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Brief Original Article

Anti-proteolytic activity of *Ganoderma lucidum* methanol extract against *Pseudomonas aeruginosa*

Nessma Ahmed El Zawawy[1], Sameh Samir Ali[1,2]

[1] Botany Department, Faculty of Science, Tanta University, Tanta, Egypt
[2] Biofuels Institute, School of the Environment and Safety Engineering at Jiangsu University, Zhenjiang, China

Abstract

Introduction: Protease enzyme is considered one of the most serious virulence factors produced by extended-spectrum β-lactamase-producing and multidrug-resistant *Pseudomonas aeruginosa* (ESβLMDRPA) clinical isolates.
Methodology: The antibacterial activity of methanol extract of *Ganoderma lucidum* fruiting bodies was tested against a protease-producing ESβLMDRPA clinical isolate, showing its mode of action.
Results: The extract showed high antibacterial activity. Its effect on purified protease indicated a reversible non-competitive protease inhibition (k_{is}= 0.45 mg/mL).
Conclusions: The *G. lucidum* extract could be a promising anti-proteolytic active against ESβLMDRPA. It may form a primary platform for further phytochemical studies and development of new drugs for therapy of skin burn infections.

Key words: *Ganoderma lucidum*; *Pseudomonas aeruginosa*; multidrug resistance; virulence factor; protease.

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Introduction

Recently, multidrug-resistant (MDR) Gram-negative bacteria have become more prevalent and are causing great problems in the treatment of infections [1]. Of clinical significance, *Pseudomonas aeruginosa* has the ability to secrete several virulence factors, which help the bacteria to adhere and invade to their host by damaging the host's immune responses and forming a barrier to antibiotics [2]. Proteases are one of the most crucial virulence factors produced by *P. aeruginosa*. These proteases work together, leading to significant damage to host tissues.

Ganoderma lucidum is an oriental fungus; it has a long history of use for promoting health and longevity in China and other Asian countries. It is a popular medicinal mushroom that has many biologically active components such as phenolics, flavonoids and polysaccharides, giving its activity as antimicrobial, antioxidant, antiviral, cardiovascular and anticancer [3,4].

Not much literature is available about the antibacterial activity of the fruiting body of *G. lucidum* for the treatment of ESβLMDRPA which produces protease as a virulence weapon. Therefore, the present study aimed to evaluate the efficiency of *G. lucidum* extract as a novel alternative and a promising method for virulence attenuation of protease-producing ESβLMDRPA.

Methodology

Detection of protease activity

An ESβLMDRPA clinical isolate of a burn infection, previously recovered and identified by Khalil *et al*. [5], was tested for its ability to produce protease enzyme as a virulence factor. Vermelho *et al*. [6] described the protease activity method.

Production of protease from ESβLMDRPA

Production of protease from ESβLMDRPA was carried out in a proteolytic liquid medium (Sigma Chemicals, St. Louis, USA). The supernatant was extracted for crude enzyme preparation [7].

Protease assay

Proteolytic activity was determined using 0.6 % Hammerstein casein solution (Merck, Darmstadt, Germany) as a substrate (50 mMTris–HCl, pH 7). For activation, 1 mL of the extracted enzyme was mixed with 5 mL of the prepared substrate and incubated at 30 °C for 30 minutes. The reaction was stopped by the

addition of 0.5 mL 20 % TCA (Pharmacia, Gothenbu, Sweden) and kept for 10 minutes at 25 °C. After 15 minutes of cooling centrifugation (6.000 rpm), the absorbance was measured at 280 nm. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg tyrosine per milliliter per minute. The experiments were carried out in triplicate and the mean value was expressed as a unit of protease activity [8].

Purification of enzyme

Ammonium sulphate precipitation

ESβLMDRPA cells were separated by centrifugation (6.000 rpm for 10 min). The supernatant was fractionated by precipitation using ammonium sulfate (50 to 70 % saturation). All precipitates were collected and dialyzed against 50 mM Tris-HCl buffer (pH 7) for 24 h using a dialysis bag. The highest ammonium sulphate fraction was applied to gel filtration chromatography [9].

Gel filtration chromatography on DEAE-Sephadex A-50

The dialyzed highest ammonium sulphate fraction was applied on a DEAE-Sephadex A-50 column (GE Health care Biosciences, Uppsala, Sweden) with dimensions 1.5 cm diameter and 20 cm length. The column was pre-equilibrated with 50 mM Tris-HCl (pH 8). The sample was eluted with the same buffer. Ten fractions were collected at a flow rate of 1 mL/minute constant intervals. The most active fractions (6–8) were dialyzed against 50 mM Tris-HCl (pH 7) at 4 °C.

Protein assay and electrophoretic analysis

The protein concentrations in different stages of enzyme purification were estimated according to Bradford [10].

Preparation of fungal extract

The fruiting bodies of *G. lucidum* were collected from around the Delta region of Gharbia governorate. The identification and extraction were done as described by Kamra and Bhatt [11].

Effect of G. lucidum extract on ESβLMDRPA

Antibacterial activity of *G. lucidum* extract was measured by using the modified agar well diffusion method [12]. One hundred microliters of inoculum (10^7 colony-forming units/mL) using standard turbidity (corresponding to 0.5 McFarland tube) was applied on Muller-Hinton agar plate (Sigma Chemicals, St. Louis, USA). For antibacterial screening, *G. lucidum* extract was dissolved in dimethyl sulfoxide to a final concentration of 0.0, 0.5, 1.0, 1.5, 2.0, 2.5 mg/mL. Regular wells were made in the inoculated agar plates by a sterile cork borer with 0.8 cm diameter. Each well was aseptically filled up with 0.1 mL volume of extract. The plates were incubated at 37 °C for 24 h. The tests were performed in triplicate.

Mode of inhibition of G. lucidum extract on protease enzyme

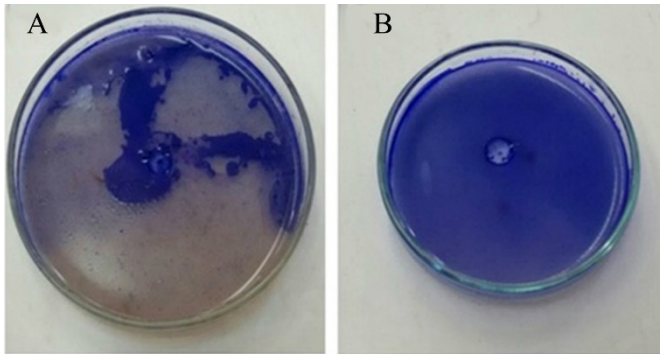
The evaluation of *G. lucidum* extract as a protease inhibitor against ESβLMDRPA was conducted in liquid protease-dependent medium (Sigma Chemicals, St. Louis, USA), as described by Ferrasson *et al.* [7]. Briefly, to each test tube containing 5 mL fractions of medium, about 50 µL of different concentrations (0.5, 1.0, 1.5, 2.0, 2.5 mg/mL) of *G. lucidum* extract were separately added. Then, the tubes were inoculated with 50 µL of ESβLMDRPA (10^7 colony-forming units/mL) and incubated at 37 °C for three days (the optimum time for protease production). The control tube was free from *G. lucidum* extract. Quantitative assay of protease activity was measured as described by Khembhavi *et al*. [8].

The mode of inhibitory action of *G. lucidum* extract against ESβLMDRPA was tested by using the purified protease dissolved in 50 mM Tris-HCl buffer (pH 7). Quantitative assay of protease activity was determined by mixing different concentrations of *G. lucidum* extract (0.5, 1.0, 1.5, 2.0, 2.5 mg/mL) and casein substrate (5, 10, 15, 20, 25%) [8]. Results were represented graphically by a reciprocal plot between enzyme activity and substrate concentration for each *G. lucidum* extract concentration [13].

Statistical analysis

In analyzing the data of ESβLMDRPA protease enzyme production and the effect of *G. lucidum* extract on enzyme activity through analysis of variance

Figure 1. Protease detection.



(A) Hydrolysis of BSA. (B) Control.

(ANOVA), one-way tests by SPSS version 17 were used to evaluate the variation in concentrations of *G. lucidum* extract on bacterial growth and its enzyme activity.

Results

The ESβLMDRPA protease enzyme was found to possess high proteolytic activity, which appeared as clear areas (Figure 1A), indicating the hydrolysis of the bovine serum albumin (BSA) compared to the control (blue color of BSA) (Figure 1B). The highest significant rate of protease production (27 units/mL) was quantitatively reported in the culture filtrate of ESβLMDRPA on the third day of growth ($p < 0.001$). In protease purification, the highest active fraction from 70 % ammonium sulphate of culture broth was adsorbed on the DEAE-Sephadex A-50 matrix (GE Health care Biosciences, Uppsala, Sweden). The bound protease was eluted with 10 mM Tris–HCl buffer (pH 7). Figure 2 shows that fractions 6–8 were the most active fractions of ESβLMDRPA protease activity, which confirmed by the appearance of a single protein band at molecular weight 40 kDa during the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Figure 3). After purification, protease enzyme indicated specific activity of 84.6 units/mg from 9 units/mg with a purification fold of nearly 9.4 times that of the crude enzyme (Table 1).

A trial was conducted to find a correlation between protease activity and high inhibition effect of *G. lucidum* extract against ESβLMDRPA as an important offensive force for the bacteria to establish the infection. The minimum inhibitory concentration (MIC) of *G. lucidum* extract against ESβLMDRPA was recorded as 2 mg/mL. The highest concentration of *G. lucidum* extract was accompanied by the lowest activity in protease, which recorded as decreasing from 27 to 3

Table 1. Purification profile of ESβLMDRPA protease.

Purification parameter	Crude culture filtrate	70% ASP	DEAE-Sephadex A-50 fraction of highest activity
Total enzyme activity (U)	5400	1950	275
Total protein (mg)	600	50	3.25
Specific enzyme activity (U/mg)	9	39	84.6
Purification fold	1	4.3	9.4
Yield (%)	100	36	5

Figure 2. Elution profile of multidrug-resistant and extended-spectrum β-lactamase-producing *Pseudomonas aeruginosa* protease on DEAE-Sephadex A-50.

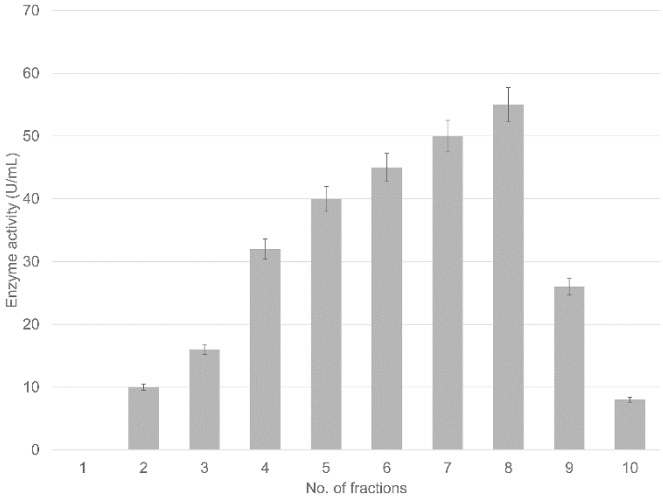
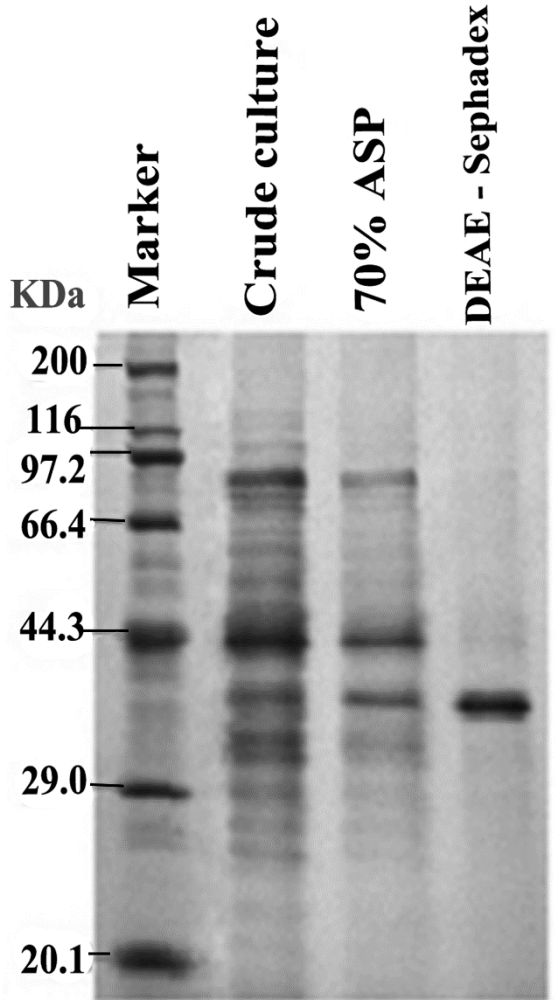


Figure 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for different stages of protease purification. ASP: ammonium sulphate precipitation



units/mL at MIC concentration 2 mg/mL (Table 2). In addition, statistical analysis revealed that the variation in the effect of different concentrations of *G. lucidum* on protease activity was highly significant ($p < 0.001$). The highest antiprotease activity of *G. lucidum* extract could explain its ability to manage infection. As shown in Figure 4A, *G. lucidum* extract was a reversible, non-competitive protease inhibitor. Its activity was decreased by increasing substrate concentrations, as confirmed by K_m (Michaelis constant) which achieved at 9.7 units/mL. This high K_m shows the low affinity between enzyme and substrate. Plotting the slope of the double reciprocal plot of *G. lucidum* extract inverse against the inverse of protease initial activity indicated an inhibition constant (k_{is}) of 0.45 mg/mL due to the effect of *G. lucidum* extract on an enzyme reaction slope (Figure 4B).

Table 2. Inhibitory effect of *G. lucidum* on ESβLMDRPA.

Concentration	Diameter of inhibition zone (mm)	Protease activity (U/mL)
0.0	0.00±0.00[a]	27.00±2.00[a]
0.5	2.00±0.25[b]	21.00±1.00[b]
1.0	2.80±0.29[c]	18.00±2.00[c]
1.5	4.60±0.32[d]	15.00±1.00[d]
2.0	7.30±0.47[e]	3.00±1.00[e]
2.5	7.80±0.25[e]	0.00±0.00[f]
Average	4.08	84
F-value	0.012	0.002
P-value	0.983	0.999

Each value is the mean of three replicates ± SD; Values with the same

Discussion

In the present work, the major protease yield of *P. aeruginosa* with ammonium sulphate is supported by the results of Kumar *et al*. [14] for *Bacillus thuringensis* with ammonium sulphate (65% saturation).

SDS-PAGE analytical studies of protease revealed that it possesses a single protein band with molecular weight of 40 kDa. This agrees with *P. aeruginosa* as described by Gupta *et al*. [15].

In a trial to explain the ability of *G. lucidum* extract to manage infection, it was observed that *G. lucidum* extract has a strong antiprotease activity. Our study determined that the kinetic properties of *G. lucidum* extract act as reversible non-competitive inhibitor for protease enzyme, in accordance with Arulpandi and Sangeetha [16], who reported reversible non-competitive inhibition of protease by using *Cassia fistula* leaf extract.

Conclusions

This novel study of *G. lucidum* as a protease inhibitor suggests that *G. lucidum* extract is a promising new active source against ESβLMDRPA, which could be a major causative agent of burn infections.

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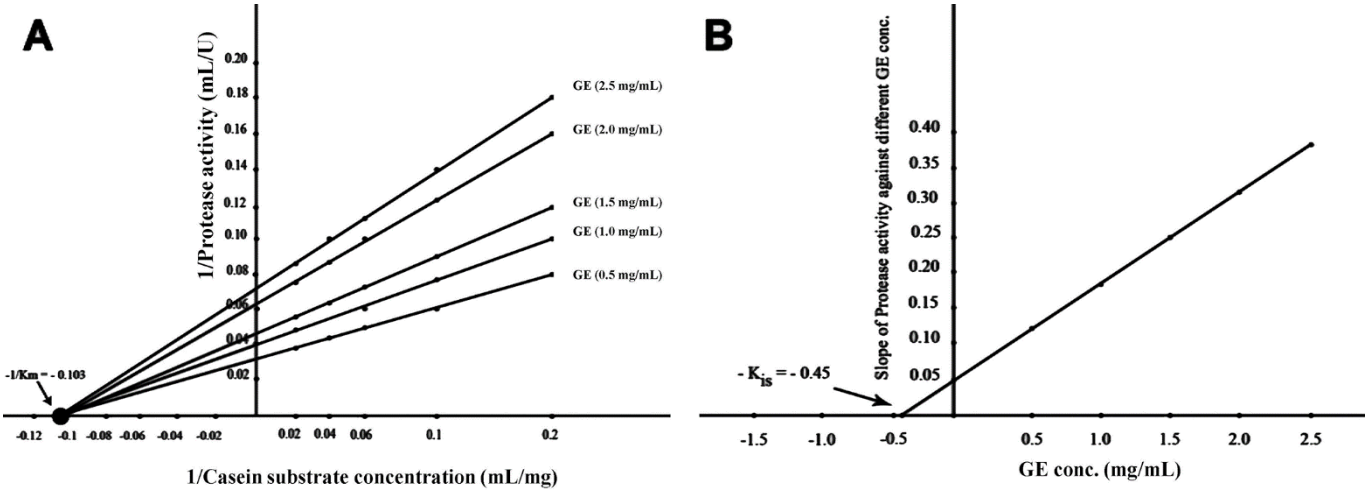
Figure 4. Inhibition mechanism of *G. lucidum* extract (GE) against protease activity of multidrug-resistant and extended-spectrum β-lactamase-producing *Pseudomonas aeruginosa*.



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Corresponding author

Nessma Ahmed El Zawawy
Botany Department, Faculty of Science
Tanta University
31527 Tanta, Egypt
Phone: (+2) 01289102444
Fax: (+2)0403350804
Email: nesma.elzawawi@science.tanta.edu.eg

Conflict of interests: No conflict of interests is declared.