytotoxic and hemolytic effects (21). It is expressed in neutrophil cells, bound to lipopolysaccharide (LPs), and targets microorganisms' membranes (19). Therefore, it can inhibit the growth of microorganisms, especially Gram-negative bacteria.

In this study, subcloning and expression of the rabbit *cap18* gene were investigated. Due to two disulfide bonds in the N-terminal region of the peptide, *Pichia Pastoris* considered a host. Our results showed that the expression and purification of rCAP18 were performed successfully, and this recombinant peptide had an antibacterial activity for Gram-positive and Gram-negative bacteria.

Materials and Methods

Microorganisms and reagents

Pichia pastoris (strain X-33), and *E. coli* (strain DH5α) were prepared from Tarbiyat Modares University (Tehran, Iran). The pPICZAα plasmid was obtained from our laboratory and used as the cloning vector. Bacterial reference strains such as *P. aeruginosa*, *S.* aureus, and *Klebsiella pneumonia* were obtained from the Iranian Research Organization for Science and Technology (Tehran, Iran). All enzymes, including restriction enzymes, T4 DNA ligase, and alkaline phosphatase, were bought from Thermo Fisher Scientific Co., Ltd. (China). DNA gel extraction and plasmid extraction kits purchased from Bioneer Inc., (China). Tricine, Zeocin antibiotic purchased from Sigma-Aldrich Chimie (France). Ni-NTA Sepharose prepared from Invitrogen Inc., (Canada). Silver nitrate, yeast extract, tryptone, agar, other chemical salts, and gel electrophoresis materials took from Merck Millipore Company (China).

Codon optimization and designing the construction of cap18 gene

We have used the coding sequence of the *cap18* gene with the M73998.1 accession number. This gene contains 516 bp of the nucleotide that 87 bp of them are translated to

a secretory signal cleaved after expression. Moreover, 426 bp of this gene encodes the main structure of the CAP18 peptide. pPICZAα vector with a secretory signal (alpha-factor signal) was considered for the secretory expression of the CAP18. As a result, we ignored the native secretory signal sequence of the *cap18* gene for the gene's desired sequence to be placed after the alpha-factor signal. Codon optimization of the *cap18* gene was done based on the *P. pastoris* codon usage. At the 5' end of the gene, the *Xho1* cleavage site was added and then followed by *kex2* and *ste13* cleavage sites. At the 3' end of the gene, a sequence relevant to histidine (6 residues) placed before the stop codon, and lastly, the *Not1* cleavage site was added (Fig. 1). The final sequence of the *cap18* gene was synthesized by Bio Basic Inc., (Ontario, Canada) and inserted into the pPUC57 vector.

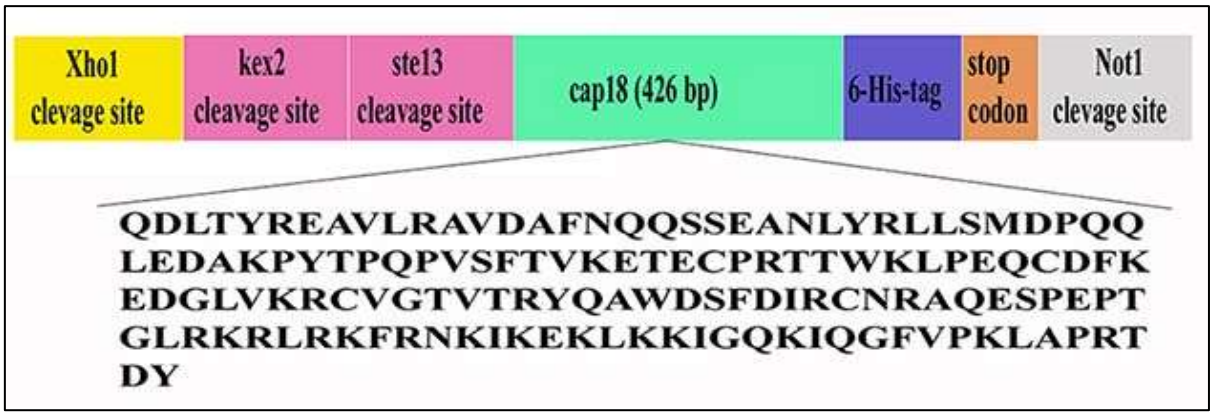

Fig. 1. Schematic gene construction of *cap18* for insertion in pPICZAα expression vector, an amino acid sequence related to the main structure of CAP18 peptide shown at the bottom of the figure.

Production of recombinant vector pPICZAα-cap18

At first, pPUC57 plasmid contains synthesized *cap18* gene transferred into DH5α competent cells and extracted from bacteria via the Bioneer plasmid extraction kit. *cap18* gene detached from pPUC57 plasmid through a double digestion reaction with *Xho1* and *Not1* endonucleases. Analysing the product was done by 1% agarose gel electrophoresis (SUB-10×7 model, Paya Pajoohesh, Tehran, Iran). The same restriction enzymes then linearized pPICZAα plasmid at 37 °C. Dephosphorylating of pPICZAα vector was performed by 1 µl alkaline phosphatase at 37 °C, 15 min. All of the experiment's enzymes were removed from the solution by the DNA precipitation method with ethanol. Prepared plasmid (75 ng) and purified *cap18* gene (225 ng) were ligated by adding T4 DNA ligase (22 °C for 2 h and finally 14 h at 4 °C). Recombinant plasmid (pPICZAα-*cap18*) was transferred to the susceptible DH5α host cells, and then bacterial cells were cultured on the plate which contains Zeocin antibiotic. To confirm the cloning steps, a single colony was cultured in 5 ml LB broth, including Zeocin antibiotic (25 µl/ml final concentrations). The plasmid was then extracted according to the Bioneer kit protocol. Single and double digestion tests were performed on the recombinant vector by restriction enzymes. Products were assessed by gel electrophoresis. Finally, the inserted gene sequencing was done by Macrogen, Inc., (Seoul, South Korea) and analysed by Chromas software (v.2.6.6, Technelysium Pty Ltd, South Brisbane, Australia; 1996).

Transferring the recombinant pPICZAα-cap18 vector into P. pastoris

The recombinant vector (pPICZAα-*cap18*) was linearized by the *Sac1* restriction enzyme (37 °C, 2 h), then the enzyme was excluded from the solution by DNA precipitation with ethanol. X-33 competent cells were prepared according to the previous protocol (22). The pPICZAα-*cap18* vector (2000 ng) was transferred into the X-33 competent cells using

the electroporation system (Gene Pulser Xcell Electroporation System, BIO-RAD Laboratories, Inc., Philadelphia, USA). After adding 1 ml of cold sorbitol (1 M), the sample was transferred into a 15 ml Falcon and incubated at 30 °C for 2.5 h. Finally, the cells were transferred to a solid YPDS medium containing 100 µg/ml Zeocin antibiotics. Plates were incubated at 28 °C until recombinant cell growth.

Expression and purification of rCAP18 protein

To investigate the expression of the rCAP18, one of the colonies that had grown on the solid plate includes antibiotics cultured in the 5 ml YPG medium overnight (30 °C, 150 rpm). The cells precipitated using Eppendorf multipurpose microcentrifuges 5430R (Eppendorf AG, Hamburg, Germany) at 8000 g for 10 min. The cellular deposition was then transferred to a fresh YPM medium, while the OD_{600} of the new medium reached 1. The expression of rCAP18 was induced by adding 0.5% (*V*/*V*) methanol every 24 h, then 1 ml of culture medium collected at a specific time after induction (6, 18, 30, 42, 48, 54, 66, 78, 90, and 96 h). The cells were precipitated by centrifuge at 11000 g, and their supernatant was stored at -70 °C. Due to evaluate the intracellular expression of rCAP18, we lysed the collected X-33 cells by ceramic beads based on the previous protocol (22). Cell debris was removed from protein solution by centrifuge at 11000 g (15 min). All samples were diluted to 1:3 and then investigated by 16% tris-tricine SDS-PAGE (Mini-PROTEIN II Electrophoresis Cell, BIO-RAD Laboratories, Inc., Philadelphia, USA). Based on the presence of His-tag in the C-terminal of rCAP18, the Ni-NTA Sepharose column was used to purify the recombinant protein. At first, the column was equilibrated by a buffer containing 0.13 mM NaH_2PO_4 and 1.25 mM NaCl at pH= 8. Then 10 ml of supernatant collected (90 h after methanol induction) slowly passed through the column. When the protein solution completely crossed through the column, resins were soaked by washing buffer containing 0.13 mM NaH_2PO_4, 0.013 mM Na_2HPO_4, and 1.25 mM NaCl 0.02 mM imidazole. The recombinant peptides are bound to the column through their polyhistidine tail. Finally, rCAP18 is released from the column by elution buffer (0.11 mM NaH_2PO_4, 0.16 mM NaH_2PO_4, 1.15 mM NaCl, and 0.25 mM imidazole at pH= 8). Collected samples were assessed by 16% tris-tricine SDS-PAGE. Then we examined the concentration of purified rCAP18 based on the Bradford protocol.

Western blotting analysis

Western blot was used to assess rCAP18 expression. Purified rCAP18 loaded on 16% tris-tricine SDS-PAGE, then electroblotting was done by semi-dry blotting set (Mini Trans-Blot® Cell, BIO-RAD Laboratories, Inc., Philadelphia, USA) with a constant current (300 mA) at 4 ºC. The nitrocellulose membrane (Sigma-Aldrich Chimie, France) was blocked in a buffer containing 150 mM NaCl, 20 mM Tris-base (pH 7.4), and 5% (W/V) skimmed milk. The blocked membrane was then subjected to the HRP enzyme conjugated anti-polyhistidine antibody for 1 h. After washing it three times during 1 h, the antibodies bounded to proteins were detected by ECL western blotting detection reagent until specific protein bands, including His-tag, were appeared.

Antibacterial activity of rCAP18

Purified rCAP18 was desalted based on the previous protocol (22). Then the antibacterial activity of recombinant peptide was estimated against pathogenic bacteria such as *E. coli* (ATCC 11229), *P. aeruginosa* (ATCC 9027), *S. aureus* (ATCC 29213), and *K. pneumonia* (ATCC 10031). Bacterial cells were grown in TSB medium overnight then diluted to 0.5 Mcfarland densities (1.5×10^8 CFU/ml). Based on the agar well diffusion method, bacteria plated on the solid plates' surface (containing TSB with agar). Wells at the centre of the plate filled with 50 µl rCAP18 protein solution. After 14 h of incubating plates at 37 °C, the halo's existence on the plates were reviewed. The antibacterial effects of the rCAP18 were

also analysed using the pour-plate method. Different concentrations of rCAP18 (4.3, 5.3, 7 µg/ml) were prepared in PBS buffer. After mixing the protein solution with the bacterial cells (5×10^4 cell), all of the suspensions were incubated at 37 °C for different time intervals (4, 6, 12 h). And then, 50 µl of them were examined based on the pour-plate method. Plates placed at 37 °C for 24 h and %CFU (colony forming unit) per one cm^2 were counted and compared with control samples for each strain.

Results

Construction of recombinant vector and transferring into x-33 cell

At first, the coding adaptation index (CAI) for the rabbit *cap18* gene was assessed to express in the *P. pastoris* by the Genscript server. The results showed a value of 0.59 that had a significant distance from the acceptable range for CAI, while the numbers between 0.8-1 are ideal for expressing the gene in the target host. After the optimization and modification of the nucleotide codons, this parameter reached 0.85. Hence, an optimized *cap18* sequence was synthesized and used.

The pPUC57-*cap18* plasmid was double-digested by two endonucleases (*Not1* and *Xho1*). Then 3 µl of the product loaded on 1% agarose gel. As shown in Fig. 2A, A single band at 479 bp on the gel determined that the isolation of the *cap18* gene was done correctly. The DNA fragment was extracted and purified from the gel. The correctness of the steps was confirmed by the electrophoresis test. Then pPICZAα vector was digested by *Xho1*/*Not1* restriction enzymes and then analysed by gel electrophoresis. The presence of an approximately 3600 bp band on the gel indicates that the pPICZAα vector has been cleaved accurately at cutting positions. The *cap18* gene's insertion at the sticky ends of the linearized plasmid was done using the T4 DNA ligase.

At last, recombinant vector pPICZAα-*cap18* was transferred into DH5α competent cells and plated on an LB-agar environment containing Zeocin antibiotic. The extracted recombinant plasmid was investigated using an enzymatic cleavage reaction. Comparison of linearized pPICZAα and pPICZAα-*cap18* on the gel showed a larger size of the recombinant plasmid due to the insertion of the *cap18* gene into it (Fig. 2B). Furthermore, double digestion of pPICZAα-*cap18* demonstrated a 479 bp band relevant to the *cap18* gene. DNA sequencing verified the absence of mutation in the *cap18* nucleotides and confirmed that it has correctly placed after the plasmid's secretary signal. Lastly, the linearized pPICZAα-*cap18* was transferred into X-33 competent cells. Recombinant *P. pastoris* cells grew on the plate that contains antibiotics after 4-5 days.

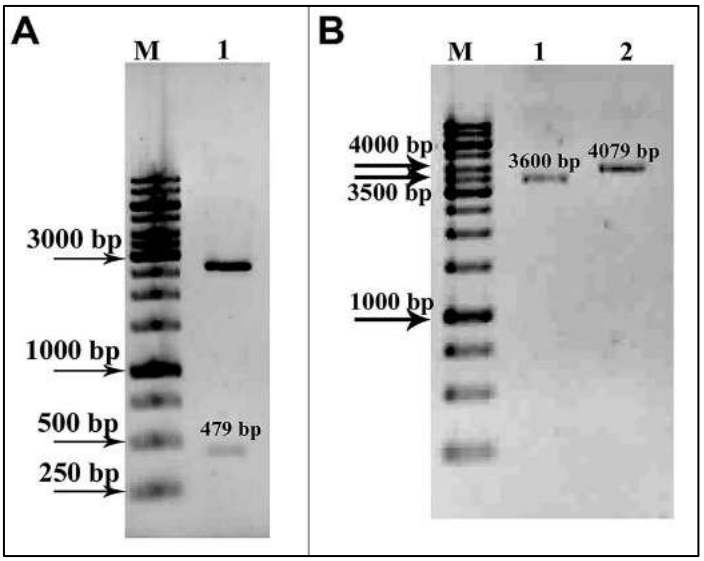

Fig. 2. (A) pPuc57-*cap18* plasmid that double-digested with *Xho1*/*Not1*. (B) single digested of pPICZAα (lane 1) and pPICZAα-*cap18* (lane 2) with *Xho1* restriction enzyme. M is DNA ladder 1 Kb and band at the location of 479 bp belongs to *cap18* gene.

Expression, purification and western blotting analysis of rCAP18

A single clone of recombinant *P. pastoris* was grown in 10 ml of YPG medium overnight and conveyed into YPM solution, including 0.5% (V/V) methanol. One ml of culture medium was removed at specific time intervals, then supernatants and cells were separated by centrifuge. The secretory and intracellular proteins were analysed using SDS-PAGE. As indicated in Fig. 3, the band's intensity belonging to the rCAP18 in the supernatant has increased during the time (17.5 kDa position). The maximum expression level of rCAP18 was seen 90 h after induction by methanol (Fig. 3, lane 8). Evaluating intracellular proteins (Fig. 3, lanes 1,3,5,7) referred that the expressed protein was secreted into the medium and had not been collected in the cells.

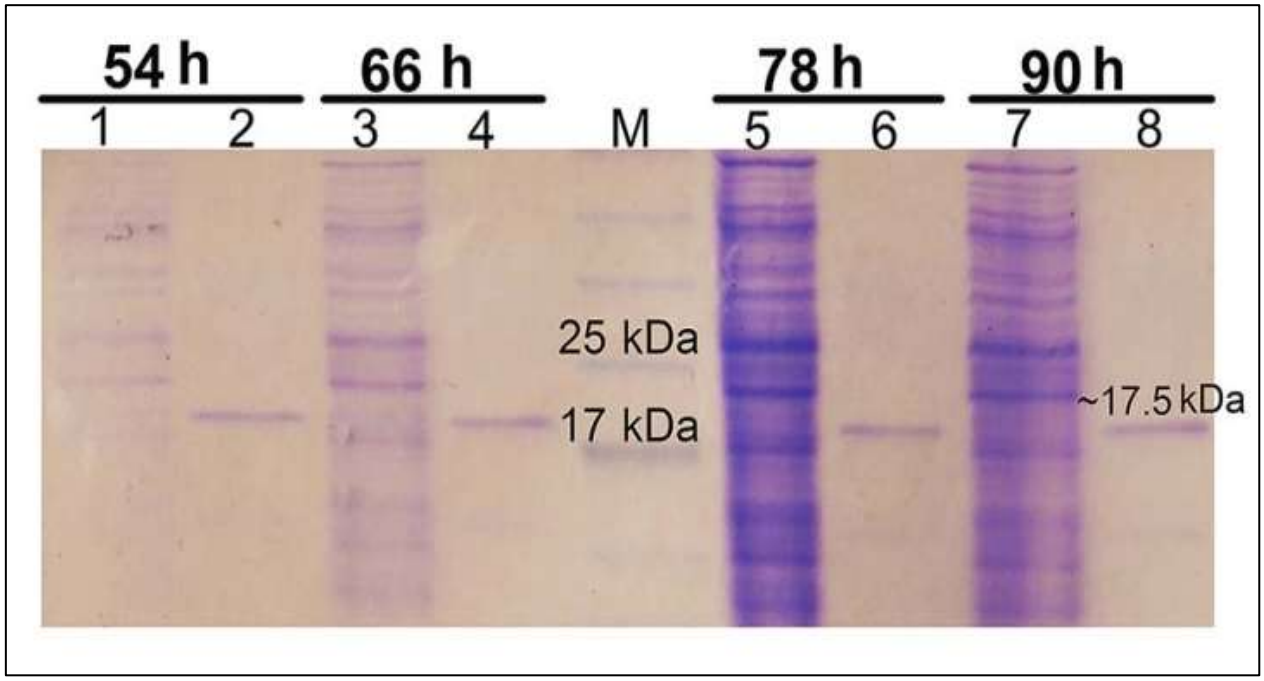

Fig. 3. 16 % tris-tricine SDS-PAGE; the extracellular and intracellular proteins were collected at different time intervals. Lanes 1, 3, 5, and 7 belong to the total intracellular protein, and lanes 2, 4, 6, and 8 belong to the total protein in the supernatant. (M) protein marker. The band at 17.5 kDa location showed the existence of rCAP18.

Since the maximum expression level of rCAP18 happened 90 h after being induced, the culture medium was collected at this time. The supernatant collected by a centrifuge was used to purify rCAP18 and cross through the column. The output of each step of the Ni-NTA Sepharose column was investigated by 16% tris-tricine SDS-PAGE. rCAP18 had attached to the column, so it was not found in the solution passed through the column. After washing the resin, non-specific proteins leave the column because of weak interaction with resin. The concentration of non-specific proteins in the prototypes is high and gradually decreases due to more column washing. rCAP18 connected to the column by polyhistidine-tag and then released by adding elution buffer, where a single band at approximately 17.5 kDa position belonged to rCAP18 (Fig. 4A, lanes 1-3). Our results showed that the concentration of rCAP18 was 33 mg/L. We also used the western blot technique to detect individual peptides, including His-tag in eluted samples. The interaction between antibody and recombinant protein occurs as low as 1 ng. As indicated in Fig. 4B, a single band was observed in the presence of an anti-His tag antibody for purified samples at the same location with SDS-PAGE. Therefore, we conclude that the purification of rCAP18 was done successfully.

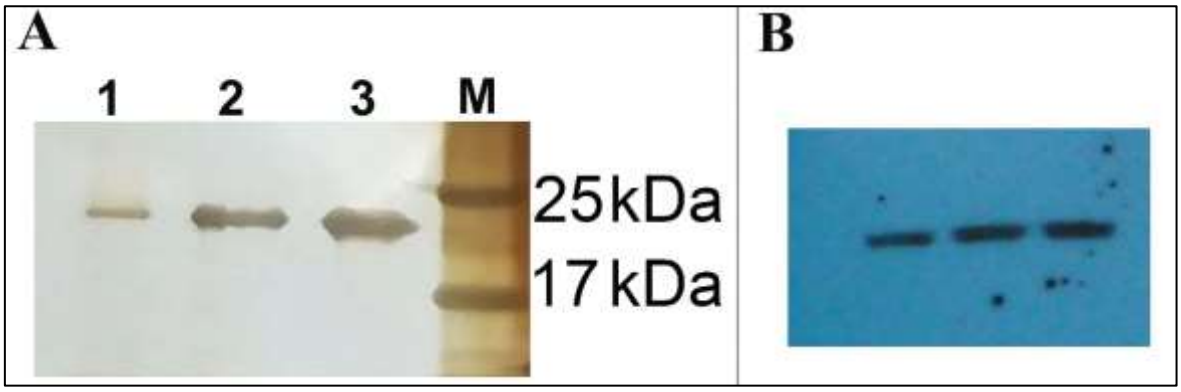

Fig. 4. (A) Silver nitrate stained 16% tris-tricine SDS-PAGE belonging to purified rCAP18 by the Ni-NTA Sepharose column. Lanes 1 to 3 correspond to eluted samples 1 to 3 (17.5 kDa). M is protein marker. (B) Western blotting analysis of purified rCAP18 showed bands relevant to the eluted samples.

Antibacterial tests

Investigation of the effect of rCAP18 on the growth of bacteria through agar well diffusion did not show antibacterial influence. The peptide's diffusion potential in the agar medium has likely decreased based on the size of rCAP18 (17.5 KDa). Our result was consistent with Choyam et al. (23), who published a paper in 2015 and showed that bacteriocin could not diffuse from the well in the agar plate. Thus, the pour-plate method was used for the antibacterial assay. Bacterial cells were treated with various concentrations of rCAP18 in different periods. Meanwhile, samples were cultured based on the pour-plate method, and the number of colonies was counted and compared. As indicated in Fig. 5A, 4.3 µg/ml rCAP18 had little effect on the growth inhibition of *E. coli*. Our results showed that increasing the during incubation time had little effect on inhibiting *E. coli*. There was only 7% of bacterial cells' growth reduction after 12 h of peptide incubation with bacteria. The use of peptide at this concentration (4.3 µg/ml) showed the greatest inhibitory effect on bacterial growth against *P. aeruginosa* strain. %CFU/cm^2 was 86%, 76%, and 62% after 4, 6, and 12 h of incubation bacteria with rCAP18, respectively. Evaluation of the effect of 4.3 µg/ml rCAP18 on growth inhibition of *S. aureus* showed a slowly decreasing trend depending on the incubation duration. As the peptide concentration increased to 5.7 µM, the more antibacterial effect was observed against different strains. As indicated in Fig. 5B, after incubation of *E. coli* with peptide at different time intervals such as 4, 6, and 12 h, %CFU/cm^2 reached 80%, 62%, and 50%, respectively. The most significant inhibitory effect of 5.7 µg/ml rCAP18 was observed on the *P. aeruginosa* strain. Our results determined that %CFU/cm^2 decreased to 30 % after the 12-hour treatment of bacteria with peptide. The concentration of 7 µm rCAP18 has played an inhibitory role for all three bacterial strains (Fig. 5C). In this case, %CFU/cm^2 was 36%, 28%, and 32% for *E. coil*, *P. aeruginosa*, and *S. aureus*, respectively. According to the results, it seems that rCAP18 has specific activity against Gram-negative bacteria.

Furthermore, in Fig. 5D, we showed the positive effect of peptide concentration on bacterial growth inhibition for the *P. aeruginosa* strain. After 4 h of treatment of bacterial cells with rCAP18, cells were cultured on the plate. As seen in the upper row of Fig. 5D, the number of single clones appearing on the plate was reduced by increasing the amount of recombinant peptide compared to the untreated sample (column 1). The difference in concentration-dependent growth inhibitor rate is becoming more pronounced with 12-hour treating of bacterial cells with the peptide (Fig. 5D, lower row).

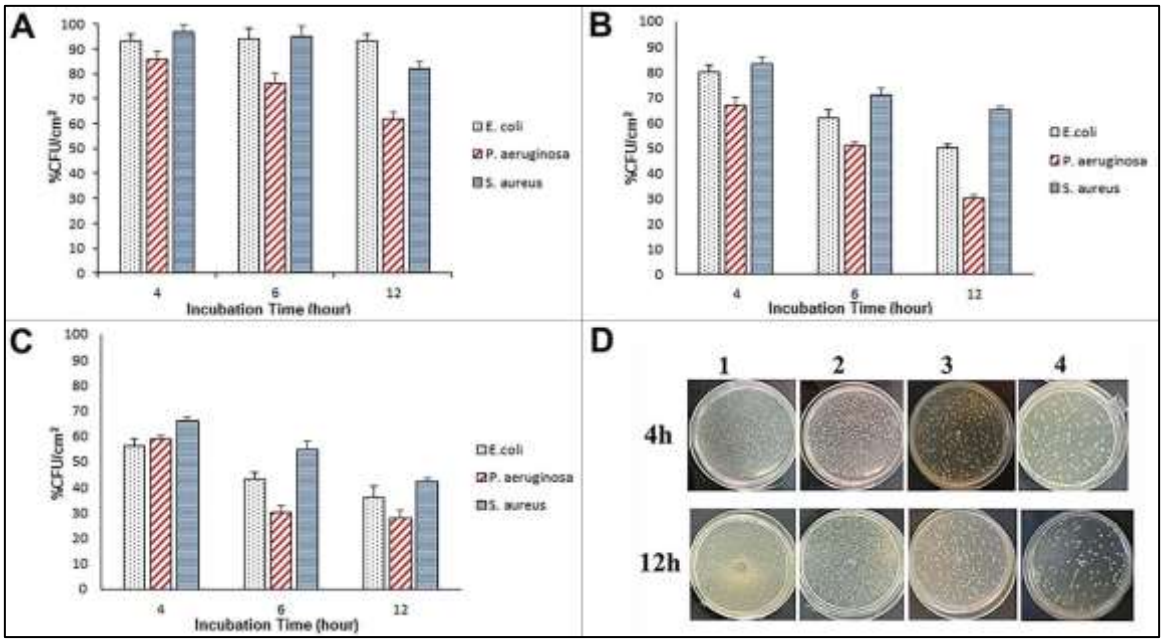

Fig. 5. Percentage of colony forming unit per one cm^2 (%CFU/cm^2) of *E. coli*, *P. aeruginosa*, and *S. aureus* after incubation of various concentrations of rCAP18 with 5×10^4 bacterial cells at 4, 6, 12 h. (A) 4.3 µg/ml, (B) 5.7 µg/ml, (C) 7 µg/ml of rCAP18. (D) Growth level of *P. aeruginosa* treated with various concentrations of rCAP1 at different incubation times; (1) positive control without rCAP18, (2) 4.3 µg/ml, (3) 5.7 µg/ml, (4) 7 µg/ml of rCAP18 was added to bacterial cells and after specific incubation time cultured on plates. The rows belong to 4 h and 12 h.

Discussion

Antimicrobial peptides are some kinds of small molecules that can inhibit the growth of different microorganisms. These peptides are found in most innate defense systems of living organisms, such as bacteria, insects, plants, and animals. Cathelicidins are critical antimicrobial peptides in mammalian that have positive charge residues and hydrophobic regions on their surface. These peptides can bind to the bacterial membrane and disrupt their membrane.

In this study, the heterologous expression of CAP18 was investigated in *P. pastoris* as host cells. The use of an expression vector with the alpha-mating factor was led to the secretion of the peptide into the culture medium and easier purification from other proteins. So far, several antibacterial peptides such as human cathelicidin (hCAP18/LL-37) (24,25), sheep beta-defensin (26), bovine lactoferrampin–lactoferricin (27), cecropin D (28), pleurocidin (29), and shrimp Ch-penaeidin (30) have been expressed in *P. pastoris*. In these studies, an expression vector had been used with alcohol oxidase (AOX) or glyceraldehyde3-phosphate dehydrogenase (pGAP) promoters (31,32). AOX promoter is strongly controlled by methanol induction. Thus, foreign protein generating is accurately controlled, especially when the peptide is toxic to the host. Human CAP18 has been expressed via vectors consist of the pGAP (24) and AOX promoters (25). In our study, the pPICZAα vector was considered for secretory expression of rCAP18 peptide in *P. pastoris*. The recombinant gene was induced by 0.5% methanol in YPM media. The maximum expression level of rCAP18 was detected after 90 h of induction. In another study, Kim et al. (25) obtained the highest amount of hCAP18/LL-37 after 72 h of induction of pPICZAα vector by 2% methanol in MM medium. We were able to purify the rCAP18 at a concentration of 33 mg/L that is consistent with Zhao et al.'s results (26) expressed sheep beta-defensin in the *P. pastoris*. They published that recombinant

peptide concentration after purification with SP-SEPHADEX (C-25) has reached 35 mg/L. In another study, Hong et al. (24) investigated the intracellular expression of LL37 in *P. pastoris* X-33 strain, but they did not describe the expression level of the recombinant peptide. In 2009, Kim et al. (25) performed cloning of the full-length hCAP18/LL-37 gene; they did not present recombinant peptide concentration, but they confirmed the antibacterial activity of the supernatant without purification. Zhao et al. (26) expressed sheep beta-defensin (msBD-1-T) in *P. pastoris* and obtained 80 mg/L recombinant peptides with 85% purity. Chen et al. (33) studied the expression of human beta-defensin in *P. pastoris* and *B. subtilis*. Their result showed a low expression level of the recombinant peptide, whereas it was only detectable by Western blot. Tang et al. (27) examined the heterologous expression and purification of bovine lactoferrampin–lactoferricin. Their results proved 91% purity among the concentration of 0.2 mg.

According to the antibacterial results, we concluded that Gram-negative bacteria are more sensitive to rCAP18. This outcome was consistent with the results of Ebbensgaard et al. (34) and demonstrated that CAP18 had specific activity against Gram-negative bacteria, especially *P. aeruginosa*. In 2009, the antibacterial activity of the CAP18 synthesized fragment of rabbit, which is equal to 106-142 amino acids from the end of the C-terminal, was investigated and verified by Larrick et al. (35). However, we selected a whole sequence of CAP18 (1-142 amino acids) for production based on recombinant DNA technology. The peptide length can influence its function, whereas increasing the size is encountered with reduced permeability. The large size of the rCAP18 peptide may be played a role in the absence of growth halo formation on the plate. By considering the fact that antibacterial peptides targeted bacterial membranes, it seems that these peptides have a high potential to become a medicinal compound. However, their formulation and conversion into an effective combination to control and treat infectious diseases require extensive research in this field.

Acknowledgements

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