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ORIGINAL PAPER

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Purification and properties of two chitinolytic enzymes of *Serratia plymuthica* HRO-C48

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Abstract The chitinolytic rhizobacterium *Serratia plymuthica* HRO-C48 was previously selected as a biocontrol agent of phytopathogenic fungi. One endochitinase (E.C. 3.2.1.14), CHIT60, and one *N*-acetyl-β-1,4-D-hexosaminidase (E.C. 3.2.1.52), CHIT100, were purified and characterized. The endochitinase CHIT60, with an apparent molecular mass of 60.5 kDa, had a N-terminal amino acid sequence highly similar to that of chitinases A from *Serratia liquefaciens* and *Serratia marcescens*. The enzyme activity had its peak at 55 °C and pH 5.4, and increased by more than 20% in the presence of 10 mM Ca^{2+}, Co^{2+} or Mn^{2+}. Activity was inhibited by 80% in the presence of 10 mM Cu^{2+}. CHIT100 appeared to be a monomeric enzyme with a molecular mass of 95.6 kDa and a pI of 6.8. Optimal activity was obtained at 43 °C and pH 6.6, and decreased by more than 90 % in the presence of 10 mM Co^{2+} or Cu^{2+}. CHIT100 (100 μg ml^{-1}) inhibited spore germination and germ tube elongation of the phytopathogenic fungus *Botrytis cinerea* by 28 % and 31.6 %, respectively. With CHIT60 (100 μg ml^{-1}), the effect was more pronounced: 78 % inhibition of of germination and 63.9 % inhibition of germ tube elongation.

Keywords *Serratia plymuthica* · Chitinase · Biocontrol

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Introduction

Chitin, an insoluble linear 1,4-β-glucosidically linked polymer of *N*-acetylglucosamine, is a major structural component of most fungal cell walls. Chitinases catalyze the hydrolysis of chitin, and several chitinolytic bacteria, e. g. *Serratia marcescens*, *Aeromonas caviae*, *Enterobacter agglomerans* and *Pseudomonas fluorescens*, have been shown to be potent biological control agents protecting plants against pathogenic fungi (Ordentlich 1988; Inbar and Chet 1991; Chernin 1995; Neiendam Nielsen and Sorensen 1999).

Serratia plymuthica strain HRO-C48 was isolated from the rhizosphere of oilseed rape and found to be an antagonist of phytopathogenic fungi such as *Verticillium dahliae* Kleb., *Rhizoctonia solani* Kühn and *Sclerotinia sclerotiorum* (Lib.) De Bary (Kalbe et al. 1996). Strain HRO-C48 was shown to be an efficacious biocontrol agent protecting strawberries against soil-borne pathogens (Berg et al. 1999; Kurze et al. 2001). *S. plymuthica* strain HRO-C48 was selected as a biocontrol agent according to the following criteria: (1) high chitinolytic activity (Frankowski et al. 1998; Berg et al. 2000); (2) production of the plant growth hormone indole-3-acetic acid (Kalbe et al. 1996); (3) relative harmlessness to human health and environment (Anonymous 1990); (4) unique molecular fingerprints (Berg 2000); and (5) low-level antibiotic resistance (Berg 2000). This bacterium produces siderophores and compounds with antibiotic activity (Berg G, unpublished observation), although the antifungal activity in dual culture assay and *ad planta* appeared to depend on the secretion of chitinolytic enzymes (Frankowski et al. 1998).

The purpose of this study was to purify and partially characterize the chitinolytic system of *S. plymuthica* HRO-C48 and to investigate the possible involvement of these enzymes in the antagonism of this bacterium against fungal plant pathogens.

Materials and methods

Cultures and growth media

Serratia plymuthica HRO-C48 (DSM 12502), isolated from the rhizosphere of oilseed rape (*Brassica napus* L.; Kalbe et al. 1996), was determined to be a strain of *S. plymuthica* according to the 16S rDNA sequence, with 98.0 % identity to the type strain (Kurze et al. 2001). Bacteria were grown at 30 °C in nutrient broth (Sifin, Berlin, Germany) or in liquid semi-minimal medium (0.5 g NaCl, 1.62 g nutrient broth, 6 g M9 salts (dissolved in 1 l distilled water; Sigma, St. Louis, USA) supplemented with 2 % colloidal chitin (w/v) (Vessey and Pegg 1973) or 2 % glucose (w/v) as the sole carbon sources. *Botrytis cinerea* 1.12 was from the culture collection of the Institut für Phytopathologie, Universität Rostock.

Enzyme and protein assays

N-acetyl-β-1,4-D-hexosaminidase activity was measured by using the chromogenic substrate *p*-nitrophenyl-β-D-*N*-acetylglucosaminide (pNP-GlcNac, Sigma) according to the method of Roberts and Selitrennikoff (1988) with modifications (Frankowski et al. 1998). Standard reaction mixtures (total volume 50 µl) contained 0.5 mM pNP-GlcNac in 50 mM potassium phosphate buffer, pH 6.7. The reaction mixture was incubated at 37 °C for 10 min, after which the reaction was stopped by adding 50 µl 0.1 M NaOH. The enzyme activity was expressed as nkatal (nmol of nitrophenol released per second). Endochitinase activity was assayed by measuring the reduction of turbidity of a suspension of colloidal chitin (Tronsmo and Harman 1993). One enzyme unit was defined as the amount of enzyme required to reduce the turbidity of the chitin suspension by 5 % after incubation at 30 °C for 24 h. Additionally, *p*-nitrophenyl-β-D-*N,N´*-diacetylchitobioside [pNP-$(GlcNac)_2$, 0.3 mM in standard reaction mixture] and *p*-nitrophenyl-β-D-*N,N´,N´´*-triacetylchitotriose [pNP-$(GlcNac)_3$, 0.2 mM in standard reaction mixture] (both from Sigma) were used for the determination of substrate specificity of purified enzymes. In the pH optimum assay, 50 mM citric acid-potassium phosphate buffer was used at the various pH values indicated in Fig. 3. Fifty mM potassium phosphate buffer, pH 6.7, was used to determine temperature optimum and examine the effects of divalent metal ions or other compounds on enzyme activity. Protein concentrations were measured with the method of Bradford (1976) in microtiter plates by using bovine serum albumin (fraction V, Sigma) as the standard.

Gel electrophoresis

SDS- and isoelectric focusing (IEF)-PAGE were performed with the PHAST-System (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's directions. SDS-PAGE broad-range standards (Sigma) and Pharmacia broad-pI markers were used to estimate the molecular masses and the isoelectric points of the chitinolytic enzymes, respectively.

Purification of chitinases

The cultures were harvested by centrifugation, the supernatant was filtered (0.2 µm; Sartorius, Göttingen, Germany). Phenylmethylsulfonyl fluoride (final concentration: 0.2 mM) and EDTA (final concentration: 1 mM) were added to inhibit protease activity. The culture filtrate was then transferred into a dialysis tube and placed in polyethylene glycol (100,000 molecular weight) at 4 °C until the volume was reduced 20- to 30-fold. The concentrated culture filtrate was dialyzed against a 100-fold volume of 25 mM imidazole-HCl buffer, pH 7.4, at 4 °C overnight and then applied to a chromatofocusing column. The column was packed with PBE 94 (Pharmacia) equilibrated with the dialysis buffer and then eluted with Polybuffer 74 (Pharmacia), pH 4.9, according to the manufacturer's recommendations. Gel filtration chromatography was applied to determine the native molecular masses of purified enzymes. A Superdex 200 HiLoad 16/60 pg column (Pharmacia) was equilibrated and eluted at 0.5 ml min^{-1} with 50 mM potassium phosphate buffer, pH 6.7, containing 200 mM NaCl. Purified enzymes were applied by using an amount corresponding to 5 nkatal of enzyme activity, together with the gel-filtration standard proteins (Bio-Rad, Hercules, Calif., USA) thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and cyanocobalamin (1.3 kDa).

N-terminal amino acid sequencing

The N-terminal amino acid sequence of the purified CHIT60 was determined at the facilities of the Universität of Osnabrück by using a model 473A sequencer (Applied Biosystems, Foster City, Calif., USA).

Bioassays with *Botrytis cinerea* spores

The antifungal properties of the purified chitinases were tested in vitro against *Botrytis cinerea* 1.12 as described by Lorito et al. (1993).

Results

Enzyme production

A strong chitinolytic activity was detected in the culture supernatant when *Serratia plymuthica* HRO-C48 was grown for 72 h in liquid semi-minimal medium with 0.2 % colloidal chitin. All of the tested substrates [colloidal chitin and pNP-$(GlcNac)_{1–3}$] were hydrolyzed by the extracellular chitinases present in the culture supernatant. No detectable extracellular chitinase activity was found when the strain was cultivated on liquid semi-minimal medium containing 0.2 % glucose as the sole carbon source.

Purification and characterization of CHIT60 and CHIT100

The concentration of the culture supernatant by dialysis against polyethylene glycol resulted in a loss of activity towards pNP-GlcNac and colloidal chitin (Table1). However, the total endochitinase activity was enhanced by about 50% after chromatofocusing relative to the total endochitinase activity detected in the concentrated dialyzed supernatant. This recovery of enzyme activity was probably caused by the dilution of one or more unknown inhibitors that were concentrated during treatment with polyethylene glycol. After chromatofocusing, two fractions with chitinase activity were selected and further analyzed.

CHIT60

One fraction showed activity by using pNP-analogues of tri- and tetramers of *N*-acetyl-β-D-glucosamine as sub-

Table 1 Purification of CHIT60 and CHIT100. Enzyme activities were assayed using colloidal chitin (CHIT60) and pNP-GlcNac (CHIT100). For assay conditions and definition of enzyme units see Materials and methods

Purification step	Volume (ml)	Total protein (μg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification (-fold)
CHIT60						
Culture supernatant	960	66200	2178	33	100	1.0
Dialyzed concentrate	25	49307	871	18	40	0.5
Chromatofocusing	40	5091	1296	255	60	7.7
CHIT100						
Culture supernatant	960	6620	162	2.4	100	1.0
Dialyzed concentrate	25	49307	49	0.9	30	0.4
Chromatofocusing	22	718	37	51.7	23	21.5

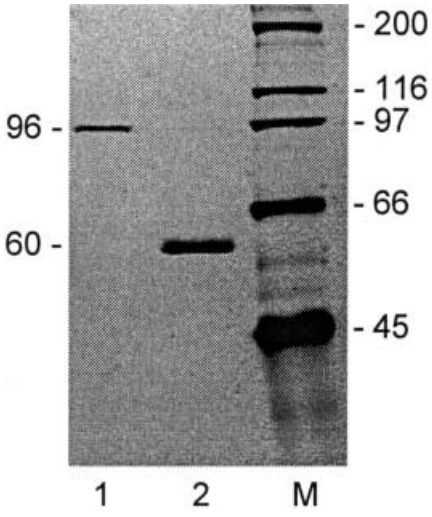

Fig. 1 SDS-PAGE analysis of purified chitinases of *Serratia plymuthica* HRO-C48. *Lane 1* CHIT100, *lane 2* CHIT60, *M* standard. Electrophoresis (7.5 % polyacrylamide) followed by silver staining followed the procedures described in the PHAST System manual (Pharmacia). The molecular masses (kDa) of the purified chitinases were calculated from a regression equation of the log of molecular mass of the standard proteins vs distance migrated at P=0.001

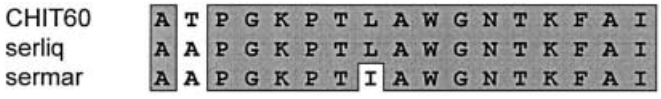

Fig. 2 Alignment of the N-terminal amino acid sequences of chitinase A mature proteins of different *Serratia* species. *CHIT60 S. plymuthica* HRO-C48 (this study), *serliq S. liquefaciens* (accession no. AAK07482), *sermar S. marcescens* (accession no. A25090). Amino acids identical to those of CHIT60 are *boxed*

strates, but not with the disaccharide analogue. Therefore the relative enzyme can be considered as an endochitinase (E.C. 3.2.1.14) (named CHIT60). SDS-PAGE of the fraction showed one band with an estimated molecular mass of 60.5 kDa (Fig. 1) and an isoelectric point of 5.0, as determined by IEF-PAGE. The analysis of the purified CHIT60 by gel filtration revealed a native molecular mass of 56 kDa, suggesting that it is a monomeric protein. The optimal conditions for the enzyme reaction of CHIT60 were 55 °C and pH 5.4 (Fig. 3A, B). The enzyme displayed at least 50 % activity in a range from 16 °C to 68 °C and pH 4.6 to pH 7.2, and at 4 °C CHIT60 still showed 30 % activity at its optimum. The activity was increased up to 240 % by adding 10 mM Ca^{2+}, Co^{2+} or Mn^{2+}, and reduced by 80 % in the presence of 10 mM Cu^{2+} (Fig. 4 A). CHIT60 chitinolytic activity was reduced by about 50 % when diacetylchitobiose at 1 mM (final concentration) was dissolved in the reaction.

The N-terminal amino acid sequence of CHIT60 was determined to be ATPGKPTLAWGNTKFAI and was found to have 94 % and 88 % identity to the N-terminal amino acid sequences of chitinases A from *Serratia liquefaciens* and *Serratia marcescens*, respectively (Fig. 2).

CHIT100

A second chromatofocusing fraction displayed activity on all the pNP-substrates tested, and therefore was considered to contain a *N*-acetyl-β-1,4-D-hexosaminidase (E.C. 3.2.1.52) (named CHIT100). SDS-PAGE of this fraction showed one band with a molecular mass of 95.6 kDa (Fig. 1) and an isoelectric point of 6.8. Analysis of purified CHIT100 by gel filtration revealed a native molecular mass of 94 kDa, suggesting that it is a monomeric protein. The optimal conditions for the enzyme activity of CHIT100 were 43 °C and pH 6.6 (Fig. 3C, D). The enzyme displayed at least 50 % of its maximum activity in a range from 24 °C to 58 °C and from pH 5.5 to pH 8.4. CHIT100 maintained 15 % of its activity at 4 °C. Divalent metal ions reduced activity by more than 90 % in the presence of 10 mM Co^{2+} and Cu^{2+} (Fig. 4B). When *N*-acetylglucosamine at 100 mM (final concentration) was dissolved in the reaction mixture, the enzyme activity on the substrates used was inhibited by about 40 %.

Bioassay

Both enzymes showed an inhibitory effect on spore germination and germ tube elongation of *Botrytis cinerea* in vitro (Fig. 5 A, B). At an enzyme concentration of 100 μg ml^{-1}, the reduction of germination and germ tube elongation caused by CHIT100 was 28 % and 31.6 %, respectively, and 78 % and 63.9 %, respectively, by CHIT60. The combination of both enzymes at concentrations of

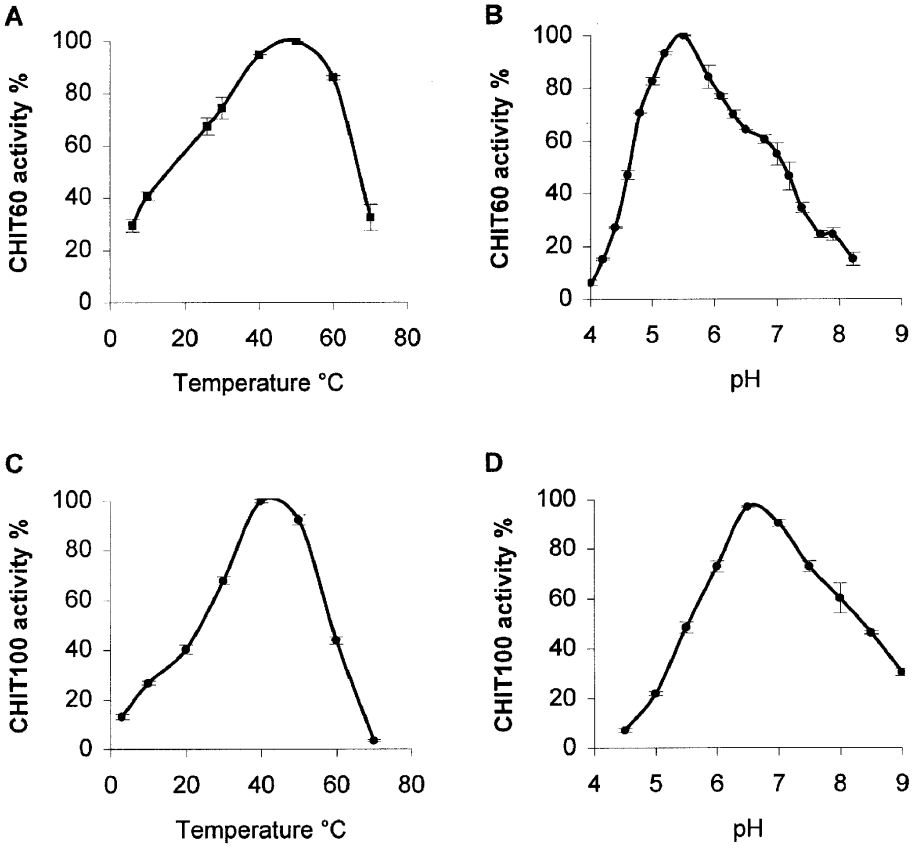

Fig. 3 Temperature and pH optimum of endochitinase CHIT60 (**A, B**) and *N*-acetyl-β-1,4-D-hexosaminidase CHIT100 (**C, D**). The *y*-axis represents the relative enzyme activity. Assay conditions: *p*-nitrophenol-β-D-*N,N′*-diacetylchitobioside (CHIT60) or *p*-nitrophenol-β-D-*N*-acetylglucosaminide (CHIT100) as substrates in 50 mM potassium phosphate buffer, pH 6.7, at various temperatures, or at 30 °C in 50 mM citric acid-potassium phosphate buffer at the pH indicated. Average results o three independent experiments with standard deviations are shown

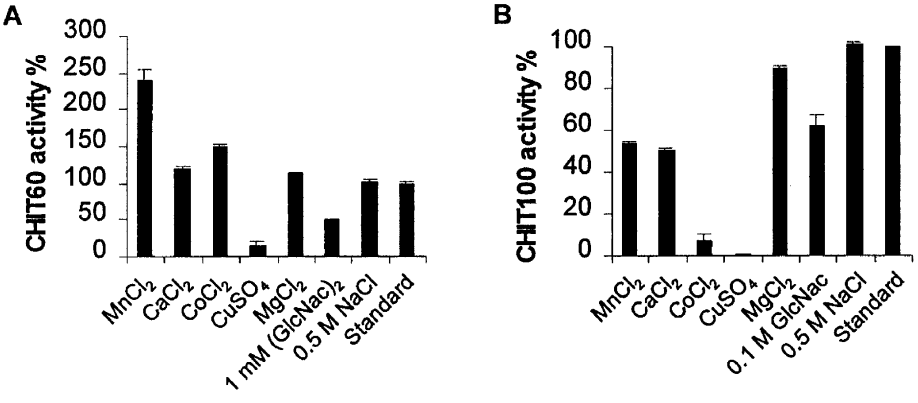

Fig. 4 Effect of divalent metal ions and other compounds on endochitinase activity of CHIT60 (**A**) or on *N*-acetyl-β-1,4-D-hexoseaminidase CHIT100 activity (**B**). *Right column* enzyme activity assayed in the presence of 10 mM of the indicated salts, relatively to the control. Assay conditions: *p*-nitrophenol-β-D-*N,N′*-diacetylchitobioside (CHIT60) or *p*-nitrophenole-β-D-*N*-acetylglucosaminide (CHIT100) in 50 mM potassium phosphate buffer, pH 6.7, at 30 °C. Average results of three independent experiments with standard deviations are shown

100 μg ml^{-1} (each) produced, respectively, a 76 % and 53.8 % inhibition of germination and hyphal elongation.

Discussion

Identification of chitinases

Serratia plymuthica HRO-C48 exhibited strong chitinolytic activity, as determined by degradation of colloidal chitin in liquid culture and by the release of pNP from chitooligosaccharide analogues. A set of three nitrophenol chitin derivatives was used to determine the chitinolytic activity of the purified proteins. According to the nomenclature suggested by Sahai and Manocha (1993), the strain *Serratia plymuthica* HRO-C48 produces two types of chitinases. CHIT60 hydrolyzed colloidal chitin and trimeric and tetrameric chitin derivatives but not the dimeric substrate, and was therefore determined to be an endochitinase (E.C. 3.2.1.14). CHIT100, which was able to release pNP from all three tested substrates, was considered to be a *N*-acetyl-β-1,4-D-hexosaminidase (E.C. 3.2.1.52). Further analysis of the reaction products accumulated by the two enzymes using TLC or HLPC is necessary to determine the enzymatic activities on natural substrates.

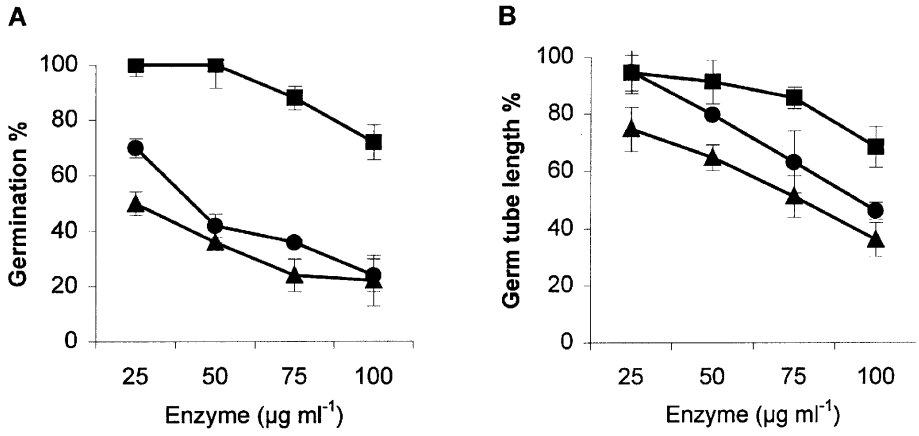

Fig. 5 Inhibition of conidial germination (**A**) and germ tube elongation (**B**) of *Botrytis cinerea in vitro* by different concentrations of the purified CHIT60 and CHIT100. *N*-acetyl-β-1,4-D-hexoseaminidase (*black squares*), endochitinase (*black triangles*) and combination of both (*black circles*). Each point represents the means (±SDs) of three replicates, with 100 conidia per replicate. Assay conditions are described by Lorito et al. (1993)

Enzymic properties of CHIT60 and CHIT100

Evidence indicating that CHIT60 is an endochitinase came not only from substrate usage tests, but also from the high similarity of the N-terminal amino acid sequence (Fig. 2) with that of the ChiA enzymes produced by *Serratia liquefaciens* (Choi et al. 2001), *Serratia marcescens* (Jones et al. 1986; Watanabe et al. 1997) and other bacteria (Chernin et al. 1997; Sitrit et al. 1995; Watanabe et al. 1990). The endochitinases CHIT60 and ChiA from *S. marcescens* (Brurberg et al. 1996) also share other characteristics, such as a broad pH optimum, a temperature optimum ranging between 50 and 60 °C and an apparent molecular mass of about 60 kDa. In contrast, the activity of CHIT60 differs from that of ChiA (Brurberg et al. 1996) by having a broader temperature optimum and a high susceptibility to divalent metal ions. The analysis of the reaction products of CHIT60 will be the subject of further studies to determine multiple chitinolytic activities, as reported for chitinase A of *S. marcescens* having exo-*N*,*N*′-diacetylchitobiohydrolase and endochitinase activity (Brurberg et al. 1996). Further work on cloning and sequencing the CHIT60-encoding gene will help to clarify the relationship of CHIT60 to other endochitinases.

N-acetyl-β-1,4-D-hexosaminidases have been found in culture filtrates of several bacteria, e.g. *Enterobacter agglomerans* (Chernin et al. 1995) and *Alteromonas* sp. (Tsujibo et al. 1995). Some of the encoding genes, including a chitobiase from *Serratia marcescens* (Kless et al. 1989), have been cloned; thus, isolation of the CHIT100 gene may reveal interesting similarities between enzymes synthesized by *Serratia* species.

Biotechnological application

The enzyme activities of CHIT60 and CHIT100 could complement each other to provide an efficient degradation of chitinous substrates (i.e. fungal cell walls) encountered in nature by *S. plymuthica* HRO-C48. In fact, the extracellular endochitinase CHIT60 may release from chitin oligomers or short polymers that are fully hydrolyzed by CHIT100 and reduced to *N*-acetylglucosamine. The major portion of the *N*-acetylhexosaminidase activity found in liquid cultures (0.2 % colloidal chitin) was actually cell-bound (data not shown), possibly supporting the efficient uptake of *N*-acetylglucosamine for nutrition. In addition, both the purified CHIT100 and CHIT60 showed a direct inhibitory activity on spore germination and germ tube elongation of the important plant pathogen *Botrytis cinerea*, but no synergistic effect could be observed when the two enzymes were combined. In contrast, synergistic effects on chitin hydrolysis were observed when different chitinases of *Serratia marcescens*, either alone (Brurberg et al. 1996) or together with a fungal endochitinase, were combined (Tronsmo and Harman 1993).

CHIT60 and CHIT100 share some general characteristics such as broad temperature and pH optima and relatively high activities at low temperatures. All of these properties are important for the applicability of *S. plymuthica* HRO-C48 or its chitinolytic enzymes in the biocontrol of plant pathogens (Kurze et al. 2001). and suggest the usefulness of additional work to characterize this new biocontrol agent fully.

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