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# Substrate specificity and antifungal activity of recombinant tobacco class I chitinases

Vivian Suarez[1], Christian Staehelin[2,3], Rafael Arango[1,4], Hauke Holtorf[1,5], Jan Hofsteenge[1] and Frederick Meins, Jr.[1,*]

[1]*Friedrich Miescher Institute, a branch of the Novartis Research Foundation, Maulbeerstrasse 66, 4058 Basel, Switzerland (*author for correspondence; e-mail: meins@fmi.ch); [2]Botanical Institute, University of Basel, Hebelstrasse 1, 4056 Basel, Switzerland; present addresses: [3]Laboratoire de Biologie Moléculaire des Plantes Supérieures, Université de Genève 1, chemin de l'Impératrice, 1292 Chambésy, Switzerland; [4]Departamento de Biología, Facultad de Ciencias, Universidad Nacional de Colombia, Sede Medellín, Autopista Norte Crs64 cl 65, Medellín, Colombia; [5]Plant Biotechnology, University of Freiburg, Sonnenstrasse 5, 79104 Freiburg, Germany*

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**Abstract**

Endochitinases contribute to the defence response of plants against chitin-containing pathogens. The vacuolar class I chitinases consist of an N-terminal cysteine-rich domain (CRD) linked by a glycine-threonine-rich spacer with 4-hydroxylated prolyl residues to the catalytic domain. We examined the functional role of the CRD and spacer region in class I chitinases by comparing wild-type chitinase A (CHN A) of *Nicotiana tabacum* with informative recombinant forms. The chitinases were expressed in transgenic *N. sylvestris* plants, purified to near homogeneity, and their structures confirmed by mass spectrometry and partial sequencing. The enzymes were tested for their substrate preference towards chitin, lipo-chitooligosaccharide Nod factors of *Rhizobium*, and bacterial peptidoglycans (lysozyme activity) as well as for their capacity to inhibit hyphal growth of *Trichoderma viride*. Deletion of the CRD and spacer alone or in combination resulted in a modest $<50\%$ reduction of hydrolytic activity relative to CHN A using colloidal chitin or *M. lysodeikticus* walls as substrates; whereas, antifungal activity was reduced by up to 80%. Relative to CHN A, a variant with two spacers in tandem, which binds chitin, showed very low hydrolytic activity towards chitin and Nod factors, but comparable lysozyme activity and enhanced antifungal activity. Neither hydrolytic activity, substrate specificity nor antifungal activity were strictly correlated with the CRD-mediated capacity to bind chitin. This suggests that the presence of the chitin-binding domain does not have a major influence on the functions of CHN A examined. Moreover, the results with the tandem-spacer variant raise the possibility that substantial chitinolytic activity is not essential for inhibition of *T. viride* growth by CHN A.

## Introduction

Endo-type chitinases (EC 3.2.1.14) are abundant proteins widely distributed in seed-plant species (reviewed by Meins *et al.*, 1992). They have been grouped into different classes on the basis of their primary structure (Neuhaus *et al.*, 1996; Hamel *et al.*, 1997). The class I, II and IV chitinases have homologous catalytic domains and correspond to glycosyl hydrolase family 19, while class III chitinases belong to glycosyl hydrolase family 18. Several functions for plant chitinases have been proposed. There is strong evidence that they play an important role in the defence of plants against fungal pathogens. Purified class I chitinases exhibit pronounced antifungal activity, particularly in combination with class I $\beta$-1,3-glucanases (e.g. Schlumbaum *et al.*, 1986; Mauch *et al.*, 1988). Moreover, plants over-expressing class I chitinase can show decreased susceptibility to infection by some fungi that have chitin-containing cell walls (Broglie *et al.*, 1991; Zhu *et al.*, 1994; Jongedijk

*et al.*, 1995). Certain plant chitinases also exhibit lysozyme activity and are able to hydrolyse the peptidoglycan of bacterial cell walls (e.g. Boller *et al.*, 1983; Brunner *et al.*, 1998; Mauch *et al.*, 1988). Possible functions of chitinases in symbiotic *Rhizobium*-legume interactions are suggested by the finding that lipo-chitooligosaccharide signals (Nod factors) produced by *Rhizobium* strains are $\beta$-1,4-linked oligomers of N-acetylglucosamine (GlcNAc) carrying a variety of substitutions. These molecules are required for nodule development in the roots of leguminous host plants (Perret *et al.*, 2000) and represent a new substrate class for plant chitinases (Staehelin *et al.*, 1994a, b; Minic *et al.*, 1998; Schultze *et al.*, 1998; Ovtsyna *et al.*, 2000). Finally, growing evidence suggests that endogenous substrates for chitinases exist in plants raising the possibility that chitinases may have a function in normal growth and plant development (Benhaumou and Asselin, 1989; de Jong *et al.*, 1995; Kragh *et al.*, 1996).

The present study focuses on vacuolar, antifungal class I chitinases that consist of an N-terminal, cysteine-rich domain (CRD), linked by a spacer region of variable length to a catalytic domain (reviewed by Neuhaus, 1999). The post-translational processing and intracellular transport of class I tobacco chitinase A (CHN A) have been studied in detail (Sticher *et al.*, 1992, 1993; Freydl *et al.*, 1995). CHN A is synthesized as a pre-pro-polypeptide with an N-terminal signal peptide for targeting to the lumen of the endoplasmic reticulum. After removal of the signal peptide but prior to sorting for secretion and vacuolar transport, the proenzyme undergoes prolyl hydroxylation at specific positions in the spacer region. The mature protein is generated late in intracellular transport by cleavage of a C-terminal heptapeptide, which is a vacuolar targeting signal (Neuhaus *et al.*, 1991b).

To clarify the contribution of the CRD and the spacer region to the catalytic activity, substrate specificity and antimicrobial activity of the enzyme, we constructed recombinants with different modifications of the tobacco class I chitinase gene *CHN48* encoding CHN A (Shinshi *et al.* 1990, van Buuren *et al.*, 1992). The recombinant and wild-type chitinases were purified from transgenic *Nicotiana sylvestris* plants. The proteins were tested for their substrate preference towards chitin, rhizobial Nod factors and bacterial peptidoglycan as well as for their capacity to inhibit hyphal growth of the fungus *Trichoderma viride*.

## Materials and methods

### *Isolation of a* N. sylvestris *chitinase genomic clone*

Unless indicated otherwise, recombinant DNA techniques were performed by standard methods (Sambrook *et al.*, 1989). Total RNA was isolated from leaves of ethylene-treated line SCIB2 empty-vector control *N. sylvestris* plants as described (Holtdorf *et al.*, 1999). Full-length cDNA clones representing a *N. sylvestris* chitinase were obtained by RT-PCR using the primers 5′-TCGGGATCCCGAAATGAGGCTT-3′ and 5′-GGACAAAGTCTTTGTTTATTATAAACG-3′ designed from conserved regions around the ATG and 3′-UTR of other class I chitinase genes. The PCR products were cloned into the pCRII plasmid (Invitrogen). Two independent cDNA clones were isolated and shown to be identical in sequence. A genomic fragment was amplified from DNA isolated from leaves of *N. sylvestris* with a plant DNAeasy kit (Qiagen) with the primers 5′-GAGAAATCGCGGCTTTCTTCG-3′ and 5′-TCCAC AAGGTCCGTAGTTGTAGTT-3′ designed from the cDNA clone. Positions of introns in the *N. sylvestris* gene were anticipated from positions of introns in the homeologous tobacco gene *CHN50* (van Buuren *et al.*, 1992).

### *Plasmid construction*

The wild-type and recombinant forms encoded by the binary vectors used are summarized in Figure 1. Plasmid pCHNA containing the coding sequence of tobacco class I chitinase gene *CHN48* encoding CHN A (Shinshi *et al.*, 1990) and plasmid p$\Delta$CS encoding a truncated CHN A lacking the CRD and the spacer region ($\Delta$CS CHN A) were constructed from pSCH10 (Neuhaus *et al.*, 1991a) and pSCM4 (Neuhaus *et al.*, 1991b), respectively, by excising the *Eco*RI fragment and cloning into the vector pBIB-HYG (Becker, 1990) containing the plant-selectable *hptII* gene. Plasmid p2S encoding CHN A with two copies of the CHN A spacer region in tandem (2S CHN A) was constructed from plasmid pSCH10 as follows. DNA encoding the chitin binding domain and the spacer region was PCR-amplified with primer 1 (5′-CATCGTTGAAGATGCCTC-3′) and primer 2 (5′-CAGGGCCCACCACCGGGTGGGGTGG-3′). The DNA encoding the spacer region and the catalytic domain was PCR-amplified with primer 3 (5′-CAGGGCCCGGTGGTCCCACACCTACACC-3′) and primer 4 (5′-TGATTTCAGCGGCATGA-3′). PCR

products were subcloned into pCR (Invitrogen), digested with *Eco*RI and *Apa*I and ligated into pUC19 to yield pUC-2SP. The *Eco*RI fragment of pUC-2SP was blunt-ended and subcloned into the *Sma*I site of pGY1 (Pietrzak *et al.*, 1986) to yield plasmid pGY-2SP. Finally, the *Eco*RI fragment of pGY-2SP was cloned into the *Eco*RI site of pBIB-HYG. Plasmid pΔS encoding CHN A with the spacer region deleted (ΔS CHN A) was constructed from pSCH10 in two PCR steps. Two separate primary PCR reactions were made with the following oligonucleotide pairs: primer 1, 5′-CATGTTGAAGATGCCTC-3′ and primer 2, 5′-GTGGTGGGCCTGGTGGTCCCAC-3′; and primer 3, 5′-TGATTTCAGCGGCATGC-3′ and primer 4, 5′-AGCCAGTGCGACCTCGGCAGT ATC-3′. The primary fragments obtained were used as target DNA for secondary PCR with primers 1 and 4. The resulting fragment was digested with *Sph*I and *Bam*HI and subcloned into the *Sph*I/*Bam*HI-digested pSCH10 to reconstruct the entire chitinase coding sequence and give an expression vector carrying the plant-selectable bacterial *nptII* gene. Modifications of the CHN A coding region were confirmed by DNA sequencing.

*Transformation and growth of plants*

Binary vectors were introduced into *Nicotiana sylvestris* plants by *Agrobacterium*-mediated leaf disc transformation and shoots were regenerated as described by Neuhaus *et al.* (1991a). Antibiotic-resistant plants were regenerated from transformed shoots and the presence of the transgenic proteins was confirmed by immunoblot analysis. Plants used for chitinase purification were raised from seed germinated in Petri dishes containing Linsmaier and Skoog (1965) agar medium without hormones supplemented with either 20 mg/l hygromycin B or 100 mg/l kanamycin, depending on the antibiotic-resistant marker used. After 5 weeks the seedlings were transferred to soil and grown to maturity in a greenhouse.

*Purification of chitinases*

Young leaves of transgenic *N. sylvestris* plants showing high levels of recombinant chitinase activity were homogenized in ascorbate buffer (500 mM sodium ascorbate pH 5.0, 1 mM EDTA). Extraction and all subsequent steps were performed at 4 °C. The extract was clarified by centrifugation (10 000 × *g*, 30 min), dialysed against acetate buffer (10 mM sodium acetate pH 5.0, 10 mM 2-mercaptoethanol, 1 mM EDTA) and concentrated by dialysis against solid PEG 6000. The concentrated extract was loaded onto a CM-Sephadex C50 column equilibrated with acetate buffer and eluted with a linear gradient of 0–300 mM NaCl in acetate buffer. The fractions with highest chitinase activity were collected, dialysed against Tris buffer (10 mM Tris-HCl pH 7.5, 10 mM 2-mercaptoethanol, 1 mM EDTA), concentrated against PEG 6000 and size-fractionated using a Bio-gel P100 column, equilibrated and eluted with Tris buffer. The fractions of highest chitinase activity were collected and applied on a DEAE-Sephacel column equilibrated with Tris buffer. The recombinant enzyme from plants carrying the ΔSC CHN A construct was eluted with the same buffer. An additional affinity chromatography step with a chitin column was required to separate the chitinases with and without the CRD (Figure 1) as recommended by Mauch *et al.* (1988). The variants ΔC CHN A and ΔCS CHN A were in the pH 8.4 run-through fraction. CHN A, ΔS CHN A and 2S CHN A were then eluted from the column with 100 mM acetic acid pH 2.8. The purified chitinase preparations were dialysed against 20 mM $NH_4HCO_3$ and stored at −20 °C.

*Inmunoblot analysis*

Leaf discs 14 mm in diameter from upper leaves of transgenic *N. sylvestris* plants were homogenized for 1 min in 200 $\mu$l of extraction buffer (50 mM Tris pH 8.0, 500 mM NaCl, 0.002% v/v Triton X 100) with a machine-driven Potter homogenizer. The extracts were clarified by centrifugation at 17 000 × *g* for 15 min (Kunz *et al.*, 1996) and equal volumes of extracts were fractionated by SDS-PAGE with 12% gels. Immunoblot analyses were performed as described previously using antibodies directed against class I tobacco chitinase that detect class I chitinases A and B of tobacco and *N. sylvestris* as well (Shinshi *et al.*, 1987; Keefe *et al.*, 1990). The protein concentration of extracts was measured according to Bradford (1976) with bovine $\gamma$-globulin as standard.

*Characterization of polypeptides*

The amount of purified protein in samples was estimated by amino acid analysis (Knecht *et al.*, 1986), with norleucine as internal standard. N-terminal amino acid sequence analysis was performed by Edman degradation in an Applied Biosystems 477A Protein Sequencer (Rennex *et al.*, 1991; Sticher *et al.*, 1992). The masses of the purified proteins were determined

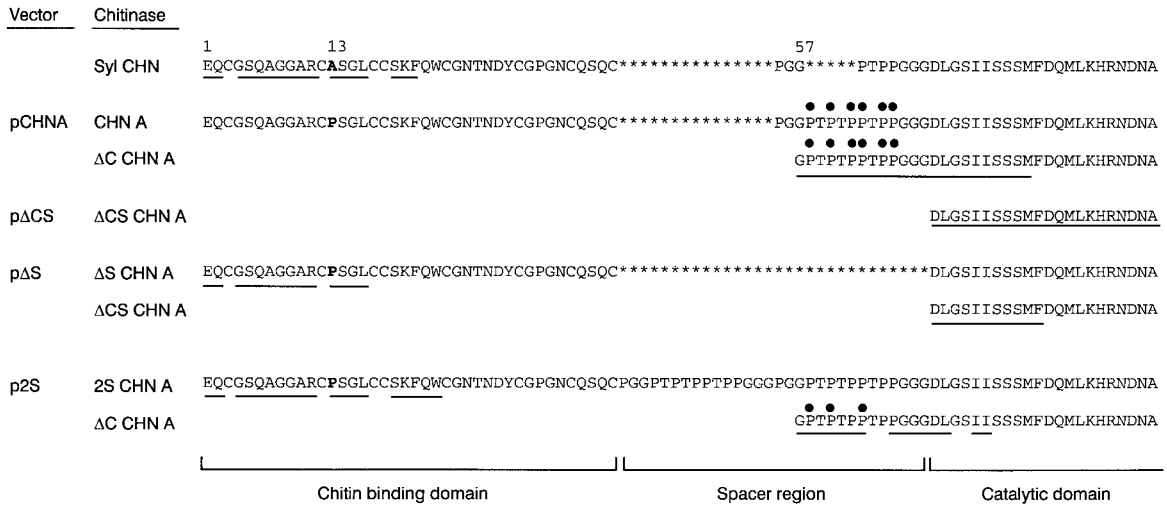


*Figure 1.* Comparison of the N-terminal amino acid sequences of CHN A, recombinant CHN A variants and *N. sylvestris* chitinase. Sequences confirmed by Edman degradation are underlined. The diagnostic amino acid for CHN A at position 13 is indicated in bold. Prolyl residues showing partial or complete 4-hydroxylation are indicated by a dot above the sequence. Data for CHN A from Sticher *et al.* (1992).

on a MALDI-TOF mass spectrometer (Micromass, UK) with dihydroxybenzoic acid as a matrix. Equine cytochrome *c*, equine myoglobin, bovine trypsinogen and bovine serum albumin were used as standards.

*Assay of enzyme activity*

For all assays, enzyme preparations were incubated at 37 °C in phosphate buffer (100 mM potassium phosphate pH 5.6, 1% w/v bovine serum albumin, 0.02% w/v sodium azide) with and without substrate. Data are reported as the mean ± SE obtained for three independent experiments with replicate samples. The stability of the enzyme during the assay procedures was confirmed by the progress-curve method of Selwyn (1965). Chitinase activity was measured radiometrically with [$^3$H]-chitin as substrate (Boller *et al.*, 1983). The amount of enzyme producing 1 pmol GlcNAc equivalents per second at infinite dilution was defined as 1 pkat of activity. Hydrolysis of Nod factors from *R. meliloti* strain 1021 (pEK327) (Schultze *et al.*, 1992) was performed as previously described (Staehelin *et al.*, 1994b), using as substrate NodRm-IV(C16:2, S) and NodRm-V(C16:2, S). After incubation, samples were extracted with *n*-butanol and fractionated by reversed-phase HPLC (Waters C18 column, particle size 5 $\mu$m), with 35% v/v acetonitrile/water, 40 mM ammonium acetate as the mobile phase. Lysozyme activity was assayed and activity expressed as described by Brunner *et al.* (1998).

*Assay of antifungal activity*

Inhibition of fungal growth was assayed using the fungus *Trichoderma viride* as described previously (Schlumbaum *et al.*, 1986). *T. viride* was grown in petri dishes containing 2% w/v malt extract agar, at 27 °C for 24 h. When the diameter of the colony was 3–4 cm, sterile filter paper discs of 6 mm diameter were placed on the agar surface 1 cm from the growing front and 20 $\mu$l of the enzyme preparation was applied to the disks. After incubation in the light at 27 °C for 12 h, the diameter of the growth inhibition zones was measured. The data are expressed as the mean mm$^2$ of the inhibition zone ± SE for three independent experiments with replicates. The inhibition-zone area was shown to increase in a linear fashion for low concentrations of the chitinases tested.

## Results

*Expression of tobacco chitinases in* Nicotiana sylvestris

*N. sylvestris* plants were transformed with the CHN A expression vectors pCHNA, pΔCS, pΔS and p2S encoding the proteins CHN A, ΔCS CHN A, ΔS CHN A and 2S CHN A, respectively, as shown in Figure 1. In addition to the full-length proteins, plants transformed with three of the vectors produced N-terminally truncated products as described below. Antibiotic-resistant

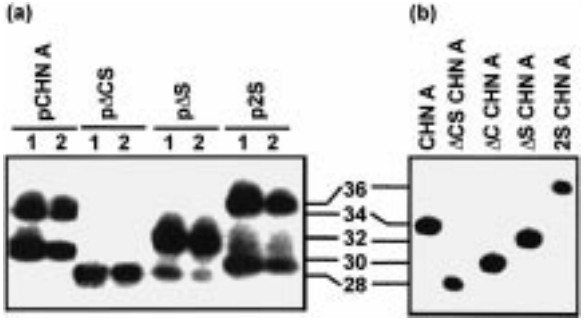


*Figure 2.* SDS-PAGE separation of CHN A and CHN A variants expressed in leaves of transgenic *N. sylvestris* plants. a. Immunoblot analyses. Lanes were loaded with 20 $\mu$l of extract with high chitinase activity prepared from replicate plants (1 and 2) transformed with the vector indicated. The antibody used detects tobacco as well as host class I chitinases. b. SDS-PAGE of purified chitinases stained with Coomassie blue. Lanes were loaded with 10 $\mu$g of protein. Scale: apparent size of polypeptides in kDa.

plants were regenerated from calluses and ca. 20 independent transformants obtained with each construct were screened for elevated chitinase activity. The chitinases in plants showing high levels of chitinase activity were partially characterized by immunoblot analysis with antibodies directed against class I tobacco chitinase, which also detect the *N. sylvestris* enzymes. As shown in Figure 2A, protein extracts from upper leaves of plants transformed with pCHNA contained abundant 30 and 34 kDa antigens corresponding in size to the full-length and truncated CHN A described earlier (Neuhaus *et al.*, 1991b). The antibodies recognized a single, abundant 28 kDa polypeptide from plants transformed with pΔCS, abundant 28 and 32 kDa polypeptides from plants transformed with pΔS, and abundant 30 and 36 kDa polypeptides from plants transformed with p2S. Under the conditions used to raise the plants, no *N. sylvestris* encoded chitinase antigens were detected in the extracts from untransformed plants (data not shown).

### *Purification and partial characterization of transgenic chitinases*

The truncated ΔCS CHN A chitinase was purified from upper leaves of the pΔCS transformant in three steps: cation-exchange chromatography, gel filtration and anion-exchange chromatography. An additional affinity chromatography step using regenerated chitin was required to separate ΔC CHN A from CHN A in extracts of the pCHNA transformant, to separate ΔS CHNA from ΔCS CHN A in extracts of the pΔS transformant, and to separate 2S CHN A from

*Table 1.* Molecular mass of chitinase CHN A variants expressed in *N. sylvestris*.

| Chitinase[1] | Molecular mass | | |
|---|---|---|---|
| | deduced (Da)[2] | MALDI-TOF (Da) | SDS-PAGE (kDa)[3] |
| CHN A | 31 958 | 32 073 | 34 |
| ΔC CHN A | 27 744 | 27 767 | 30 |
| ΔCS CHN A | 26 629 | 26 613 | 28 |
| ΔS CHN A | 30 690 | 30 681 | 32 |
| 2S CHN A | 33 227 | | 36 |

[1]The truncated chitinases ΔC CHN A and ΔCS CHN A were purified from extracts of the p2S and pΔCS transformants, respectively.
[2]Calculated from the amino acid sequence of the mature polypeptide deduced from the nucleotide sequence of the coding region.
[3]Apparent molecular mass determined by comparing mobility of the polypeptide relative to protein standards. Note that mobility of chitinases is retarded due to hydroxylation of prolyl residues in the spacer region.

ΔC CHN A in extracts of the p2S transformant. Chitinases without the CRD domain were present in the pH 8.4 run-through fraction from the column, whereas chitinases with the CRD, which were tightly bound to the column, were eluted in 100 mM acetic acid, pH 2.8 indicating that binding of the proteins to chitin required the CRD. The yield of purified materials, which showed single bands on SDS gels (Figure 2B), was about 35% of total chitinase activity in the crude extracts.

A gene encoding the *N. sylvestris* class I chitinase SYL-CHN B was cloned (EMBL accession number AJ301671) and the amino acid sequence of the polypeptide deduced from alignment of the nucleotide sequence with that of a cDNA. Mature SYL-CHN B and CHN A are very similar in sequence. There is a single replacement of the proline at position 13 in CHN A with alanine and a deletion of 5 amino acid residues in the spacer region, which serve to distinguish CHN A from the host chitinase (Figure 1).

Microsequencing of the regions where SYL-CHN B and the CHN A polypeptides differ in sequence confirmed that the polypeptides designated CHN A, ΔS CHN A and 2S CHN A have N-terminal sequences expected for chitinases with CRDs, and that these polypeptides are encoded by the transgene. The results also confirmed that the chitinase accumulating in the pΔCS transformant is a truncated variant starting at the catalytic domain (Figure 1). Unexpectedly, truncated chitinases were found in full-length

pCHNA, pΔS and p2S transformants. Microsequencing showed that the N-terminus of the polypeptides from the pCHNA and p2S transformants, designated ΔC CHN A, is the glycine at position 57 near the start of the spacer region. In contrast, the N-terminal sequence of the truncated form found in the pΔS transformant, designated ΔCS CHN A, corresponds to the start of the catalytic domain.

Table 1 shows that there was good agreement between the $M_r$ calculated from the deduced amino acid sequences and the $M_r$ of the purified chitinases estimated by MALDI-TOF. Deviations of the deduced and measured values obtained with the polypeptides with a spacer region are likely to reflect heterogeneity due to variable prolyl hydroxylation of the spacer (Sticher *et al.*, 1992). Prolyl hydroxylation was also shown earlier to result in the anomalously high apparent $M_r$ values when estimated from SDS-PAGE. Prolyl hydroxylation of the spacer region was confirmed for CHN A and ΔC CHN A by microsequencing (Figure 1).

*Enzymatic activities of recombinant chitinases*

The purified chitinase variants were assayed for hydrolytic activity and the values expressed as specific activity per mole of protein quantitated by amino acid composition. We verified by the progress-curve method of Selwyn (1965) that, over a period of at least 60 min, the 5 chitinases did not lose enzymatic activity (data not shown). Thus, the CRD and spacer did not appear to influence the stability of CHN A in the enzymatic assays. Table 2 shows that all of the chitinases tested were able to hydrolyse regenerated chitin, but that they differed in specific activity. Wild-type CHN A exhibited the highest activity, while the truncated enzymes lacking the CRD (ΔC CHN A) or both the CRD and the spacer region (ΔCS CHN A) showed ca. 50% lower values. Interestingly, 2S CHN A with two spacer sequences in tandem exhibited a dramatic 13-fold reduction in activity relative to CHN A.

The chitinases were further analysed for their capacity to hydrolyse Nod factors from *Rhizobium meliloti*. When the tetrameric NodRm-IV(C16:2, S) was used as a substrate, no hydrolysis was detected (not shown), while all tested chitinases could release the lipo-trisaccharide from the pentameric NodRm-V(C16:2, S) (Figure 3), as shown previously for class I chitinases (Staehelin *et al.*, 1994b; Schultze *et al.*, 1998). The NodRm-V(C16:2, S) was cleaved by the full-length and the truncated chitinases at similar hydrolytic rates, whereas the 2S CHN A showed only weak activity (Figure 3e).

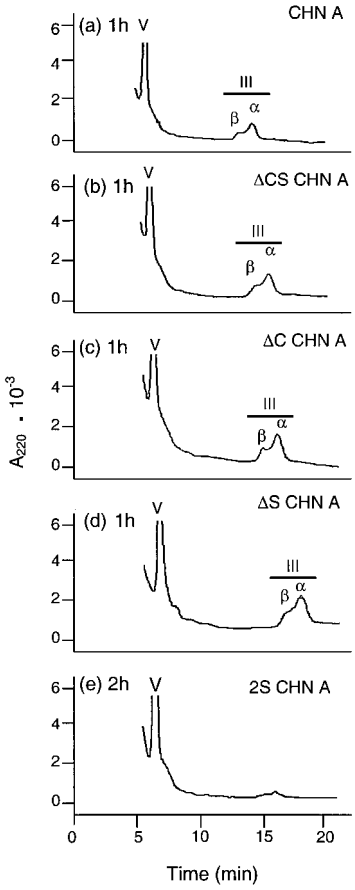


*Figure 3.* Hydrolysis of pentameric lipo-chitooligosaccharide NodRm-V(C16:2, S) by CHN A and CHN A variants. Reversed-phase HPLC of reaction mixtures containing 1 $\mu$g of substrate (V) and 1 $\mu$g of chitinase after incubation for the time indicated. The acetylated product, NodRm-III(C16:2), is separated into its anomers (III$\alpha$ and III$\beta$).

To examine the lysozyme activity of the recombinant chitinases, lysis of cell walls of *Micrococcus lysodeikticus* was monitored (Table 2). As calculated from the initial rate of decrease in absorbance, the specific activity of CHN A was ca. 100-fold lower than that of hen egg white lysozyme (data not shown). Removal of the CRD, or the CRD plus spacer region, reduced activity by ca. 60–67% whereas removal of the spacer alone reduced activity by 43%. In contrast, 2S CHN A exhibited activity comparable to that of CHN A indicating that increasing the length of the spacer reduces hydrolysis of chitin and Nod factors, but not that of bacterial peptidoglycans.

*Table 2.* Hydrolytic and antifungal activity of recombinant chitinases.

| Expression vector | Chitinase | Chitinase activity | | Lysozyme activity | | Antifungal activity | |
|---|---|---|---|---|---|---|---|
| | | sp. act.[1] | rel. act. | sp. act.[2] | rel. act. | sp. act.[3] | rel. act. |
| pCHNA | CHN A | 383. ± 11.9 | 1.00 | 855. ± 25.7 | 1.00 | 46.7 ± 6.7 | 1.00 |
| pΔCS | ΔCS CHN A | 204. ± 2.7 | 0.53 | 286. ± 9.1 | 0.33 | 6.4 ± 0.69 | 0.14 |
| pΔS | ΔS CHN A | 219. ± 13.4 | 0.57 | 574. ± 5.8 | 0.67 | 7.8 ± 1.04 | 0.17 |
| p2S | 2S CHN A | 29.7 ± 2.0 | 0.08 | 854. ± 20.4 | 1.00 | 70.8 ± 1.43 | 1.51 |
| | ΔC CHN A | 209. ± 8.2 | 0.53 | 353. ± 7.1 | 0.41 | 9.9 ± 0.72 | 0.21 |

[1]Mean kat per mole protein ± SE for 3 independent experiments with [$^3$H]-chitin as substrate.
[2]Mean units per mole protein × $10^3$ ± SE for 3 independent experiments with *M. lysodekticus* as substrate.
[3]Mean size of the fungal inhibition zone ($m^2$) per mmole protein ± SE with *T. viride* as the test fungus.


*Figure 4.* Inhibition of *T. viride* growth by CHN A and CHN A variants. Filter paper discs impregnated with 20 $\mu$l of test solution were placed 1 cm from the growing front of the fungus. Cultures were photographed after 12 h incubation. Amounts of chitinase were chosen to give roughly the same size inhibition zones: 1, 3.8 nmol ΔCS CHNA; 2, 1.8 nmol ΔC CHN A; 3, 0.63 nmol CHN A; 4, 3.3 nmol ΔS CHN A; 5, 0.6 nmol 2S CHN A; 6, water.

### *Antifungal activity of recombinant chitinases*

Chitinases are able to inhibit growth of certain fungi, such as *T. viride* (Schlumbaum *et al.*, 1986). To examine the effect of the different chitinase A variants on growth of *T. viride*, paper filter discs with purified proteins were placed near the front of the growing hyphae. After incubation, the size of the zone with inhibited hyphal growth was measured. Although all chitinase variants were able to inhibit growth, inhibitory activity expressed on a per mole protein basis was markedly reduced by 79–86% for CHN A with the CRD, CRD plus spacer region, or spacer region deleted (Figure 4, Table 2). The 2S CHN A variant, which exhibited appreciably less activity when assayed using chitin and Nod factors as substrates, showed 50% higher activity than wild-type CHN A.

## Discussion

Structural classes of plant chitinases have been shown to differ in enzymatic activity, substrate specificity, and antifungal activity (Sela-Buurlage *et al.*, 1993; Brunner *et al.*, 1998; Schultze *et al.*, 1998). We examined the functional role of the CRD and spacer region in class I chitinases by comparing wild-type CHN A with several informative structural variants. The proteins were expressed in *N. sylvestris* plants, purified to near homogeneity, and their structures confirmed by mass spectrometry and partial sequencing. Use of the *N. sylvestris* expression system offered important experimental advantages. The developmentally regulated host class I chitinase, SYL-CHN B, is not detectable in upper leaves of the mature plant (Shinshi *et al.*, 1987; Neuhaus *et al.*, 1991a; Hart *et al.*, 1992). Thus, the concentration of transgene-encoded proteins was routinely at least 100-fold higher than that of the host homologue. Moreover, in contrast to chitinase expression in certain yeast cells (Kunze *et al.*, 1998), CHN A undergoes correct post-translational prolyl hydroxylation and cleavage of the C-terminal extension and is correctly targeted to the vacuole (Neuhaus *et al.* 1991b).

Two truncated forms of CHN A were detected in extracts of transgenic *N. sylvestris* leaves: a ca. 28 kDa polypeptide found in pΔS, which corresponds to ΔCS CHN A encoded by pΔCS and the Tob ΔH vector described earlier (Iseli *et al.*, 1993) and a ca. 30 kDa polypeptide found in pCHNA and p2S transformants, which was shown to be a ΔC CHN A starting near the proximal end of the spacer region. No transcripts encoding a truncated form were detected in either *N. sylvestris* or in transformants carrying the full-length CHN A transgene (data not shown) indicating that these forms were not the result of either alternative splicing or the transcription of other

unidentified host chitinase genes. This suggests that the truncated forms arise by proteolytic cleavage in the plant or during the purification procedure. It is of interest in this regard that proteases cleave class IV chitinase at several sites in the spacer region in bean plants infected with the fungal pathogen *Fusarium solani* (Lange *et al.*, 1996). On the other hand, no truncated forms of CHN A were detected in pulse-chase experiments using CHN A transformants of tobacco (Sticher *et al.*, 1993).

Domains closely related to the CRD of class I chitinases are found in several proteins including chitin-binding lectins from cereals and stinging nettle (for a review, see Neuhaus, 1999). Based on similarity to known lectins and the finding that CHN A, but not ΔCS CHN A, was retained on regenerated chitin columns, Iseli *et al.* (1993) concluded that the CRD is a chitin-binding domain. Our results provide strong support for this hypothesis. We showed that CHN A, ΔS CHN A and 2S CHN A with a CRD bind chitin, whereas ΔC CHN A and ΔCS CHN A without this domain do not. Thus, the CRD is necessary and sufficient for chitin-binding activity of a class I chitinase.

The functional significance of CRD-mediated chitin binding is less clear. Studies of purified ΔCS CHN A and the susceptibility of ΔCS CHN A transformants of *N. sylvestris* to *Rhizoctonia solani* (Vierheilig *et al.*, 1993) have led to the proposal that while chitin binding is not essential for chitinolytic activity or antifungal activity, it contributes to both activities (Iseli *et al.*, 1993). According to this view, the CRD might anchor the enzyme to insoluble substrates and, hence, increase the concentration of substrate at the hydrolytic site as been reported for the lectin domain of cellulases (for a review, see Henrissat, 1994). The CRD might also have intrinsic antifungal activity unrelated to hydrolysis of substrates, as reported for hevein and chitin-binding stinging nettle lectin, which do not have chitinolytic activity and consist, respectively, of one- and two-CRD related domains (Broekaert *et al.*, 1989; van Parijs *et al.*, 1991).

The present studies show that deletion of the CRD and spacer alone or in combination resulted in a modest $<$50% reduction of hydrolytic activity relative to CHN A using colloidal chitin or *M. lysodeikticus* walls as substrates, whereas antifungal activity was reduced by up to 80%. Of particular interest was the finding that the tandem-spacer variant 2S CHN A, which binds chitin, exhibited a unique substrate preference. Relative to CHN A, this hydrolase showed very low hydrolytic activity towards chitin and the lipo-oligosaccharidic Nod factors, but comparable lysozyme activity and enhanced antifungal activity. Among the variants tested neither hydrolytic activity, substrate specificity nor antifungal activity were strictly correlated with the CRD-mediated capacity to bind chitin. Thus, the presence of the CRD per se in these hydrolases is not a major factor in determining activity. We speculate that the spatial arrangement of the CRD and spacer could influence the accessibility and conformation of functional sites in the catalytic domain.

Although *T. viride* and other fungi susceptible to class I chitinases contain chitin in their cell walls, our results suggest that the antifungal activity of 2S CHN A is only partly due to degradation of fungal chitin. One possible explanation is that alternative substrates may exist in fungi. For example, the chitin in fungal cell walls can differ in extent of N-acetylation (Wessels and Sietsma, 1981). Class I chitinases hydrolyse chitin and deacetylated chitin at similar efficiencies, whereas class II chitinases with a catalytic domain similar to that of the class I enzymes but lacking the CRD and spacer show greatly reduced hydrolysis of the deacetylated form (Brunner *et al.*, 1998). Similarly, *Medicago sativa* hydrolases can cleave Nod factors, but not the N-deacetylated forms (Staehelin *et al.*, 2000). Although CHN A and its variants showed similar stability in tests of chitinase activity, we cannot rule out the possibility that the structural modifications alter susceptibility of the proteins to proteolytic activity in fungus-inhibition tests that last for 12 h. A more interesting possibility is that inhibition of *T. viride* growth does not depend on chitin hydrolysis. This hypothesis could be tested by comparing the inhibitory activity of wild-type CHN A with that of the variant described by Iseli-Gamboni *et al.* (1998), which lacks hydrolytic activity due to mutation of putative catalytic residues.

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