

Basic Study

Crude extract of maggots: Antibacterial effects against *Escherichia coli*, underlying mechanisms, separation and purification

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the crude extract of maggots to assess the antibacterial effects of the active ingredients in the crude extract.

METHODS: Different concentrations of the crude extract of maggots were incubated with *E. coli* (O157:H7) and cultured. The optical density (OD) was measured at different time points to plot the OD-T curve. The effects of different concentrations of the crude extract on bacterial membrane permeability were determined by fluorescence probe technique. The effects of different concentrations of the crude extract on plasmid DNA replication were determined by agarose gel electrophoresis. DEAE-Sepharose ion exchange chromatography and Sephacryls-200HR gel filtration chromatography were used to separate and purify the crude extract of maggots. The molecular weight of proteins in the purified crude extract was determined by SDS-PAGE electrophoresis, and its antibacterial effects were determined by turbidimetric method.

RESULTS: The antibacterial effects of the crude extract of maggots at concentrations > 0.5 mg/mL were significant. The antibacterial effects of the crude extract at concentrations of 1.0, 1.5 and 2.0 mg/mL did not differ significantly. Fluorescence probe analysis showed that the rate of membrane permeability change was 1223.1% in bacteria incubated with 2 mg/mL of the crude extract, and 1300.0% in those incubated with 80 mg/mL of the crude extract. Plasmid DNA was undetectable in *E. coli* incubated with 2 and 80 mg/mL of the crude extract. A low molecular weight protein band (about 15 kDa) was detected in the crude extract of maggots and eluent, but not in eluent, from DEAE-Sepharose ion exchange chromatography. The antibacterial effects of the crude extract of maggots and eluent were superior to those of eluent, with the antibacterial effects of eluents being better than those of the crude extract of maggots. Of 24 tubes of

Abstract

AIM: To investigate the antibacterial effects of a crude extract of maggots against *Escherichia coli* (*E. coli*) and the underlying mechanisms, and to separate and purify

filtrates, the antibacterial effects of filtrates in tubes 4, 5 and 11 were significantly higher than those of the control. The molecular weight of the protein in filtrates in tubes 4, 5 and 11 was about 15 kDa.

CONCLUSION: The crude extract of maggots exhibits obvious, dose-dependent antibacterial effects. The crude extract exerts antibacterial effects by changing the bacterial membrane permeability and inhibiting plasmid DNA replication. The protein that has antibacterial effects in the crude extract of maggots has a molecular weight of about 15 kDa.

Key words: Maggots; Antibacterial peptide; Antibacterial mechanism; *Escherichia coli*; Colorectal

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Core tip: Previous studies have found the antibacterial activity of the maggots, but studies on the underlying mechanisms are still lacking. In this study, we separated and purified the crude extract of maggots using DEAE-Sepharose ion exchange chromatography and Sephacryls-200HR gel filtration chromatography. Agarose gel electrophoresis and fluorescence probe technique were used to investigate the antibacterial mechanism of the crude extract of maggots. We found that the protein of maggots weighing around 15 KD had the antibacterial effect and the crude extracts played the antibacterial role by destroying the cell membrane of *Escherichia coli* and disturbing the DNA replication of plasmids.

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INTRODUCTION

Maggots are the larvae of *Lucilia sericata*, *Chrysomya megacephala* and their relatives, belonging to the Calliphoridae family^[1,2]. According to the records in "Bencao Biandu" and "Bencao Qiuyuan", maggots are nontoxic and cold-natured. After being dried and ground to a powder, they can be externally applied to treat ecthyma and lip pustules^[3-5]. In 1929, the American doctor Baer^[6] reported, for the first time, the successful treatment of more than 100 cases of chronic osteomyelitis with live maggots *in vivo*. In recent years, the abuse of antibiotics has led to the emergence of a large number of drug-resistant bacterial strains, and therefore, there is an urgent need to develop a new generation of antimicrobial drugs. Studies have found that active antimicrobial

substances secreted by maggots can effectively control wound infection^[7-10], and they have become the new hot spot of antimicrobial drug research.

Current research on antimicrobial substances of maggots is focused on small molecular weight antimicrobial peptides. Antimicrobial peptides not only have non-specific anti-bacterial, anti-fungal, anti-viral and anti-parasitic effects^[11,12] but also can exert anti-tumor effects^[13], thus having broad application prospects. Their main site of action is located on the membrane, and they can change the structure of the bacterial membrane and kill bacteria, with advantages of fast action and not easy to produce drug resistance^[14]. However, their exact mechanisms of action remain to be studied. Wang *et al.*^[15,16] have purified a single antimicrobial peptide from maggot secretions by ion exchange chromatography and gel filtration chromatography. However, there is still a lack of experimental studies on antibacterial mechanisms of maggots.

This study investigated the antibacterial effects of a crude extract of maggots and preliminarily explored the antibacterial mechanisms of the crude extract using agarose gel electrophoresis and fluorescent probe technique. In addition, we separated and purified the crude extract of maggots by ion exchange chromatography and gel filtration chromatography and detected its antibacterial effects. The results obtained will provide a theoretical basis for the development of new biological antibiotics for treatment of infections.

MATERIALS AND METHODS

Materials

Maggots used in the present study were the third instar larva of *Lucilia sericata* that were raised under sterile conditions. Maggots were cultured as previously described by Wang *et al.*^[17]. *Escherichia coli* (*E. coli*) strain (O157:H7) was provided by the Department of Microecology, Dalian Medical University. The bacteria were inoculated into 200 mL of broth, cultured with shaking at 37 °C for 12 h and centrifuged at 8000 rpm for 10 min. The pellet was re-suspended in broth and diluted to 10⁸-10¹⁰ CFU/mL.

Preparation of a crude extract of maggots

One hundred maggots were selected and 10 mL of TNE buffer (5 mmol/L EDTA, 10 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.5) precooled at 4 °C was added. After sufficient homogenization, the homogenate was centrifuged at 12000 r/min for 10 min at 4 °C. The supernatant was transferred to a new tube, and three volumes of TNE buffer were added. The mixture was boiled for 2 min, cooled and centrifuged at 12000 r/min at 4 °C for 10 min. The resulting supernatant was a crude extract of maggots. The crude extract was filtered through

a membrane with a pore size of 0.22 μm , and the filtrate was frozen at $-80\text{ }^{\circ}\text{C}$. The freeze-dried powder was stored in a refrigerator at $-20\text{ }^{\circ}\text{C}$ for further use.

Determination of protein content of the crude extract of maggots by Coomassie brilliant blue assay

The protein content of the crude extract was measured using the Coomassie brilliant blue assay system and adjusted to 2 mg/mL.

Determination of antibacterial effects of different concentrations of the crude extract of maggots

Two hundred microliters of the crude extract of maggots at a protein concentration of 0.1, 0.5, 1.0, 1.5 or 2.0 mg/mL were mixed with 22 μL of 10% sterile tryptone, respectively. The mixture (150 μL) was added into a sterile 96 well plate, followed by the addition of 30 μL of the bacterial suspension. The plate was shaken to mix the liquid evenly and then incubated at $37\text{ }^{\circ}\text{C}$. The optical density (OD) at 589 nm was measured with an automated microplate reader at 0, 3, 6, 9, 12, 15, 18, 21 and 24 h after zeroing the absorbance with a blank solution. The OD-T curve was plotted with time on X-axis and OD value on Y-axis.

Determination of effects of different concentrations of the crude extract of maggots on bacterial membrane permeability by fluorescence probe technique

The co-cultured broth of the crude extract of maggots and *E. coli* was centrifuged, washed with sterile normal saline, re-suspended with 1 mmol/L of Fluo-3/AM, and adjusted to a final concentration of 3 $\mu\text{mol/L}$. After incubation and centrifugation (10000 g, 5 min), the supernatant was discarded and the pellet was washed and re-suspended in 3 mL of normal saline. The sample was scanned between 380 and 430 nm using 405 nm as the excitation wavelength, and an absorption peak was detected at 408 nm. Then, changes in intracellular calcium were monitored with the excitation wavelength set at 408 nm. The rate of membrane permeability change was calculated as $[(\text{sample fluorescence intensity} - \text{blank fluorescence intensity})/\text{fluorescence intensity of blank}] \times 100\%$. Gentamicin was used as a control.

Determination of effects of different concentrations of the crude extract of maggots on plasmid DNA replication by agarose gel electrophoresis

For gel preparation, 0.2 g of agarose was mixed with 20 mL of $1 \times \text{TAE}$ and heated for 1 min. While the agarose was cooling, 1 μL of EB was added. The cooled agarose was then poured into a casting tray. Ten microliters of the samples (crude extract 2 mg/mL + plasmid, crude extract 80 mg/mL + plasmid, TNE + plasmid, plasmid alone, gentamicin 50 mg/mL + plasmid, and gentamicin 100 mg/mL

+ plasmid) were mixed with 2 μL of $6 \times$ loading buffer and loaded into slots. Three microliters of 1 kDa DNA marker was also loaded. After loading, electrophoresis was conducted at a voltage of 80 V for about 20 min.

Separation and purification of the crude extract of maggots

DEAE-Sephacryl Fast Flow ion exchange chromatography and Sephacryl-200HR gel filtration chromatography were used to separate and purify the crude extract of maggots^[18-20]. The eluent and eluant were obtained.

Determination of antibacterial effects of the crude extract of maggots, eluent and eluant by turbidimetric method

Two hundred microliters of the crude extract of maggots, eluent or eluant were mixed with 22 μL of 10% sterile tryptone, respectively. The mixture (150 μL) was added into a sterile 96 well plate, followed by the addition of 30 μL of diluted bacterial suspension. 1% sterile tryptone was used as a negative control. The plate was incubated at $37\text{ }^{\circ}\text{C}$, and the OD at 589 nm was measured with an automated microplate reader at 0, 3, 6, 9, 12, 15, 18, 21 and 24 h after zeroing the absorbance with a blank solution. The OD-T curve was plotted with time on X-axis and OD value on Y-axis.

RESULTS

Antibacterial effects of different concentrations of the crude extract of maggots

As shown in Figure 1, the antibacterial effects of the crude extract of maggots at concentrations > 0.5 mg/mL were significant. The antibacterial effects of the crude extract of maggots at concentrations of 1.0, 1.5 and 2.0 mg/mL did not differ significantly. However, the antibacterial effects of the crude extract of maggots at a concentration of 0.5 mg/mL were significantly poorer than those at a concentration of 1.0 mg/mL. The antibacterial effect of the crude extract of maggots at a concentration of 0.1 mg/mL was the poorest.

Effects of different concentrations of the crude extract of maggots on bacterial membrane permeability

As shown in Figure 2, the results of fluorescence probe analysis showed that the rate of membrane permeability change was 35.4% in the 20 mg/mL gentamicin group, 40.0% in the 50 mg/mL gentamicin group, 42.3% in the 100 mg/mL gentamicin group, 1223.1% in the 2 mg/mL crude extract group, and 1300.0% in the 80 mg/mL crude extract group. The rates of membrane permeability change were significantly higher in the crude extract

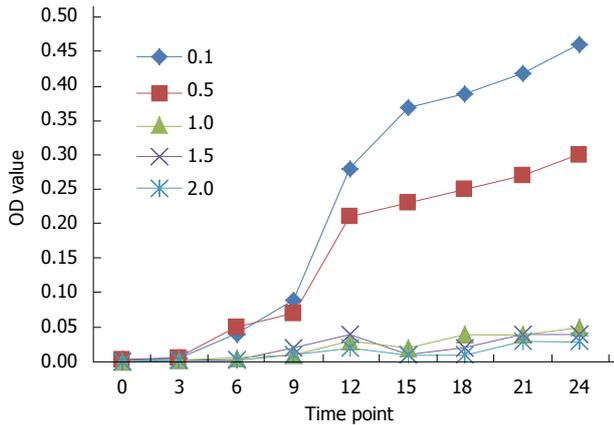


Figure 1 Antibacterial effects of different concentrations of the crude extract of maggots.

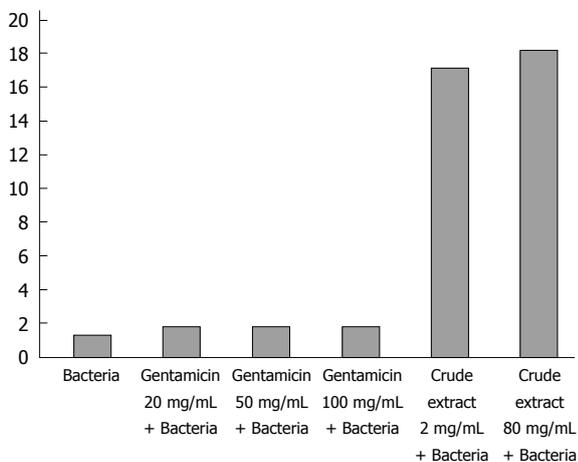


Figure 2 Determination of the effects of different concentrations of the crude extract of maggots on bacterial membrane permeability by fluorescence probe technique. Antibiotic, crude extract, unit: mg/mL; bacteria: 1×10^8 CFU/mL.

groups than in the gentamicin groups.

Effects of different concentrations of the crude extract of maggots on plasmid DNA replication

As shown in Figure 3, no plasmid DNA band was detected in the two lanes containing 2 mg/mL and 80 mg/mL of the crude extract.

Antibacterial effects of the crude extract of maggots, eluent and eluant obtained from DEAE-Sepharose ion exchange chromatography

As shown in Figure 4, a lower molecular weight protein band (about 15 kDa) was detected in the crude extract of maggots and eluent, but not in eluant from DEAE-Sepharose ion exchange chromatography. The antibacterial effects of the crude extract of maggots and eluent were superior to those of eluant, with the antibacterial effects of eluents being better than those of the crude extract of maggots.

Antibacterial effects of filtrates obtained from sephacryls-200HR gel filtration chromatography

As shown in Figure 5, of 24 tubes of filtrates obtained, the antibacterial effects of filtrates in tubes 4, 5 and 11 were significantly higher than those of the control. The molecular weight of proteins in filtrates in tubes 4, 5 and 11 was about 15 kDa.

DISCUSSION

The discovery of antibiotics is a milestone in the battle of humans against infectious diseases. However, the abuse of antibiotics in recent years has led to the emergence of multidrug resistant strains, and even super-resistant strains, bringing an enormous obstacle to clinical management of infections^[21]. This makes the development of new antibacterial drugs become an urgent task in the field of medical research. With the extraction of small molecule proteins with antimicrobial activity (antimicrobial peptides) from various insects by both domestic and foreign scientists, the direction of antibiotic research has gradually shifted from conventional antibacterial drugs to biological antibacterial drugs. The antibacterial effects of maggots have become a research hotspot. *E. coli* colonizes the intestine of humans and is a major member of the human intestinal flora, forming a symbiotic relationship with the body. However, when the body's defense declines, for example in cases of hepatitis, cirrhosis, cancer and immune system disorders, patients are prone to opportunistic infections with *E. coli*, such as spontaneous bacterial peritonitis and *E. coli* bacteremia. In recent years, the overuse of antibiotics has led to the generation of multidrug resistant *E. coli*. For this reason, the present study investigated the antibacterial effects of a crude extract of maggots against *E. coli* and explored the underlying mechanisms by co-culturing the crude extract and *E. coli*.

This study firstly co-cultured different concentrations of the crude extract of maggots and *E. coli* to observe the antibacterial effects of the crude extract, and then detected the cell membrane permeability and DNA synthesis in *E. coli*. We experimentally found that as the concentration of the crude extract increased, the antibacterial effects became increasingly significant. When the concentration of the crude extract reached 1.0 mg/mL, the strongest antibacterial effects were achieved and no obvious bacterial growth was further observed. This finding suggests that the crude extract of maggots can exert antibacterial effects against *E. coli* in a concentration-dependent manner. The finding, however, raises the following questions: How do maggots inhibit the growth of *E. coli*? Which ingredients of maggots inhibit the growth *E. coli*?

In order to investigate the mechanism of anti-

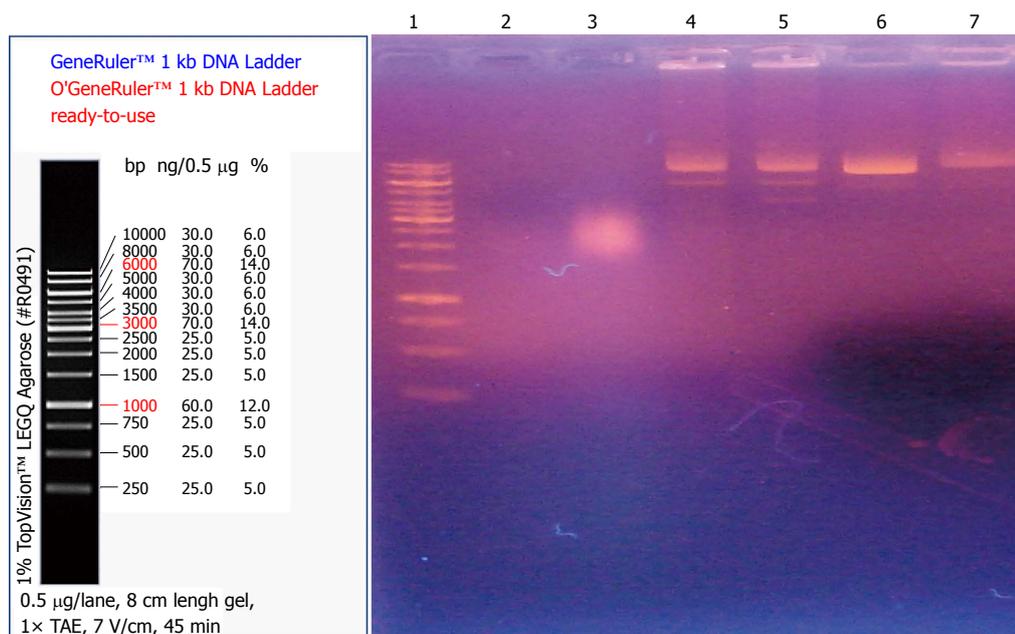


Figure 3 Determination of the effects of different concentrations of the crude extract of maggots on plasmid DNA replication by agarose gel electrophoresis. The left-most lane: DNA marker; 1: Marker; 2: Crude extract 2 mg/mL + plasmid; 3: Crude extract 80 mg/mL + plasmid; 4: TNE + plasmid; 5: Plasmid alone; 6: Gentamicin 50 mg/mL + plasmid; 7: Gentamicin 100 mg/mL + plasmid. Antibiotic, crude extract; unit: mg/mL.

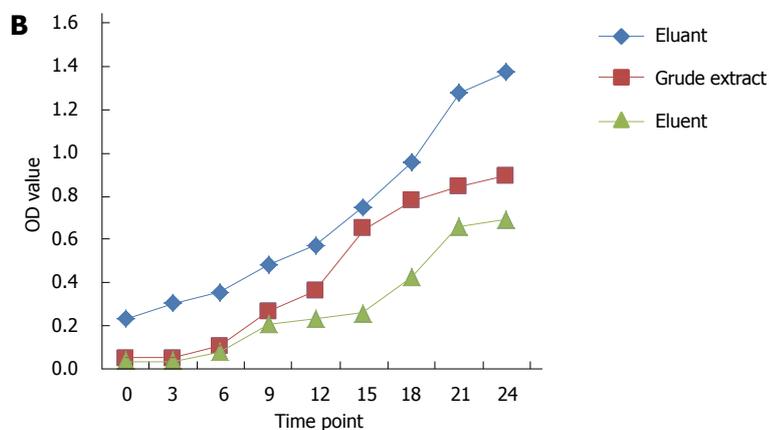
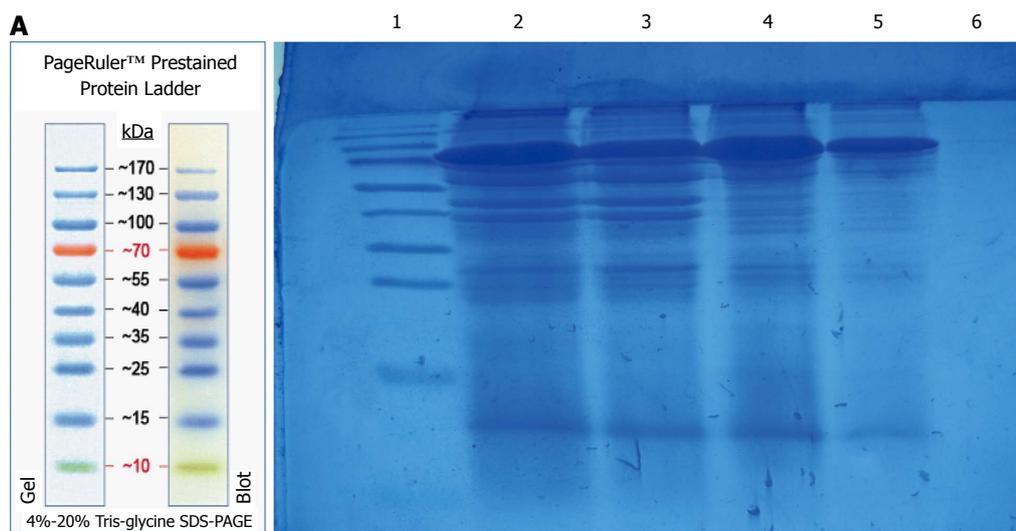


Figure 4 Antibacterial effects of the crude extract of maggots, eluent and eluant obtained from DEAE-Sepharose ion exchange chromatography. A: SDS-PAGE analysis of the crude extract of maggots, eluent and eluant. 1: Marker; 2: Crude extract 1 mg/mL; 3: Crude extract 0.5 mg/mL; 4: Eluent 1 mg/mL; 5: Eluent; 0.5 mg/mL; 6: Eluant 1 mg/mL; the left-most lane: marker (unit: kDa); B: Antibacterial effects of the crude extract of maggots 1 mg/mL, eluent and eluant.

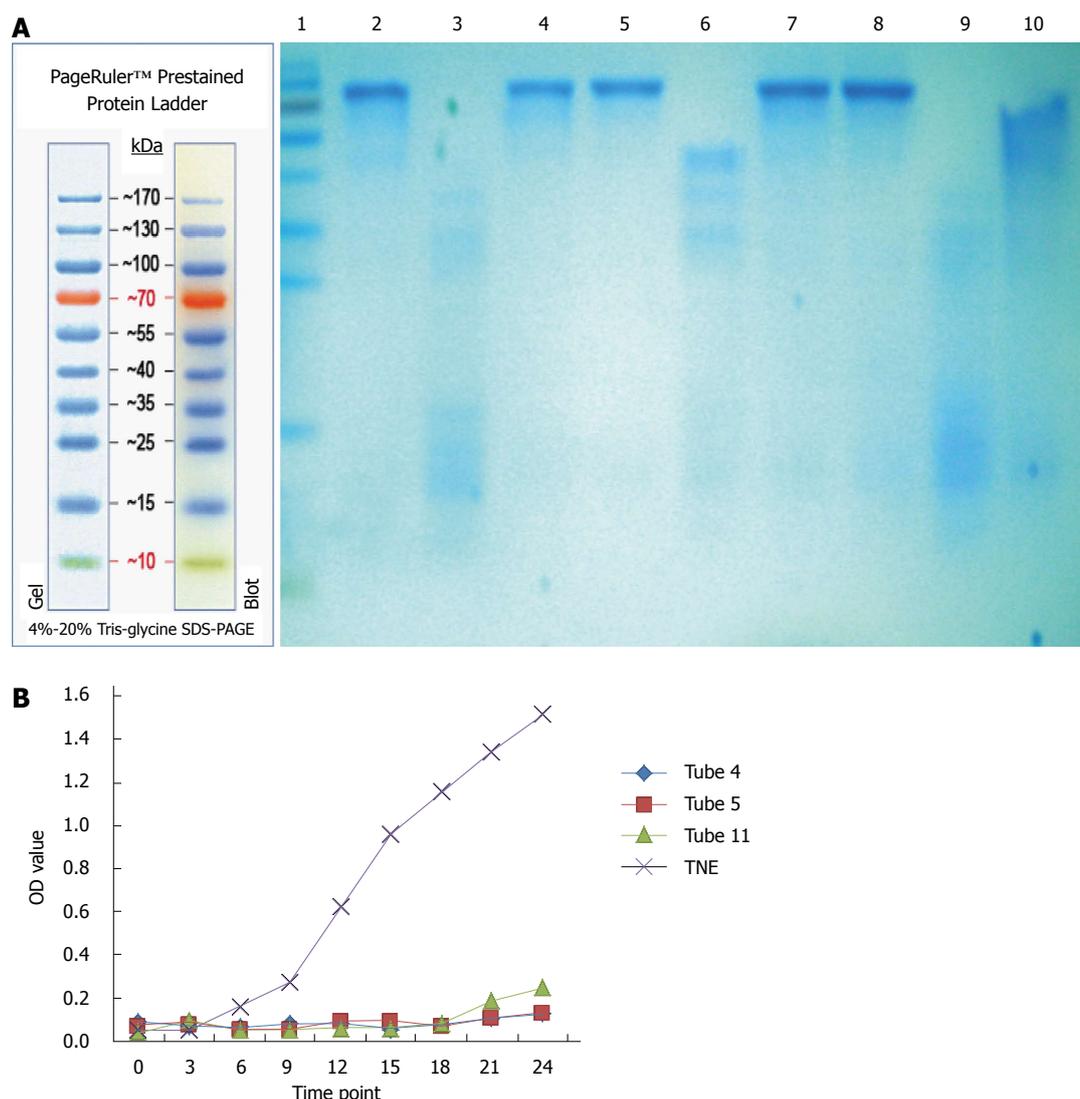


Figure 5 Antibacterial effects of filtrates obtained from sephacryls-200HR gel filtration chromatography. A: SDS-PAGE analysis of filtrates from Sephacryls-200HR gel filtration chromatography. 1: Marker (kDa); 2: tube 12; 3: tube 11; 4: tube 10; 5: tube 9; 6: tube 8; 7: tube 7; 8: tube 6; 9: tube 5; 10: tube 4; B: Antibacterial effects of filtrates from sephacryls-200HR gel filtration chromatography. OD: Optical density.

bacterial effects of maggots, we conducted the following two experiments. First, we examined the effects of different concentrations of the crude extract and gentamicin on the rate of membrane permeability change of *E. coli* by fluorescence probe technique. The experimental results showed that the rate of membrane permeability change was 35.4% in the 20 mg/mL gentamicin group, 40.0% in the 50 mg/mL gentamicin group, 42.3% in the 100 mg/mL gentamicin group, 1223.1% in the 2 mg/mL crude extract group, and 1300.0% in the 80 mg/mL crude extract group. This finding suggests that compared with gentamicin, the crude extract of maggots can significantly change the membrane permeability of *E. coli*. By changing the membrane permeability of *E. coli*, the crude extract of maggots can affect the exchange of substances inside and outside the bacteria, thereby inhibiting bacterial growth. Since gentamicin exerts antibacterial effects by inhibiting

bacterial protein synthesis, its impact on bacterial growth was less significant. Second, we performed agarose gel electrophoresis to detect the effects of different concentrations of the crude extract of maggot and gentamicin on plasmid DNA replication of *E. coli*. Plasmids are a class of covalently closed double-stranded DNA molecules that are physically separate from chromosomal DNA and can replicate autonomously in bacterial or fungal cells. They often contain a variety of genes that confer bacterial resistance or are related with bacterial metabolism. Therefore, inhibition of plasmid replication can inhibit bacterial metabolism to achieve bacteriostatic and bactericidal effects. Our experimental results showed that no plasmid DNA bands were observed in the two lanes containing 2 mg/mL and 80 mg/mL of the crude extract of maggots. In other words, the levels of plasmid DNA were low in *E. coli* incubated with 2 mg/mL and 80 mg/mL of the crude extract,

suggesting that the above two concentrations of the crude extract can inhibit plasmid DNA synthesis and thereby inhibit bacterial growth.

In addition to exploring the antibacterial mechanism of the crude extract of maggots, this study also preliminarily separated and purified the crude extract of maggots. First, we separated the crude extract by DEAE-Sepharose ion chromatography. DEAE-Sepharose Fast Flow is an anion exchanger, and the technical principle of DEAE-Sepharose ion-exchange chromatography is that the isolated protein (as the crude extract of maggots in this study) carries a net negative charge in Tris-HCl buffer (pH = 8.0) and may be bound by the anion exchanger in the column. When gradient elution was conducted, exchange of ions occurred between the proteins bound by the ion exchanger and the elution solution, and the proteins were finally eluted into the solution. Due to variations of the charges of different proteins, their affinities to the ion exchanger are not the same^[19]. Therefore, they are eluted in different orders and thereby separated^[20]. In this study, we selected 1 mg/mL of the crude extract, 0.5 mg/mL of the crude extract, eluent and eluant obtained from ion exchange chromatography to conduct SDS-PAGE and antibacterial experiments. The experimental results showed that a approximately 15 kDa protein band was detected in the crude extract of maggots and eluent, but not in eluant. Antibacterial experiments with 1 mg/mL of the crude extract, eluent and eluant showed that the antibacterial effects of the crude extract of maggots and eluent were superior to those of eluant, with the antibacterial effects of eluent being better than those of the crude extract of maggots. This finding suggests that both the crude extract and eluent contained the active antibacterial ingredient and that the content of the antibacterial ingredient in the eluate was higher. Second, we used Sephacryls-200HR gel filtration chromatography for the separation and purification of the crude extract. Gel filtration chromatography, also known as molecular sieve^[22,23], separates proteins based on the molecular weight of proteins, does not require binding between proteins and columns, and can significantly reduce the loss and inactivation of proteins caused by irreversible binding. When different substances in the sample pass through the gel column with the eluent, molecules with a size larger than the pore size of the gel can not enter the pores of the gel and have to enter the gap between the gel particles, therefore having the same flow rate as the eluent. If the molecule is smaller than the gel pore size, it can enter the pores of the gel and therefore does not have the same flow rate as the eluent. Based on this principle, molecules of different sizes can be separated^[19]. In this study, we collected the eluents at different time points, detected their molecular

weights by SDS-PAGE electrophoresis, and assessed their antibacterial effects. The experimental results showed that of 24 tubes of eluents, a suspicious 15 kDa band was detected in tubes 4, 5 and 11, and that the filtrates in tubes 4, 5 and 11 had significant antibacterial effects, indicating that the molecular size of the active ingredient of the crude extract of maggots may be approximately 15 kDa.

In short, this study found that the crude extract of maggots can inhibit *E. coli* growth, exhibiting obvious, dose-dependent antibacterial effects. The crude extract exerts bacteriostatic effects by changing the bacterial membrane permeability and inhibiting plasmid DNA replication, but their specific targets and mechanisms of action need to be further studied. In addition, whether maggots exert antibacterial effects through other mechanisms also needs further study. The present study also separated and purified the crude extract of maggots by ion exchange chromatography and gel filtration chromatography and found that a protein with a molecular weight of about 15 kDa had significant antibacterial effects. The identity of this protein as well as its structure and composition remains to be studied. The research on maggots as a novel biological antimicrobial drug is still in the initial stage, and there are many problems awaiting us to solve. Our efforts in research of maggots may create a new research area for biological antimicrobial drugs.

COMMENTS

Background

Currently abuse of antibiotics results in resistance of bacteria, which brings along great difficulties in treatment of clinical infectious diseases. The maggots are proven to have antibacterial effects, but the antibacterial substances and mechanisms are still not clear.

Research frontiers

In this study, DEAE-Sepharose ion exchange chromatography and Sephacryls-200HR gel filtration chromatography were used to isolate and purify the crude extract of the maggots.

Innovations and breakthroughs

The authors obtained small molecular proteins of the maggots with antibacterial effects and explored the antibacterial mechanisms of the maggots.

Applications

The purified antimicrobial peptides from the maggots can be used to treat infectious diseases in future.

Terminology

Maggots are the larvae of *Lucilia sericata*, *Chrysomya megacephala* and their relatives, belonging to the *Calliphoridae* family. Maggots are nontoxic and cold-natured. After being dried and ground to a powder, they can be externally applied to treat ecthyma and lip pustules. Antimicrobial peptide is a general name of short peptides and constitutes of the main part of natural immunity. It has characteristics of broad-spectrum, high efficiency, selective toxicity, stability and not being easy to cause drug resistance.

Peer-review

This study obtained small molecular peptides from maggots using advanced molecular biological techniques and elucidated the antibacterial mechanisms of the peptides, which provides a theoretical basis for clinical treatment of infectious diseases.

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