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Purification and characterization of an antibacterial protein from dried fruiting bodies of the wild mushroom *Clitocybe sinopica*

Suyue Zheng[1,2], Qinghong Liu[1], Guoqing Zhang[1], Hexiang Wang[1]✉ and Tzi Bun Ng[3]✉

[1]State Key Laboratory for Agrobiotechnology and Department of Microbiology, China Agricultural University, Beijing, China; [2]Hebei Engineering University, Handan, China; [3]School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China

A novel antibacterial protein with a molecular mass of 44 kDa has been isolated from dried fruiting bodies of the wild mushroom *Clitocybe sinopica*. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis showed that the protein was composed of two subunits each with a molecular mass of 22 kDa. Its N-terminal amino-acid sequence, SVQATVNGDKML, has not been reported for other antimicrobial proteins. The purification protocol included ion exchange chromatography on DEAE-cellulose, CM-cellulose and Q-Sepharose, and gel filtration by fast protein liquid chromatography on Superdex 75. The antibacterial protein was adsorbed on all three ion exchangers. The antimicrobial activity profile of the protein against tested bacterial and fungal strains disclosed that it possessed potent antibacterial activity against *Agrobacterium rhizogenes, A. tumefaciens, A. vitis, Xanthomonas oryzae* and *X. malvacearum* with a minimum inhibitory concentration mostly below 0.6 μM. However, it had no antibacterial activity against *Pseudomonas batatae, Erwinia herbicola, Escherichia coli*, and *Staphylococcus aureus*, and no antifungal activity against *Setosphaeria turcica, Fusarium oxysporum, Verticillium dahliae, Bipolaris maydis*, and *B. sativum*. The antibacterial antivity against *A. tumefaciens* was stable after exposure to 20–60°C for 30 min and to pH 4–9 for 1 h.

Keywords: mushroom, fruiting bodies, antibacterial protein, *Clitocybe sinopica*

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INTRODUCTION

Mushrooms have long been appreciated for their flavor and texture. They need to produce antibacterial and antifungal compounds in order to survive in their natural environment. It is therefore not surprising that antimicrobial compounds could be isolated from many mushrooms (Lindequist *et al*., 2005). Antimicrobial proteins (AMPs), as important antibacterial and antifungal compounds, have attracted attention of a large number of investigators.

AMPs are largely distributed among living organisms including plants, animals, fungi and some single-celled microorganisms (Jenssen, 2005). AMPs, which directly interfere with the growth, multiplication and spread of microbial organisms and permit plants and animals to resist infection by environmental microbes, represent important components of the defense system (Lehrer & Ganz, 1999). To date, different proteins with antibacterial or antifungal activity have been reported. Most of these proteins were isolated from animals (Steiner *et al*., 1981; Lemaître *et al*., 1996; Destoumieux *et al*., 1997; Krishnakumari & Nagaraj, 1997; Torres-Larios *et al*., 2000), plants (Huynh *et al*., 1992; Cammue *et al*., 1995; Talas, 2004) and bacteria (Barja *et al*., 1989; James *et al*., 1996; Longeon *et al*., 2004). Only few of them came from fungi (Mygind *et al*., 2005; Hu *et al*., 2006), especially macrofungi.

Antifungal peptides and proteins have been isolated from the fungi *Aspergillus giganteus* (Lacadena *et al*., 1995), *A. niger* (Lee *et al*., 1999), *Zygosaccharomyces bailii* (Weiler *et al*., 2003), *Hypsizigus marmoreus* (Lam & Ng, 2001b), *Pleurotus erygii* (Wang & Ng, 2004), *Lyophyllum shimeji* (Lam & Ng, 2001a), and *Tricholoma giganteum* (Guo *et al*., 2005). However, the literature pertaining to antibacterial peptides and proteins is scanty.

Clitocybe sinopica is a well-known wild edible mushroom species found in China. To the best of our knowledge, no research has been reported on the chemical composition and biological activities of *C. sinopica* extract. Therefore, the aim of the present work was to evaluate the antimicrobial potential of this mushroom extract on several phytopathogenic microorganisms.

MATERIALS AND METHODS

Materials. The fruiting bodies of the wild mushroom *Clitocybe sinopica* were collected from Heilongjiang province, in the northeast region of China. Fifteen bacterial pathogenic strains were used in this study: *Agrobacterium rhizogenes* (NL24-2, NL5-4, MG 12-1, HBT 6-1, SX073, NL12-2) *A. vitis* (MI3-2, MI23-1), *A. tumefaciens* (MG10-1), *Xanthomonas oryzae*, *X. malvacearum*, *Pseudomonas batatae*, *Erwinia herbicola*, *Escherichia coli* and *Staphylococcus aureus*. Five fungal strains were also tested: *Setosphaeria turcica*, *Bipolaris maydis*, *Fusarium oxysporum*, *Verticillium dahliae* and *Bipolaris sativum*. All tested bacterial and fungal strains were obtained from the Department of Plant Pathology of the China Agriculture University (Beijing, China PR).

Isolation procedure. Dried fruiting bodies of *C. sinopica* were homogenized in 0.15 M NaCl at 4 °C overnight. The supernatant was centrifuged at 8000 × *g*

✉e-mail: Tzi Bun Ng: b021770@mailserv.cuhk.edu.hk; Hexiang Wang: hxwang@cau.edu.cn

Abbreviations: AMP, antimicrobial proteins; cfu, colony-forming unit; FPLC, fast protein liquid chromatography; MIC, minimum inhibitory concentration.

for 15 min before $(NH_4)_2SO_4$ was added to 80% saturation. The precipitate was collected by centrifugation at 8 000 × *g* for 15 min again. Then the precipitate was dissolved and dialyzed to remove $(NH_4)_2SO_4$ before ion exchange chromatography on a DEAE-cellulose (Sigma) column (2.5 cm × 20 cm) in 10 mM sodium phosphate buffer (pH 7.5). Unadsorbed proteins (fraction D1) were eluted with the buffer while bound ones were desorbed sequentially with 100 mM NaCl, 200 mM NaCl and 1 M NaCl to form fractions D2, D3 and D4, respectively. Fraction D3 with antibacterial activity was dialyzed and then chromatographed on a column of CM-cellulose (Sigma) (1.5 × 20 cm) in 10 mM sodium phosphate buffer (pH 6.2). After elution of unadsorbed proteins (CM1) with the same buffer, the bound fractions CM2, CM3 and CM4 were eluted with the same buffer containing 50 mM NaCl, 150 mM NaCl and 1 M NaCl, respectively. The active peak (CM3) was then subjected to ion exchange chromatography on a column of Q-Sepharose (GE Healthcare) (1 cm × 10 cm) in 10 mM sodium phosphate buffer (pH 6.5). After removal of unadsorbed protein (fraction Q1), the bound material was eluted with a linear NaCl concentration gradient (0–500 mM) in the same buffer. The active fraction (Q2) was then gel-filtered on a Superdex 75 HR 10/30 column (GE Healthcare) by fast protein liquid chromatography (FPLC) in 0.2 M NH_4HCO_3 buffer (pH 8.5) using an AKTA Purifier (GE Healthcare). Two peaks were obtained. The first peak (SU1) with antibacterial activity was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) which was conducted according to the method of Laemmli and Favre (1973). N-terminal sequencing was carried out using a Hewlett-Packard (HP) Edman degradation unit and an HP 1000 HPLC System.

Assay of antibacterial activity. The assay for antibacterial activity was conducted using sterile Petri plates (90 mm × 15 mm) containing 15 ml suitable medium agar (1.8% agar). Five milliliters of warm nutrient agar (0.7%) containing the bacterium were poured into the plates. A sterile blank paper disk (6.25 mm in diameter) or Oxford cup was placed on the agar. The antibacterial protein (40 µl of a 1 mg/ml solution) in 10 mM sodium phosphate buffer (pH 7.0) was added to one of the disks. Only sterile buffer was added to the control disk. The plate was incubated at 28°C for 24 h. A transparent ring around the paper disk signifies antibacterial activity (Lam *et al*., 2000).

Assay of minimum inhibitory concentration (MIC). The MIC for each bacterial strain tested was measured by the liquid culture medium dilution method using Luria Bertani or modified 523 medium, depending on the strain. Nine hundred milliliters of medium were placed in a sterilized test tube to which 90 µl of the tested sample and 10 µl of the cultured bacterial solution (final bacterial count of 1×10^6 cfu/ml) were added. The tube was cultured under suitable conditions. The minimum concentration of added samples in which no bacterial growth was observed was defined as the MIC (Hirasawa *et al*., 1999).

Assay of antifungal activity. The assay was conducted as detailed by Wang *et al.* (2004).

Test for thermostability and pH stability. The antibacterial protein was exposed to various temperatures ranging from 20°C to 80°C at 10°C intervals for 30 min. After heat treatment, samples were centrifuged (12 000 × *g*, 5 min) and the supernatants were used for assay of antibacterial activity. The antibacterial activity of each sample was compared with that of a control treated at 4°C for 30 min.

The test for pH stability was conducted as described above. The antibacterial protein was treated at various pH values from 3 to 9 for 1 h.

Determination of ribonuclease activity. The activity of the antibacterial protein toward yeast tRNA was determined as described by Wang and Ng (1999).

Determination of haemagglutinating activity. The assay was conducted as described by Wang *et al.* (1996).

RESULTS

Protein purification

Antibacterial activity was located mainly in fraction D3 adsorbed on DEAE-cellulose which was eluted with 0.2 M NaCl. The larger unadsorbed fraction D1 and other adsorbed fractions were devoid of antibacterial activity. D3 was fractionated on CM-cellulose into an unadsorbed fraction CM1 and three adsorbed fractions CM2, CM3, and CM4. Antibacterial activity resided in fraction CM3. Fraction CM3 was collected for further purification on a Q-Sepharose column. The activity was eluted with a linear gradient of 0–0.5 M NaCl in 10 mM phosphate buffer (pH 6.5). Two peaks were obtained. Antibacterial activity was detected in fraction Q2 (Fig. 1). Fraction Q2 was resolved on Superdex 75 into two fractions, SU1 and SU2 (Fig. 2). The first and larger fraction, SU1, was enriched in antibacterial activity. It demonstrated a molecular mass of 44 kDa in gel filtration (Fig. 2) and 22 kDa in SDS/PAGE (Fig. 3). Thus the antibacterial protein is most likely composed of two subunits with the same molecular mass of 22 kDa. The yields of the various chromatographic fractions are presented in Table 1. The N-terminal sequence of the protein, SVQATVNGDKML, is not found in any reported antimicrobial protein. It is dissimilar to those of previously isolated mushroom antimicrobial proteins (Table 2).

Assays of antimicrobial and other activities

The antimicrobial activity of the isolated protein was tested against different bacterial and fungal strains. The antimicrobial activity was tested at the dose of 40 µg, it showed potent antimicrobial activity against most bacteria (Fig. 4). The minimal inhibitory concentration (MIC)

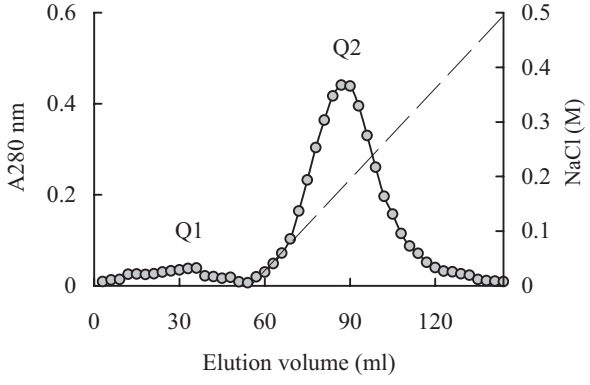

Figure 1. Cation exchange chromatography of fraction CM3 on Q-Sepharose
Starting buffer: 10 mM sodium phosphate buffer (pH 6.5).

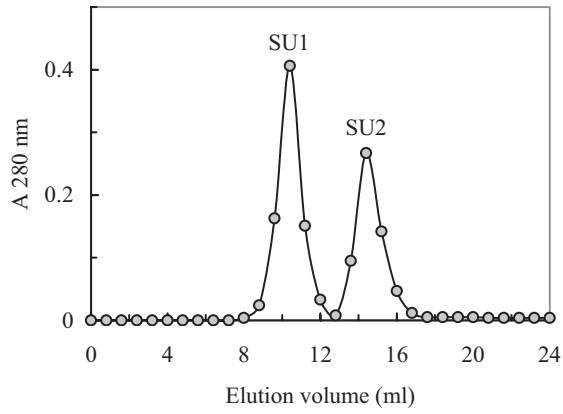

Figure 2. Gel filtration of Q2 on Superdex 75 HR10/30
The column had been calibrated with molecular mass markers from GE Healthcare including phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14.4 kDa).

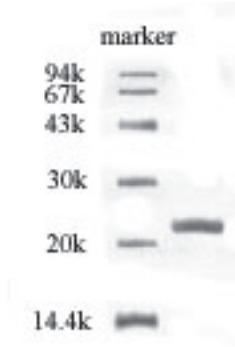

Figure 3. SDS/PAGE analysis of *C. sinopica* antibacterial protein
Left**:** molecular mass markers. Right**:** fraction SU1 (8.5 µg) from Superdex 75 column representing purified antibacterial protein.

of the antibacterial protein toward different bacterial strains is presented in Table 3. However, antifungal, ribonuclease and hemagglutinating activities were not detected (not shown).

Influence of pH and heat treatments

Figure 5A shows the antibacterial effects of the protein against *Agrobacterium tumefaciens* after exposure to 20–80°C for 30 min. The protein was heat-labile. Almost no change was observed in the antibacterial effect after heat treatment at 60°C for 30 min. After exposure to 70°C for 30 min, the antibacterial activity was reduced to 80%. Following treatment at 80°C for 30 min, the protein was completely inactivated.

Figure 5B shows the antibacterial effects of the protein against *A. tumefaciens* after exposure to pH 3.0–9.0 for 1 h. After treatment at pH 3.0 for 1 h, most of the antibacterial activity was lost. After treatment at pH 4.0–9.0 for 1 h, there was no change in the antibacterial activity. The data show that the antibacterial activity of the protein was stable over a fairly wide pH range.

Table 1. Protein yields and MIC toward *A. tumefaciens* of various chromatographic fractions
Extract from 100 g dried mushroom.

Fraction	Yield (mg)	MIC (µM)	Purification fold
Extract	7230	15.8	1
D1	1560	–	–
D2	880	–	–
D3	920	2.7	5.9
D4	1480	–	–
CM1	20	–	–
CM2	40	–	–
CM3	290	1.17	13.5
CM4	70	–	–
Q1	20	–	–
Q2	90	0.5	31.5
SU1	18	0.14	112.3
SU2	15	–	–

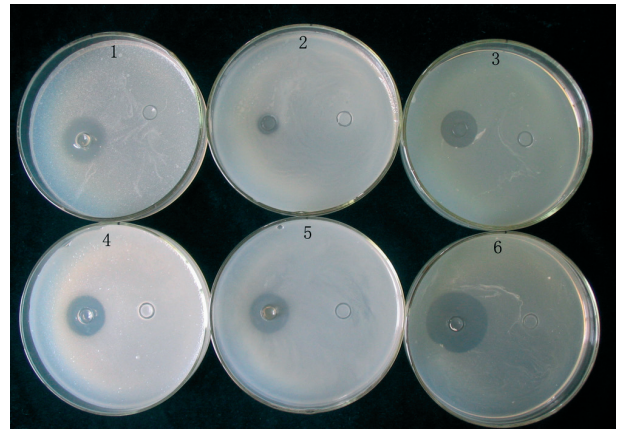

Figure 4. Photomicrograph showing antibacterial activity of isolated antibacterial protein toward various bacterial strains
The dosage of the antibacterial protein was 40 µg. Dishes 1, 2, 3, 4, 5 and 6 represent *A. rhizogenes* (NL24-2), *A. rhizogenes* (NL5-4), *A. rhizogenes* (MG 12-1), *A. rhizogenes* (HBT 6-1), *A. vitis* (MI3-2) and *A. tumefaciens* (MG10-1), respectively.

DISCUSSION

Antibacterial peptides and proteins have great economic implications because they protect crops from the devastating damage brought about by bacterial infections. Antibacterial peptides/proteins which are different in physiological and biochemical characteristics such as molecular mass, MIC and antibacterial spectrum have been isolated from a variety of organisms. Kisugi *et al.* (1992) purified from *Dolabella auricularia* a large, 250-kDa antibacterial glycoprotein consisting of four subunits and showing both antibacterial and antineoplastic activities. Another large antibacterial protein of 190-kDa was isolated from *Ciona intestinalis*. The protein consisted of more than one subunit (James *et al.*, 1996). The sea hare *Aplysia dectylomela* produced a 60-kDa monomeric antibacterial protein with hemagglutinating activity. It exerted activity against *S. aureus* with a MIC of 0.25 µM (Melo *et al.*, 2000). The silkworm *Bombyx mori* produced an antibacterial peptides with a higher activity against Gram-negative and -positive bacteria than cecropin B1, a major antibacterial peptide of *B. mori*. Its lethal concentration against *S. aureus* was less than 0.44 µM (Hara & Yamakawa, 1995a; 1995b). Ebran *et al.* (1999; 2000)

Table 2. Comparison of N-terminal sequence of *C. sinopica* antibacterial protein with those of other antimicrobial proteins

Name	Source	N-terminal sequence	Da	Reference
Antibacterial protein	*C. sinopica*	SVQATVNGDKML	44 000	This study
	Marine bacterium	MNLKIHPSVGAXLGNRQM	90 000	Barja *et al.*, 1989
	Cordyceps sinensis	ALATQHGAP	35 000	Hu *et al.*, 2006
	Bullfrog	GVVKVSLRKGESLRARL	1 865	Minn *et al.*, 1998
	Bullfrog	IIKVPLKKFKSMRGVMRDHGIKAPVV	3 691	Minn *et al.*, 1998
	Scylla serrata	GQALNKLMPKIVSAIIYMVG	10 800	Huang *et al.*, 2006
	Bombyx mori–I	DLRFLYPRGKLPVPXPPPFNPKPITIDMGNRY	5 500	Hara & Yamakawa, 1995a; 1995b
	Bombyx mori–II	AKIPIKAIKTVGKAVGKGLRAZNIASTANDVFN-FLKPKKRKH	4 500	Hara & Yamakawa, 1995a; 1995b
Trichogin	*Tricholoma giganteum*	QVHWPMF	27 000	Guo *et al.*, 2005
Alveolarin	*Polyporus alveolaris*	GVCDMADLA	28 000	Wang *et al.*, 2004
Eryngin	*Pleurotus eryngii*	ATRVVYCNRRSGSVVGGDDTVYYEG	10 000	Wang & Ng, 2004
Antifungal protein	*Lyophyllum shimiji*	AGTEIVTCYNAGTKVPRGPSAXGGAIDFFN	20 000	Lam & Ng, 2001a

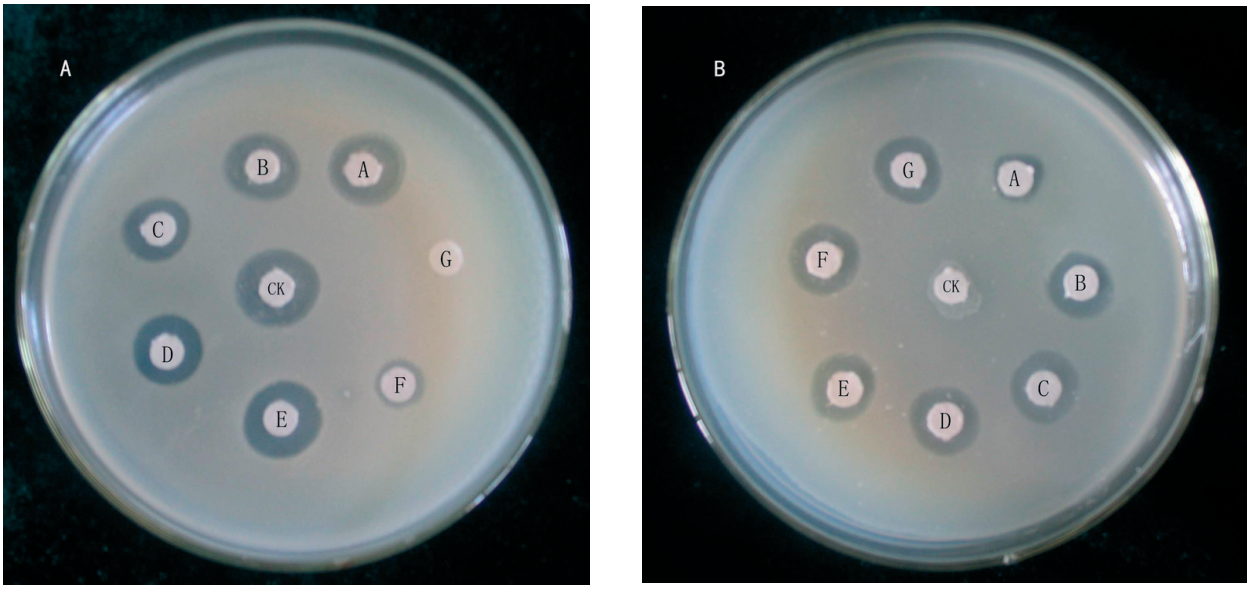

Figure 5. Effect of heat and pH treatments on the antibacterial activity of isolated antibacterial protein against *A. tumefaciens*
(A) Heat treatments (30 min). A 20 μg sample of the protein was applied to each paper disk in sterile water. After treatment at 20°C, 30°C, 40°C, 50–60°C, 70°C and 80°C for 30 min (A–G), (CK) represents control incubated at 4°C for 30 min. **(B)** pH treatment (1 h). A 20 μg sample of the protein was applied to each paper disk in sterile water. After treatment at pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 for 1 h (A–G), (CK) represents control of sterile water.

reported antibacterial proteins with a molecular mass of 27-kDa and 31-kDa and activity against *P. aeruginosa*, *P. fluorescens*, *Aeromonas hyarophila* and *S. aureus* from carp mucus. Their MIC ranged from 0.16 μM to 4.8 μM against *S. aureus*. The bullfrog produced a 1865-Da peptide and another 3691-Da one both with antibacterial activity. They exerted antibacterial activity against *B. subtilis* and *E. coli* with a MIC of 1.6–10.7 μM (Minn *et al.*, 1998). A 35-kDa antibacterial protein with a MIC of 14.3 μM to 28.6 μM was isolated from *Cordyceps sinensis* (Hu *et al.*, 2006).

In this study, we describe the isolation and properties of an antibacterial protein with a molecular mass of 44 kDa from dried fruiting bodies of the wild mushroom *C. sinopica*. This protein displayed a strong antibacterial activity on *Agrobacterium* spp. and *Xanthomonas* spp., but there was no obvious inhibitory effect against fungi. A comparison of the N-terminal sequence and the molecular mass of the *C. sinopica* antibacterial protein with the aforementioned antibacterial proteins reveals that they are distinctly different (Table 3). So it can be regarded as a novel antibacterial protein.

The behavior of the *C. sinopica* protein on the various ion exchange chromatographic media employed in the isolation procedure was different from that reported for other antimicrobial proteins. It was adsorbed on DE-AE-cellulose whereas other antimicrobial proteins were not (Lam & Ng, 2001a; Wang & Ng, 2004; Wang *et al.*, 2004; Guo *et al.*, 2005), but they were all adsorbed on CM-cellulose.

Table 3. Antimicrobial activity of *C. sinopica* antibacterial protein

Strain	MIC (μM)
A. rhizogenes (NL24-2)	0.28
A. rhizogenes (NL5-4)	2.27
A. rhizogenes (MG 12-1)	0.28
A. rhizogenes (HBT 6-1)	0.28
A. rhizogenes (SX073)	0.14
A. rhizogenes (NL12-2)	0.28
A. vitis (MI3-2)	0.28
A. vitis (MI23-1)	1.14
A. tumefaciens (MG10-1)	0.14
X. oryzae	0.56
X. malvacearum	0.56

The *C. sinopica* antibacterial protein was devoid of ribonuclease or lectin activities. This is important in view of the reports that some ribonucleases (Ng & Wang, 2000; Wang & Ng, 2000) and lectins (Broekaert *et al*., 1989; Gozia *et al*., 1995; Ye *et al*., 2001) exhibit antimicrobial activity.

In conclusion, our research demonstrated that *C. sinopica* fruiting bodies produce an antibacterial protein that shows a broad spectrum of activity against a number of plant pathogenic bacteria, including strains of the genera *Agrobacterium* and *Xanthomonas*. Its high potency and the broad spectrum of antibacterial activity suggest that the expression of its gene in transgenic plants may confer protection against bacterial pathogens.

Further work aims at the elucidation of the mechanism of action and cloning of the gene encoding the antibacterial protein for possible pratical application in the future.

Acknowledgements

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