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Efficient Screening of a Novel Antimicrobial Peptide from *Jatropha curcas* by Cell Membrane Affinity Chromatography

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ABSTRACT: A novel method named cell membrane affinity chromatography was used to screen antimicrobial peptides from *Jatropha curcas*. A cationic antimicrobial peptide (KVFLGLK, JCpep7) was successfully isolated and identified. Antimicrobial assays indicated that JCpep7 was active against the tested microorganisms (*Salmonella typhimurium* ATCC 50013, *Shigella dysenteriae* ATCC 51302, *Pseudomonas aeruginosa* ATCC 27553, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 23631, and *Streptococcus pneumoniae* ATCC 49619) with minimal inhibitory concentration (MIC) values ranging from 24 to 64 μg/mL. The antimicrobial mechanisms based on Fourier transform infrared (FTIR) spectroscopy and transmission electron microscopy (TEM) techniques showed that JCpep7 killed microbes principally via breaking of their cell walls and membranes, followed by cell lysis. The results indicated that cell membrane affinity chromatography could be a promising approach for high-throughput screening of antimicrobial peptides from *J. curcas*.

KEYWORDS: Cell membrane affinity chromatography, antimicrobial peptides, *Jatropha curcas*

INTRODUCTION

The rise of antimicrobial resistance in pathogens represents an increasing challenge to medicine and public health.[1] With this problem growing, there is a clear need for the development of alternative therapies. Antimicrobial peptides (AMPs), which can physically permeate and destroy microbe cell membranes to kill microbes, may serve as potential and novel antibiotics to overcome the problem.[2−4] However, in comparison to most antibiotics, low yield hinders AMP application. Therefore, the purification methods of AMPs must be developed to be efficient, reliable, and cost-effective.[5,6] Although a lot of purification schemes have been described for AMPs, these purification methods require multiple steps.[7−11]

Cell membrane affinity chromatography, a high specificity separation technique, can screen the component binding to the cell membrane and its receptor according to its chromatographic retention characteristic. At present, this technique has been successfully used to screen the lead compound for drugs.[12−15] Most know that AMPs can bind to bacterial cell membranes as the first step to exert their antimicrobial activity. On the basis of this membrane-binding activity, cell membrane affinity chromatography can be used to screen AMPs. However, until now, there have been few reports on a high-throughput screening of AMPs by this method.

Plants are constantly exposed to a diverse array of pathogenic organisms, and their survival in these conditions relies on different defense mechanisms, which include constitutive and inducible physical barriers and numerous defense molecules, such as cysteine-rich AMPs.[16−19] *Jatropha curcas* has proven to be an opportunistic crop in tropical areas, particularly in unfavorable environments. Over the past decade, a novel cyclic octa-peptide has been isolated from *J. curcas* and the anti-inflammatory activity was investigated.[20] Juan et al.[21] reported the antitumor effects of curcin from *J. curcas*.

In this paper, on the basis of ligand−biological receptor binding activity, a novel method named cell membrane affinity chromatography was developed to isolate AMPs from *J. curcas*. Moreover, the antimicrobial model based on Fourier transform infrared (FTIR) spectroscopy and transmission electron microscopy (TEM) techniques was studied.

MATERIALS AND METHODS

Materials. The powder of *J. curcas* meal protein and silica carrier were obtained from the School of Food Science and Technology, Jiangnan University, Wuxi, China. Adult Wistar rats (210−290 g) of either sex were obtained from the Experimental Animal Center of Jiangnan University. All of the reference strains were provided by the Wuxi Disease Prevention and Control Center (Wuxi, China). They are Gram-negative bacteria (*Salmonella typhimurium* ATCC 50013, *Shigella dysenteriae* ATCC 51302, and *Pseudomonas aeruginosa* ATCC 27553), Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 23631, and *Streptococcus pneumoniae* ATCC 49619), *Penicillium expansum* ATCC 1117, *Aspergillus niger* ATCC 16404, and *Saccharomyces cerevisiae* ATCC 40075. All strains were maintained as frozen stocks in appropriate broth plus 20% (v/v) glycerol.

Preparation for the Extracts of *J. curcas* Peptides. The experiment was performed according to Tang et al.,[22] with some modifications. The powder (100 g) of *J. curcas* meal protein was homogenized in a colloid mill (IKA T-18 ULTA-TURRAX) in a 1 L

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beaker of phosphate-buffered saline (PBS, 50 mM, pH 7.2) with 35 μg/mL phenylmethylsulfonyl fluoride, 0.2 mg/L ethylenediaminetetraacetic acid, and 2% 2-mercaptoethonal (5 mM). The homogenate was centrifuged at 5000*g* for 20 min (Eppendorf), followed by heat treatment at 100 °C for 5 min with continuous agitation, and then centrifuged at 12000*g* for 30 min at 4 °C. The same volume of ethyl acetate was added to the supernatant with continuous agitation and then centrifuged at 12000*g* for 10 min at 4 °C. After centrifugation, the water fraction containing peptides was freeze-dried and stored at −20 °C until needed.

Cell Membrane Preparation. The experiment was performed according to He et al.,[13] with some modifications. Adult Wistar rats were killed, and the atrium was removed immediately. The atrium was washed thoroughly, cut into small pieces, and added to ice-cold PBS buffer (50 mM, pH 7.2). The tissue suspension was homogenized by using a polytron homogenizer. The homogenate was digested with 0.25% trypsin and then centrifuged (1000*g* for 10 min) to obtain the precipitate. The precipitate was resuspended for 30 min in deionized water, then sonicated for 30 min, and centrifuged at 1000*g* for 10 min. The obtained precipitate was again centrifuged at 12000*g* for 20 min. The resulting precipitate was the cell membrane. All procedures were performed at 4 °C.

Cell Membrane Affinity Chromatography Experiment. The experiment was performed according to Charcosset,[23] with some modifications. A total of 0.5 g of activated silica was added to a 15 mL reaction tube, and then the suspension solution of the cell membrane was slowly added under the evacuation conditions at 4 °C. The adsorption of the cell membrane on the activated silica surface was taken for 5 h until equilibrium was reached. Then, the supernatant in the tube was removed by centrifugation, and the cell membrane stationary phase (CMSP) was washed with 50 mM PBS buffer until there was no residually free cell membrane on it. CMSP was packed in the chromatography column (50 × 2 mm inner diameter) and equilibrated with 50 mM PBS buffer at least 3 days before runs.

The extracts of *J. curcas* peptides (20 μL, 5 mg/mL) were loaded onto a cell membrane affinity chromatography column (50 × 2 mm inner diameter). The mobile phase (50 mM PBS, pH 7.2) was pumped through the column at a flow rate of 0.3 mL/min, and the elution pattern was monitored by measuring the absorbance at 220 nm. Each peak was manually collected, and the antimicrobial activities were tested. Then, the most effective peak was manually collected, freeze-dried, and used in the subsequent experiments.

Detection the Purity of the Active Peak. The active peak (2 mg/mL) obtained from cell membrane affinity chromatography was manually collected and rechromatographed to reversed-phase high-performance liquid chromatography (RP-HPLC) (Amethyst C_{18}, 250 × 4.6 mm, 5 μm) to detect the purity. The two solvent reservoirs contained the following eluents: (A) 0.05% (v/v) trifluoroacetic acid (TFA) and (B) 100% acetonitrile. The elution program consisted of a gradient system (5−80% B in 40 min) with a flow rate of 0.5 mL/min.

Peptide Sequence Analysis. A mass spectrometric experiment was performed on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI−TOF MS) (4700 proteomics analyzer, Applied Biosystems, Carlsbad, CA) according to ref 24. All spectra were measured under the following conditions for MS: reflector positive; CID, off; mass range, 400−3200 Da; focus mass, 1200 Da; fixed laser intensity, 6000; and digitizer bin size, 1.0 ns; and for MS/MS: 1 kV positive; CID, on; precursor mass windows, relative 80 resolution [full width at half maximum (fwhm)]; fixed laser intensity, 7000; and digitizer bin size, 0.5 ns. α-Cyano-4-hydroxycinnamic acid (Aldrich, Steinheim, Germany) was used as a matrix. The concentration of peptide solution was 2 mg/mL. The amount of 0.5 mL of the peptide solution plus 0.5 mL of matrix was deposited on the sample slide and left to dry at room temperature. The resulting spectra were analyzed and compared.

Assay for Antimicrobial Activity. *Microorganism Growth Conditions.* Throughout the whole experiment, all tested strains were subcultured every 2 weeks on agar media to keep the microorganisms viable and kept at 4 °C. Before use in experiments, cultures were propagated twice in liquid media overnight. All media were sterilized by autoclaving at 121 °C for 20 min.

Minimal Inhibitory Concentration (MIC). MIC was determined with a liquid growth antimicrobial assay.[25,26] The overnight culture was washed and resuspended in PBS (10 mM, pH 7.4) by centrifugation at 3000*g* for 10 min to attain the final microbial density of 10^7 colony forming units (CFU)/mL. Peptide stock solution (192 μg/mL) was prepared with PBS (10 mM, pH 7.4). The peptide solution was sterilized by filtration (Millipore 0.22 μm) and diluted 2-fold serially in sterile PBS (10 mM, pH 7.4). A total of 50 μL of peptide solution was incubated in sterilized 96-well plates with 100 μL of media and 100 μL of the test microorganisms disposed, as described above. A total of 50 μL of PBS (10 mM, pH 7.4) tested under the same conditions was used as the control. MIC was considered the lowest peptide concentration that showed no increase in the optical density (OD_{600}) read at the microplate reader (Multiskan MK3, Thermo Fisher Scientific, Waltham, MA) after 24 h of incubation.

Model of Antimicrobial Action of JCpep7. *Antibacterial Activity Kinetics.* The experiment was performed by a liquid growth inhibition assay according to Bulet et al. and Hultmark et al.,[27,28] with some modifications. The overnight culture of *S. aureus* ATCC 25923 was adjusted to the final density of 10^6 CFU/mL. Then, the MIC of JCpep7 was incubated in sterilized 96-well plates with 100 μL of media and 100 μL of the test microorganisms for 0, 0.5, 1, 2, 3, 4, 5, 6, 7, and 8 h at 37 °C, and aliquots of PBS (10 mM, pH 7.4) tested under the same conditions were used as the control. After these times, the optical density (OD_{600}) was read at the microplate reader. The antibacterial activity units (U) were estimated by the following formula:

$$U = \left(\frac{A_0 - A}{A_0}\right)^{1/2}$$

where A was the absorbance of the sample and A_0 was the absorbance of the control.

Activity on the Structure of the Cell Wall Analyzed by FTIR Spectroscopy. The sample of *S. aureus* ATCC 25923 preparation was carried out as described previously,[29] with some modifications. After the cultivation on agar plates, a platinum loop was used to remove bacterial biomass from the agar plate and the biomass was dissolved in 200 μL of distilled water. Then, 25 μL suspensions were transferred to an IR transparent optical crystal (ZnSe) in a multi-sample cuvette (FTIR spectrometer, Thermo Electron Corporation). The samples were dried under moderate vacuum (0.3 bar) using anhydrous silica gel (Prolabo, China) in a desiccator to form films suitable for FTIR analysis. The spectra were recorded in the region between 4000 and 400 cm^{-1}, with a spectral resolution of 4 cm^{-1} and an aperture of 5.0 mm. For each spectrum, 32 scans were averaged.

Activity on Membrane Permeability. The experiment was performed according to Tang et al.,[30] with some modifications. The overnight culture of *S. aureus* ATCC 25923 at 37 °C was washed and resuspended in PBS (10 mM, pH 7.4) to reach the final density of 10^7 CFU/mL. Strains were incubated with JCpep7 at the MIC for different times (0, 30, 60, 120, 180, 240, 300, 360, 420, and 480 min). The mixture was filtered through 0.22 μm to remove the bacteria cells. The filtrate was then diluted appropriately, and the optical density at 260 nm was recorded (UV-2800, Unico) at room temperature (25 °C). Strains incubated with 10 mM PBS (pH 7.4) were used as the control.

TEM. The effect of the purified antimicrobial peptide on the ultrastructural morphology of *S. aureus* ATCC 25923 was assessed using TEM (Hitachi H-7000, Japan).[31] After treatment with JCpep7 at the

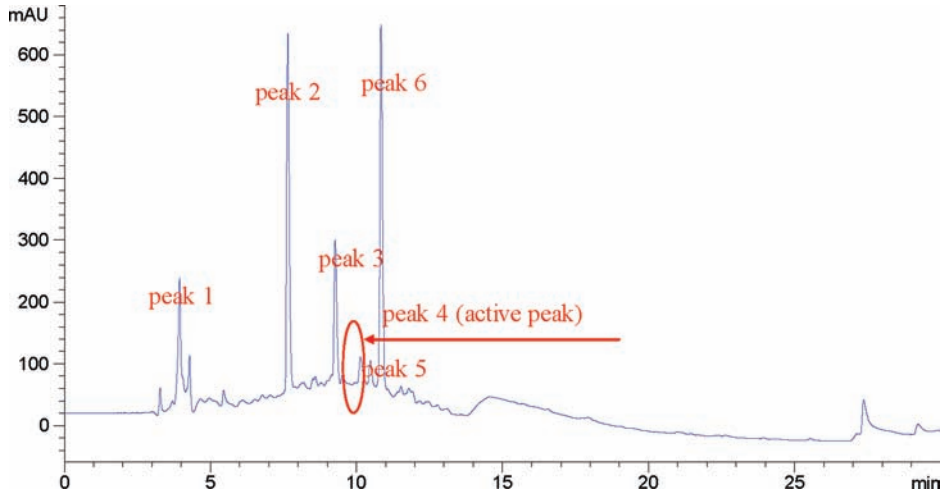

Figure 1. Elution profile of cell membrane affinity chromatography.

MIC for different times (60 and 240 min), *S. aureus* ATCC 25923 cells were immediately washed 3 times with PBS and fixed with 2.5% (v/v) glutaraldehyde.

Statistical Analysis. All of the experiments were performed in triplicate. The average value and standard deviation were calculated. The data were analyzed using SPSS 13.0 statistical software.

RESULTS AND DISCUSSION

Isolation and Purification of Peptide JCpep7. The elution profile of the cell membrane affinity chromatogram of the sample was shown in Figure 1. Six peaks were obtained, and the antimicrobial activity test (data not shown) demonstrated that peak 4 highlighted in red was the most effective among all peaks. The active peak (peak 4) obtained from cell membrane affinity chromatography was subjected to RP-HPLC to detect the purity. As shown in Figure 2a, the purity of the active peak was 99.1%. The active peak was eluted at about 65% acetonitrile by RP-HPLC, suggesting that the molecule was hydrophobic. As known, the hydrophobic interactions of AMPs with the cell membrane are important. AMPs can coat the surface of the bacterial membrane with the hydrophobic face toward the lipid components and the polar residues binding to the phospholipid head groups.[5,32]

The MS analysis of the active peak was shown in Figure 2b. The purified fraction was found to be a cationic antimicrobial peptide (Lys-Val-Phe-leu-Gly-leu-Lys), with total net charge of +2 and total hydrophobic ratio of 57%. Cationic antimicrobial peptides are likely to be attracted first to the net negative charges on bacterial surfaces.[33,34] There is no matching point between the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) and Antimicrobial Peptide Database (APD, http://aps.unmc.edu/AP/main.html), which suggesting that the active peak is a novel peptide, designated as "JCpep7".

Antimicrobial Activity. As shown in Table 1, JCpep7 was active against both Gram-negative bacteria (*S. typhimurium* ATCC 50013, *P. aeruginosa* ATCC 27553, and *S. dysenteria* ATCC 51302) and Gram-positive bacteria (*S. aureus* ATCC 25923, *B. subtilis* ATCC 23631, and *S. pneumoniae* ATCC 49619), with MIC values ranging from 24 to 56 μg/mL, except fungi (*P. expansum* ATCC 1117 and *A. nige* ATCC 16404) and yeast (*S. cerevisiae* ATCC 40075). *B. subtilis* ATCC 23631 was the most sensitive to JCpep7. The MIC of JCpep7 for *B. subtilis* ATCC 23631 was 24 μg/mL. The data showed that Gram-positive bacteria were more sensitive than Gram-negative bacteria to JCpep7. It may because of the subtle differences in the compositions of the Gram-positive and Gram-negative bacterial cell membranes.[35−38] The results suggested that JCpep7 could control some bacteria efficiently.

Model of Antimicrobial Action. *Antibacterial Activity Kinetics.* Fast and efficient antibacterial activity is essential for the antibacterial peptides. As shown in Figure 3, the significant increase in antimicrobial activity was observed after the original 30 min of incubation. In addition, we observed that longer incubation times ranging from 1 to 8 h did not induce further significant inhibitory effects, which suggested that the cell growth was completely inhibited. These results showed that JCpep7 could limit the increase in viable cell numbers rapidly.

Activity on the Structure of the Cell Wall Analyzed by FTIR Spectroscopy. The measurement of antibacterial activity kinetics only ensured that cell proliferation was affected. However, the cellular changes were also important to the effect of AMPs. We detected cellular changes by FTIR spectroscopy. FTIR spectroscopy, with unprecedented specificity and sensitivity to molecular and structural changes, has been widely recognized.[39−41] FTIR spectra of microorganisms are usually divided into five regions.[42] These regions contain different cell component information: (1) 3000−2800 cm^{-1}, fatty acids in the bacterial cell membrane; (2) 1800−1500 cm^{-1}, amide bands from proteins and peptides; (3) 1500−1200 cm^{-1}, mixed region of proteins and fatty acids; (4) 1200−900 cm^{-1}, polysaccharides within the cell wall; and (5) 900−500 cm^{-1}, "true" fingerprint region containing bands.

As seen from Figure 4, high-quality absorption spectra of the bacterial cell as well as the differences between the control and tested bacterial cells were obtained from 4000 to 400 cm^{-1}. The differences were observed in the FTIR absorption spectra, mainly concerning the spectral shape of the band of amide bands from proteins and peptides (1800−1500 cm^{-1}), proteins and fatty acids (1500−1200 cm^{-1}), polysaccharides within the cell wall (1200−900 cm^{-1}) and the mannan and global glucan contents (790−925 cm^{-1}). Therefore, it could be concluded that, once close to the microbial surface, JCpep7 can traverse the cell wall.

Activity on Membrane Permeability. Membrane attachment was the second step involved in the antimicrobial peptide

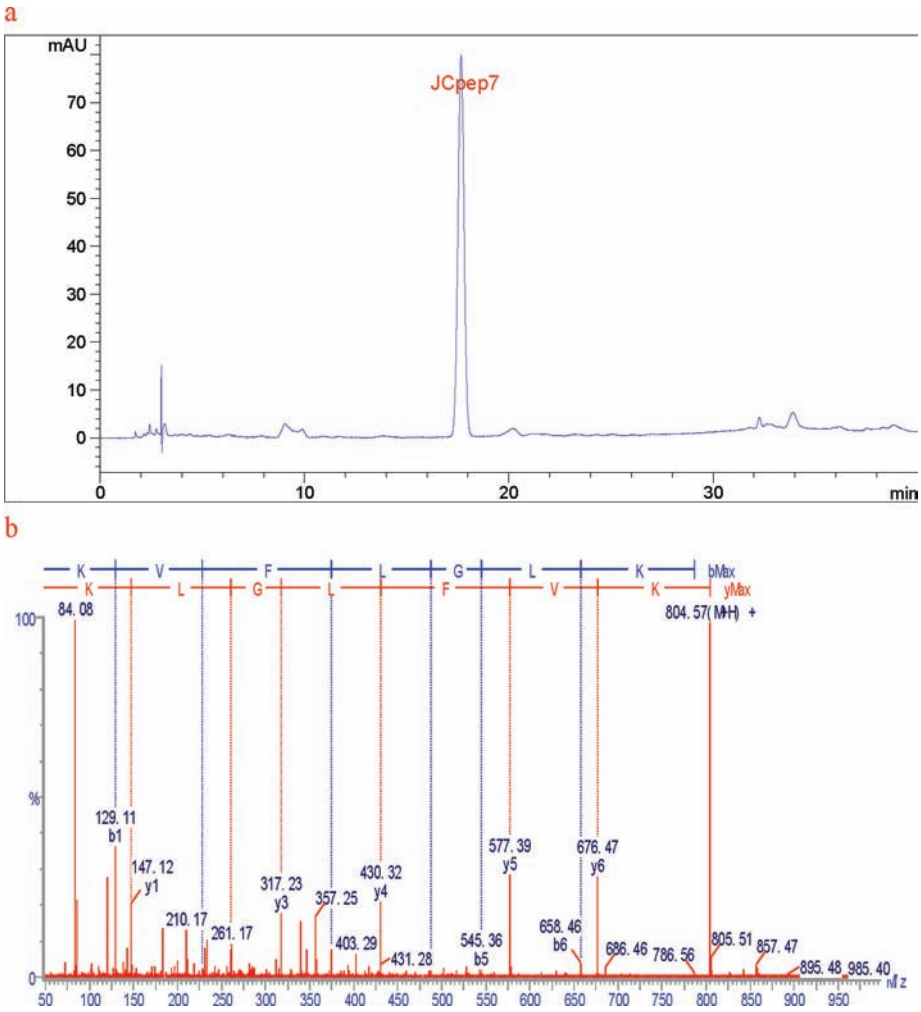

Figure 2. (a) RP-HPLC chromatographic profile of active peak. (b) MS/MS spectrum of JCpep7 (MALDI−TOF MS, reflector positive-ion mode).

Table 1. MICs of JCpep7

microorganisms	MIC (μg/mL)
Gram-Negative Bacteria	
S. typhimurium ATCC 50013	48
P. aeruginosa ATCC 27553	64
S. dysenteriae ATCC 51302	48
Gram-Positive Bacteria	
S. aureus ATCC 25923	40
B. subtilis ATCC 23631	24
S. pneumoniae ATCC 49619	56
Fungi	
P. expansum ATCC 1117	*a*
A. niger ATCC 16404	*a*
Yeast	
S. cerevisiae ATCC 40075	*a*

[a] Antimicrobial activity not detected up to the concentration of 560 μg/mL.

interaction process, and the above results (Figure 4) demonstrated that JCpep7 could significantly attract bacterial surfaces. To explore when an effective concentration of peptide binding to the cell membrane was reached, whether the plasma membrane may be broken apart, leading to the death of the cell or not, a membrane permeability assay was performed.[32] Total nucleotide leakage from bacterial cells after incubation with JCpep7 from different times was shown in Figure 5. When JCpep7 was interacted with bacterial cells, the total leakage content was dependent upon the incubation time. For *S. aureus* ATCC 25923, there were three stages for membrane permeability. First, there was no significant increase in OD_{260} within 60 min by the addition of JCpep7 to *S. aureus* ATCC 25923. This was the initial steps for membrane binding. Then, a fast increase to 0.197 in OD_{260} was observed (60−360 min). At this step, JCpep7-treated cells showed a significant increase of nucleotide leakage, indicating that JCpep7 can bind to the membrane lipid and cause membrane permeabilization to varying extents. Finally, no increase was shown afterward. The results suggested that there were at least three steps involved in the membrane disruption process. In view of this, the cytoplasmic cell membrane may be the target for JCpep7, which can exert its antimicrobial action by disrupting and disintegrating bacterial cell membranes, leading ultimately to the loss of cytoplasmic membrane integrity. Similar results was also reported by Tang et al.[22]

To further elucidate the nature of the killing mechanisms of JCpep7, *S. aureus* ATCC 25923 treated with JCpep7 for 60 and

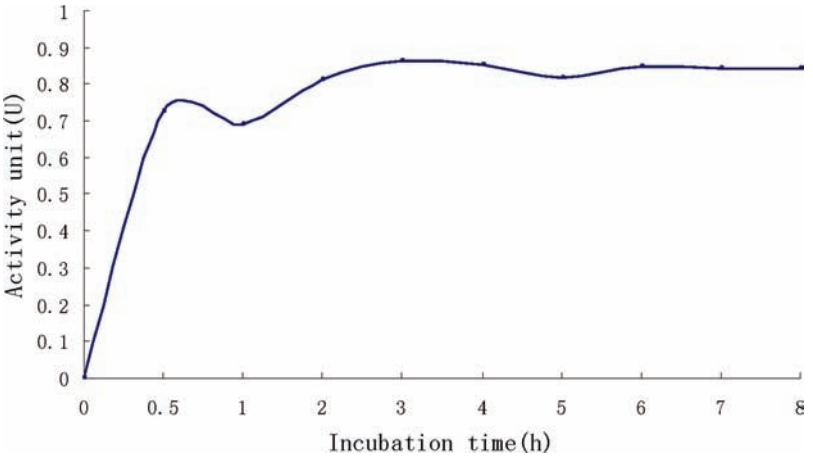

Figure 3. Antimicrobial activity kinetics of JCpep7 for *S. aureus* ATCC 25923.

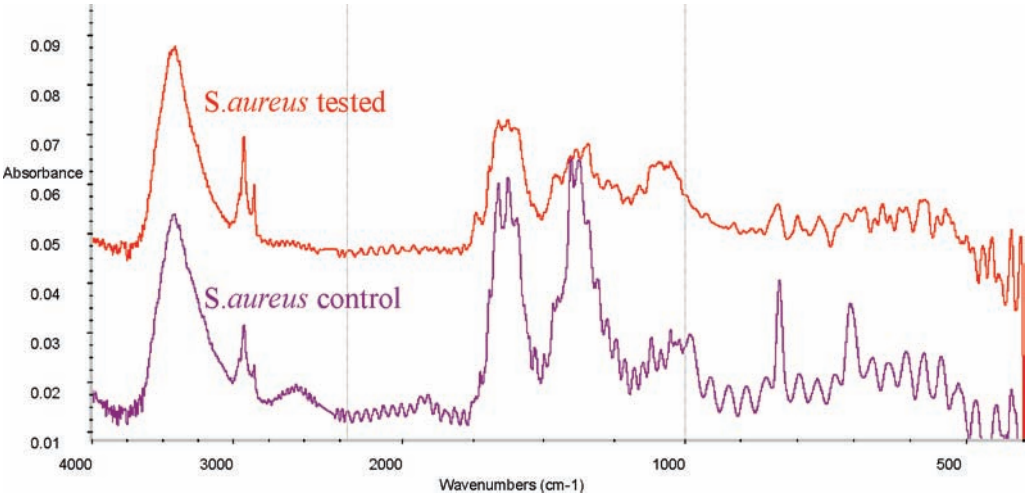

Figure 4. FTIR absorption spectrum of *S. aureus* ATCC 25923: (purple) control cell and (red) cell after treated with JCpep7 for 30 min.

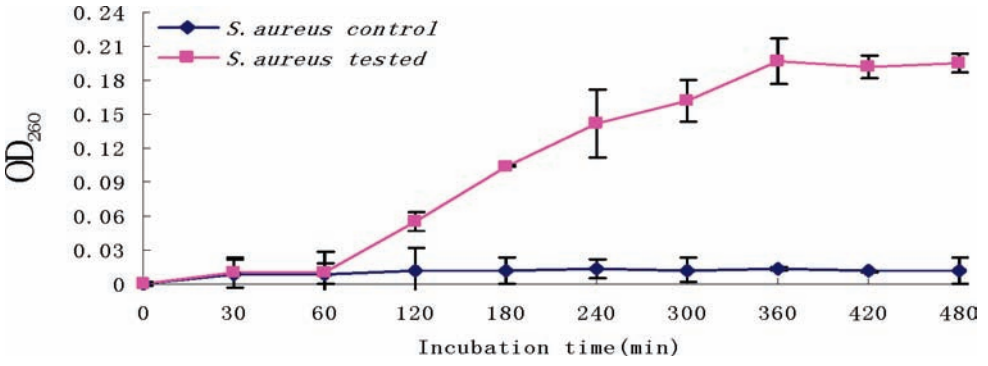

Figure 5. Total nucleotide leakage from *S. aureus* ATCC 25923 treated with JCpep7.

240 min was analyzed by TEM. As shown in Figure 6, in comparison to the control, the treated cell had clear morphological changes. After 60 min of treatment, pores were found at the bacterial surface. After up to 240 min of treatment, a ghost-like appearance and the lysed cell were observed. These data correlated with the rapid and remarkable increase in the nucleotide leakage after peptide treatment (Figure 5).

In conclusion, cell membrane affinity chromatography, a new bioaffinity chromatography technique, can be used to effectively study the interaction between drugs and receptors by the chromatographic characteristics of drugs on the stationary phase prepared by immobilizing the cell membrane onto the surface of the silica carrier. It has demonstrated that the chromatographic retention characteristic of the active component in the model of

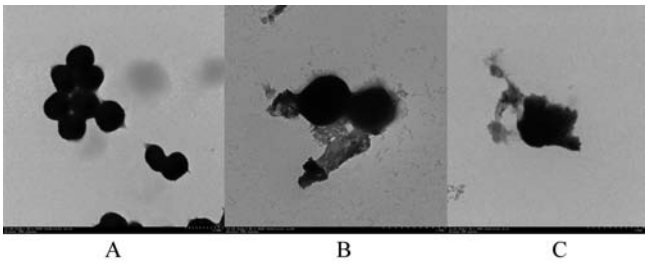

Figure 6. Morphological changes of *S. aureus* ATCC 25923 upon incubation with JCpep7: (A) control cell, (B) 60 min of treatment, with pore formation at the bacterial surface, and (C) 240 min of treatment, with a "ghost-like" appearance of the *S. aureus* ATCC25923 cell.

cell membrane affinity chromatography correlates with biological activity. In our study, a tailor-made monolithic-based bioaffinity chromatography was developed for the isolation and purification of antimicrobial peptides. The purity of the isolated antimicrobial peptide (JCpep7) was very high, and our new procedure provided a way of isolating antimicrobial peptides, which was much faster and more efficient than the conventional chromatography. The data from our study confirm earlier observations and demonstrate that the cell membrane affinity chromatography approach can be employed to screen AMPs from *J. curcas*.

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