Chimeric proteins of stinging nettle lectin, chitinase and beta-1,3-glucanase

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Chapter 5

A Chimera of *Urtica dioica* Agglutinin and Tobacco Chitinase Displays Both Agglutination and Chitinase Activity

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ABSTRACT

To obtain a protein with agglutination activity as well as chitinase activity, a fusion protein was designed in which *Urtica dioica* agglutinin (UDA)-isolectin I and the catalytic domain of tobacco (*Nicotiana tabacum* cv. Samsun NN) chitinase I were assembled. A construct was made containing sequences encoding the signal peptide and the isolectin sequence of the precursor to UDA-isolectin I, followed by the linker and the catalytic domain of tobacco chitinase I. Due to the introduction of a stop codon, the precursor to this chimera lacked the seven carboxyl-terminal amino acids necessary for vacuolar targeting of tobacco chitinase I. The construct was expressed in transgenic tobacco (*Nicotiana tabacum* cv. Samsun NN) under control of the cauliflower mosaic virus 35S promoter. Analysis of transgenic plants showed that the fusion protein UDA-Chi is targeted extracellularly. Both crude leaf extracts of transgenic tobacco and purified fusion protein showed agglutination activity on trypsin-treated rabbit erythrocytes. The molar agglutination activity of the UDA-Chi chimera was shown to be similar to that of mature UDA. The chimera has chitinase activity that differs from that of tobacco chitinase I.
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INTRODUCTION

_Urtica dioica_ agglutinin (UDA) consists of two cysteine-rich chitin-binding domains (Peumans et al., 1984; Beintema and Peumans, 1992; Does et al., 1999b). Both are homologous to hevein, a small chitin-binding protein of 43 amino acids from the rubber tree (Archer, 1960; Walujono et al., 1975). The presence of a carbohydrate-binding site in each chitin-binding domain enables UDA to agglutinate erythrocytes (Peumans et al., 1984; Horn et al., 1995). In most stinging nettle (_Urtica dioica_ L.) ecotypes, UDA is present as a mixture of isolectins (Van Damme and Peumans, 1987; Does et al., 1999b). Each isolectin is processed from a precursor (Fig. 1A), which comprises an amino-terminal (N-terminal) signal peptide followed by two chitin-binding domains, a hinge region of 16 amino acids and a carboxyl-terminal (C-terminal) chitinase domain of 244 amino acids (Lerner and Raikhel, 1992; Does et al., 1999b). A vacuolar sorting determinant has been shown to be present within the 25 C-terminal amino acids of the UDA-precursor (Does et al., 1999a). The signal peptide, the hinge region as well as the chitinase domain are processed from the precursor to yield mature UDA (Fig. 1A). Because of the presence of the two chitin-binding domains and the homology of the chitinase domain with other plant chitinases, the precursor to UDA is classified as a plant class V or Chia5 chitinase (Meins et al., 1994; Neuhaus et al., 1996). Recently, processing of the precursor to UDA-isolectin I was studied in transgenic tobacco plants (Does et al., 1999a). UDA purified from these tobacco plants had retained its chitin-binding activity, agglutination activity and antifungal activity against chitin-containing plant pathogenic fungi (Peumans et al., 1984; Broekaert et al., 1989; Does et al., 1999a). Fungal growth-inhibition by UDA does not occur by lysis of the fungal cell wall. It does, however, depend on the phase of fungal growth and is temporal, suggesting that fungi adapt to the presence of UDA (Does et al., 1999a).

Mature tobacco chitinase I is comprised of one chitin-binding domain fused to a linker and a chitinase domain (Linthorst et al., 1990b; Shinshi et al., 1990). The structure of the precursor to this chitinase is similar to the UDA-precursor and consists of a signal peptide, the single chitin-binding domain, a linker, the catalytic domain of 244 amino acids and a C-terminal propeptide of seven amino acids (Fig. 1B). This propeptide has been shown to be necessary and sufficient for vacuolar targeting (Neuhaus et al., 1991b). Removal of this vacuolar sorting determinant causes secretion of the mature basic chitinase into the extracellular space (Neuhaus et al., 1991b; Melchers et al., 1993; Sticher et al., 1993). Depending on the isoform of the tobacco chitinases I, the linker consists of 10 (chitinase A) or 15 (chitinase B) amino acids (Van Buuren et al., 1992). Unlike the hinge region of the UDA-precursor, the linker of tobacco chitinase I is rich in glycine and proline residues, some of which have been shown to be 4-hydroxylated (Sticher et al., 1992b).
Tobacco chitinase I displays antifungal activity on several fungi that contain chitin in their cell walls. Unlike UDA, tobacco chitinase I causes lysis of the hyphal tips as a result of its hydrolase activity (Sela-Buurlage et al., 1993). Also the range of susceptible chitin containing fungi and the amount of each protein needed to obtain 50% growth-inhibition differ for both these chitin-binding proteins (Van Parijs et al., 1991; Broekaert et al., 1992).

In this paper, we report the production of a fusion protein in transgenic tobacco (Nicotiana tabacum cv. Samsun NN), in which we have brought together the different biological activities of both UDA and tobacco chitinase I. The UDA-Chi chimera consists of the mature UDA-isolectin I sequence fused to the linker and the catalytic domain of tobacco chitinase I (chitinase B). We show that the purified fusion protein displays both agglutination and chitinase activity.

**Figure 1.** Schematic representation of how the precursors to UDA (A), tobacco chitinase I (B) and the UDA-Chi (C) fusion are processed. The amino acid sequence around the fusion point is depicted (C). Known processing sites are indicated by vertical arrows. The location of the introduced stop codon is indicated by an asterisk. CB, chitin-binding domain; CHI, chitinase domain; CHICAT, catalytic chitinase domain; H, hinge region; L, linker; SP, signal peptide; V, vacuolar targeting signal.
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MATERIALS AND METHODS

Cloning Procedures

The chimeric UDA-ChiΔCT7 fusion gene containing the UDA-isolectin I leader sequence followed by the sequences encoding the signal peptide and UDA-isolectin I of the UDA-precursor, the linker and the catalytic domain of tobacco chitinase I, was made by the following procedure.

The gene encoding the precursor to UDA-isolectin I (UdChia5.1.1) from stinging nettle ecotype Weerselo was cloned between the cauliflower mosaic virus 35S promoter and the nopaline synthase terminator on vector pMOG181 as described previously (Does et al., 1999a,b). To isolate the UDA-isolectin I sequence, polymerase chain reaction (PCR) was performed on this clone using the primers 35S1 (5'-CGACACGCTTGTCTACTCC-3') and UD9 (5'-GCAGCGGTACTGGCATTTG-3'). PCR amplification was performed on 100 pg of plasmid DNA in a 100 μL reaction mixture containing 2.5 U Pfu DNA polymerase (Stratagene), 200 μM of each of the dNTPs (Pharmacia) and 100 pmol of both primers, using a DNA Thermal Cycler (Perkin Elmer). The PCR fragment was obtained after an initial step of 4 min at 95°C, followed by 30 cycles of 1 min at 95°C, 2 min at 57°C and 2 min at 72°C, and a final step of 8 min at 72°C. Subsequently, the fragment was digested with BamHI.

To isolate the tobacco chitinase sequences, a clone was used containing the tobacco (N. tabacum cv. Samsun NN) basic chitinase cDNA on vector pMOG181 (Melchers et al., 1993). PCR was performed using the primers Chi5 (5'-CCTGGTGGTCCCACACC-3') and LS19 (5'-TTCCCCAGTCACGACGTGT-3') on 100 pg plasmid DNA as already described. The chitinase PCR fragment was obtained after an initial step of 4 min at 95 °C, followed by 35 cycles of 1 min at 95°C, 2 min at 52°C and 2 min at 72°C, and a final step of 8 min at 72°C. This fragment was digested with HindIII.

The BamHI-blunt (UDA-isolectin I) and blunt-HindIII (chitinase) PCR fragments were together cloned in the expression cassette on pMOG181. For extracellular targeting of the fusion protein, a stop codon was introduced in the sequence of the chitinase domain. Therefore, a vacuolar targeting mutant chitinase construct (pMOG189) was used which had been created previously (Melchers et al., 1993). This construct encodes the basic tobacco chitinase I lacking the C-terminal vacuolar targeting signal of seven amino acids. The mutated chitinase gene was cloned from pMOG198 into pMOG181 (ChiΔCT7). The stop codon was introduced into the UDA-ChiΔCT7 fusion by exchanging the PstI/HindIII fragment from the UDA-Chi fusion by the stop codon containing PstI/HindIII fragment from ChiΔCT7. The construct was sequenced using the dideoxynucleotide chain-termination method (Sanger et al., 1977). The EcoRI/HindIII fragment from pMOG181:UDA-ChiΔCT7 was cloned into the binary vector pMOG402 (Jongedijk et al., 1995). Cloning procedures were performed as described (Sambrook et al., 1989).
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Tobacco Transformation

Transfer of the pMOG402:UDA-ChiΔCT7 construct to *Agrobacterium tumefaciens* and tobacco (*N. tabacum* cv. Samsun NN) transformation were performed as previously described (Does et al., 1999a). Seeds of primary transformants and of their progeny were selected on plates containing kanamycin (200 μg mL⁻¹).

Western Analysis of Transgenic Plants

Total leaf extracts were isolated by grinding leaf samples in sodium acetate buffer (50 mM NaOAc, pH 5.2). Extracellular washing fluid was isolated according to De Wit and Spikman (1982), using sodium acetate buffer. Protein concentrations were determined by the Bradford method (Bradford, 1976) using BSA as standard. Protein electrophoresis was performed using 12.5% SDS-polyacrylamide gels (Laemmli, 1970) or using 20% tricine-SDS-polyacrylamide gels (Schägger and Von Jagow, 1987). Immunological detection by α-UDA antibodies was performed as described previously (Does et al., 1999b). For immunological detection with α-CHI antibodies (Melchers et al., 1993), antibodies were diluted 5000-fold and incubated with the blot overnight at room temperature.

Purification of the UDA-Chi Fusion Protein from Transgenic Tobacco

The UDA-Chi fusion protein was isolated from a homozygous high-expressor line. Leaves were de-veined and homogenized in ice-cold extraction buffer (50 mM NaOAc pH 5.2, 0.1% ascorbic acid) using a blender. Per 200 mL extraction buffer, one teaspoon of charcoal was added. The mixture was squeezed through four layers of gauze and kept on ice. Subsequently, the filtrate was centrifuged for 30 min at 3,200g at 4°C. Supernatant was filtered through eight layers of gauze and centrifuged at 20,000g for 60 min at 4°C. The supernatant was again filtered through eight layers of gauze and brought to pH 4.0 using 2 N acetic acid. NaCl was added to a concentration of 0.1 M and the mixture was kept on ice for at least 1 h. After centrifugation for 60 min at 20,000g at 4°C, the supernatant was filtered through eight layers of gauze.

The filtrate was applied to a chitin affinity column (± 100 mL), which was prepared as described previously (Peumans et al., 1984) and equilibrated with buffer (50 mM NaOAc, 0.1 M NaCl, pH 4.0). The column was washed with 1000 mL of the same buffer. Bound proteins were eluted with 0.5 N acetic acid and brought to pH 4.0 immediately.

The pool of eluted chitin-bound proteins was subjected to cation-exchange chromatography, using a HiLoad SP Sepharose Fast Flow column (26/10, Pharmacia)
equilibrated with 50 mM NaOAc, pH 4.0. Elution was performed using a gradient of 0 to 0.25 M NaCl in 50 mM NaOAc, pH 4.0. Fractions were tested for the presence of fusion protein UDA-Chi by western analysis using α-UDA antibodies. Fractions containing fusion protein were pooled and concentrated by ultrafiltration through an YM10 Diaflo ultrafiltration membrane (Amicon) using a 400 mL stirred cell (Amicon).

The concentrate was dialysed against PBS and applied to a Superdex 75 gel-filtration column (HiLoad™16/60, Pharmacia), equilibrated with PBS. Gel-filtration occurred at 0.8 mL min⁻¹. Fractions containing the fusion protein were pooled and gel-filtration was performed repeatedly, until fractions with pure fusion protein were obtained. Pooled fractions were concentrated and dialysed against 50 mM potassium phosphate buffer pH 6.0.

Protein concentration was determined using a bicinchoninic acid protein assay kit (Sigma). Purity of the fusion protein was checked by 12.5% SDS-PAGE and both immunological detection using α-UDA antibodies and α-CHI antibodies, and silverstaining using the Silverstain plus kit (Biorad).

**Purification of Tobacco Chitinase I from Transgenic Tobacco**

Homozygous seeds of transgenic tobacco expressing the gene encoding tobacco chitinase I (Melchers et al., 1993) were obtained from Zeneca MOGEN. Chitinase I was isolated from transgenic tobacco leaves according to the procedure described previously (Sela-Buurlage et al., 1993). Protein concentration was determined using the bicinchoninic acid protein assay kit (Sigma). Purity was checked by 12.5% SDS-PAGE and immunological detection using α-UDA antibodies and α-CHI antibodies, and silverstaining.

**Agglutination Assays**

Agglutination assays using crude leaf extracts were performed on microscope slides, with squashed leaf samples to which trypsin-treated rabbit erythrocytes (Peumans et al., 1984) and PBS were added.

Agglutination tests with purified UDA-Chi fusion protein and purified tobacco chitinase I were performed as follows: to 30 μL trypsin-treated rabbit erythrocytes, 0.25, 0.5, 1.0, 2.0, 3.0, and 4.0 μg of purified protein was added. Five times concentrated PBS was added to a final volume of 60 μL.

To inhibit possible proteolytic activity from residual trypsin or other proteolytic enzymes, tests were also performed in the presence of protease inhibitors. Therefore, 2.4 μL of a 25× stock solution of a protease inhibitor cocktail (Complete, Boehringer Mannheim) in PBS
Chimeric protein with agglutination and chitinase activity was first added to 30 μL erythrocytes, followed by the different amounts of fusion protein. The final volume was brought to 60 μL with five times concentrated PBS.

Chitinase Assay

Chitinase activity measurements were carried out using carboxymethyl-chitin-Remazol-Brilliant-Violet 5R (CM-chitin-RBV, LOEWE Biochemica, Sauerlach, Germany) as a substrate (Wirth and Wolf, 1990) as described previously (Melchers et al., 1994).

RESULTS

UDA-ChiΔCT7 Precursor Construct

Construct UDA-ChiΔCT7 encoding the precursor to the mature chimera UDA-Chi (Fig. 1C) was made by fusion of sequences coding for the signal peptide and the mature isolectin of the precursor to UDA-isolectin I (Does et al., 1999b) (Fig. 1A) to the coding sequence for the linker and the catalytic domain of tobacco chitinase I (Melchers et al., 1993) (Fig. 1B). Due to the introduction of a stop codon, the precursor construct UDA-ChiΔCT7 lacks the C-terminal propeptide of seven amino acids (ΔCT7) which has been shown to be necessary for the targeting of tobacco chitinase I to the vacuoles (Neuhaus et al., 1991b). Because of this deletion, we expected the fusion protein to be targeted extracellularly, like the tobacco chitinase (Melchers et al., 1993). Compared with the tobacco chitinase I, the mature fusion protein UDA-Chi contains an additional chitin-binding domain (Fig. 1). Mature tobacco chitinase I consists of 294 amino acids, while the mature fusion protein consists of 340 amino acids.

Construct UDA-ChiΔCT7 was placed in an expression cassette, between the cauliflower mosaic virus 35S promoter and the nopalin synthase transcription terminator, and transformed into tobacco using Agrobacterium.

Production and Extracellular Targeting of the Fusion Protein UDA-Chi in Transgenic Tobacco

Primary transformants were selected for expression of the transgene by western analysis (not shown). For immunological detection, α-UDA antibodies were used that had
been raised against a synthetic peptide consisting of 15 sequential amino acids of the mature UDA-isolectin I sequence (Does et al., 1999b).

In an agglutination assay, crude extracts of several high-expressor lines agglutinated trypsin-treated rabbit erythrocytes (data not shown), indicating that an agglutinating protein was present in these extracts. A crude extract of a nontransformed tobacco did not agglutinate rabbit erythrocytes.

![Western analysis of the extracellular washing fluid from the F1 progeny using α-UDA antibodies (A) and α-CHI antibodies (B). Proteins were electrophorized on 20% tricine-SDS-polyacrylamide gels. Lanes 1-3, 5-6, 9-17, 19-20 and 23-28, extracellular washing fluid from different F1 progeny; lanes 4 and 18, nontransformed control (C); lanes 7 and 22, purified UDA from stinging nettle; lanes 8 and 21, purified tobacco chitinase I. The UDA-Chi fusion, UDA and tobacco chitinase I are indicated by horizontal arrows. CHI-I, tobacco chitinase I.](image)

Seeds of several primary transformants were germinated and extracellular washing fluid of kanamycin resistant F1 progeny was analysed by western analysis using α-UDA antibodies (Fig. 2A) and α-CHI antibodies (Fig. 2B). As is shown in Figure 2A, α-UDA antibodies recognized purified UDA (lanes 7 and 22) and the fusion protein UDA-Chi in the extracellular washing fluid from the F1 plants (lanes 1-3, 5-6, 9-17, 19-20, 23-28), but they did not cross-react with purified tobacco chitinase I (lanes 8 and 21). Upon longer exposure a weak signal at the same height as that of UDA was found in some high-expressor lines (Fig. 2A, lanes 10, 11, 12 and 24), suggesting that a small amount of the fusion protein was
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being processed in transgenic tobacco. When the same blot was probed with α-CHI antibodies, the fusion protein was recognized as well (Fig. 2B, lanes 1-3, 5-6, 9-17, 19-20, 23-28). Some cross-reacting bands are present. The two bands of approximately 26 and 28 kD most likely represent endogenous class II chitinases (Sela-Buurlage et al., 1993), known to be induced by stress. The UDA-Chi chimera is clearly larger than tobacco chitinase I (Fig. 2B, lanes 8 and 21), due to the presence of an extra chitin-binding domain of about 4.5 kD. The chimera was not present in the extracellular washing fluid from control plants (Fig. 2A and B, lanes 4 and 18). The precursor UDA-ChiACT7 to the mature fusion protein UDA-Chi lacks the vacuolar targeting signal. The presence of the fusion protein in the extracellular washing fluid indicates extracellular targeting of UDA-Chi, as expected.

Agglutination and Chitinase Activities of Purified Fusion Protein UDA-Chi and Tobacco Chitinase I

Fusion protein UDA-Chi was purified to homogeneity from the total extract of a homozygous high-expressor F₂ line. Figure 3A shows the immunological detection of different amounts of purified UDA-Chi by α-UDA antibodies. One single band was detected for 100 ng of the fusion protein, even upon longer exposure time. The same blot was used for detection with α-CHI antibodies (Fig. 3B). No contamination by endogenous chitinase was detected using these antibodies. Upon longer exposure time of this blot, 5 ng fusion

![Figure 3. Western analysis of purified fusion protein UDA-Chi using α-UDA antibodies (A) and α-CHI antibodies (B). Lanes 1, 100 ng of UDA-Chi; lanes 2, 50 ng of UDA-Chi; lanes 3, 5 ng of UDA-Chi; lanes 4, 20 ng of purified tobacco chitinase I.](image)

![Figure 4. Silverstained protein gel with 250 ng of purified fusion protein UDA-Chi.](image)
protein became clearly visible, while no endogenous chitinase was detected in the sample of 100 ng fusion protein. Purity of the fusion protein was also checked by silverstaining, shown in Figure 4.

Tobacco chitinase I was purified from homozygous transgenic tobacco plants, expressing the protein at high level (Melchers et al., 1993). Purity was verified by western analysis using α-CHI antibodies (Fig. 3) and silverstaining of the protein gel (not shown).

Agglutination activity was tested for both proteins. The tobacco chitinase I did not agglutinate trypsin-treated rabbit erythrocytes (data not shown). In contrast, UDA-Chi displayed a low but significant agglutination activity at a concentration of 8.3 μg mL⁻¹ (0.5 μg per 60 μL). Increased agglutination activities were shown for higher concentrations of the fusion protein (Fig. 5). To exclude the possibility that UDA-Chi was being processed by residual trypsin or proteases from the erythrocytes, proteinase inhibitors were added to the agglutination mixtures. No differences were detected between the assays with or without the protease inhibitors (data not shown).

\[ \text{μg UDA-Chi} \]
\[
\begin{array}{ccccccc}
0 & 0.25 & 0.5 & 1.0 & 2.0 & 3.0 & 4.0 \\
\end{array}
\]

Figure 5. Agglutination assay with fusion protein UDA-Chi using trypsin-treated rabbit erythrocytes. The amounts of UDA-Chi added to a total volume of 60 μL are indicated.

Purified UDA-isolectin I from transgenic tobacco agglutinated trypsin-treated rabbit erythrocytes as effectively at a concentration of 2.5 μg mL⁻¹ (Does et al., 1999a) as UDA-Chi at a concentration of 8.3 μg mL⁻¹. Since the molecular mass of the UDA-Chi fusion protein is 36,546 D and the molecular mass of UDA-isolectin I is 9,408 D, 8.3 μg of fusion protein corresponds to approximately the same number of UDA-containing molecules as 2.5 μg UDA-isolectin I. Therefore we conclude that the molar agglutination activities of UDA-isolectin I and the UDA-Chi fusion are similar.

Chitinase activities of purified UDA-Chi, tobacco chitinase I and purified UDA-isolectin I were measured using the dye-labelled substrate CM-chitin-RBV (Table I). Using this substrate, the chitinase activity of the fusion protein appeared to be 3.6-fold lower than that of tobacco chitinase I. UDA-isolectin I did not display chitinase activity. Fusion of
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UDA-isolectin I to the catalytic domain of tobacco chitinase I therefore seemed to have changed the chitinase activity of the molecule on CM-chitin-RBV.

Table I. Chitinase activities of UDA-isolectin I, tobacco chitinase I and fusion UDA-Chi, purified from transgenic tobacco.

<table>
<thead>
<tr>
<th></th>
<th>Chitinase activity (ODμ/μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDA-isolectin I</td>
<td>0</td>
</tr>
<tr>
<td>Tobacco chitinase I</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td>Fusion UDA-Chi</td>
<td>1.0 ± 0.2</td>
</tr>
</tbody>
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CM-chitin-RBV was used as the substrate. Chitinase activity is represented as units of optical density (ODμ) at 550 nm per μg protein.

DISCUSSION

A chimeric protein UDA-Chi, consisting of the UDA-isolectin I sequence fused to the linker and catalytic domain of tobacco chitinase I, has been produced in transgenic tobacco. In high expressor-lines, a protein with a similar molecular weight to that of UDA was detected by western analysis using α-UDA antibodies. This protein could be a processing product of the fusion protein. Recently, two truncated class I chitinases have been identified in NaCl-adapted tobacco cells (Yun et al., 1996). Both chitinases lacked the chitin-binding domain, but still contained a partial linker. These linkers resembled those of both tobacco chitinase 1 isoforms A and B. It was suggested that the truncated proteins were most probably products of new genes. However, it cannot be excluded they were products of class I chitinases that had been processed between the first two glycine residues of the linker. Hence, the UDA-like protein we have detected might be the product of a similar processing event in the linker of the UDA-Chi chimera.

Agglutination activity was detected in crude leaf extracts of high expressor-lines producing the fusion protein. Some of this activity might have been caused by the UDA-like protein, if processed from the UDA-Chi chimera. However, since processing hardly occurs, most if not all of the agglutination activity should be attributed to the fusion protein. Purified UDA-Chi displays agglutination activity, which was not detected for tobacco chitinase I. On a molar basis, the agglutination activity of the fusion protein was similar to that of mature UDA. Apparently, fusion of UDA to the chitinase domain by a flexible linker does not affect agglutination activity. This suggests that the folding and exposure of sugar-binding sites
(Hom et al., 1995) in both chitin-binding domains of the fusion protein and mature UDA are identical.

Using CM-chitin-RBV in a colorimetric assay, the enzymatic activity of the fusion protein was lower than that of the chitinase I (1.0±0.2 ODu μg⁻¹ vs 3.6±0.3 ODu μg⁻¹). This does not necessarily mean that the actual chitinase activity was lower. It has been shown that different classes of tobacco chitinases display distinct hydrolysing and lysozyme activities, depending on the substrate (Brunner et al., 1998). Melchers et al. (1994) have shown differences in activity between tobacco class I chitinase and class II chitinase on two substrates, CM-chitin-RBV and tritiated-chitin. Although the enzymatic activity of class I chitinase was 10,000 times higher on tritiated chitin, the activity on CM-chitin-RBV was 30 times lower than that of the class II chitinase. Therefore, the enzymatic activity of the UDA-Chi chimera on tritiated-chitin could be higher than that of tobacco chitinase I. We therefore conclude that the chitinase activity of the fusion protein differs from that of tobacco chitinase I.

Previously, it has been shown that the enzymatic activity of the catalytic domain of tobacco chitinase I on tritiated-chitin is modified by the addition of the chitin-binding domain (Iseli et al., 1993). Using CM-chitin-RBV, tobacco chitinase I with and without a chitin-binding domain showed similar activities at pH 5.2 (Sela-Buurlage et al., 1996). Although the presence of one chitin-binding domain did not affect the enzyme activity of the catalytic domain of tobacco chitinase I on CM-chitin-RBV substrate, the fusion protein was 3.6-fold less active compared with the tobacco chitinase I. Actually, the UDA-Chi fusion can be seen as a tobacco chitinase I with an extra chitin-binding domain added to it. Because of this addition, the interaction of the chitin-binding domains and the chitinase domain during hydrolysis might have changed.

The structure of the UDA-Chi fusion resembles that of the UDA-precursor. Whether the UDA-precursor has agglutination activity and chitinase activity is unclear, because the UDA-precursor has not been isolated yet. With the exception of the UDA-precursor, the chitinase domains of plant chitinases that belong to the family 19 of glycosyl hydrolases contain two glutamate residues at conserved positions. For chitinase activity of tobacco chitinase I, these two glutamate residues in the catalytic domains have been shown to be necessary (Iseli-Gamboni et al., 1998). Removal of either of them results in loss of chitinase activity. The UDA-precursor lacks both these corresponding glutamate residues and therefore it has been suggested it has no chitinase activity (Iseli-Gamboni et al., 1998). However, two glutamate residues are present in the chitinase domain of the UDA-precursor, albeit four amino acids C-terminally of the conserved positions, and at the same distance from each other as in the amino acid sequence of tobacco chitinase I. The chitinase domain of the UDA-precursor is only 45% identical to the chitinase domain of tobacco class I.
chitinases. Therefore, it is possible that folding of the chitinase domain of the UDA-precursor differs from folding of other plant chitinase domains, keeping the catalytic site intact. This suggestion is supported by results obtained by Lerner and Raikhel (1992), who expressed both the precursor to UDA and the chitinase domain in Escherichia coli. Crude extracts of both E. coli strains showed significant chitinase activity.

UDA and tobacco chitinase I both display antifungal activity on chitin-containing plant pathogenic fungi. However, the range of susceptible chitin-containing fungi and the amount of each protein needed to obtain 50% growth-inhibition differ for both these chitin-binding proteins (Van Parijs et al., 1991; Broekaert et al., 1992). For example, UDA is known to inhibit the growth of Botrytis cinerea (Broekaert et al., 1989; Does et al., 1999a), whereas tobacco chitinase does not affect this fungus (Van Parijs et al., 1991). For inhibition of Colletotrichum lindemuthianum, very high concentrations of tobacco chitinase were needed (Broekaert et al., 1992), whereas much lower concentrations of UDA were sufficient (Broekaert et al., 1989, 1992; Does et al., 1999a). On the contrary, growth of Trichoderma hamatum was inhibited by low concentrations of chitinase and high concentrations of UDA (Van Parijs et al., 1991; Broekaert et al., 1992). UDA and tobacco chitinase I have been shown to act synergistically on T. hamatum (Broekaert et al., 1989). Since the fusion protein binds to chitin, and shows agglutination and chitinase activity, it may display antifungal activity as well. One may speculate that the UDA-Chi fusion has a growth-inhibiting effect on each fungus that is susceptible for either chitinase or UDA, and therefore has potential to be used as an antifungal on a broad range of chitin-containing fungi.

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