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INHIBITION OF CHITINOLYTIC ACTIVITIES FROM TREE SPECIES AND ASSOCIATED FUNGI

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Key Word Index—*Pinus sylvestris*; Pinaceae; *Eucalyptus pilularis*; Myrtaceae; roots; fungi; β-*N*-acetylglucosaminidase; chitinase; inhibition; allosamidin; (2-acetamido-2-deoxy-D-glucopyranosylidene)aminophenylcarbamate.

Abstract—Effects of two inhibitors, allosamidin and (2-acetamido-2-deoxy-D-glucopyranosylidene) amino phenylcarbamate (PUGNAC), have been assessed on chitinolytic activities of two plants, *Pinus sylvestris* L. and *Eucalyptus pilularis* Sm., and of seven fungi. *Pinus sylvestris* and *E. pilularis* root endochitinase activities were inhibited by allosamidin. Activities of *P. sylvestris* were more sensitive to inhibition than those of *E. pilularis*. The mechanism of inhibition varied with the plant species and the enzyme involved. PUGNAC inhibited β-*N*-acetylglucosaminidase and exochitinase activities in root extracts from both plant species. In all cases PUGNAC acted as a reversible competitive inhibitor. Both inhibitors also affected chitinolytic activities from the fungi screened. Allosamidin inhibited endochitinase activities from both the mycorrhizal and pathogenic fungi tested. In addition, exochitinase activity from the ectomycorrhizal fungus *Paxillus involutus* (Batsch) Fr. was inhibited by allosamidin. PUGNAC inhibited β-*N*-acetylglucosaminidase activity from all the fungi tested. PUGNAC was also a potent inhibitor of both exo- and endochitinase activities from the fungi, except *P. involutus*. Competitive inhibition was the most common form. These findings show allosamidin does inhibit endochitinase activity in plants and the ability of PUGNAC to inhibit not only β-*N*-acetylglucosaminidase activity but also fungal endochitinase activity may be useful to distinguish between host and fungal endochitinase activities in symbiotic or pathogenic dual systems.

INTRODUCTION

Chitinase activities (EC 3.2.1.14) have been reported in a wide variety of taxonomically diverse plant species despite the absence of chitin. Plant chitinase activity is induced in response to a wide variety of abiotic or physical stresses, but it is the increased activity in response to pathogens as part of the pathogenesis-related (PR) response which has received most attention [1]. Root systems of higher plants are also infected by mutualistic symbiotic microorganisms, most notably those forming mycorrhizal associations [2], but there have been only a limited number of studies on host chitinase in symbiotic systems. Chitinolytic activities have been reported to increase transiently during establishment of the arbuscular mycorrhizal association [3,4]. In contrast, host chitinase has been postulated to aid in the development of the Hartig net structure during formation of the ectomycorrhizal association [5,6].

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Apart from a few exceptions, chitin is ubiquitous in fungi [7] and all chitin-containing fungi investigated thus far produce chitinases [8]. Fungi also produce β-*N*-acetylglucosaminidase (EC 3.2.1.30) activity [9] which, although not a chitinase enzyme *per se*, forms part of the chitinolytic enzyme system necessary for the complete degradation of chitin. We have previously shown that β-*N*-acetylglucosaminidase activity produced by the host may be an important response to fungal invasion [10]. In order to investigate the role of host chitinolytic activities when invaded by either mutualistic or pathogenic fungi it is important that activities from both sources can be distinguished when in dual culture. In this study we assessed the potential of two inhibitors, allosamidin, a specific chitinase inhibitor [11,12], and (2-acetamido-2-deoxy-D-glucopyranosylidene)aminophenylcarbamate (PUGNAC) a synthetic analogue of *N*-acetylglucosamine reported to be an effective inhibitor of β-*N*-acetylglucosaminidase activity [13], to discriminate between chitinolytic activities from *Pinus sylvestris* L. and *Eucalyptus pilularis* Sm. and several pathogenic and mycorrhizal fungi commonly associated with these two plant species. Assays used four fluorogenic substrates, 4-methylumbelliferyl-glucosides of *N*-acetylglucosamine,

its dimer, trimer and tetramer, (4MU-CGlcNAc)$_{1-4}$. β-*N*-Acetylglucosaminidase, exochitinase and endochitinase activities are indicated by hydrolysis of 4MU-GlcNAc, 4MU-(GlcNAc)$_2$ and 4MU-(GlcNAc)$_{3,4}$, respectively, following published definitions [14, 15].

RESULTS

Enzyme activities

All extracts tested showed hydrolytic activity towards 4MU-GlcNAc, and all except those from *P. cinnamomi* showed hydrolytic activity towards 4MU-(GlcNAc)$_2$, 4MU-(GlcNAc)$_3$ and 4MU-(GlcNAc)$_4$.

Inhibition by allosamidin

The effects of various concentrations of allosamidin on chitinolytic activities are illustrated by Figs 1 and 2. The estimated IC_{50} values are summarized in Table 1. Allosamidin did not inhibit β-*N*-acetylglucosaminidase activity from any of the species tested (Figs 1 and 2). It was also relatively ineffective against exochitinase activity (i.e. activity against 4MU-(GlcNAc)$_2$) with only that from *P. involutus* being affected. For all species, addition of allosamidin resulted in a marked decrease in one or both of the endochitinase activities (i.e. activity against 4MU-(GlcNAc)$_{3,4}$). In the root extract of the two plant species, i.e. *P. sylvestris* and *E. pilularis*, activity against both 4MU-(GlcNAc)$_{3,4}$ was inhibited. Activity against 4MU-(GlcNAc)$_4$ was less sensitive to allosamidin than activity against 4MU-(GlcNAc)$_3$ for both plant species (Table 1). Endochitinase activity from *E. pilularis* root extract was less sensitive to allosamidin than that of *P. sylvestris*. At the higher allosamidin concentrations, *P. sylvestris* root endochitinase activities were completely inhibited whereas those from *E. pilularis* were not. This suggests *E. pilularis* has a complex endochitinase system, composed of two or more enzymes, which have different sensitivities to allosamidin. Thus, as allosamidin concentration increased the sensitive enzymes became inhibited but the resistant ones were still functional and maintained a level of activity (see Fig. 1, *E. pilularis* activity against 4MU-(GlcNAc)$_3$). This may also explain the higher IC_{50} values required for *E. pilularis* compared to *P. sylvestris*.

Allosamidin had a range of inhibitory effects on the chitinases of the fungi tested. It inhibited endochitinase activities of the ectomycorrhizal fungi against both 4MU-(GlcNAc)$_{3,4}$. Activity against 4MU-(GlcNAc)$_2$ was also inhibited for *P. involutus*, the only species to exhibit such an effect. Extracts of *B. cavipes* and *S. variegatus* were the most sensitive of the fungi tested to inhibition by allosamidin. Extracts of the two pathogenic fungi tested, *A. ostoyae* and *H. annosum*, were generally insensitive to inhibition by allosamidin. In both species only the activity against 4MU-(GlcNAc)$_4$ was inhibited sufficiently to obtain an IC_{50} value. These values were 58 μM allosamidin for *A. ostoyae* and 70 μM allosamidin for *H. annosum*, and were approximately 120–145 times the IC_{50} value for *P. tinctorius* and 10–12 times the IC_{50} value for *E. pilularis*, the most resistant ectomycorrhizal fungi and plant spp. tested against the same substrate (Table 1).

At higher allosamidin concentrations, endochitinase activities from *P. involutus* were completely inhibited. Endochitinase activities of the other ectomycorrhizal fungi, with the exception of *P. tinctorius* activity against 4MU-(GlcNAc)$_4$ appeared to be reaching a state where further inhibition was not obtained.

Double-reciprocal plots of enzyme activity of the extracts with and without allosamidin at its estimated IC_{50} value allowed the mode of inhibition of allosamidin on the test species to be determined. The results showed that allosamidin could act in four different inhibitory fashions depending on the extract and substrate used (Table 1).

Inhibition by PUGNAC

PUGNAC inhibited β-*N*-acetylglucosaminidase activity in all the species (i.e. activity against 4MU-GlcNAc) and activity against other substrates in many of the species (Figs 3 and 4a, b). The estimated IC_{50} values are

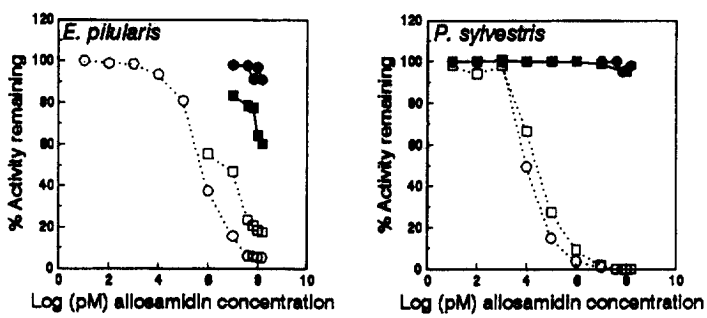

Fig. 1. Percentage inhibition of chitinolytic activity from the root extract (soluble fraction) at pH 5.0 (0.1 M MES buffer) with allosamidin, assayed against each of the four substrates: (●) 4MU-GlcNAc; (■) 4MU-(GlcNAc)$_2$; (○) 4MU-(GlcNAc)$_3$; (□) 4MU-(GlcNAc)$_4$. The results are expressed as a percentage of activity remaining where the activity in absence of allosamidin is taken as 100%. Results are mean values of duplicate wells.

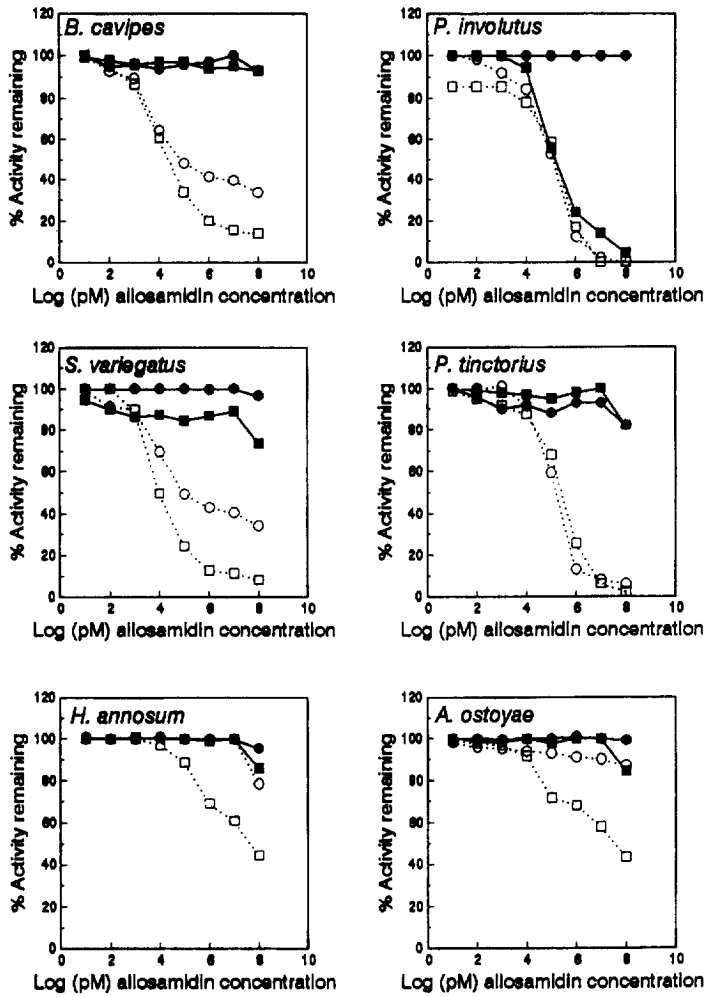

Fig. 2. Percentage inhibition of chitinolytic activity of the soluble cell-bound extract of the six fungal spp. at pH 5.0 (0.1 M MES buffer) with allosamidin, assayed against each of the four substrates (symbols are as for Fig. 1). The results are expressed as a percentage of activity remaining where the activity in absence of allosamidin is taken as 100%. Results are mean values of duplicate wells.

summarised in Table 2. PUGNAC inhibited activity against 4MU-GlcNAc and 4MU-$(GlcNAc)_2$ in *P. sylvestris* and *E. pilularis* root extracts, but did not affect endochitinase activities. Against 4MU-GlcNAc the IC_{50} values required for a 50% drop in enzyme activity were 650 nM for *P. sylvestris* and 600 nM for *E. pilularis*. The IC_{50} values obtained against 4MU-$(GlcNAc)_2$ were lower, i.e. 410 nM for *P. sylvestris* and 270 nM for *E. pilularis*. The IC_{50} values obtained against exochitinase activity from *P. tinctorius*, *S. variegatus*, *B. cavipes* and *H. annosum* were also lower than those obtained for the corresponding β-*N*-acetylglucosaminidase activities. For *A. ostoyae* activities against 4MU-$(GlcNAc)_{1-3}$ were inhibited by PUGNAC, but in this case as the size of the 4MU-$(GlcNAc)_{1-4}$ substrate increased the potency of PUGNAC decreased (Table 2). In the case of *P. involutus*, PUGNAC was only effective at inhibiting activity against 4MU-GlcNAc (Fig. 4b). PUGNAC inhibited β-*N*-acetylglucosaminidase activity from *P. cinnamomi* (Fig. 4a) with an estimated IC_{50} value of 240 nM. Generally the IC_{50} values suggest that PUGNAC is a more potent inhibitor of exochitinase activity than β-*N*-acetylglucosaminidase activity (Table 2). The potency of PUGNAC as an inhibitor of fungal endochitinase activities differed according to the species; e.g. *P. tinctorius* endochitinase activities were less inhibited by PUGNAC than its β-*N*-acetylglucosaminidase activity whereas for *S. variegatus* the reverse was observed. At the higher concentrations β-*N*-acetylglucosaminidase activity from the fungal species, except *S. variegatus* and *H. annosum*, was inhibited the most, and endochitinase activity the least, except for *H. annosum* (Fig. 4b).

The amount of PUGNAC required for a 50% decrease in enzyme activity (IC_{50} value) was lowest against the chitinolytic activities from *P. tinctorius*. These results show that *P. tinctorius* was the most sensitive to inhibition by PUGNAC of the fungi tested. Inhibition of β-*N*-acetylglucosaminidase from *P. cinnamomi* required

Table 1. Effect of allosamidin on chitinase activities

Source	Substrate	IC_{50}*(nM)	Mode of inhibition
Plant			
Pinus sylvestris (root)	4MU-$(GlcNAc)_3$	10	Non-competitive
	4MU-$(GlcNAc)_4$	25	Mixed
Eucalyptus pilularis (root)	4MU-$(GlcNAc)_3$	560	Mixed
	4MU-$(GlcNAc)_4$	6000	Competitive
Fungi			
Paxillus involutus	4MU-$(GlcNAc)_2$	270	Competitive
	4MU-$(GlcNAc)_3$	150	Competitive
	4MU-$(GlcNAc)_4$	290	Competitive
Pisolithus tinctorius	4MU-$(GlcNAc)_3$	290	Competitive
	4MU-$(GlcNAc)_4$	480	Competitive
Suillus variegatus	4MU-$(GlcNAc)_3$	100	Competitive
	4MU-$(GlcNAc)_4$	10	Competitive
Boletinus cavipes	4MU-$(GlcNAc)_3$	90	Uncompetitive
	4MU-$(GlcNAc)_4$	45	Mixed
Armillaria ostoyae	4MU-$(GlcNAc)_4$	58000	Mixed
Heterobasidion annosum	4MU-$(GlcNAc)_4$	70000	Uncompetitive

*Concentration giving 50% inhibition.

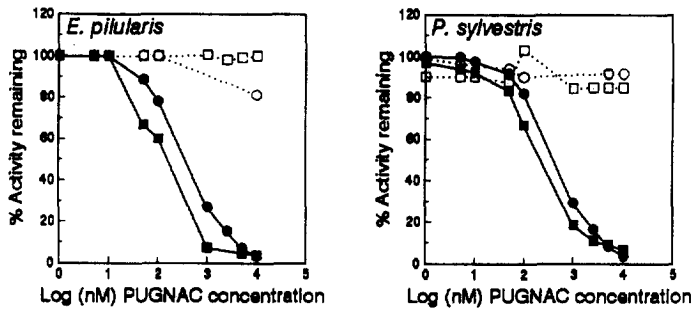

Fig. 3. Percentage inhibition of chitinolytic activity from the root extract (soluble fraction) at pH 5.0 (0.1 M MES buffer) with PUGNAC, assayed against each of the four substrates (symbols as for Fig. 1). The results are expressed as a percentage of activity remaining where the activity in absence of PUGNAC is taken as 100%. Results are mean values of duplicate wells.

a relatively low PUGNAC concentration (i.e. 240 nM) compared to this corresponding activity in the other fungi. Activity of *P. involutus* was the least inhibited with only activity against 4MU-GlcNAc affected, which required a higher concentration of PUGNAC than the other fungi, except *H. annosum*. The two pathogenic basidiomycetes required the highest concentration of PUGNAC for inhibition of activity against 4MU-$(GlcNAc)_{1-2}$ for *H. annosum* and 4MU-$(GlcNAc)_3$ for *A. ostoyae*.

Double-reciprocal plots of crude extract with and without PUGNAC at its estimated IC_{50} value allowed the mode of inhibition of PUGNAC on the test species to be determined. As observed for allosamidin, PUGNAC could act in four different inhibitory modes depending on the extract and substrate used (Table 2), with competitive inhibition the most common form.

DISCUSSION

The potency of allosamidin as an inhibitor of chitinolytic activities from the ectomycorrhizal fungal species used in this study is similar to that reported in the literature for other fungi [16–18]. However, against the two pathogenic basidiomycetes, *A. ostoyae* and *H. annosum*, the IC_{50} values for inhibition of 4MU-$(GlcNAc)_4$ were approximately 36 and 44 times those recorded for *Neurospora crassa* [19].

A study reported that allosamidin had no effect on the chitinolytic activities of yam when used at concentrations up to 100 μM [20]. In contrast the work described here showed that allosamidin is a potent inhibitor of endochitinase activities of *P. sylvestris* root extract, and also an inhibitor of the activities of *E. pilularis* root extract at concentrations between one and two orders of magnitude greater.

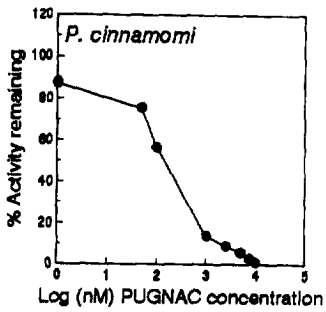

Fig. 4a. Percentage inhibition of β-*N*-acetylglucosaminidase activity from the soluble cell-bound extract of *P. cinnamomi* assayed against 4MU-GlcNAc (●) with PUGNAC and 0.1 M MES buffer (pH 5.0). The results are expressed as a percentage of activity remaining where the activity in absence of PUGNAC is taken as 100%. Results are mean values of duplicate wells.

Allosamidin had no inhibitory affect on β-*N*-acetylglucosaminidase activities from any of the sources used which is consistent with the findings of Koga *et al.* [20]. Generally only endochitinase activities, with the exception of exochitinase activities from *P. involutus*, were affected.

β-*N*-Acetylglucosaminidases are ubiquitous glycoprotein-processing enzymes occurring in a wide range of sources that have no apparent chitinase system *per se*. They occur frequently as isoenzymes, i.e. multiple forms occurring in a single species [21]. These multiple forms may play different roles such as cell wall processing [22,23], nutrition [9] and pathogenesis [24].

PUGNAC inhibited β-*N*-acetylglucosaminidase activities from all the test species, but in most cases inhibition was more potent against chitinase, particularly exochitinase, activities. The results in this study suggest

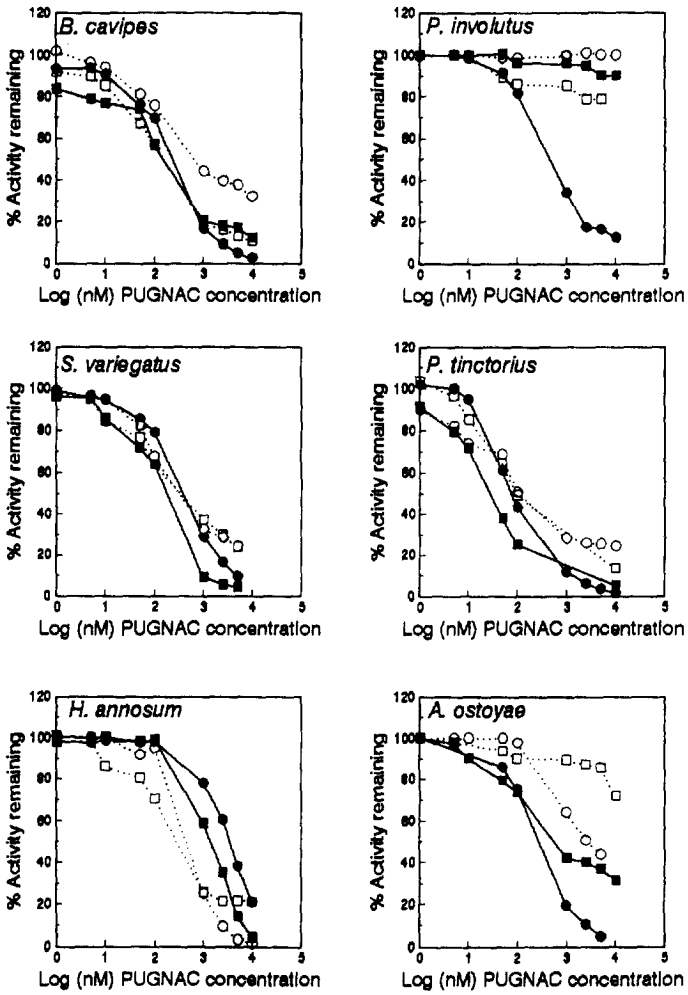

Fig. 4b. Percentage inhibition of chitinolytic activity of the soluble cell-bound extract of the six fungal spp. at pH 5.0 (0.1 M MES buffer) with PUGNAC, assayed against each of the four substrates (symbols are as for Fig. 1.). The results are expressed as a percentage of activity remaining where the activity in absence of PUGNAC is taken as 100%. Results are mean values of duplicate wells.

Table 2. Effect of PUGNAC on chitinolytic activities

Source	Substrate	IC_{50}* (nM)	Mode of inhibition
Plant			
Pinus sylvestris (root)	4MU-GlcNAc	650	Competitive
	4MU-$(GlcNAc)_2$	410	Competitive
Eucalyptus pilularis (root)	4MU-GlcNAc	600	Competitive
	4MU-$(GlcNAc)_2$	270	Competitive
Fungi			
Paxillus involutus	4MU-GlcNAc	700	Uncompetitive
Pisolithus tinctorius	4MU-GlcNAc	80	Competitive
	4MU-$(GlcNAc)_2$	35	Competitive
	4MU-$(GlcNAc)_3$	100	Non-competitive
	4MU-$(GlcNAc)_4$	95	Competitive
Suillus variegatus	4MU-GlcNAc	610	Competitive
	4MU-$(GlcNAc)_2$	320	Competitive
	4MU-$(GlcNAc)_3$	550	Uncompetitive
	4MU-$(GlcNAc)_4$	550	Mixed
Boletinus cavipes	4MU-GlcNAc	430	Non-competitive
	4MU-$(GlcNAc)_2$	250	Non-competitive
	4MU-$(GlcNAc)_3$	820	Non-competitive
	4MU-$(GlcNAc)_4$	250	Competitive
Armillaria ostoyae	4MU-GlcNAc	500	Competitive
	4MU-$(GlcNAc)_2$	800	Non-competitive
	4MU-$(GlcNAc)_3$	2500	Competitive
Heterobasidion annosum	4MU-GlcNAc	3600	Competitive
	4MU-$(GlcNAc)_2$	1500	Competitive
	4MU-$(GlcNAc)_3$	680	Competitive
	4MU-$(GlcNAc)_4$	500	Competiti8ve
Phytophthora cinnamomi	4MU-GlcNAc	240	Competitive

*Concentration giving 50% inhibition.

PUGNAC is not a specific inhibitor of β-*N*-acetylglucosaminidase activity. These findings are in contrast to the results of Arnold [25] who observed that PUGNAC was a specific inhibitor of β-*N*-acetylglucosaminidase activities from the fungus *Pneumocystis carinii* and the nematode, *Heligmosomoides polygyrus*. Arnold [25] obtained these results by use of the 4MU-$(GlcNAc)_{1-4}$ substrates as employed in the present study. This would suggest that the differences observed for PUGNAC specificity are due to origin of the extract and not differences in structure of the fluorogenic substrates tested. However, there is some evidence that chitinolytic activities that degrade naturally occurring chitin oligomers may not exhibit the same preference for artificial substrates [21, 26]. Hence it is possible that some activity may have been undetected if the chitinolytic activities from the fungi and plant species used in this study differed in their ability to cleave the artificial 4MU-$(GlcNAc)_{1-4}$ substrates.

PUGNAC has been reported to be a more potent inhibitor of β-*N*-acetylglucosaminidase activities than other synthetic analogues [13]. Concentrations of PUGNAC required to cause a 50% decrease in β-*N*-acetylglucosaminidase activities in this study were between two and four orders of magnitude greater than the IC_{50} values reported using extracts from *P. carinii* (0.07 nM) and *H. polygyrus* (0.34 nM) [25]. However the K_i value reported (i.e. 100 nM) for PUGNAC against *Canavalia ensiformis* [13] is of the same order of magnitude to the IC_{50} values obtained for the two plants used in this study. Horsch *et al.* [13] reported competitive inhibition. This was also the predominant mode observed in this study although, as observed for allosamidin, the other three types were also observed.

In conclusion, this study showed that allosamidin is an effective inhibitor of both plant and fungal endochitinase activities. The synthetic inhibitor PUGNAC inhibited endochitinase activities from several of the fungi but was ineffective against plant endochitinase activity, therefore suggesting PUGNAC could be used to distinguish between host and certain fungal endochitinase activities when in association.

EXPERIMENTAL

Tree seedlings. Pinus sylvestris L. (87 (4008), from UK Forestry Commission) and *Eucalyptus pilularis* Sm. (1449 DC 6, from Dr Anne E. Ashford, Faculty of Biological & Behaviourial Sciences, University of New South Wales, Australia) were used for all experiments. Seeds were taken from refrigerated storage and maintained for 5 days at 4° in sterile distilled water before surface sterilization by shaking with 30% H_2O_2 for 15 min

(*P. sylvestris*) [27] or 40% H_2O_2: 60% ethanol (*E. pilularis*). Seeds were rinsed with 3 l sterile distilled H_2O, transferred to Petri dishes containing 1.5% water agar and germinated at 20° in the dark.

When radicles were approximately 6 mm in length seedlings were aseptically transferred to appropriate growing systems. A modified version of the slit Petri dish technique [28] was used for *P. sylvestris* seedlings and the enclosed seedling technique [29] for *E. pilularis* seedlings. Petri dishes (90 × 15 mm) were filled with 50 ml of the sugar-free medium of Wong and Fortin [28]. The medium was solidified with 1.5% agar and buffered with 25 mM MES (2-(*N*-morpholino)-ethanesulphonic acid) with pH adjusted to 5.5 using NaOH prior to autoclaving to give a final pH of 5.4. A 90 mm circle of boiled and autoclaved Cellophane was placed on top of the agar and the seedling laid on top of the Cellophane. A hot spatula was used to cut a slit into the side of the Petri dishes and their covers for *P.* sylvestris seedlings so that the shoot protruded but the root remained in aseptic conditions. Autoclaved lanolin (Sigma Chemical Co., St Louis, MO, USA) was used to seal the slit. The root systems of both species were overlain with sheets of Whatman No. 1 filter paper (70 mm diameter). All Petri dishes were sealed using adhesive tape.

Plants were then transferred to Fitotron growth cabinets where a combination of fluorescent tubes and incandescent bulbs provided a photon flux density of approximately 250 $\mu E\,m^{-2}\,s^{-1}$ at plant level. The relative humidity was set at maximum (95%) with an 18 hr, 18° day and 6 hr, 8° night for *P. sylvestris* and a 25° day, 8° night for *E. pilularis*.

Fungal cultures. The fungi studied were *Pisolithus tinctorius* (Pers.) Coker and Couch, *Paxillus involutus* (Batsch) Fr., *Suillus variegatus* (Fr.) O. Kuntze, *Boletinus cavipes* (Opat.) Kalchbr. (ectomycorrhizal basidiomycetes); *Heterobasidion annosum* (Fr.) Karst., *Armillaria ostoyae* Romagn. (root-infecting basidiomycete pathogens); and *Phytophthora cinnamomi* Rands. Cultures were maintained on modified Melin–Norkans (MMN) agar [30] at 20° in the dark. For experimentation they were grown on an overlay of Cellophane.

Enzyme extraction. Root systems of 7-week-old *P. sylestris* and 9-week-old *E. pilularis* seedlings were excised, ground with a pestle in a mortar containing liquid nitrogen and suspended in 4 ml 0.1 M sodium citrate buffer (pH 5.0) g^{-1} fresh weight of root tissue. The buffer contained PVPP (BDH, Poole, Dorset, UK) at a concentration of 250 mg g^{-1} fresh weight of tissue [31], 5 mM EDTA and an intensive phenolic removal treatment suggested by Gegenheimer [32]. The homogenate was centrifuged (11 600 × *g* for 20 min) and the supernatant collected and desalted using Sephadex gel (G-25, Sigma). This desalted supernatant was assayed for β-*N*-acetylglucosaminidase, exochitinase and endochitinase activities using a range of fluorogenic 4-methylumbelliferyl substrates (4MU-$(GlcNAc)_{1-4}$) [15].

The same protocol was used for enzyme extraction from fungal tissue except that the intensive phenolic removal treatment was not used. Cultures aged 5 weeks were used with the exception of *P. cinnamomi* (5 days).

Enzyme substrates and inhibitors. The fluorogenic substrates were 4-methylumbelliferyl glycosides of *N*-acetylglucosamine oligosaccharides (4MU-$(GlcNAc)_{1-4}$) [15] supplied by Sigma (monomer, dimer and trimer) and Janssen Biochimica, Belgium (tetramer). Stock solutions of monomer, dimer and trimer were prepared at a concentration of 0.8 mM in distilled water. Tetramer (0.8 mM) was prepared in 50% ethanol. Stocks were stored at − 20°.

The inhibitors, allosamidin, obtained from Dr S. Suzuki (Tokyo, Japan), and PUGNAC, obtained from CarboGen (Zurich, Switzerland) were each dissolved as a 1 mM stock solution in sterile distilled water. All inhibitor solutions were stored at − 20° until required.

Chitinolytic assays. Amounts of inhibitor stock solutions were added to each of four wells of a microtitre plate containing 0.1 M MES buffer, pH 5.0, to obtain required final concentrations in a total assay volume of 150 μl. The volume of test sample (fungal or plant root extract) was added in 5 μl portions to each test well, along with 5 μl of the appropriate fluorogenic substrate (4MU-$(GlcNAc)_{1-4}$) to initiate the reaction. Controls consisted of buffer blanks with allosamidin or PUGNAC. Fluorescence was monitored (excitation 355 nm, emission 460 nm) using a Fluoroskan II Microtitre plate fluorometer. The microtitre plate was incubated at 37°. As maximum fluorescence of 4-methylumbelliferone (4MU) is obtained at alkaline pH the reaction was terminated after 60 min by the addition of 100 μl of 1 M NaOH and fluorescence was measured immediately. This gave a single point assay of the maximum fluorescence due to 4-methylumbelliferone released by chitinolytic activity.

In the second part of the study to obtain Lineweaver–Burk plots, substrate concentration was varied whilst maintaining the total assay volume of 150 μl. The allosamidin or PUGNAC concentration used in the second part of this study was the IC_{50} value (i.e. the inhibitor concentration found to cause a 50% drop in enzyme activity) obtained from the first part of the study. Specific activity of chitinolytic enzymes was expressed in terms of mg protein. Protein contents of plant and fungal extracts were determined by the method of Bradford [33].

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