

Evaluation of thermal stability of an antifungal protein from *Bacillus subtilis* isolated in Vietnam

Đánh giá tính bền nhiệt của protein có hoạt tính kháng nấm từ chủng Bacillus subtilis phân lập ở Việt Nam

Research article

Do Thi Tuyen^{1,2*}, Le Thanh Hoang¹, Nguyen Thi Thao¹, Nguyen Thi Trung², Nguyen Sy Le Thanh¹, Vũ Thị Bích Ngọc¹

¹Institute of Biotechnology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Cau Giay Distr., 10600 Ha Noi, Vietnam; ²Graduate University of Science and Technology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Cau Giay Distr., 10600 Ha Noi, Vietnam

Antifungal proteins were isolated from the crude bacterial supernatant using ammonium sulfate salt precipitation followed by passage over DEAE –cellulose and Biogel P100 columns. The purified protein had an apparent molecular mass of 14 kDa. Its antifungal activity was retained even at 100°C, for 60 min. The results of protein identification using MALDI –TOF/TOF mass spectrometer suggested that the purified protein is indeed a chitin binding protein that has 206 acid amine containing chitin –bind –3 region with a relative molecular mass of 22230 Da.

Protein có hoạt tính kháng nấm được tinh sạch từ dịch ngoại bào chủng vi khuẩn Bacillus subtilis sau khi qua ba bước tinh sạch: tủa muối ammonium sulphate 30-70%, qua cột sắc ký trao đổi ion DEAE – cellulose và cột sắc ký lọc gel Biogel P100. Protein tinh sạch có khối lượng phân tử đạt 22 kDa trên điện di SDS-PAGE. Hoạt tính kháng nấm của protein tinh sạch vẫn còn duy trì khi ủ ở 100°C trong 60 phút. Kết quả nhận dạng bằng khối phổ MALDI –TOF/TOF đã chỉ ra rằng protein bền nhiệt này là chitin binding protein được mã hóa bởi 206 acid amin cùng với khối lượng phân tử là 22230 Da. trị an toàn đối với Al và Atrazie trong môi trường nước tự nhiên về khía cạnh bảo vệ sức khỏe sinh thái.

Keywords: Antifungal protein, *Bacillus subtilis*, Chitin binding protein, Purification

1. Introduction

In the 20th century the number of pesticides, based on biocidal molecules, are the main tool to increase production and quality of food and fiber products. Due to health issues and the environment, the use of many chemicals has caused controversy and needs to be replaced. In addition to self-resistance of cereal crops, mainly other biocontrol measures are based on whole body to suppress pests. Some other approaches to biologically active substances to be tested and some commercial preparations increased. However, it still requires a lot of bio-products using high biotechnology to replace chemicals drug. Currently biocontrol preparations can not be compared with the chemical because of effectiveness, market and other factors, but they still have

a promising future, especially the use of the genetically modified strains [1].

To produce biological preparations against plant pathogenic fungi one can conduct the following researches: (1) Screening of antagonistic microorganisms. (2) Studying the effect of culture supernatants of antagonistic microorganisms to plant pathogenic fungi, plants, animals and humans. (3) Optimization of production of antagonistic microorganism. (4) Production of antagonistic preparations used as antifungal reagents or fertilizer additive. (5) Study on chemical structures and effects of secondary compounds in cell culture fragments and antagonistic microorganisms against pathogens. (6) Improving the production of secondary compounds by recombinant DNA technology. (7) Study on the

mechanism of action of pure secondary compounds against pathogens.

In the world, research topics are all from (1) to (7), but in Vietnam due to lower development we only focus on the simple topics including (1) to (4). Because of a large number (165 thousands) of publications in this topic with the keyword antagonist in Medline, so I just concentrated overview of topic (5) in the world that we will do and topics (1) to (4) in Vietnam.

Bacillus is a Gram-positive, rod-shaped bacterium commonly found in soil. It has a high thermal tolerance, grows rapidly in liquid medium, and produces many spores. The bacterium can also produce antibiotics that inhibit or kill other microorganisms and have a positive or negative impact on bacterial and fungal diseases. Recently, a number of *B. subtilis* strains were used to control plant diseases [10, 17]. In addition, The *Rhizoctonia solani* and *Fusarium oxysporum* are two widespread phytopathogenic fungi. Both of them can survive for a long time in soil and infect many agricultural and industrial plants including leguminous crops, solanaceous crops, cucurbits, cotton plants, etc. These pathogenic fungi caused black root rot symptoms, ulcer roots, stem rot, rotting leaves. Indeed, they can infect seedlings and may remain on the plants until the first harvest [2, 3].

Investigation of the thermal stability of antifungal compounds from the *Bacillus* will help to the bio-products restricted oxidation process under the effect of the conditions such as light, high temperature, pH ... Here, we purified and assessed of antifungal protein from *Bacillus subtilis* isolated in Vietnam. The purified antifungal protein was also identified and investigated for its thermal stability to help bio-products.

2. Materials and methods

2.1 Microorganisms

The *F. oxysporum* and *R. solani* strains were provided by the Plant Diseases Division at the Institute of Plant Protection, Tu Liem, Ha Noi.

Bacillus subtilis was supplied by the Department of Enzyme Biotechnology at the Institute of Biotechnology, Vietnam Academy of Science and Technology. *Bacillus subtilis* was grown in NYD medium (pH 7.5), which comprised glucose (10 g/L), beef extract (8 g/L), and yeast extract (5 g/L).

2.2 Chemicals

Sodium chloride and D-glucose were purchased from Merck (Darmstadt, Germany). Minisart membranes and agar were purchased from Biotech (Vietnam). DEAE – cellulose, Biogel P100, Trisbase and Bovine serum albumin (BSA) were supplied by Sigma–Aldrich (St. Louis, MO, USA). NaHPO₄, Na₂HPO₄, meat extract, and yeast extract were purchased from BioBasic Inc. (Ontario, Canada).

2.3 Methods

2.3.1. Antifungal protein purification

The bacterial culture was centrifuged at 12500 rpm for 10 min. Ammonium sulfate (30–70% saturation) was then slowly added to 200 ml of crude protein solution with constant stirring. The mixture was left overnight and then centrifuged at 12500 rpm for 10 min. The supernatant was then carefully decanted, and the precipitate was re-dissolved in 0.02 M phosphate buffer (pH 6.8) and dialyzed to remove the salt. The dialyzed fraction (6 ml) was then applied to a DEAE –cellulose column (2.6 × 6 cm) pre-equilibrated with 0.02 M phosphate buffer (pH 6.8) at a flow rate of 25 ml/hr until the OD 280 nm was <0.01. The column was then eluted with 0.02 M phosphate buffer (pH 6.8) containing 1 M NaCl. The eluted fractions of 1.5 ml were collected (27 fractions). The antifungal activity of each purified fraction was tested against *F. oxysporum* and *R. solani*. Fractions with strong antifungal activity were continuously purified on a Biogel P100 column. The column was then eluted with 0.02 M phosphate buffer (pH 6.8) at a flow rate of 25 ml/hr and 1.5 ml fractions were collected. The antifungal activity of the purified fractions was once again tested against the two above pathogenic fungi. All purification steps were carried out at 4°C unless specified otherwise.

2.3.2 Antifungal activity of the purified protein determination

To test antifungal for purification and characterization of an antifungal protein, ampicillin (0.1%) was added to petri plates (90 × 15 mm) containing 10 ml of PDA (a mycelial plug from 4-day-old cultures of *F. oxysporum* or *R. solani* was placed in the center of the PDA plates), and filter papers (0.5 cm in diameter) soaked with 15 µl of purified protein were placed on the surface. The agar plates were then incubated at 30°C for 3 –5 days (Incubator, Sanyo, Japan). As a control, filter papers were soaked in 0.02 M phosphate buffer (pH 6.8).

2.3.3 SDS-PAGE and protein concentration

SDS-PAGE was carried out as described by Laemmli (1970) [4] with Bio-Rad equipment. SDS-PAGE was usually performed on gels containing 12.5% (w/v) acrylamide according to the manufacture's recommendation. The gels were stained with Coomassie Brilliant Blue R-250 for protein. Protein was estimated by the method of Bradford with the bovine serum albumin as standard [5].

2.3.4 Thermal stability

The thermal stability of the chitin binding protein was determined by incubating purified protein (20 µl) at 80°C for 5 –60 min or at 100°C for 60 min. Antifungal activity was then determined as described above.

2.3.5 Protein identification

The purified antifungal protein was identified by MALDI –TOF MS. The sample was trypsin digested and peptides

extracted according to standard techniques. Peptides were analysed by MALDI –TOF/TOF mass spectrometer using a 5800 Proteomics Analyzer [AB Sciex]. Spectra were analysed to identify protein of interest using Mascot sequence matching software [Matrix Science] with MSPnr100 Database. Peptide fragments showing ion scores > 59 were identified uniquely or high –similarly (p < 0.01).

2.3.6 Statistical analysis

All measurements were carried out in triplicate. The means were presented for averages of experiments.

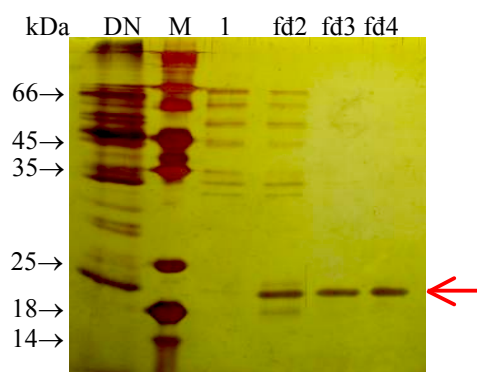
3. Results

3.1. Purification and identification of antifungal protein

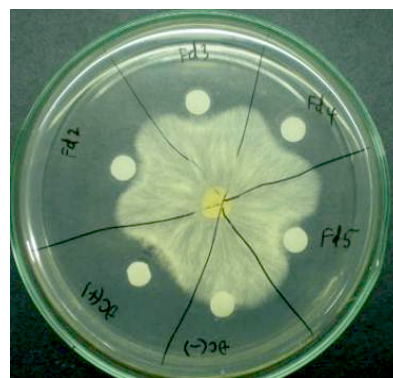
After 120 hr of cultivation, the crude supernatant of *B. subtilis* was purified by addition of 30– 70% ammonium sulfate, followed by passage over a DEAE– cellulose column. Three range of fractions containing high protein concentration were observed (corresponding to peaks 1, 2, and 3). These fractions were tested for antifungal activity against *F. oxysporum* and all were positive (data not shown). Fractions 5–11 from peak 1 were then mixed and passed over a Biogel P100 column. The purity and relative molecular mass of the proteins were assessed by 12.5% polyacrylamide gel electrophoresis with Fermentas protein standards, followed by silver staining. A protein

of ~22 kDa was observed (Fig. 1A) with the yield of the purification process was 11.9% (data not show).

In order to find out the element responsible for the antifungal activity of *B. subtilis*, the supernatant was precipitated by addition of 30– 70% ammonium sulfate, and then was continuously purified using ion exchange chromatography on DEAE-cellulose and Biogel P100 chromatography. These methods also used for the purification some protein from bacteria and fungi by other studies [6, 7]. Indeed, ion exchange chromatography on DEAE-cellulose, followed by Biogel P100 chromatography, has been used to extract other proteins with antifungal activity [8, 9]. In our study, the purified protein with molecular weight of 22 kDa has activity against both of *F. oxysporum* and *R. solani* (Fig. 1A and 1B). The molecular weight of this antifungal protein was different from that of other antifungal proteins purified from *B. subtilis*. Liu et al. (2007) extracted bacisubin from *B. subtilis* B916, which has a molecular weight of 41.9 kDa and exhibits antifungal activity against *F. oxysporum* and *R. solani* [9]. Li et al. (2009) extracted protein B29I from *B. subtilis* strain B29, which has a molecular weight of 42.9 kDa and shows antifungal activity [10]. Tan et al. (2013) purified antifungal protein with molecular weight of 38 kDa from *B. subtilis* B25[11]. The difference in antifungal peptides produced by different strains of *B. subtilis* may be due to the fact that “biosynthesis of antibiotics from microorganisms is often regulated by nutritional and environmental factors” [12].



A



B

Figure 1. (A) Protein profile on SDS -PAGE of the crude supernatant (Lane 1), fractions from peak 1 after passage through the Biogel P100 column (Lane 2), M: molecular mass of standard proteins (Fermentas, Thermo Fisher Scientific Inc., Waltham, USA); (B) Anti – *F. oxysporum* activity of the purified protein (DC(-): 20 mM sodium phosphate buffer, pH 6.8; DC(+): the crude supernatant; Fd 2-Fd 4: the fraction purified protein from fraction 2- 4.

3.2 Protein identification

The purified protein was then identified by MALDI-TOF mass spectrometry. Peptide fragments showing ion scores above 59 identified uniquely or high –similarly with p < 0.01. These peptides were 100% identical to corresponding fragments of the chitin binding protein (Table 1).

Chitin binding protein from *Bacillus* (Accession number: WP_003154023) has 206 acid amine containing chitin –bind –3 region such as "Chitin binding domain;

pfam03067" from acid amine position 28 to 202 with a relative molecular mass of 22230 Da. Using MALDI-TOF mass spectrometry to identify the purified protein, we found that it is a chitin-binding protein. This result was matched with other reports that chitin-binding proteins have play an important role in the protection of plants against pathogen infection [13, 14]. The mechanisms against fungal attack of chitin binding proteins was supposed to take part in degrading chitin and/or adhering to the chitinous surfaces of host cells [15, 16].

Table 1. Peptides exceeding the identity threshold

Query	Observed	Peptide mass	Score	Peptide sequence
151	450.2341	898.4508	62	ADTNLTHK
223	521.2684	1040.5040	67	GFPAAGPPDGR
312	587.8201	1173.6142	64	QTLGWATAAAQK
491	795.4013	1588.7733	97	YGSVIDNPQSVEGPK
492	795.4013	1588.7733	99	YGSVIDNPQSVEGPK
522	825.4164	1648.8057	146	IASANGGSGQIDFGLDK
527	830.9003	1659.7740	106	DEFELIGTVNHDGSK
669	881.1017	2640.2820	141	IASANGGSGQIDFGLDKQTADYWVK

3.3 Effect of temperature and thermostability

The effect of temperature on the chitin binding protein was studied at 80°C for 5 –60 min and at 100°C for 60

min. The results show that the chitin binding protein retained activity when heated to 80°C and even to 100°C for 60 min (Fig. 2A and 2B).

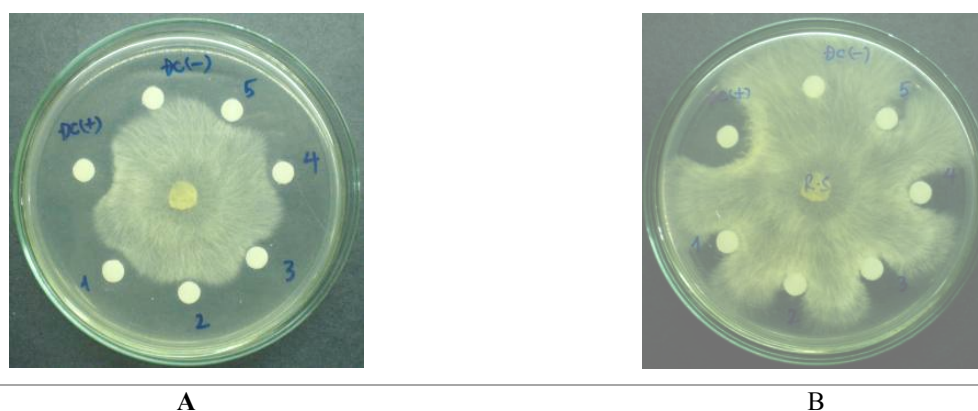


Figure 2. Effects of temperature on the antifungal activity of the chitin binding protein against *F. oxysporum* (A) and *R. solani* (B), (DC(-): control (20 µl of 20 mM sodium phosphate buffer, pH 6.8); DC(+): the crude supernatant; 1 –4: Samples treated at 80°C for 5–60 min; 5: sample treated at 100°C for 60 min)

The chitin binding protein is also quite thermostable as it retained activity when heated to 80°C and even to 100°C for 60 min. This result was coincident with some previous studies. Zang et al. (2008) purified a novel protein-BTL- from *Bacillus* strain B-TL2 from tobacco stems was also thermostable, retaining almost 100% activity when heated to 100°C for 15 min [17]. Chitarra et al. (2003) reported that an antifungal compound produced by *B. subtilis* YM 10-20 was heat stable. The protein inhibited the growth of *P. roqueforti* after heating to 70°C or 100°C for 1 hr [18]. The above mentioned result of MALDI –TOF indicated that the purified protein TOF was chitin binding protein may give us logical explanation for this thermal stability.

4. Conclusions

We successfully purified chitin binding protein from *Bacillus subtilis*. The chitin binding protein is also very thermostable as it retained activity when heated to 80°C and even to 100°C for 60 min.

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