Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

Purification and characterization of an extracellular serine protease from the nematode-trapping fungus *Dactylella shizishanna*

R.B. Wang, J.K. Yang, C. Lin, Y. Zhang and K.Q. Zhang

Laboratory for Conservation and Utilization of Bio-resources, Yunnan University, Kunming, China

Keywords
Dactylella shizishanna, nematicidal analysis, purification, serine protease.

Correspondence
K.Q. Zhang, Laboratory for Conservation and Utilization of Bio-resources, Yunnan University, Kunming 650091, China.
E-mail: kqzhang111@yahoo.com.cn

2005/0985: received 1 September 2005, revised 12 December 2005 and accepted 20 December 2005

doi:10.1111/j.1472-765X.2006.01908.x

Abstract

Aims: To evaluate the production of an extracellular serine protease by *Dactylella shizishanna* and its potential as a pathogenesis factor.
Methods and Results: An extracellular alkaline serine protease (Ds1) was purified and characterized from the nematode-trapping fungus *D. shizishanna* using cation-exchange chromatography and hydrophobic interaction chromatography. The molecular mass of the protease was approximately 35 kDa estimated by SDS–PAGE. The optimum activity of Ds1 was at pH 10 and 55°C (over 30 min). The purified protease could degrade purified cuticle of *Penagrellus redivivus* and a broad range of protein substrates. The purified protease was highly sensitive to phenylmethyl sulfonyl fluoride (PMSF) (0·1 mmol l^{-1}), indicating it belonged to the serine protease family. The N-terminal amino acid residues of Ds1 are AEQTDSTWGL and showed a high homology with Aozl and PII, two serine proteases purified from the nematode-trapping fungus *Arthrobotrys oligospora*.
Conclusions: Nematicidal activity of *D. shizishanna* was partly related to its ability to produce extracellular serine protease.
Significance and Impact of the Study: In this report, we purified a new serine protease from *D. shizishanna* and provided a good foundation for future research on infection mechanism.

Introduction

Plant-parasitic nematodes have been reported to cause severe damages to agriculture all over the world (Huang *et al.* 2005); and chemical pesticides can cause a series of environmental problems. Nematode-trapping fungi, natural enemies of nematodes, which can immobilize and digest nematodes, have been suggested as a potential biocontrol agent to control the harmful nematodes. Infection of nematodes by nematode-trapping fungi proceeds by adherence and trapping of the nematode followed by penetration and immobilization and finally digestion of the nematode (Jansson and Nordbring-Hertz 1988; Tunlid and Jansson 1991; Yang *et al.* 2005), and penetration of the nematode cuticle has been assumed to be due to the combination of mechanical activity and hydrolytic enzymes including protease, chitinase and collagenase. However, the molecular background of the infection process is still obscure.

Among the above hydrolytic enzymes, serine proteases are of special interest because it is believed that proteases are important for infecting nematodes and they are produced during the first step of infection (Dackman *et al.* 1989; Tunlid and Jansson 1991). So far, six extracellular proteases (PII, Aozl, VCP1, pSP-3, P32 and Ver112) have been isolated and characterized as virulence factors from nematophagous fungi (Lopez-Llrca and Robertson 1992; Segers *et al.* 1994; Tunlid *et al.* 1994; Bonants *et al.* 1995; Zhao *et al.* 2004; Yang *et al.* 2005). These proteases are medium-sized subtilisin-like serine proteases, with broad substrate specificity, and have effects on degradation of nematode cuticles and eggshells (Jansson *et al.* 1997). Moreover, collagenase and chitinase are two important enzymes involved in infection process

(Schenck *et al.* 1980; Kang *et al.* 1999; Tikhonov *et al.* 2002).

Dactylella shizishanna is an autochthonal fungus and can capture nematodes with its special hypha of three-dimensional networks (Liu and Zhang 2003), but little is known about its extracellular enzymes and other factors involved in the infection. In this report, we describe the purification, biochemical characterization, nematicidal activity and N-terminal amino acid analysis of a cuticle-degrading serine protease, Ds1, from *D. shizishanna*.

Materials and methods

Organisms and growth conditions

Dactylella shizishanna was originally isolated from soil samples in Hubei Province (Liu and Zhang 2003) and had been deposited in China General Microbiological Culture Collection Center (CGMCC no. 1311). The isolate was incubated on potato dextrose agar (PDA) plate at 28°C for 10–15 days. The protease was produced by *D. shizishanna* in liquid medium LMZ (Zhao *et al.* 2004). The isolate was carried out in 250 ml shaking flask containing 60 ml medium at 26°C and 200 rev min^{-1} for 7 days on a rotary shaker.

Panagrellus redivivus and *Bursaphelenchus xylophilus* (conserved in Laboratory for Conservation and Utilization of Bio-resources, Yunnan University) were maintained as described (Zhao *et al.* 2004; Li *et al.* 2005). Nematodes were separated and washed thoroughly with 50 mmol l^{-1} sodium phosphate buffer (pH 7·0) before being used in the assays.

Nematicidal activity analysis

The effect of protease on nematodes was investigated by *in vitro* assays as follows: approximately 50 nematodes were added to solutions of purified protease Ds1, culture filtrate, crude enzyme and LMZ medium respectively. The mixture was incubated at 26°C for 12–36 h, and numbers of dead nematodes were counted under a light microscope. The experiment was repeated three times.

Protein concentration and protease activity analysis

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

Protease activity was assayed by a modified caseinolytic method (Peterson 1977). Hundred microlitres protease solution was added to 200 μl of 1·0% casein solution (prepared in 20 mmol l^{-1} Tris–HCl, pH 7·0) and incubated at 37°C for 20 min. The reaction was stopped by adding 300 μl of 10% trichloroacetic acid (TCA). Non-digested proteins were removed by centrifugation at 10 000 ***g*** for 5 min. Then 500 μl of the clear supernatant was mixed with 2·5 ml 0·55 mol l^{-1} Na_2CO_3 and 0·5 ml Folin-phenol agent, followed by incubation at 30°C for 15 min. The absorbance value of the resulting supernatant was measured at 680 nm against a blank control. One unit (U) of protease activity was defined as the amount of enzyme that hydrolyzed the substrate and produced 1 μg tyrosine in 1 min under the assay conditions.

Purification of the protease

Culture filtrate was collected by vacuum filtration and was precipitated with ammonium sulfate to 85% saturation, then centrifuged at 7500 ***g*** for 15 min and the supernatant was discarded. The precipitate was resuspended in a minimal amount of sodium phosphate buffer (pH 6·5). The solution was dialysed against 10 mmol l^{-1} sodium phosphate buffer (pH 6·5) and stored at 4°C until further purification.

Solutions and sample were filtered through 0·22 μm membranes before application to the column. Buffer A_1, 10 mmol l^{-1} sodium phosphate buffer (pH 6·5); B_1, 10 mmol l^{-1} sodium phosphate buffer (pH 6·5) with 1 mol l^{-1} NaCl. The sample was loaded to a HiTrap^{TM} SP FF column (Amersham, Uppsala, Sweden) connected to the Amersham FPLC system. Bound proteins was eluted with a three stage isocratic elution of 6% B_1 for 8 min, 30% B_1 for 10 min and 100% B_1 for 10 min. Fractions (5 ml each) were collected at a flow rate of 2 ml min^{-1} and assayed for protease activity.

The fractions with high protease activity were pooled and purified with hydrophobic interaction chromatography (HIC). Buffers used were: A_2, 10 mmol l^{-1} sodium phosphate buffer (pH 6·5) with 1 mol l^{-1} ammonium sulfate; B_2, 10 mmol l^{-1} sodium phosphate buffer (pH 6·5). The sample was loaded to a HiLoad 16/10 Phenyl Sepharose column and was eluted with a three stage isocratic elution of 30% B_2 for about 60 min, 50% B_2 for about 60 min and 100% B_2 for 60 min with a flow rate of 2 ml min^{-1}. Elution of proteases was followed by monitoring the absorbance at 280 nm.

Molecular mass determination

SDS–PAGE was performed according to the method of Laemmli (1970) under reducing conditions on a 12% polyacrylamide gel.

Effect of temperature and pH on enzyme activity

Optimal assay temperature was determined by performing a standard activity assay in a temperature range from 25

to 75°C. In order to determine optimal pH, the enzymic assay was carried out at different pH values, at 55°C. Buffer used as barbital sodium–NaOH buffer system at pH values between 3 and 12.

Hydrolysis of protein substrates and nematode cuticle

The purified protease was incubated with different protein substrates at pH 10 and 55°C for 10 min, respectively, and protease activity was quantitatively assayed. Nematode cuticle was extracted according to the method of Cox *et al.* (1981).

Effect of metal ions on the enzyme activity

The purified protease was incubated with different metal ions including Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+} and Mn^{2+} for 20 min and then the protease activity was measured.

Effect of protease inhibitors on purified enzyme activity

The purified protease Ds1 was assayed against casein in the presence of protease inhibitors. Purified protease was preincubated with different protease inhibitors for 10 min at room temperature. Residual activity was determined as a percentage of the activity in control samples without inhibitors.

N-Terminal amino acid sequence analysis

The N-terminal amino acid sequence of the purified protease sample was determined on an ABI Procise 491 protein sequencer (Applied Biosystems, Foster City, CA, USA).

Results

Purification of the protease

Following ammonium sulphate precipitation, the crude protease was purified with cation-exchange chromatography after dialysis in 10 mmol l^{-1} sodium phosphate buffer (pH 6·5). Most of the proteases were absorbed in the HiTrap™ SP FF column (Amersham) and eluted by buffer B_1 (Fig. 1). The active fractions were pooled and applied to the HiLoad 16/10 Phenyl Sepharose column and eluted by buffer B_2 (Fig. 2). Purification factors and yields at each step are summarized in Table 1.

Molecular mass determination

The purified protease was found to be homogeneous on SDS–PAGE. Based on the electrophoretic mobility, the

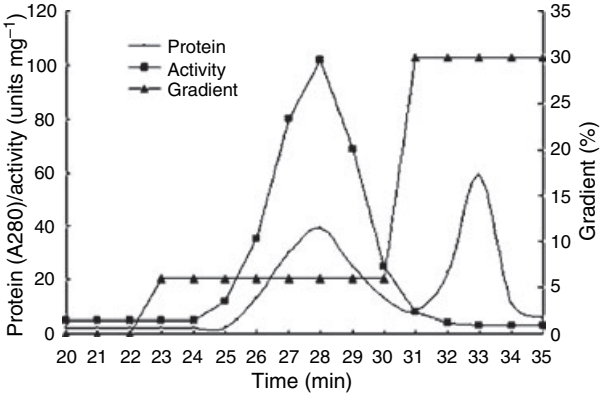

Figure 1 Elution profile from cation-exchange chromatography.

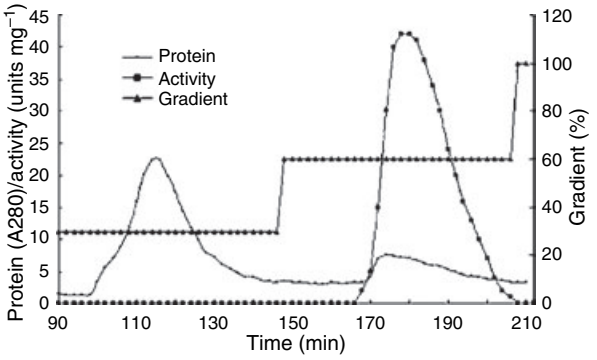

Figure 2 Elution profile from hydrophobic interaction chromatography.

Table 1 Purification of the extracellular serine protease (Ds1) from *Dactylella shizishanna*

Purification procedure	Volume (ml)	Total protein (mg)	Total activity (U‡)	Specific activity (U mg^{-1})	Yield (%)	Purification factor
Culture filtrate	740	2450·9	25 929·6	10·6	100	1·0
Crude extract*	26·5	1008·3	12 847·9	12·7	49·5	1·2
Cation-exchange	25	48·0	3388·7	70·6	13·1	6·7
Purified Ds1†	50	3·1	745·0	240·3	2·9	22·7

*Culture filtrate concentrated by ammonium sulfate precipitation.
†Protease purified by hydrophobic interaction chromatography.
‡Proteolytic units. For definition see Materials and methods.

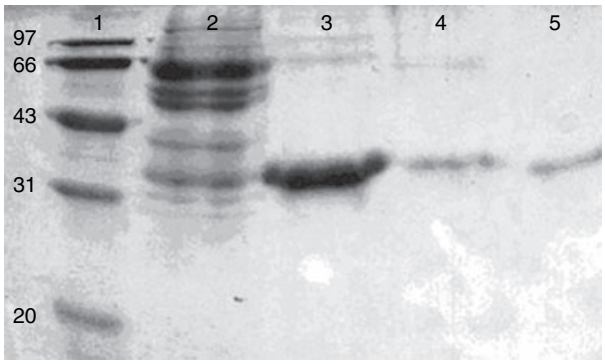

Figure 3 SDS–PAGE electrophoresis gel by 12%. Lane 1, molecular mass marker; lane 2, crude extract; lane 3, fraction after cation-exchange chromatography; lanes 4 and lane 5, fraction after hydrophobic interaction chromatography.

molecular mass of the purified enzyme was approximately 35 kDa (Fig. 3).

Effect of pH and temperature on enzyme activity

The optimum pH of the purified enzyme was pH 10. At pH values below this, the observed activity progressively fell as the pH was lowered. Only 37·5% of the original activity remained at pH 5·0. The enzyme appeared to be stable in alkaline conditions with 73% activity being retained after 2 h exposure at pH values of 8–12. Below pH 7, the enzyme became progressively unstable as the pH was lowered.

The optimum reaction temperature for Ds1 was 55°C. But the thermal stability experiments indicated that the purified protease retained about 70% activity at 35°C after 30 min of incubation and it losses activity at 55°C, it is a 'trade-off' between the Arrhenius equation and thermostability of the enzyme.

Hydrolysis of protein substrates and nematode cuticle

From the results of Table 2, the protease Ds1 was particularly effective at degrading casein and skimmed milk, whilst being less effective at hydrolysing BSA, gelatin and nematode cuticle. However, it was almost no effective at degrading collagen and denatured collagen.

Effect of metal ions on the enzyme activity

Protease Ds1 was stable in the presence of calcium and magnesium cations, exhibiting residual enzyme activities of 91% and 107% respectively. Both zinc and ferrous cations caused mild deactivation of Ds1 as observed by residual activities of 72% and 42% respectively. The presence of cupric cations caused severe inactivation of Ds1 with a residual activity of only 12% being observed.

Table 2 Hydrolysis of various protein substrates by the serine protease

Substrate	Enzyme activity as % of control (A_{680})
Casein	100*
Bovine serum albumin	12·5 ± 0·5
Skimmed milk	61 ± 2·0
Gelatin	15 ± 1·0
Collagen	1 ± 0·2
Collagen (denatured)	2 ± 0·2
Nematode cuticle	14·9 ± 0·5

*The proteolytic activity in control incubated without the inhibitors (corresponding to 100%) was 240·3 U, same as Table 3.

Table 3 Effect of Inhibitors on the enzyme activity

Inhibitor	Concentration	Enzyme activity as % of control (A_{680})
None		100
PMSF	1·0 mmol l^{-1}	5 ± 0·5
	0·1 mmol l^{-1}	8 ± 0·5
EDTA	1·0 mmol l^{-1}	87 ± 2·0
Pepstain A	10 μmol l^{-1}	103 ± 1·5
Leupeptin	100 μmol l^{-1}	111 ± 2·0
Aprotinins	1 μg ml^{-1}	111 ± 2·0

Effect of protease inhibitors on purified enzyme activity

The effects of various protease inhibitors on the enzyme activity were determined under standard conditions. Only in the presence of PMSF, the enzyme activity was strongly inhibited. Other serine protease inhibitors (leupeptin and aprotinins), the metal chelator (EDTA) and aspartic protease inhibitor (pepstatin A) had a limited influence on the protease Ds1 (Table 3).

Nematicidal activity analysis

From the results of Table 4, both the crude extract and the purified protease had obvious nematicidal effect on *P. redivivus* and *B. xylophilus*, but Ds1 had more effect on *P. redivivus* than *B. xylophilus*. More than 60% tested nematodes (*P. redivivus*) were killed and degraded after being treated with crude extract or the purified enzyme for 12 h. But in control samples (including LMZ medium sterilized, ddH_2O and boiled purified protease) <15% tested nematodes were killed. Furthermore, the crude extract has much more effect on nematodes than the purified enzyme, which suggests that other enzymes may play roles in the infection process.

Table 4 The effect of the protease from *Dactylella shizishanna* on *Panagrellus redivivus* and *Bursaphelenchus xylophilus*

		PR† Lethal rate‡ (%)			*BX*§ Lethal rate‡ (%)		
Sample	PA* (U ml^{-1})	12 h	24 h	36 h	12 h	24 h	36 h
Control 1¶	0	10 ± 2	20 ± 2	30 ± 5	5 ± 2	7 ± 3	12 ± 3
Control 2**	0	10 ± 2	10 ± 2	20 ± 5	5 ± 2	10 ± 2	15 ± 3
Control 3††	0	10 ± 2	10 ± 2	30 ± 5	ND	ND	ND
Culture filtrate	12·7	10 ± 3	15 ± 5	30 ± 5	ND	ND	ND
Crude extract	70·6	90 ± 2	95 ± 2	100	30 ± 2	50 ± 3	70 ± 2
Purified protease	240·3	60 ± 5	80 ± 5	85 ± 5	30 ± 3	45 ± 2	65 ± 3

*Protease activity.
†*Panagrellus redivivus*.
‡*Dead nematodes (%).*
§Bursaphelenchus xylophilus.
¶LMZ medium sterilized.
**ddH_2O.
††Boiled purified protease.
ND, not determined.

N-terminal amino acid sequence analysis

The N-terminal amino acid residues of the purified protease were AEQTDSTWGL. Alignment result showed Ds1 had 50–100% homologies with the N-terminal region of serine proteases from other nematophagous fungi.

Discussion

Nematode-trapping fungi, as natural enemies of nematodes, are very important sources of biological control agents. *Dactylella* Grove is one of important genus including many important biocontrol fungi. In this report, a cuticle-degrading serine protease Ds1, secreted by *D. shizishanna*, share several similar biochemical properties with PII and Aozl, two serine proteases from nematode-trapping fungus *A. oligospora* (Tunlid *et al.* 1994; Zhao *et al.* 2004). They share similar molecular masses (35–39 kDa), shows highly sensitivity to PMSF and they have optimum activity in neutral or alkaline conditions. Which suggested that *D. shizishanna* and *A. oligospora*, two nematode-trapping fungi share not only similar predacious pattern: capture (three-dimensional nets), killing, and degradation of the nematode (Rosenzweig and Pramer 1980), but also produce similar virulence factor in infection process.

The N-terminal amino acid residues analysis also showed that these cuticle-degrading proteases isolated from nematophagous fungi shared high degree of similarity. Proteases (P11 and Aoz1) isolated from nematode-trapping fungi had a high homology (100%) towards the 10 amino acid Ds1 peptide. Proteases (pSP-3, VCP1 and Ver112) isolated from egg-parasitic or nematode parasitic fungi had a lower homology to the Ds1 peptide (50–60%). Further study of nematode-trapping fungi proteases would be helpful in distinguishing between different nematophagous fungi.

This is the first report on isolation and purification of an extracellular protease from nematode-trapping fungus *D. shizishanna*. The purified protease exhibits obvious nematicidal activity, and we also find that crude enzyme can degrade and kill the nematode more effectively than purified protease (Table 4), which suggests that penetration process requires the synergistic action of several different enzymes because of the complex componential nature of the nematode cuticle and eggshell. In fact, collagenase and chitinase had been isolated from some nematophagous and entomophagous fungi, and they could also hydrolyze the eggshell and cuticle of nematode (Kang *et al.* 1999; Tikhonov *et al.* 2002; Khan *et al.* 2004). Therefore, further studies are necessary to ascertain the role of enzyme and other factors in infection.

Acknowledgements

The work was funded by the Department of Science and Technology of Yunan Province (approved nos 2004C0001Z, 2005NG03 and 2005NG05). We are grateful to Dr Xiaowei Huang, Dr Minghe Mo and Dr Linqian Dong for their help in preparing this manuscript.

References

Bonants, P.J., Fitters, P.F., Thijs, H., Den-Belder, E., Waalwijk, C. and Henfling, J.W.D.M. (1995) A basic serine protease from *Paecilomyces lilacinus* with biological activity against *Meloidogyne hapla* eggs. *Microbiology* **141**, 775–784.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantitation of microgram

quantities of protein utilizing the principle of protein-dye binding. *Annu Rev Biochem* **72**, 248–254.

Cox, G.N., Kusch, M. and Edgar, R.S. (1981) Cuticle of *Caenorhabditis elegans*: its isolation and partial characterization. *J Cell Biol* **90**, 7–17.

Dackman, C., Chet, I. and Nordbring-Hertz, B. (1989) Fungal parasitism of the cyst nematode *Heterodera schachtii*: infection and enzymatic activity. *FEMS Microbiol Lett* **62**, 201–208.

Huang, X.W., Zhao, N.H. and Zhang, K.Q. (2005) Extracellular enzymes serving as virulence factors in nematophagous fungi involved in infection of the host. *Res Microbiol* **115**, 811–816.

Jansson, H.B. and Nordbring-Hertz, B. (1988) Diseases of nematodes: infection mechanisms in the fungus-nematode system. In *Diseases of Nematodes*, vol. 2, ed. Poinas, G.O. Jr and Jansson, H.-B. pp. 59–72. Boca Raton, FL: CRC Press.

Jansson, H.B., Tunlid, A. and Nordbring-Hertz, B. (1997) *Fungal Biotechnology: Biological Control of Nematodes*. Weinheim: Chapman & Hall pp. 38–50.

Kang, S.C., Park, S. and Lee, D.G. (1999) Purification and characterization of a novel chitinase from the entomopathogenic fungus, *Metarhizium anisopliae*. *J Invertebr Pathol* **73**, 276–281.

Khan, A., Williams, K.L. and Nevalainen, H.K.M. (2004) Effects of *Paecilomyces lilacinus* protease and chitinase on the eggshell structures and hatching of *Meloidogyne javanica* juveniles. *Biol Control* **31**, 346–352.

Laemmli, E.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Li, G.H., Shen, Y.M. and Zhang, K.Q. (2005) Nematicidal activity and chemical component of *Poria cocos*. *J Microbiol* **43**, 17–20.

Liu, X.F. and Zhang, K.Q. (2003) *Dactylella shizishanna* sp. nov. from Shizi Moutain, China. *Fungal Divers* **14**, 103–107.

Lopez-Llrca, L.V. and Robertson, W.M. (1992) Immumocytochemical locolization of a 32-kDa protease from the nematophagous fungus *Verticillium suchlasporium* in infected nematode eggs. *Exp Mycol* **16**, 261–267.

Peterson, G.L. (1977) A simplification of the protein assay method of Lowry et al., which is more generally applicable. *Annu Rev Biochem* **83**, 346–356.

Rosenzweig, W.D. and Pramer, D. (1980) Influence of cadmium, zinc, and lead on growth, trap formation, and collagenase activity of nematode trapping fungi. *Appl Environ Microbiol* **40**, 694–696.

Schenck, S., Chase, T. Jr, Rosenzweig, W.D. and Pramer, D. (1980) Collagenase production by nematode-trapping fungi. *Appl Enviro Microbiol* **40**, 567–570.

Segers, R., Butt, T.M., Kerry, B.R. and Peberdy, J.F. (1994) The nematophagous fungus *Verticillium chlamydosporium* produces a chymoelastase-like protease which hydrolyses host nematode proteins *in situ*. *Microbiology* **140**, 2715–2723.

Tikhonov, V.E., Lopez-Llorca, L.V., Salinas, J. and Jansson, H.B. (2002) Purification and characterization of chitinasas from the nematophagous fungi *Verticillium chlamydosporium* and *V. suchlasporium*. *Fungal Genet Biol* **35**, 67–78.

Tunlid, A. and Jansson, S. (1991) Proteases and their involvement in the nematophagous fungus *Arthrobotrys oligospora*. *Appl Environ Microbiol* **57**, 2868–2872.

Tunlid, A., Rosen, S., Ek, B. and Rask, L. (1994) Purification and characterization of an extracellular serine protease from the nematode-trapping fungus *Arthrobotrys oligospora*. *Microbiology* **140**, 1687–1695.

Yang, J.K., Huang, X.W., Tian, B.Y., Wang, M., Niu, Q.H. and Zhang, K.Q. (2005) Isolation and characterization of a serine protease from the nematophagous fungus, *Lecanicillium psalliotae*, displaying nematicidal activity. *Biotechnol Lett* **27**, 1123–1128.

Zhao, M.L., Mo, M.H. and Zhang, K.Q. (2004) Characterization of a neutral serine protease and its full-length cDNA from the nematode-trapping fungus *Arthrobotrys oligospora*. *Mycologia* **96**, 16–22.

