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**Original Research Article**

# Purification and characterization of an antimicrobial protein from *Gastrodia elata* Blume tubers

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## Abstract

***Purpose:*** *To purify and characterize a novel antimicrobial protein from the Gastrodia elata Blume (Bl.) plant, which has long been used in herbal medicine.*
***Methods:*** *The procedure for isolation and purification of Gastrodia elata protein (GEP) involved phosphate buffer extraction, ammonium sulfate precipitation, ion-exchange chromatography, and gel-filtration chromatography. Sodium dodecyl sulfate - polyacrylamide gel electrophoresis was employed to detect the apparent molecular mass and determine homogeneity, while paper disc diffusion was used to measure the antibacterial activity of GEP. A hemolytic assay was performed on rabbit red blood cells. The effect of pH, salt concentration, and temperature on the antibacterial activity of GEP was evaluated by minimum inhibitory concentration assay.*
***Results:*** *GEP was a 14-kDa monomer and displayed antimicrobial activity against Staphylococcus aureus and Candida albicans, with 8.0-mm and 9.4-mm zones of inhibition, respectively, but no antibacterial activity was observed against Escherichia coli. GEP had little hemolytic activity on red blood cells even at a concentrations of up to 200 mg/ml. GEP was thermally stable at temperatures below 70 °C for 30 min, and displayed higher antibacterial activity in the pH range 5.0 to 7.0.*
***Conclusion:*** *GEP protein is relatively thermostable and possesses antimicrobial activity. The results suggest that GEP protein has potential agricultural and industrial applications, such as in transgenic plants.*

***Keywords:*** *Antimicrobial protein, Gastrodia elata, Protein characterization*

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## INTRODUCTION

Plants exhibit a variety of defenses to combat bacteria in the environment. Despite not having an integrated immune system, plants produce proteins and peptides with antibacterial activity to combat pathogenic invasion [1]. Transgenic plants that express antibacterial proteins are expected to be resistant to bacterial infections. Therefore, several investigators have identified and characterized the antibacterial properties of proteins and peptides derived from the leaves,

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flowers, seeds, roots, and stems of plants [2,3]. These proteins and peptides display antibacterial activity against phytopathogens, as well as bacteria that are pathogenic to humans. Thus, plant-derived antibacterial proteins and peptides are considered promising antibiotic compounds.

Gastrodiae Rhizoma (Chinese name: *Tian-ma*) is the dried tuber of the *Gastrodia elata* Bl. plant, which is a member of the Orchidaceae family [4]. *Gastrodia elata* Bl. has been used in herbal medicine for over 2,000 years in many countries, including China, Japan, Korea, and India [5], as a traditional medicine for the treatment of headaches, dizziness, migraine, rheumatism, epilepsy, and nervous disorders [6]. Additionally, *Gastrodia elata* Bl. has also been used in food, health tea, and as a food additive. The biological functions of its chemical constituents, including gastrodin [7], polysaccharide [8], and lectin, have been reported.

However, whether *Gastrodia elata* Bl. protein extracts possess antibacterial properties are obscure.

## EXPERIMENTAL

### Materials

Fresh *Gastrodia elata* Bl. tubers were purchased from Lueyang County, Shaanxi province, China, and stored at –20 °C. Lueyang County has been a major area for *Gastrodia elata* Bl. cultivation for over 50 years.

### Chemicals and reagents

Diethylaminoethyl (DEAE)-52 cellulose and Sephadex G-50 were purchased from GE Healthcare Company (Gothenburg, Sweden). The molecular mass marker was purchased from Sangon Biotech Co., Ltd (Shanghai, China). N, N, N, N-tetramethyl-ethylenediamine (TEMED) was purchased from Sigma-Aldrich (Saint Louis, Missouri, USA).

### Crude extract preparation

Fresh *Gastrodia elata* Bl. tubers were thoroughly cleaned with tap water and peeled. The tubers were then homogenized at 4°C for 2 min in two volumes of 0.05 mol/L phosphate buffer (pH 7.0), stirred at 4°C overnight, filtered through a cheesecloth, and centrifuged at 5000 rpm for 20 min. The supernatant was collected and concentrated by freeze-drying to yield the crude extract of *Gastrodia elata* Bl. tubers for further purification.

### Ammonium sulfate precipitation

The supernatant was subjected to precipitation using 40–80% saturated ammonium sulfate at 4°C, and the supernatant was discarded, while the precipitate was harvested by centrifugation at 5000 rpm for 20 min. The precipitate containing the protein fraction was resuspended in phosphate-buffered saline (PBS) buffer and dialyzed extensively in distilled water for 48 h to remove ammonium sulfate. The antibacterial activity of each fraction was determined using indicator bacteria.

### Primary purification by ion-exchange chromatography

The dialysate was filtered through 0.45-μm filters and loaded onto a 1.6 × 20 cm DEAE-cellulose anion-exchange column equilibrated with 50 mmol/L Tris–HCl (pH 7). The column was then washed with the same buffer to remove unbound proteins. Adsorbed proteins were desorbed using stepwise elution with increasing sodium chloride (NaCl) concentrations of 0.1, 0.2, 0.4, and 0.6 mol/L. The flow rate was 0.5 mL/min and the eluate was monitored at 280 nm. Elution fractions were pooled, concentrated, and dialyzed overnight with distilled water. The buffer used for dialysis was changed two to three times to ensure the complete removal of salts. The antibacterial activity of each fraction was also determined. The first fraction (D1) demonstrating antibacterial activity was lyophilized and stored at –4°C for further purification.

### Secondary purification by gel-filtration chromatography

The lyophilized fraction from the anion-exchange column was resuspended in ultrapure water, centrifuged at 10,000 rpm for 10 min, and filtered through a 0.45-μm filter prior to chromatography. The sample was added to a gel-filtration chromatograph using Sephadex G-50 beads pre-equilibrated with 100 mmol/L PBS solution (pH 7). Then, elution was performed with PBS at a regulated flow rate of 30 mL/h into 6 mL collection tubes. The absorbance of the eluate was detected at 280 nm, and the active components were lyophilized.

### Concentration measurement and homogeneity analysis of the purified protein

During the purification process, protein concentrations were measured by the Bradford method using a Coomassie Protein Assay Kit from Applygen Technologies Inc. (Beijing, China). Bovine serum albumin was used as the

standard. Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis was used to detect the apparent molecular mass and homogeneity of the purified antibacterial proteins. All samples were heated for 5 min in boiling water prior to SDS-PAGE. The protein bands were visualized by staining with R-250.

**Antibacterial activity determination**

Paper disc diffusion was utilized to detect the antibacterial activity of the crude extract, ion-exchange chromatography fractions, and gel-filtration chromatography fractions [9]. A suspension of each indicator strain ($10^7$ CFU/mL) was spread on LB agar plates (Φ = 90 mm). Four paper discs (Φ = 6 mm) were placed in each agar plate using sterile forceps. Each protein sample (30 mg/mL) was transferred at 6 µL/well, and sterile water was used as negative control. All bacterial plates were incubated for 12 h at 37°C. The diameter of the minimum zone of inhibition was measured in millimeters. The test strains were *Escherichia coli* (ATCC25922), *Staphylococcus aureus* (ATCC25923), and *Candida albicans* (ATCC2002).

The minimum inhibitory concentration (MIC) of the antibacterial protein-of-interest was detected using microdilution methods [10] with an initial strain concentration of $5 \times 10^5$ CFU/mL. The antibacterial activity was examined in sterile 96-well plates at final volumes of 100 µL, 90 µL, and 10 µL of the antibacterial protein. The purified protein (50 µL) was added to the 96-well culture plates along with LB medium. A total of 50 µL of bacteria ($5 \times 10^5$ CFU/mL) was added to each well, and incubated at 37°C for 24 h. Bacterial growth was detected by absorbance at 600 nm. MIC was calculated as the lowest protein concentration that reduced bacteria growth by 50% relative to the control. All experiments were performed in duplicate with three replicates each.

**Hemolytic assay**

Hemolytic assay was performed using rabbit red blood cells [11]. Briefly, fresh rabbit red blood cells were washed with 0.85% saline, limiting the final concentration to 2% (v/v). The antibacterial protein was added in serial dilution, and incubated for 30 min at 37°C. After centrifugation, absorbance of the supernatant was detected. The control experiment for maximum hemolysis was performed by adding 1% Triton X-100 to the red blood cells. The hemolytic assay was repeated three times.

**Effect of pH, salt concentration, and temperature**

The effect of pH on the antibacterial activity of the protein was measured in the pH range of 5 to 10. MIC was used to determine antibacterial activity at different pH values after incubating at room temperature for 30 min.

The effect of salt concentration on the antimicrobial activity of the protein was detected by evaluating the MIC at different concentrations. NaCl at varying concentrations (from 0–300 mmol/L) was added to the Mueller-Hinton broth used in the assay.

The thermal stability of the protein was tested by heating the protein to temperatures ranging from 30 to 90 °C for 30 min. After cooling to room temperature, the MIC of the protein was measured as described above. The test was repeated three times.

## RESULTS

**Purification of the antibacterial protein**

The protein was purified from *Gastrodia elata* Bl. tubers using ammonium sulfate precipitation, ion-exchange chromatography using DEAE-52 columns, and gel-filtration chromatography using Sephadex G-50 columns (Table 1). The protein yield was 9.33%; i.e., the yield from 100 g of fresh *Gastrodia elata* Bl. tubers after multiple steps of purification was 9.33 g of protein. The crude extract was precipitated with ammonium sulfate (40, 50, 60, 70, and 80% saturation). The results indicated that 80% saturation achieved the highest yield of crude extract, i.e., 40.77%. The 80% ammonium sulfate fraction was separated into four fractions using DEAE-cellulose, as indicated in Figure 1A. The D1 fraction contained the active protein, and this was further purified on a Sephadex G-50 gel-filtration column. The D1 fraction was further divided into three fractions (G1, G2, and G3) on a Sephadex G-50 gel-filtration column. Only the G3 fraction exhibited antibacterial activity (Figure 1B). The isolated protein was demonstrated to be a 14 kDa monomer by SDS-PAGE analysis (Figure 2).

**Antimicrobial activity**

The antibacterial activity of the protein (GEP) purified from *Gastrodia elata* Bl. tubers was examined by paper disc diffusion against the three indicator strains shown in Table 3. The crude extract, ion-exchange fractions, and gel-filtration chromatography fractions displayed

clear antibacterial activity against *C. albicans* ATCC 2002, and moderate activity against *S. aureus* ATCC 25923. However, they did not exhibit antibacterial activity against *E. coli* ATCC 25922.

**Table 1:** Purification of the antibacterial protein from 500 g fresh *Gastrodia elata* Bl tubers

| Step | Total protein (mg) | Yield (%) |
|---|---|---|
| Extract | 5200 | 100 |
| Ammonium sulfate | 2120 | 40.77 |
| CM-52 fraction C1 | 1020 | 19.62 |
| Sephadex G-50 fraction G3 | 485 | 9.33 |

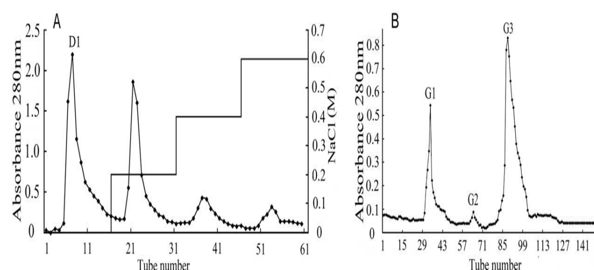


**Figure 1:** Column chromatography purification of antibacterial protein from *Gastrodia elata* Bl. tubers. A. Ion-exchange chromatography on DEAE-cellulose. B. Gel-filtration on Sephadex G-50

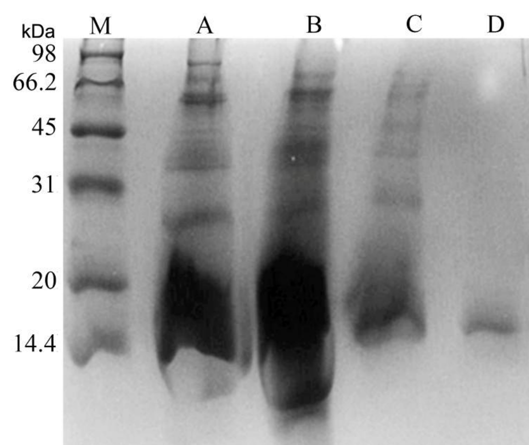


**Figure 2:** SDS-PAGE analysis of antibacterial protein during successive purification steps. M, protein marker. A, crude extract of *Gastrodia elata Bl* tubers. B, dialyzed proteins (precipitated by ammonium sulfate). C, purified proteins after chromatography on DEAE-52 (ion-exchange). D, purified protein after chromatography on Sephadex G-50 (gel-filtration)

## Hemolytic activity

The purified antibacterial protein obtained from *Gastrodia elata* Bl. displayed moderate hemolytic activity. Compared with the control – 1% Triton X-100 – which can induce 100% hemolysis, the antibacterial protein obtained from *Gastrodia elata* Bl. resulted only in 4% hemolysis even at the high concentration of 200 mg/mL. Thus, the GEP protein may show potential as a novel antimicrobial drug that is safe to use in animals.

**Table 3:** Antibacterial activity of antibacterial protein from *Gastrodia elata Bl* tubers against indicator strains

| Test material | Zone of inhibition (mm; mean ± SD; n = 3) | | |
|---|---|---|---|
| | ***E. coli*** | ***S. aureus*** | ***C. albicans*** |
| Water | – | – | – |
| Extract | – | 6.0±0.10 | 7.5±0.31 |
| D1 | – | 7.2±0.27 | 9.0±0.41 |
| G3 | – | 8.0±0.36 | 9.4±0.29 |

- = indicates no detectable zone of inhibition

## Effect of pH, salt, and temperature

To determine the optimal pH range for the antimicrobial activity of the protein of interest, the MIC of the GEP protein against *S. aureus* and *C. albicans* was determined at a range of pH values. Different pH conditions had profound effects on the antibacterial activity of the GEP protein. The MIC was greater in alkaline conditions than in acidic conditions, and the antibacterial activity was determined to be optimal at pH 6 (Figure 3).

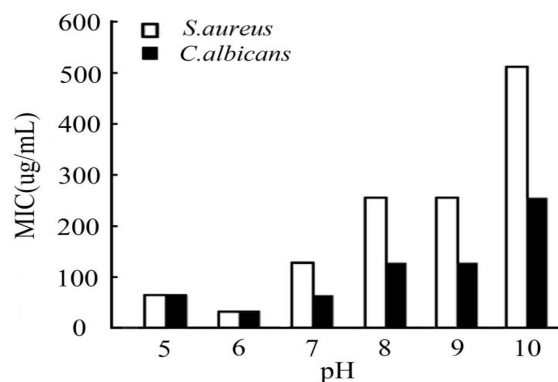


**Figure 3:** Effect of pH on the MIC of the purified protein against *S. aureus* and *C. albicans*

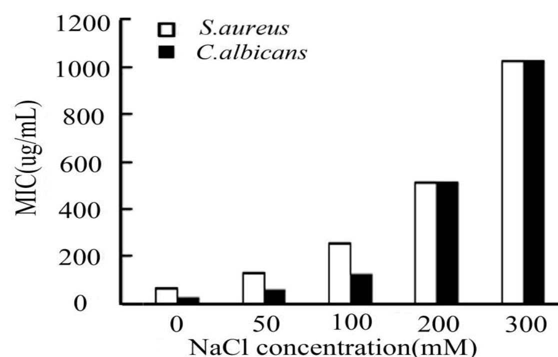


**Figure 4:** Effect of NaCl concentration on the MIC of the purified protein against *S. aureus* and *C. albicans*

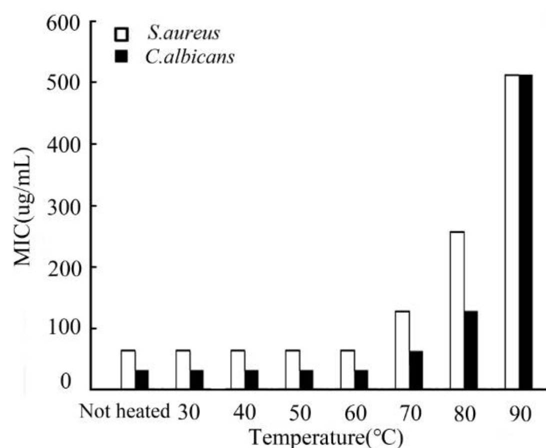


**Figure 5:** Effect of temperature on the MIC of the purified protein against *S. aureus* and *C. albicans*

The salt sensitivity of the protein was established by detecting the MIC of the protein against *S. aureus* and *C. albicans* in the presence of NaCl concentrations from 0 to 300 mmol/L. The MIC of the GEP protein was inhibited at high ionic concentrations of salt (Figure 4).

To investigate the thermal stability of the protein of interest, the antibacterial protein was exposed to different temperatures for 30 min (Figure 5). GEP retained high levels of antibacterial activity after incubation at 30, 40, 50, or 60°C for 30 min as assessed by the MIC assay against *S. aureus* and *C. albicans*. Importantly, the antibacterial protein exhibited a 16-fold increase in MIC against *S. aureus* and an 8-fold increase in MIC against *C. albicans* on heating to 90°C for 30 min, when compared to an unheated control.

## DISCUSSION

A *Gastrodia elata* antibacterial protein was successfully isolated using a procedure that has proven useful for its purification. The methods used here include ammonium sulfate precipitation, ion exchange chromatography, gel filtration chromatography, preparative liquid chromatography, affinity chromatography and combined chromatography techniques, all of which have previously been used for the isolation and purification of antibacterial peptides/proteins from plants [12]. Lam *et al* purified an antifungal protein from mushroom (*Lyophyllum shimeji*) using ammonium acetate extraction, CM–cellulose ion exchange chromatography, Affi-Gel blue gel affinity chromatography, and Mono S high performance liquid chromatography [13]. Boleti *et al* purified a 14-kDa antifungal protein from *Pouteria torta* seeds using Sephacryl S-100 size exclusion chromatography, DEAE-Sepharose ion exchange chromatography, and C18 reverse-phase HPLC [14]. Ruan *et al.* reported purifying an antifungal protein with a molecular mass of 14 kDa from *Fagopyrum tataricum* seeds using ammonium sulfate precipitation, affinity chromatography, and centrifugal ultrafiltration [15].

The antibacterial protein isolated from *Gastrodia elata* Blume tubers in the present study has a molecular weight of 14 kDa, similar to that of antibacterial proteins previously isolated from other plant-derived materials. An antifungal protein isolated from the mushroom *Lyophyllum shimeji* exhibited a molecular mass of 14 kDa [13]. Antifungal proteins from *Pouteria torta* seed and *Fagopyrum tataricum* seed each have a molecular mass of 14 kDa [14,15]. Pr-2, an antifungal protein from pumpkin rind has a molecular mass of 14.8 kDa [16].

Many plant-derived proteins have been shown to display antibacterial activity against phytopathogens and against organisms that are pathogenic to animals and humans, including bacteria, viruses, fungi, parasites, and neoplastic cells [17]. In the present study, the GEP displayed significant antibacterial activity against fungi, and moderate activity against gram-positive bacteria. However, they did not exhibit antibacterial activity against gram-negative bacteria. Interestingly, the results are consistent with those reported for an antifungal protein purified from the *Ginkgo biloba* seed.

Some studies have confirmed that antibacterial proteins from plant seeds, fungi, and bacteria exhibit significant hemolytic activity, and are therefore harmful to erythrocytes [18]. However, GEP had little hemolytic activity on rabbit red blood cells even at protein concentrations up to 200 mg/ml.

Our research showed that the GEP protein retains significant antibacterial activity at pH values up to 10.0, and displays good thermal stability up to 60°C. However, Sakthivel *et al* [12] purified an antibacterial protein from *Bauhinia purpurea* L., which had no antibacterial activity at pH values below 3.0 and above 9.0. Zheng *et al.* [2] found that an antibacterial protein from *Clitocybe sinopica* had no activity at temperatures above 80°C.

Some antibacterial proteins lose their antibacterial activity at high salt concentrations. This is in accordance with previous studies, which found that the antibacterial activity of such proteins is greatly influenced by salt concentration, and no antibacterial activity was observed under high salt concentrations.

## CONCLUSION

A novel antibacterial 14-kDa GEP protein has successfully been purified from *Gastrodia elata* Bl tubers. GEP exhibits antimicrobial activity against *S. aureus* and *C. albicans*, but has no antibacterial activity against *E. coli*. The protein is thermally stable and displays potent antibacterial activity under acidic conditions. Thus, the GEP protein derived from *Gastrodia elata* Bl. tubers is a potential candidate for further drug development.

## DECLARATIONS

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### *Conflict of Interest*

No conflict of interest associated with this work.

### *Contribution of Authors*

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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