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NOTE

Antibacterial Activity of Recombinant hCAP18/LL37 Protein Secreted from *Pichia pastoris*

Soon-ja Kim[1]*, Renshu Quan[1], Sung-Jin Lee[2], Hak-Kyo Lee[3], and Joong-Kook Choi[1]*

[1]Department of Biochemistry, College of Medicine, Chungbuk National University, Cheongju 361-763, Republic of Korea
[2]Department of Animal Life Resource Science, Kangwon National University, Chuncheon 200-701, Republic of Korea
[3]Gyeonggi Regional Research Center, Hankyong National University, Ansung 456-749, Republic of Korea

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Human antimicrobial peptide CAP18/LL37 (hCAP18/LL37) was expressed in *Pichia pastoris* and its antibacterial activity was tested against pathogenic bacteria. The full length ORF of hCAP18/LL37 was cloned into the pPICZαA vector followed by integration into the genomic *AOX1* gene of *P. pastoris*. Agar diffusion assay demonstrated that the different hCAP18/LL37 transformants showed various antibacterial activities against *Staphylococcus aureus*, *Micrococcus luteus*, and *Salmonella gastroenteritis*. The secreted form of hCAP18/LL37 exhibited its maximum activity after 72 h incubation with 2% methanol in MM media, not in BMM. This result suggests that the yeast secreted expression system can be used as a production tool of antimicrobial peptides for industrial or pharmaceutical application.

***Keywords*:** hCAP18/ LL37, antimicrobial peptide (AMP), antibacterial activity, *Pichia pastoris*

Antimicrobial peptides (AMP) are an evolutionarily conserved component found in the natural defense systems of most living organisms (Hancock and Scott, 2000; Thevissen *et al.*, 2003). AMPs have been isolated from various organisms such as bacteria (Liu and Hansen, 1990; Luders *et al.*, 2003), insects (Steiner *et al*., 1981), plants (Colilla *et al.*, 1990; Mendez *et al.*, 1990), animals (Zasloff, 1987; Nakamura *et al.*, 1988; Selsted *et al.*, 1993; Iwanaga *et al.*, 1998) and humans (Schnapp *et al.*, 1998). They are cationic and amphipathic with variable length, sequence, and structure, but most have relatively small (<10 kDa) molecular weights (Reddy *et al.*, 2004). These peptides present a broad spectrum of biological properties, including bactericidal, fungicidal, virucidal, and anticancer properties (Bals, 2000).

Cathelicidin is one of the several groups of antimicrobial peptides. In human, hCAP18/LL37 is the only member of cathelicidin (Turner *et al.*, 1998) expressed in neutrophils and epithelial cells of the respiratory, digestive, and reproductive tracts (Cowland *et al.*, 1995; Gudmundsson *et al.*, 1996; Frohm *et al.*, 1997; Bals *et al.*, 1999; Agerberth *et al.*, 2000; Zanetti, 2004). Human CAP18 is stored as an inactive propeptide in the secretory granules of neutrophils and released as an active form (LL37), following proteolysis from the C-terminal end (Gudmundsson *et al.*, 1996; Frohm *et al.*, 1997; Sorensen *et al.*, 2001). The LL37 displays a spectrum of activities against Gram-positive, Gram-negative bacteria, and fungi (Turner *et al*., 1998), which functions via the bacterial membrane-disruption route by interacting with phospholipids of the membrane (Henzler-Wildman *et al*., 2003, 2004). In addition to directly killing microbes, LL37 has several other beneficial biological functions such as binding and inactivating LPS (Bals *et al.*, 1999), chemotaxis of neutrophils, monocytes, mast cells, and T cells, induction of mast cells degranulation, stimulation of wound vascularization and re-epithelialization of healing skin (Zanetti, 2004).

These findings suggest that LL37 might be a highly potent and effective novel therapeutic agent, but the cost of producing LL37 by using solid phase chemical synthesis could be very expensive. Producing antimicrobial peptides using a microorganism could be an efficient alternative. Since the expression of recombinant LL37 using *E. coli* by the GST (Glutathione S-transferase) fusion system, additional enzymatic (thrombin or Factor Xa) or chemical (CNBr, formic acid) cleavage steps are required to harvest bioactive recombinant LL37 (Yang *et al*., 2004; Moon *et al*., 2006). In addition, the prokaryotic expression system lacks post-translational modifications such as protein glycosylation, processing, and folding that occurs in an eukaryotic, yeast expression system. Recently, methylotrophic yeast, *Pichia pastoris* has been developed as an excellent host for the high expression heterologous proteins under the *AOX1* (alcohol oxidase1) gene promoter (Cereghino and Cregg, 2000; auley-Patrick *et al.*, 2005), which is tightly regulated by methanol. Therefore, this study was an attempt to effectively produce the hCAP18/LL37 peptide in *P. pastoris*. To do so, hCAP18/LL37 was cloned into pPICZαA vector, through which the cloned hCAP18/LL37 in *P. pastoris* can be secreted into cul-

* To whom correspondence should be addressed.
(Tel) 82-43-261-2844; (Fax) 82-43-273-9710
(E-mail) J.-K. Choi; jkchoi@chungbuk.ac.kr
S.-j Kim; hankyol2@hanmail.net

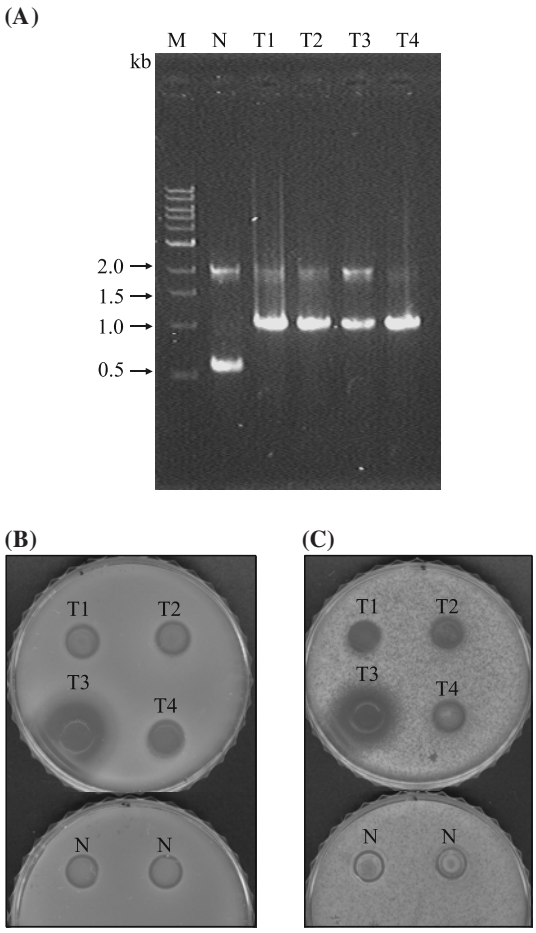

Fig. 1. Identification of the integration pPICZαA::hCAP18/LL37 recombinant plasmid into *Pichia pastoris AOX1* gene (Fig. 1A) and antibacterial activity of secreted recombinant hCAP18/LL37 protein against *Staphylococcus aureus* KCTC 1621 (Fig. 1B) and *Micrococcus luteus* KCTC 1056 (Fig. 1C). Genomic DNAs were isolated from control strain with empty vector, pPICZαA (N) and four transformants with pPICZαA::hCAP18/LL37 (T1, T2, T3, and T4) and used as PCR template. PCR analysis was performed with the specific *AOX1* gene primers. Lane M, Molecular DNA Marker (1 kb DNA ladder).

ture media following methanol induction. Antibacterial activity of secreted hCAP18/LL37 recombinant protein was determined by agar diffusion assay. Two culture conditions were also examined; methanol concentration and pH of media for higher production of LL37.

First, pPICZαA::hCAP18/LL37 was constructed to express and secrete a human antimicrobial peptide hCAP18/LL37 in *P. pastoris*. The full cDNA clone of hCAP18/LL37 (NM_004345) was obtained from the KRIBB Gene Bank (Korea Research Institute of Bioscience and Biotechnology) and amplified by PCR using the following primers: forward primer (F1); 5'-CGC GAA TTC ATG AAG ACC CAA AGG GAT-3'/ reverse primer1 (R1); 5'-CGC TCT AGA AAG GAC TCT GTC CTG GGT AC. The PCR product (513 bp) of the full length ORF of human cathelicidin (hCAP18/LL37) was cleaved with *Eco*RI/*Xba*I restriction enzymes (NEB, USA) and was cloned into the *P. pastoris* expression vector pPICZαA (Invitrogen, USA). The resulting vector (pPICZαA::hCAP18/LL37) contained the full length ORF of hCAP18/LL37 at downstream of the secretion signal sequence of *Saccharomyces cerevisiae* α-factor prepropeptide and Zeocin™ resistance gene as a selection marker. In order to integrate the pPICZαA::hCAP18/LL37 plasmid into the 5'-*AOX1* region of *P. pastoris* (X33), we linearized the pPICZαA::hCAP18/LL37 with *Sac*I restriction enzyme (NEB). Transformation was performed by the lithium chloride method according to the manufacturer's protocols (Invitrogen, USA).

To determine whether the pPICZαA::hCAP18/LL37 plasmid integrated into *P. pastoris*, four individual transformants (T1, T2, T3, and T4) with pPICZαA::hCAP18/LL37 plasmid and a control transformant (N) with empty vector (pPICZαA) were picked. After the genomic DNAs were isolated by the manufacturer's protocols (Invitrogen), the presence of the integrated hCAP18/LL37 was confirmed by PCR using the

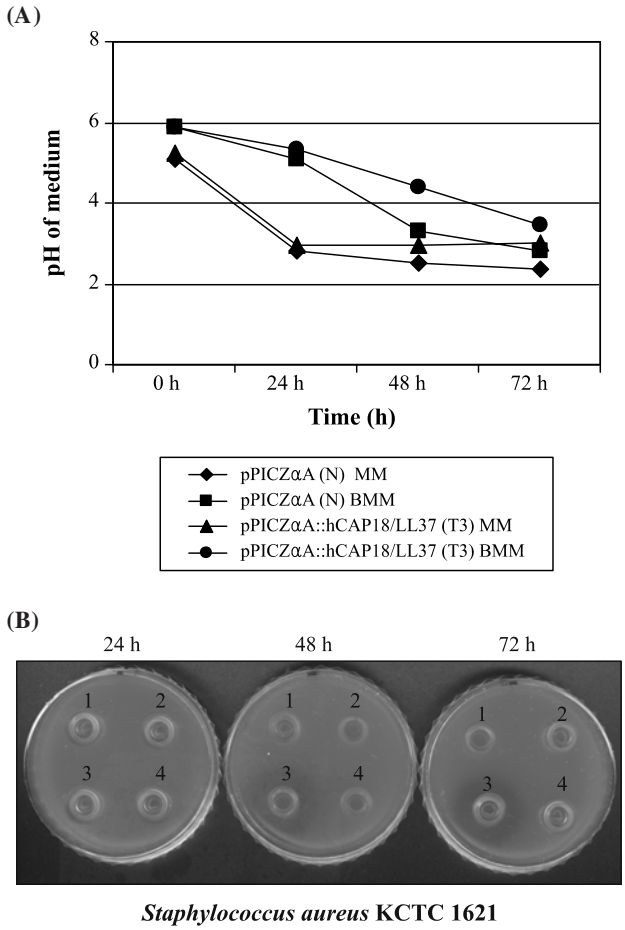

Fig. 2. The pH change of culture media. The pH change of each culture was measured after growing control strain (N) and transformant (T3) in MM (unbuffered minimal methanol) and BMM (buffered minimal methanol) for the indicated time (Fig. 2A) and comparison of the antibacterial activity of secreted recombinant LL 37 protein (T3) in the same media (Fig. 2B). 1, 2; control strain (N) and 3, 4; transformant (T3). 1, 3; culture supernatant from MM media and 2, 4; culture supernatant from BMM media.

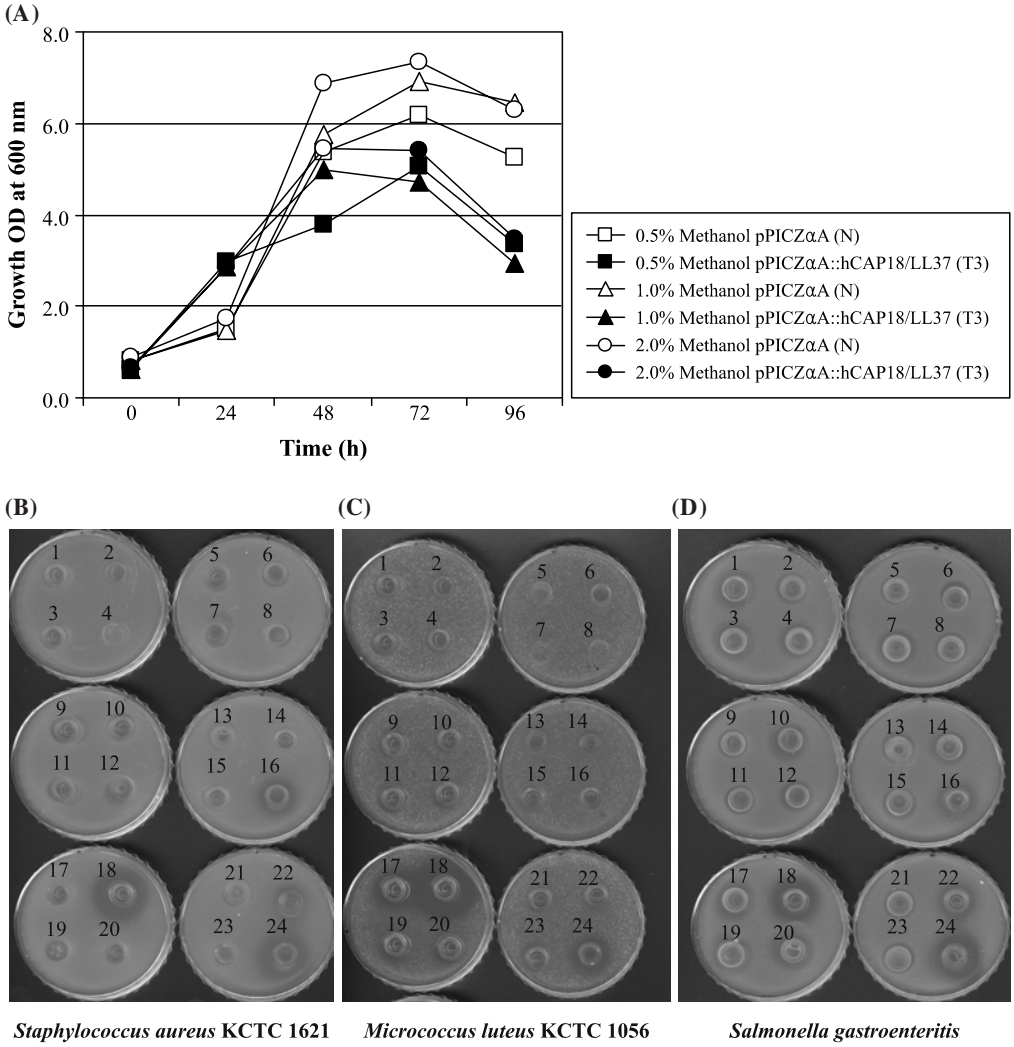

Fig. 3. Growth curve of the T3 transformant and control strain (N) under different methanol concentrations (Fig. 3A) and antibacterial activity (Fig. 3B, C, and D) of their respective culture supernatants. For these experiments, T3 and N were grown in different concentrations of methanol media (0.5%, 1%, and 2%) for the indicated time before measuring the cell growth by spectrometric analysis of the culture density (Fig. 3A). Antibacterial activity was analyzed by agar diffusion assay using the culture supernatants as follow: 1&2, 7&8, 13&14, and 19&20; culture supernatant from 0.5% methanol media for 24 h, 48 h, 72 h, and 96 h respectively. 3&4, 9&10, 15&16, 21&22 culture supernatant from 1% methanol media for 24 h, 48 h, 72 h, and 96 h respectively. 5&6, 11&12, 17&18, and 23&24 culture supernatant from 2% methanol media for 24 h, 48 h, 72 h, and 96 h respectively. Odd and even numbers indicate control strain (N), transformant (T3), respectively.

specific primers of *AOX1* gene: forward primer (F2); 5'-GAC TGG TTC CAA TTG ACA AGC-3'/ reverse primer (R2); 5'-GCA AAT GGC ATT CTG ACA TCC-3'. Transformants (Fig. 1A. lane N and lane T1∼T4) showed three kinds of bands as expected; the 2,200 bp fragment of the wild type *AOX1* gene, the 588 bp fragment of PCR product made from empty pPICZαA vector, and the 1,101 bp fragment of full length ORF of hCAP18/LL37 cloned into pPICZαA (513 bp+588 bp=1,101 bp).

Next to determine if the transformed cells (T1∼T4) were able to secrete the recombinant hCAP18/LL37 as an active form with antibacterial activity, the transformants (T1∼T4) and control (N) were grown in 20 ml of MM (Minimal methanol; 1.34% yeast nitrogen base (YNB), 0.5% methanol, 4×10^{-5}% biotin) media at 30°C for 96 h. The cell culture supernatant was collected by removing the pellet after centrifuging at 3,000 rpm for 5 min and was subjected to the antibacterial activity assay by determining the size of clear zones, i.e. growth inhibition zone against the lawn of bacterial strains tested; *Staphylococcus aureus* KCTC 1621, *Micrococcus luteus* KCTC 1056 and *Salmonella gastroenteritis.* These bacteria were either purchased from the KRIBB Gene Bank or kindly provided by Dr. Choi, Kang-Duk at Han-Kyong Nat'l University.

Bacterial culture was grown to early log phase (OD at 600 nm ≈ 0.5, $1\sim2\times10^{8}$/ml CFU) and then poured on the test media, followed by placing a cylindrical plastic tube (6 mm inner diameter×8 mm high) on top of the lawn of bac-

teria culture. Thereafter, 180 μl of the supernatant was loaded inside the tube. The plates were incubated at 37°C overnight and the size of the clear zone around each cylindrical tube was observed. One transformant (Fig. 1B and C. T3) showed strong antibacterial activity against *S. aureus* KCTC 1621 (Fig. 1B) and *M. luteus* KCTC 1056 (Fig. 1C), but the clear inhibition zones were absent in the other three transformants (Fig. 1B and C. T1, T2, and T4) and the control (Fig. 1B and C. N). The difference of antibacterial activity between the transformants (T1 *vs* T2, T3, and T4) may be due to either the multiple gene insertion events at the *AOX1* loci or the difference of the processing of the transcriptional, translational regulation.

As the expression of the heterologous protein in *P. pastoris* proceeded in unbuffered media (MM), the pH of the media dropped to 3 or below, inactivating many neutral pH proteases (Brierley *et al*., 1994). However, the growth of *P. pastoris* was not affected by the low pH of the media because of the resistance to acidic pH. In contrast, the inhibition of extracellular protease occurred in the media buffered at pH 6.0 and the yield of the mouse epidermal growth factor increased (Clare *et al.*, 1991). Therefore, we asked whether the secreted LL37 recombinant protein was susceptible to neutral pH protease or extracelluar protease. The T3 transformant was grown in 2 ml MD media (1.34% YNB, 4× 10^{-5}% biotin, 2% dextrose) at 30°C overnight and harvested by centrifugation. The cells were cultured in MM (same as MD medium but containing methanol instead of dextrose) and BMM (MM media plus 100 mM potassium phosphate pH 6.0) at 30°C for 72 h. As expected, the pH of MM and BMM media went down to around 3, even though the pH decline of BMM cultures was slower compared to that of the MM culture (Fig. 2A). With this, the antibacterial activity of secreted recombinant LL37 protein showed a clear inhibition zone after 48 h and 72 h incubation in MM culture, but not in BMM culture (Fig. 2B). Therefore, the secreted LL37 recombinant protein in *P. pastoris* seems to be affected by neutral pH protease.

Lastly, we investigated several different methanol concentrations to establish the culture conditions for the highest production of LL37. The T3 transformant was cultured in 20 ml of MM media at 30°C, 200 rpm for 96 h. Different volumes of 100% methanol were added into the cultures to a final concentration of 0.5%, 1%, and 2% every day. Samples were harvested every 24 h and the LL37 production was determined by agar diffusion assay. As shown in Fig. 3A, the growth rates of the control (N) strain and transformant (T3) were depended on methanol concentration of the culture media. The growth of the transformant (T3) was higher in 2% methanol than in 0.5% or 1% at 48 h (Fig. 3A). The antibacterial activity of the transformant (T3) reached up to its maximum after 72 h incubation with 2% methanol and to a lesser extent at the 72 h and 96 h time points with 1% methanol concentration (Fig. 3B, C, and D). Based on the results, the production of LL37 seems to be related to the growth of the strain, but not directly proportional.

We found that the transformant (T3) had a good antibacterial activity against bacterial stains tested on a small-scale expression. The active LL37 recombinant protein was successfully expressed and secreted from *P. pastoris* transformant (T3). The results in our study would provide the groundwork for a large-scale expression to produce useful antimicrobial peptides.

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