

Study of Biological Activity of *Trichoderma harzianum* Isolate T9

การศึกษาคุณสมบัติทางชีวภาพของ *Trichoderma harzianum* Isolate T9

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ABSTRACT

Trichoderma harzianum is a fungus that are important in the trade as a biocontrol. However, the identified species and the structure of the active substance which played a role in biocontrol is not clear. So, in this study the antimicrobial activity and biochemical properties of crude cell extract and culture supernatant from *Trichoderma harzianum* strain T9 were analyzed. The results showed that both of crude cell extract and culture supernatant were able to inhibit growth of 10 microorganisms. Furthermore, SDS-PAGE was used to study the protein expression from both samples. The result showed many protein bands from both sources were detected. However, the SDS-PAGE displayed high protein concentration from culture supernatant. Both culture supernatant and crude cell extract showed heat-stable antimicrobial activity after sterilization at 100 °C for 15 minutes and 121 °C for 15 min, respectively. Both of them are also resist to pronase digestion. These results indicate that the antibacterial substance from *Trichoderma harzianum* isolate T9 might not be a protein in nature.

บทคัดย่อ

Trichoderma harzianum เป็นเชื้อราที่มีความสำคัญทางการค้าซึ่งใช้เป็น biocontrol แต่อย่างไรก็ตามยังไม่มีการระบุชัดเจนถึงชนิดและโครงสร้างของ active ingredient ที่มีบทบาทสำคัญในกระบวนการควบคุมที่ชัดเจน ในงานวิจัยนี้จึงได้มีการศึกษาคุณสมบัติทางชีวภาพและชีวเคมีเบื้องต้นของสารออกฤทธิ์ทำลายเชื้อแบคทีเรียจาก *T. harzianum* isolate T9 จากผลการศึกษาพบว่าเมื่อนำสารสกัดจากทั้งสองส่วนไปทดสอบความสามารถในการยับยั้งเชื้อจุลินทรีย์พบว่าสามารถยับยั้งเชื้อทดสอบทั้ง 10 ชนิดได้ โดย culture supernatant สามารถยับยั้งการเจริญของเชื้อได้ดีกว่าสารสกัดจาก pellet cell และผลการศึกษาการแสดงออกของโปรตีนโดย SDS-PAGE พบแถบโปรตีนจำนวนมากทั้งในส่วน crude cell extract และ culture supernatant โดยพบว่าในส่วนของ culture supernatant จะมีปริมาณความเข้มข้นของโปรตีนสูงกว่า crude cell extract ในการทดสอบ thermal stability พบว่าสารสกัดที่ได้ยังคงสามารถยับยั้งการเจริญของเชื้อจุลินทรีย์ที่ใช้ทดสอบได้ โดย culture supernatant สามารถทนความร้อนได้ที่ 100 °C เป็นเวลา 15 นาทีและสารสกัดจาก crude cell extract

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สามารถทนความร้อนได้ที่ 121 °C เป็นเวลา 15 นาที การทดสอบ pronase stability พบว่าสารสกัดจากทั้งสองส่วนยังคงสามารถยับยั้งการเจริญของเชื้อจุลินทรีย์ทดสอบได้ จากผลการศึกษาสามารถระบุเบื้องต้นว่า antibacterial substance จาก *Trichoderma harzianum* isolate T9 อาจจะไม่ใช้โปรตีนในธรรมชาติ

Key Words : Antimicrobial substances, Biological activity, Biochemical properties, *Trichoderma harzianum*
คำสำคัญ : สารออกฤทธิ์ยับยั้งจุลินทรีย์ คุณสมบัติทางชีวภาพ คุณสมบัติทางชีวเคมี

Introduction

Trichoderma harzianum are free-living fungi which highly interactive in root, soil and foliar environments and commonly used for biocontrol of soil borne plant pathogens. The mechanisms of biocontrol are composed of 3 main mechanisms, the first mechanism is production of cell-wall degrading enzyme such as chitinase, cellulose protease, glucanase (Harman *et al.* 2004). CWDEs attack cell wall of phytopathogenic fungi to cause cell lysis and subsequent death. The second mechanism is competition for nutrients or space. The last mechanism of mycoparasitism is not completely understood (Chet, 1987). *T. harzianum* can inhibit or kill the target microorganism through production of antimicrobial, antibiotic and cell-wall degrading enzyme. However, the structure and function fully understand. So, in this report, we focus on the biological activity and biochemical properties of crude cell extracted and culture supernatant from *T. harzianum* strain T9.

Materials and Methods

Strain and culture condition

Trichoderma harzianum isolate T9 was isolated from Faculty of Agriculture, Khon Kaen University. It was maintained on potato dextrose agar at 30°C for 7 days and incubated in PDB media at 25 °C. It was shaken in incubator shaker at 125 rpm for 15 days. The cell culture and culture

supernatant were withdrawn at 3, 6, 9, 12 and 15 days after incubation.

Antimicrobial activity assay

Cell culture and culture supernatant were filtrated by Whatman® No. 1. Cell cultures were extracted following Vizcaino *et al.* (2005) with minor modification. The 100% methanol was used to extract by shaking for 15 min and then centrifuged at 1500 g for 15 min. Supernatants were collected and concentrated by rotary evaporation. Both cell extract and culture supernatant were screened for their antimicrobial activity by disc diffusion assay. The target microorganism for tested consisted of Gram positive bacteria such as *Bacillus subtilis* TISTR008, *Bacillus subtilis* TISTR6633, *Bacillus amyloliquefaciens* TISTR1045, *Bacillus cereus* ATCC11778, *Bacillus licheniformis* TISTR1010, *Staphylococcus aureus* ATCC25923, *Staphylococcus aureus* ATCC1466 and Gram negative bacteria such as *Escherichia coli* 0157:H7, *Vibrio cholerae* (clinical) and *Salmonella typhi* ATCC5784. Inhibition zone in the paper disc was measured after 6–8 h of incubation. A PDB media and methanol was used as a negative control. For positive control broad-spectrum antibiotic streptomycin disc were used. Protein content was determined by Bradford's method (Bradford, 1976) and analyzes the protein expression by 13.5% SDS-PAGE (Vizcaino *et al.*, 2005).

Biochemical properties assay

For thermal stability, the cell extract and culture supernatants were heated at difference temperature ranging for 60 °C – 121 °C for 15 min. Then the samples were tested by disc diffusion assay. For enzyme stability, both crude cell extract and culture supernatant were digested with protease and tested by disc diffusion assay (Anderson and Yu, 2003).

Results

Electrophoresis of antimicrobial substance

The protein expression profile of both crude cell extract and culture supernatant were analyzed at different day of culture process at day 3, 6, 9, 12 and 15 by SDS-PAGE. The expressed protein band molecular weight at around 19, 35, 38, 40 and 66 kDa from crude culture supernatant showed intent major bands. The same as crude cell extract, protein bands molecular weight at around 14.4 to 97 kDa are obtained. From SDS-PAGE, all culture day process of *T. harzianum* isolate T9. The protein concentration of culture supernatant is more than crude cell extracted (Figure 1).

Antimicrobial activity of antimicrobial substance using disc diffusion assay

Antimicrobial activities of substance from both sources are able to inhibit growth of microorganism. The substances from both sources are able to inhibit growth of microorganism such as *Bacillus subtilis* TISTR008, *Bacillus subtilis* ATCC6633, *Bacillus cereus* ATCC11778, *Bacillus licheniformis* TISTR1010, *Bacillus amyloliquefaciens* TISTR1045, *Staphylococcus aureus* ATCC1466, *Staphylococcus aureus* ATCC25923, *Escherichia coli* 0157:H7, *Vibrio*

cholerae and *Salmonella typhi* ATCC5784. On the other hand, culture supernatant showed broad-spectrum activity. As the data showed in table 1.

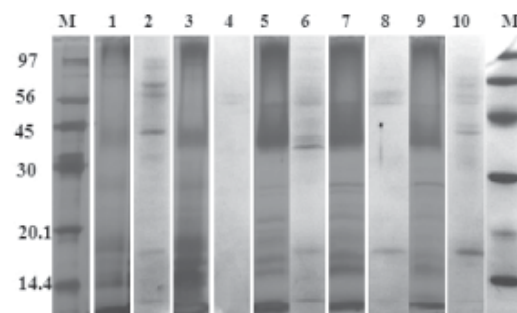


Figure 1 SDS-PAGE analysis of substance from *Trichoderma harzianum* isolate T9 on 13.5% acrylamide gel.

Lane M : Marker (low molecular weight)

Lane 1,3,5,7,9 : crude cell extracted extracted during culture process at 3, 6, 9, 12 and 15 days.

Lane 2,4,6,8,10 : culture supernatant during culture process at 3, 6, 9, 12 and 15 days.

Biochemical properties of antimicrobial substance by thermal stability and enzyme stability

Antimicrobial substance from both sources showed thermal stability and stable to pronase digestion after tested with all conditions. For thermal stability assay, both culture supernatant and crude cell extracted showed heat-stable antimicrobial activity after sterilization at 100 °C for 15 minutes and 121 °C for 15 min, respectively (Figure. 2). For enzyme stability, both of them are also resist to pronase digestion (Figure. 3). These results indicate that the antibacterial substance from *T. harzianum* isolate T9 might not be a protein in nature.

Table 1 Antimicrobial activity from *Trichoderma harzianum* isolate T9 on the target microorganism

microorganism	Inhibition Zone									
	Pellet cell					Culture supernatant				
	TS3	TS6	TS9	TS12	TS15	TS3	TS6	TS9	TS12	TS15
<i>B. subtilis</i> TISTR008	–	–	++	++	+++	+	+	+	++	++
<i>B. subtilis</i> TISTR6633	+	+	++	++	++	+	+	+	++	++
<i>B. cereus</i> ATCC11778	++	++	+++	+++	+++	++	++	+++	+++	+++
<i>B. amyloliquefaciens</i> TISTR1014	+	+	++	++	+++	+	+	++	++	++
<i>B. licheniformis</i> TISTR1010	+	+	++	++	++	+	+	++	++	++
<i>S. aureus</i> ATCC1466	–	–	+	++	++	++	++	++	++	++
<i>S. aureus</i> ATCC25923	–	–	+	+	++	++	++	++	++	++
<i>E. coli</i> 0157:H7	–	–	–	–	++	+	+	+	++	++
<i>V. cholerae</i> (clinical)	+	+	+++	+++	+++	–	+	++	++	++
<i>S. typhi</i>	+	+	+++	+++	+++	+++	+++	+++	+++	+++

Activities were classified according to the diameter of the inhibition zones around the disc : – non activity (6 mm), + inhibition zone 7 – 12 mm, ++ inhibition zone 13–19 mm, +++ inhibition zone 20–27 mm

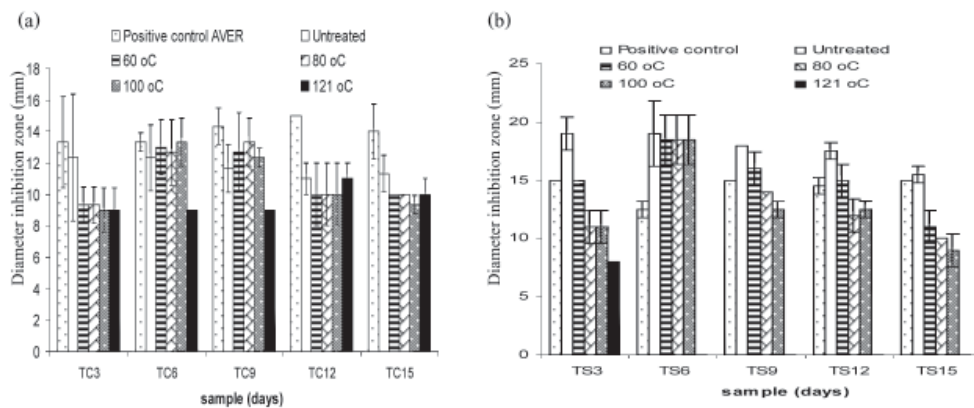


Figure 2 Thermal-stability, inhibition clear zone grown on microorganism
a) culture supernatant and b) crude cell extract tested by disc diffusion assay.

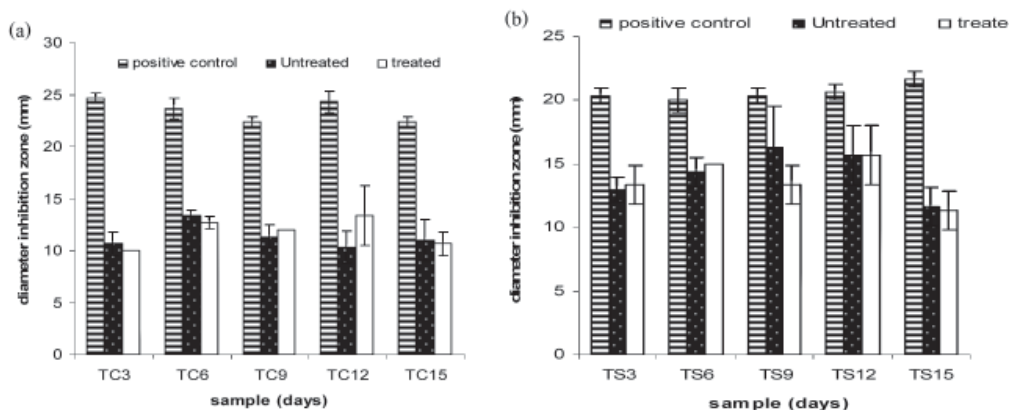


Figure 3 Pronase-stability, inhibition clear zone grown on microorganism
a) culture supernatant and b) crude cell extract tested by disc diffusion assay.

Discussion

In this study, our result indicates the source of antimicrobial substances might be secreted outside the cell. These substances can inhibit all of 10 microorganisms. Our result coincide with Vizcaino *et al.* (2005) that found the antimicrobial activity of culture filtrate from *T. harzianum* isolate T9 can inhibit both of bacteria and fungi.

Furthermore, the antimicrobial substance of both source showed thermal stability at 100 °C for culture supernatant and 121 °C for crude cell extract from *T. harzianum* isolate T9. These substances from *T. harzianum* isolate T9 showed higher thermal stability than *Trichoderma* spp. such as *T. viride* and *T. lignorum* (Betina, 1984). The antimicrobial substance from culture supernatant and crude cell extract was characterized by the effect of pronase digestion. The results suggest that the antimicrobial substance from both might be not the protein in nature.

Conclusions

The antimicrobial substances from *Trichoderma harzianum* isolate T9 are able to inhibit growth of Gram positive and Gram negative bacteria. The antibacterial substance from both source showed resistant to heat and pronase digestion.

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References

- Anderson, RC., and Yu, PL. 2003. Isolation and characterization of proline/arginine-rich cethelcidin peptides from ovine neutrophils. *Biochem Biophys Res Com.* 312:1139–1146.
- Betina, V. 1984. Mycotoxin production, isolate separation and purification. Elsevier Science Publishing Company. New York.
- Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye-binding. *Anal Biochem.* 72:248–254.
- Chet, I. 1987. *Trichoderma*: application, mode of action and potential as a biocontrol agent of soilborne plant pathogenic fungi. In *Innovative Approaches to Plant Disease Control*. New York. 137–160.
- Herman, GE., Howell, CR., Viterbo, A., Chet, I., and Lorito, M. 2004. *Trichoderma* species—opportunistic, avirulent plant symbionts. *Nat. Rev.* 2:43–56.
- Vizcaino, JA. *et al.* 2005. Screening of antimicrobial activities in *Trichoderma* isolates representing three *Trichoderma* sections. *Mycological Research.* 109 (12): 1397–1406.