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Mode of action and antifungal properties of two cold-adapted chitinases

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Abstract The mode of action of two chitinases from the Antarctic *Arthrobacter* sp. strain TAD20 on *N*-acetyl-chitooligomers and chitin polymers has been elucidated. Identification of the length of chitin oligomers following enzymatic hydrolysis was verified by using HPLC-based analysis. It was observed that the length of the oligomer is important for enzyme action. The enzymes cannot effectively hydrolyze chitin oligomers with a degree of polymerization lower than four. *Ar*ChiA is an endochitinase which hydrolyzes chitin substrates randomly, whereas *Ar*ChiB is an exochitinase which degrades chitin chains and *N*-acetyl-chitooligomers from the nonreducing end, releasing*N*-*N*′-diacetyl-chitobiose. *Ar*ChiB (100 μg/ml) inhibited spore germination and hyphal elongation of the phytopathogenic fungus *Botrytis cinerea* by 15% and 30%, respectively. A more pronounced effect was observed with *Ar*ChiA (100 μg/ml) resulting in 70% inhibition of spore germination and 60% inhibition of germ tube elongation. A slight additive effect was observed, when the two enzymes were used in combination, only on the inhibition of germ tube elongation.

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Introduction

Chitinases are glycosyl hydrolases that catalyze the degradation of chitin, a fibrous, insoluble polysaccharide, consisting of β-1,4-*N*-acetyl-glucosamine residues (GlcNAc). Based on their sequence similarities, chitinases have been grouped in two distinct families in the glycosyl-hydrolase classification system (Henrissat and Bairoch 1996): family 18 which includes chitinases from bacteria, fungi, animals and certain plants, consists of a catalytic domain of (β/α_8) topology; and family 19, comprising mainly chitinases from plants, which resemble the structure of lysozyme. The structures of several family-18 chitinases have been elucidated (Hollis et al. 2000; Perrakis et al. 1994; Terwisscha van Scheltinga et al. 1994; van Aalten et al. 2000) and their catalytic mechanisms investigated (Papanikolau et al. 2001; Sakuda 1996; van Aalten et al. 2001; Watanabe et al. 2001). Furthermore, according to the mode of action of these enzymes on chitooligomers and chitin polymers, they can be classified as endochitinases, exochitinases, and β-*N*-acetyl-glucosaminidases (Gooday 1994). Endochitinases cleave randomly β-1,4-glycosidic bonds of chitin; exochitinases cleave the chitin chain either from the reducing or the nonreducing end, producing*N*,*N*′-diacetyl-chitobiose ($GlcNAc_2$); and β-*N*-acetyl- glucosaminidases hydrolyze $GlcNAc_2$ and *N*-acetyl-chitooligosaccharides from the nonreducing end, producing GlcNAc (Gooday 1994).

Many chitinolytic bacteria such as *Serratia marcescens* and *Aeromonas* spp., have been reported to produce more than one type of chitinase (Brurberg et al. 1996; Shiro et al. 1996). Efficient chitin degradation is assumed to be performed by the synergistic action of chitinases exhibiting a different mode of action on chitin substrates (Brurberg et al. 1996). A synergistic effect is also quite

common among several other classes of glycosyl-hydrolases (Boisset et al. 2000; Gaudin et al. 2000).

Chitinolytic enzymes have been studied as potential antifungal agents against pathogens (Frankowski et al. 2001). Genes encoding cell-wall-degrading enzymes, especially chitinases, have been used to increase plant resistance to fungal pathogens. The gene *ech42* from the mycoparasitic fungus *Trichoderma harzianum* (coding for an endochitinase exhibiting antifungal activity), transferred to tobacco and potato, was found to improve disease resistance of the transgenic plants to a variety of fungal pathogens (Lorito et al. 1998).

Cloning and sequencing of the *archi*A and *archi*B genes from the Antarctic bacterium *Arthrobacter* strain TAD20 has been previously reported (Lonhienne et al. 2001a). The enzymes *Ar*ChiA and*Ar*ChiB belong to glycosyl-hydrolase family 18 and consist of a catalytic domain and two and one chitin binding domains, respectively. In this study the mode of action of *Ar*ChiA and *Ar*ChiB on *N*-acetyl-chitooligomers and chitin polymers was examined. The direct antifungal effect of the two purified enzymes, applied both alone and in combination, on spore germination and germ tube elongation was also investigated.

Materials and methods

Enzyme purification

Arthrobacter sp. strain TAD20 was obtained from sea sediments at the Dumont d'Urville Antarctic station (60°40′ S, 40°01′ E) and the native form of *Ar*ChiA was purified from *Arthrobacter* sp. TAD20 as previously described (Lonhienne et al. 2001a). Expression of the gene encoding *Ar*ChiB was performed in BL21(DE3) *E. coli* cells (Novogen, Berkshire, UK) according to the manufacturer's instructions. A 4-l culture was grown at 20°C and induced with 0.1 mM IPTG when the culture reached an OD_{600} 0.6–0.8. The culture was centrifuged after 12 h and the supernatant containing *Ar*ChiB was concentrated to a final volume of 200 ml. Proteins were precipitated using 1.8 M $(NH_4)_2SO_4$and the sample was centrifuged for 1 h at 10,000 rpm. The pellet was re-suspended in 50 mM HEPES buffer pH 7.4, and $(NH_4)_2SO_4$was added to a final concentration of 1 M. The sample was centrifuged and the supernatant was loaded to a Phenyl-Sepharose fast flow column, previously equilibrated with a buffer containing 1 M $(NH_4)_2SO_4$. A $(NH_4)_2SO_4$ degreasing gradient (1.0 to 0 M) in a buffer containing 50 mM HEPES pH 7.4 was applied. Fractions exhibiting chitinase activity were pooled and concentrated to a final protein concentration of 1.5 mg/ml. Enzyme assays were performed as previously described (Lonhienne et al. 2001a). Both enzymes were stored at −20°C, in 20% glycerol, 0.5 mM EDTA, and protease inhibitors (PMSF, leupeptine, benzamidine, all at 1 mM final concentrations). Prior to use they were dialyzed against 5 mM HEPES pH 7.3.

Separation of*N*-acetyl-chitooligosaccharides

All reactions were performed at 20°C, in a buffer containing 5 mM HEPES pH 7.5 buffer. *Ar*ChiA or *Ar*ChiB (1 μg) was incubated with each chitooligosaccharide substrate ($GlcNAc_2$ to $GlcNAc_6$, 10 μg) for 1 and 16 h in a final volume of 30 μl. In the case of polymeric chitin substrates, 25 μg of either colloidal chitin prepared from crab shell chitin (Jeuniaux 1966) or poly-*N*-acetylglucosamine from diatoms (a kind gift from Dr. J. Vournakis) were incubated with 1 μg of either of the two enzymes for 1 and 28 h. Separation of reaction products was performed using a Waters 600/480 HPLC system on a μ-Bondapak-NH_2 column (Waters, Milford, MA, USA) by using a mobile phase of distilled H_2O : acetonitrile 25:75 at a flow rate of 3 ml/min and a pressure of −1300 psi.

Separation of the anomeric forms of *N*-acetyl-chitooligosaccharides

Separation of the anomeric forms of *N*-acetyl-chitooligosaccharides ($GlcNAc_n$,$n = 1$–6; Sigma) was performed on a TSK-Amide 80 column (Tosoh Biosep, Stuttgart, Germany) by using a mobile phase of distilled H_2O : acetonitrile 20:80 at a flow rate of 1 ml/min and a pressure of −1,500 psi. Elution of *N*-acetyl-chitooligosaccharides from the column was monitored at 210 nm. Samples containing *N*-acetyl-chitooligosaccharides ($GlcNAc_2$-$GlcNAc_6$, 10 μg) in a total volume of 30 μl were injected onto the column in order to estimate the retention time for each sugar. *Ar*ChiA or *Ar*ChiB (1 μg) were incubated with $GlcNAc_6$ (50 μg) for 30 and 60 min in a total volume of 90 μl, and reaction products were analyzed by HPLC on the TSK-Amide-80 column. Incubation of *Ar*ChiB with poly-*N*-acetyl-glucosamine resulted in the production of $GlcNAc_2$. This product was separated initially by HPLC using a μ-Bondapak-NH_2 column, concentrated to a volume of 30 μl with a Speedvac and further analyzed on the TSK-Amide-80 column.

Bioassays

The antifungal properties of purified chitinases were tested in vitro against *Botrytis cinerea* strain 309 as previously described (Lorito et al. 1993). Flat-bottomed ELISA plates containing assay mixtures with the enzymes and fungal spores were observed at different times using an inverted microscope for evaluation of spore germination and germ tube elongation. All assays included controls containing sterile water and multiple replicates of treatments and controls. Each experiment was repeated on two separate days and the results were combined for calculating final mean and standard deviations. The control values for spore germination and germ tube elongation were considered as no inhibition; and percentage inhibition was the value from other treatments divided by control value, multiplied by 100. Dose response curves and ED_{50} values (50% effective dose) were calculated for each treatment by probit analysis. Limpel's formula as described by Richer (1987) was used to determine antifungal synergistic interactions between the two chitinolytic enzymes. Limpel's formula is $E_e = X + Y - (XY/100)$, where E_e is the expected effect from additive responses of two inhibitory agents, and X and Y are the percentages of inhibition relative to each single agent. Therefore, synergism exists if the combination of the two agents produces any value of inhibition greater than E_e.

Results and discussion

In this report we present the mode of action of two chitinases from the Antarctic *Arthrobacter* sp. strain TAD2O on both *N*-acetyl-chitooligomers and chitin polymers as well as the antifungal properties of the enzymes.

Mode of action of ArchiA and ArchiB on chitin polymers

When ArchiA was incubated (28 h) with either fully acetylated chitin from diatoms (poly-*N*-acetylglucosamine) or colloidal chitin,*N*-acetyl-chitooligomers of various degrees of polymerization were identified (Fig. 1B). This is in agreement with an endochitinase

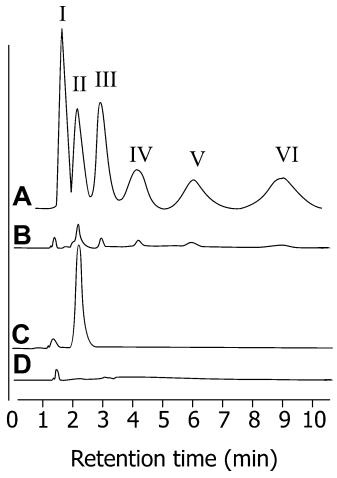

Fig. 1A–D HPLC analysis of the reaction products of poly-*N*-acetyl-glucosamine with *Ar*ChiA and *Ar*ChiB. **A** Resolution of control samples of *N*-acetyl-chitooligosaccharides (*I* GlcNAc, *II* $GlcNAc_2$, *III* $GlcNAc_3$, *IV* $GlcNAc_4$, *V* $GlcNAc_5$, *VI* $GlcNAc_6$). **B** Resolution of the reaction products of *Ar*ChiA with poly-*N*-acetylglucosamine. **C** Resolution of the reaction products of *Ar*ChiB with poly-*N*-acetylglucosamine. **D** Elution profile of poly-*N*-acetylglucosamine incubated with inactivated *Ar*ChiA and *Ar*ChiB

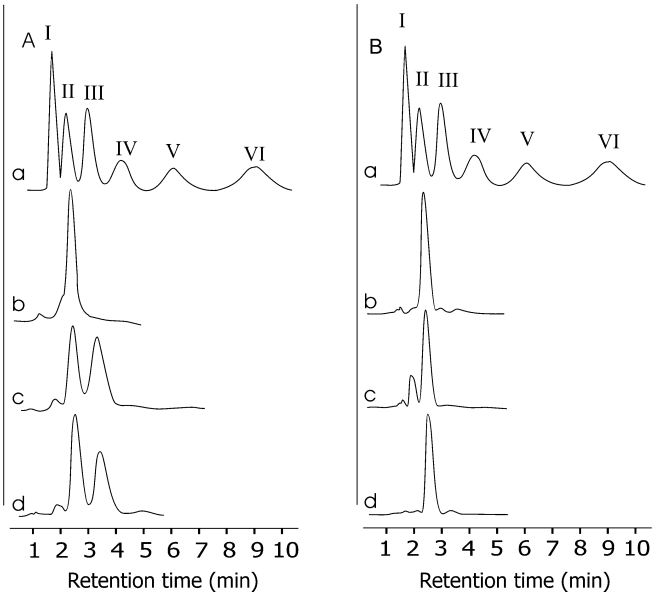

Fig. 2 HPLC analysis of the reaction products of *N*-acetyl-chitooligosaccharides with *Ar*ChiA (**A**) and *Ar*ChiB (**B**) after 16 h. *a* Resolution of control samples of*N*-acetyl-chitooligosaccharides (*I* GlcNAc, *II* $GlcNAc_2$, *III* $GlcNAc_3$, *IV* $GlcNAc_4$, *V* $GlcNAc_5$, *VI* $GlcNAc_6$). *b*, *c*, *d* Resolution of the reaction products of *Ar*ChiA and *Ar*ChiB with $GlcNAc_4$ (*b*), $GlcNAc_5$ (*c*), $GlcNAc_6$ (*d*)

mode of action (endo-type action) whereas the enzyme hydrolyzes chitin substrates in a random fashion. However, when ArchiB was incubated with the same chitin polymers, $GlcNAc_2$ was identified from the early up until the late stages of the reaction, indicating that this enzyme is an exo-chitinase which releases $GlcNAc_2$ in a processive fashion (exo-type action) from one end of the substrate (Fig. 1C). The term processivity is used to indicate that an enzyme degrades a polymer chain without dissociating from it between successive catalytic events.

Mode of action of ArchiA and ArchiB on *N*-acetyl chitooligosaccharides

ArchiA or ArchiB could not effectively hydrolyze chitin oligomers with a degree of polymerization lower than four, although $GlcNAc_3$ could be hydrolyzed by either of the two enzymes but only after prolonged incubation (16 h, data not shown). ArchiA hydrolyzed $GlcNAc_4$ to produce only $GlcNAc_2$ (Fig. 2Ab). Furthermore $GlcNAc_5$ and $GlcNAc_6$ were hydrolyzed by this enzyme to produce $GlcNAc_2$ and $GlcNAc_3$ (Fig. 2Ac,d). $GlcNAc_6$ was initially hydrolyzed by ArchiA to $GlcNAc_3$ and $GlcNAc_4$. $GlcNAc_4$ was further hydrolyzed to produce $GlcNAc_2$ (Fig. 3B).

These results are in agreement with the endo-type mode of action of this enzyme shown on chitin polymers. ArchiB hydrolyzed $GlcNAc_4$ and $GlcNAc_6$ to produce $GlcNAc_2$ (Fig. 2Bb,d). Furthermore, the enzyme hydrolyzed $GlcNAc_5$ to produce $GlcNAc_2$ and $GlcNAc_3$ (Fig. 2Bc). These results are consistent with the exo-type mode of action of the enzyme shown on chitin polymers.

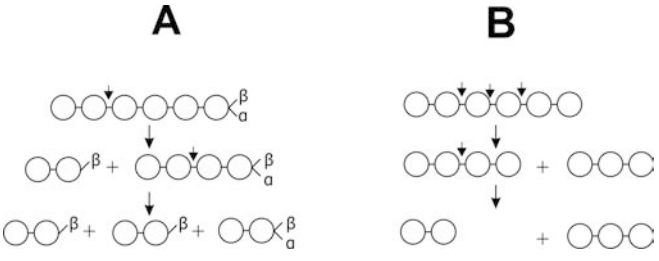

Fig. 3 Hydrolysis products of $GlcNAc_6$ by ArChiB and ArChiA. $GlcNAc_6$ is initially hydrolyzed by ArChiB to $GlcNAc_2$ exhibiting the β anomeric conformation and $GlcNAc_4$ exhibiting both the α and β conformation. $GlcNAc_4$ is further hydrolyzed to produce $GlcNAc_2$ exhibiting mainly the β anomeric conformation (**A**). $GlcNAc_6$ is initially hydrolyzed by ArChiA to $GlcNAc_4$ and $GlcNAc_3$. $GlcNAc_4$ is further hydrolyzed to produce $GlcNAc_2$ (**B**)

Identification of a and β anomeric forms of *N*-acetyl chitooligosaccharides

In order to reveal the orientation of hydrolysis of chitin polymers and *N*-acetyl-chitooligosaccharides by *Ar*ChiB, we further examined the anomeric conformation of the *N*-acetyl-chitooligosaccharides resulting from hydrolysis of chitinous substrates. Family 18 chitinases are retaining enzymes (Armand et al. 1994), therefore the products resulting from hydrolysis of*N*-acetyl-chitooligomers and chitin polymers by *Ar*ChiA and *Ar*ChiB should retain the β anomeric conformation. Only the products containing the original reducing end -OH should exhibit both the α and β anomeric conformations in a ratio of 1:0.6 (Koga et al. 1998). Each *N*-acetyl-chitooligosaccharide

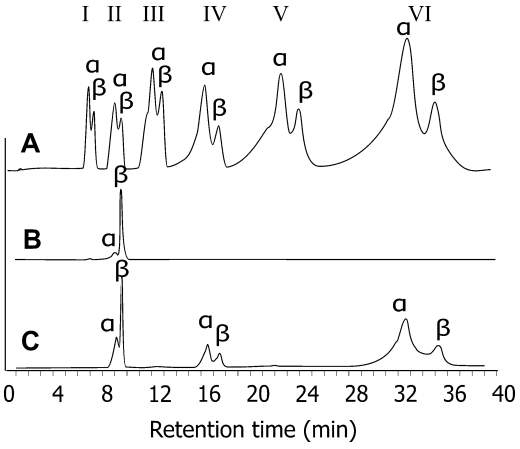

Fig. 4A–C HPLC analysis of anomeric forms of *N*-acetyl-chitooligosaccarides resulting from hydrolysis of poly-*N*-acetylglucosamine and $GlcNAc_6$ with *Ar*ChiB. **A** Retention time of control samples of *N*-acetyl-chitooligosaccharides (*I* GlcNAc, *II* $GlcNAc_2$, *III* $GlcNAc_3$, *IV* $GlcNAc_4$, *V* $GlcNAc_5$, *VI* $GlcNAc_6$). **B** Resolution of the anomeric forms of the $GlcNAc_2$ following incubation of *Ar*ChiB with poly-*N*-acetylglucosamine. **C** Resolution of the anomeric forms of *N*-acetyl-chitooligosaccharides released following incubation of *Ar*ChiB with $GlcNAc_6$

Table 1 *N*-acetyl-chitooligosaccharide products resulting from hydrolysis of various chitinous substrates by *Ar*ChiA and *Ar*ChiB

Substrate	*Ar*ChiA End products	*Ar*ChiB End products
Poly-*N*-acetyl glucosamine	Several chitooligosaccharides	$GlcNAc_2$
Colloidal chitin	Several chitooligosaccharides	$GlcNAc_2$
$GlcNAc_4$	$GlcNAc_2$	$GlcNAc_2$
$GlcNAc_5$	$GlcNAc_2$	$GlcNAc_2$
	$GlcNAc_3$	$GlcNAc_3$
$GlcNAc_6$	$GlcNAc_2$	$GlcNAc_2$
	$GlcNAc_3$	

was eluted from the TSK- Amide-80 column into two neighboring peaks, representing the α anomeric form (earlier peak) and the β anomeric form (later peak) (A/Banat at al 1999; Koga et al. 1998) (Fig. 4A). If *Ar*ChiB was an exochitinase releasing $GlcNAc_2$ from the nonreducing end of the chitin chain then the $GlcNAc_2$ produced should exhibit only the β anomeric conformation (Fig. 3A). However, if the enzyme was an exochitinase acting in a processive fashion from the reducing end, then the released $GlcNAc_2$ should exhibit both the α and β anomeric conformations. The end products resulting from hydrolysis of chitin polymers and*N*-acetyl-chitooligomers ($GlcNAc_4$ –$GlcNAc_6$) by *Ar*ChiA and*Ar*ChiB are presented in Table 1. Analysis of $GlcNAc_2$ resulting from hydrolysis of poly-*N*-acetylglucosamine by *Ar*ChiB revealed that it exhibited almost exclusively the β anomeric conformation (Fig. 4B). Furthermore, analysis of the initial reaction products $GlcNAc_2$ and $GlcNAc_4$ resulting from hydrolysis of $GlcNAc_6$ by*Ar*ChiB revealed that $GlcNAc_2$exhibited the β anomeric form while $GlcNAc_4$ exhibited both anomeric conformations with ratios similar to the α and β anomeric forms of $GlcNAc_6$ (Fig. 4C). $GlcNAc_6$ is initially hydrolyzed by *Ar*ChiB to $GlcNAc_2$ and $GlcNAc_4$ (Fig. 3A). Subsequently, $GlcNAc_4$ is further hydrolyzed by this enzyme to $GlcNAc_2$ (Fig. 3A). These results suggest that *Ar*ChiB cleaves both poly-*N*-acetylglucosamine and $GlcNAc_6$ from the nonreducing end in a processive fashion, producing mainly β-$GlcNAc_2$.

Recently, the structural basis of the mode of action of two exochitinases from *Seratia marcescens* has been elucidated (van Aalten et al. 2001). *Sm*ChiA and *Sm*ChiB contain one chitin binding domain (ChBD) and cleave chitin in a processive fashion to produce *N*,*N*′-diacetyl-chitobiose from the reducing and the nonreducing end, respectively. Similarly to *Sm*ChiB, *Ar*ChiB contains one ChBD and cleaves chitin from the nonreducing end producing *N*-*N*′-diacetyl-chitobiose. It is reasonable to assume that exochitinases require only one ChBD, which results in a more efficient hydrolysis of chitin substrates from either the reducing or the nonreducing end of the chitin chain (Watanabe et al. 1993).

Bioassays

Both purified enzymes ArchiA and ArchiB exhibited an inhibitory effect on spore germination and germ tube elongation of the important plant pathogen *B. cinerea* in vitro. The inhibitory effect of ArchiA was more pronounced than that of ArchiB. This is in agreement with previous reports according to which endochitinases have stronger antifungal activity than exochitinases (Lorito et al. 1998). Using an enzyme concentration of 100 μg/ml, the conidial germination and germ tube elongation were reduced by 70% and 60%, respectively, by ArchiA, and by 15% and 30%, respectively, by ArchiB (Fig. 5A, B). A concentration of ArchiA of 200 μg/ml inhibited both conidial germination and hyphal elongation by more than 80%. This enzyme had an ED_{50} value estimated by probit analysis of 45 μg/ml for inhibition of spore germination and 65 μg/ml for germ tube elongation, while for ArchiB the ED_{50} values were 185 μg/ml and 130 μg/ml, respectively (Fig. 5A, B). An apparent, although not very high, additive effect was observed for the inhibition of germ tube elongation, but not spore germination, by using a combination of the two enzymes (Fig. 5). A combination of both enzymes, at a concentration of 100 μg/ml each, resulted in about 70% reduction of spore germination (ED_{50} of 60 μg/ml) and germ tube elongation (ED_{50} of 40 μg/ml). Application of Limpel's formula indicates that there was no significant additive effect with the enzyme mixture on the inhibition of conidial germination. A slight additive effect at 200 μg/ml enzyme concentration for both ArchiA and ArchiB was observed for the inhibition of germ tube elongation (E_e of about 70% vs observed effect of about 90%). Since the enzyme concentrations required for a strong

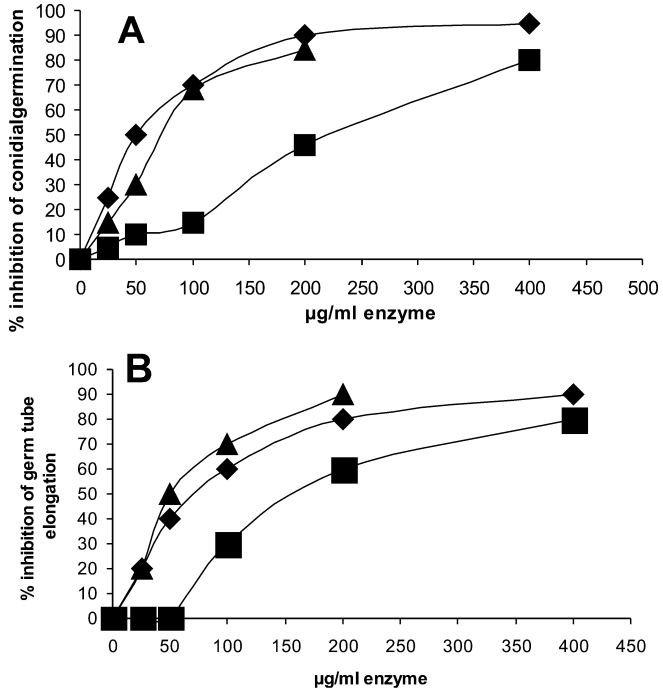

Fig. 5 Inhibition effect of different concentrations of *Ar*ChiA (*diamonds*), *Ar*ChiB (*squares*) and a combination of both enzymes (*triangles*) on **A** conidial germination, and **B** germ tube elongation of *Botrytis cinerea* strain 309. ($P < 0.10$ for all experiments combined, for each condition.) The in vitro tests were previously described by Lorito et al. (1993). On both graphs, standard deviation varied from 1.5 to 9.5% inhibition units

antifungal effect appear to be high, it is doubtful whether these bacteria have a significant mycotoxic or antagonistic activity.

The additive effect of chitinases *Ar*ChiA and *Ar*ChiB could result in an efficient degradation of chitinous substrates (i.e., fungal cell walls or epicuticle of decapod crustaceans) encountered in the environment of this psychrophilic *Arthrobacter* sp. strain (Frankowski et al. 2001). The extracellular endochitinase *Ar*ChiA may hydrolyze chitin to produce chitooligomers or short polymers that are further cleaved by *Ar*ChiB to produce *N*,*N*′-diacetyl-chitobiose. *N*,*N*′-diacetyl-chitobiose is further hydrolyzed to *N*-acetyl-glucosamine by chitobiase from the same bacterial strain which has been found to be cell-bound, thus possibly supporting the uptake of GlcNAc (Lonhienne et al. 2001b). Both chitinases exhibit typical features of psychrophilic enzymes, i.e., an increased catalytic efficiency at low temperatures and a lower thermal stability (Gerday et al. 2000; Zecchinon et al. 2001).

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