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. © 1999 Published by Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Fusion protein; Stinging nettle lectin; Tobacco chitinase I; Agglutination activity; Chitinase activity

1. Introduction

*Urtica dioica* agglutinin (UDA) consists of two cysteine-rich chitin-binding domains [1–3]. Both are homologous to hevein, a small chitin-binding protein of 43 amino acids from the rubber tree [4,5]. The presence of a carbohydrate-binding site in each chitin-binding domain enables UDA to agglutinate erythrocytes [1,6]. In most stinging nettle (*U. dioica* L.) ecotypes, UDA is present as a mixture of isolectins [3,7]. 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 [3,8]. A vacuolar sorting determinant has been shown to be present within the 25 C-terminal amino acids of the UDA precursor [9]. 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 [10,11]. Recently, processing of the precursor to UDA-isolectin I was studied in transgenic tobacco plants [9]. UDA purified from these tobacco plants had retained its chitin-binding activity, agglutination activity and antifungal activity against chitin-containing plant pathogenic fungi [1,9,12]. 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 [9].
Mature tobacco chitinase I is comprised of one chitin-binding domain fused to a linker and a chitinase domain [13,14]. 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 [15]. Removal of this vacuolar sorting determinant causes secretion of the mature basic chitinase into the extracellular space [15–17]. Depending on the isoform of the tobacco chitinases I, the linker consists of 10 (chitinase A) or 15 (chitinase B) amino acids [18]. 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 [19]. 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 [20].

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-isolecitin 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.

2. Materials and methods

2.1. Cloning procedures

The chimeric UDA–ChiACT7 fusion gene containing the UDA-isolecitin I leader sequence followed by the sequences encoding the signal peptide and UDA-isolecitin I of the UDA precursor, the linker and the catalytic domain of tobacco chitinase I (chitinase B), was made by the following procedure.

The gene encoding the precursor to UDA-isolecitin I (UdChia5.1.1) from stinging nettle ecotype Weerselo was cloned between the cauliflower mosaic virus 35S promoter and the nopalin synthase terminator on vector pMOG181 as described previously [3,9]. To isolate the UDA-isolecitin 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 [16]. PCR was performed using the primers Chi5 (5’-CCTGGTGGTCCCA-CACC-3’) and LS19 (5’-TTCCCCAGT-CAGCAGTGTG-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-isoelectin I) and blunt-HindIII (chitinase) PCR fragments were together cloned in the expression cassette on pMOG181. For extracellular targeting of the fusion protein, a stopcodon was introduced in the sequence of the chitinase domain. Therefore, a vacuolar targeting mutant chitinase construct (pMOG189) was used which had been created previously [16]. 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 (ChiDCT7). The stopcodon was introduced into the UDA–ChiDCT7 fusion by exchanging the PstI/HindIII fragment from the UDA–Chi fusion by the stopcodon-containing PstI/HindIII fragment from ChiDCT7. The construct was sequenced using the dideoxynucleotide chain-termination method [21]. The EcoRI/HindIII fragment from pMOG181:UDA–ChiDCT7 was cloned into the binary vector pMOG402 [22]. Cloning procedures were performed as described elsewhere [23].

2.2. Tobacco transformation

Transfer of the pMOG402:UDA–ChiDCT7 construct to Agrobacterium tumefaciens and tobacco (N. tabacum cv. Samsun NN) transformation were performed as previously described [9]. Seeds of primary transformants and of their progeny were selected on plates containing kanamycin (200 µg/ml).

2.3. 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 (EWF) was isolated according to De Wit and Spikman [24], using sodium acetate buffer. Protein concentrations were determined by the Bradford method [25] using bovine serum albumen as standard. Protein electrophoresis was performed using 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels [26] or using 20% Tricine-SDS-polyacrylamide gels [27]. Immunological detection by α-UDA antibodies [3] was performed as described previously [3]. For immunological detection with α-CHI antibodies [16], antibodies were diluted 5000-fold and incubated with the blot overnight at room temperature.

2.4. 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. One teaspoon of charcoal was added per 200 ml extraction buffer. The mixture was squeezed through four layers of gauze and kept on ice. Subsequently, the filtrate was centrifuged at 3200 × g for 30 min at 4°C. Supernatant was filtered through eight layers of gauze and centrifuged at 20,000 × g 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 20,000 × g and 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 [1] 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 0–0.25 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 over a YM10 Diaflo ultrafiltration
membrane (Amicon, Inc.) using a 400 ml stirred cell (Amicon, Inc.).

The concentrate was dialyzed against phosphate buffered saline (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. 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 dialyzed 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-polyacrylamide gel electrophoresis and both immunological detection using α-UDA antibodies and α-CHI antibodies, and silverstaining using the Silverstain plus kit (Biorad).

2.5. Purification of tobacco chitinase I from transgenic tobacco

Homozygous seeds of transgenic tobacco expressing the gene encoding tobacco chitinase I [16] were obtained from ZENECA MOGEN. Chitinase I was isolated from transgenic tobacco leaves according to the procedure described previously [20]. Protein concentration was determined using the bicinchoninic acid protein assay kit (Sigma). Purity was checked by 12.5% SDS-polyacrylamide gel electrophoresis and immunological detection using α-CHI antibodies, and silverstaining.

2.6. Agglutination assays

Agglutination assays using crude leaf extracts were performed on microscope slides, with squashed leaf samples to which trypsin-treated rabbit erythrocytes [1] 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 x stock solution of a protease inhibitor cocktail (Complete™, Boehringer Mannheim) in PBS 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.

2.7. 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 [28] as described previously [29].

3. Results

3.1. UDA–ChiACT7 precursor construct

Construct UDA–ChiACT7 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 [3] (Fig. 1A) to the coding sequence for the linker and the catalytic domain of tobacco chitinase I [16] (Fig. 1B). Due to the introduction of a stop codon, the precursor construct UDA–ChiACT7 lacks the C-terminal propeptide of seven amino acids (ACT7), which has been shown to be necessary for the targeting of tobacco chitinase I to the vacuoles [15]. Because of this deletion, we expected the fusion protein to be targeted extracellularly, like the tobacco chitinase [16]. 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–ChiACT7 was placed in an expression cassette, between the cauliflower mosaic virus 35S promoter and the nopaline synthase transcription terminator, and transformed into tobacco using Agrobacterium.

3.2. 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 [3].

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.

Seeds of several primary transformants were germinated and EWF of kanamycin resistant F1 progeny was analysed by Western analysis using \( \alpha \)-UDA antibodies (Fig. 2A) and \( \alpha \)-CHI antibodies (Fig. 2B). As is shown in Fig. 2A, \( \alpha \)-UDA antibodies recognized purified UDA (lanes 7 and 22) and the fusion protein UDA–Chi in EWF 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 level 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 being processed in transgenic tobacco. When the same blot was probed with \( \alpha \)-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 [20], 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 EWF from control plants (Fig. 2A,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 EWF indicates extracellular targeting of UDA–Chi, as expected.

3.3. Agglutination and chitinase activities of purified fusion protein UDA–Chi and tobacco chitinase I

Fusion protein UDA–Chi was purified to homogeneity from total extract of a homozygous high expressor F2 line. Fig. 3A shows the immunological detection of different amounts of purified UDA–Chi by \( \alpha \)-UDA antibodies. One single band was detected for 100 ng of the fusion protein, even upon longer exposure time. The same blot was
Samsun NN) plants, expressing the protein at high level [16]. 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 (0.5 µg/60 µl). Increased agglutination activities were shown for higher concentrations of 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).

Purified UDA-isolectin I from transgenic tobacco agglutinated trypsin-treated rabbit erythrocytes as effectively at a concentration of 2.5 µg/ml [9] as UDA-Chi at a concentration of 8.3 µg/ml. Since the molecular mass of the UDA-Chi fusion protein is 36,546 Da and the molecular mass of UDA-isolectin I is 9408 Da, 8.3 µg 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 1). 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 UDA-isolectin I to the catalytic domain of tobacco chitinase I therefore
Table 1
Chitinase activities of UDA-isolectin I, tobacco chitinase I and fusion UDA–Chi, purified from transgenic tobacco

<table>
<thead>
<tr>
<th></th>
<th>Chitinase activitya (ODu/µg)</th>
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<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>
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*a CM-chitin-RBV was used as the substrate. Chitinase activity is represented as units of optical density (ODu) at 550 nm per µg protein.

seemed to have changed the chitinase activity of the molecule on CM-chitin-RBV.

4. 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 z-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 [30]. Both chitinases lacked the chitin-binding domain, but still contained a partial linker. These linkers resembled those of both tobacco chitinase I 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. The molar 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 [6] 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 tobacco chitinase I (1.0 ± 0.2 ODu/µg vs 3.6 ± 0.3 ODu/µg). 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 [31]. Melchers et al. [29] 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 addition of the chitin-binding domain [32]. Using CM-chitin-RBV, tobacco chitinase I with and without a chitin-binding domain showed similar activities at pH 5.2 [33]. 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. Due to this addition, the interaction of the chitin-binding domains and the chitinase domain during hydrolysis might have changed.

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 [34,35]. For example, UDA is known to inhibit the growth of *Botrytis cinerea* [9,12], whereas tobacco...
chitinase does not affect this fungus [34]. For inhibition of *Colletotrichum lindemuthianum*, very high concentrations of tobacco chitinase were needed [35], whereas much lower concentrations of UDA were sufficient [9,12,35]. On the contrary, growth of *Trichoderma hamatum* was inhibited by low concentrations of chitinase and high concentrations of UDA [34,35]. UDA and tobacco chitinase I have been shown to act synergistically on *T. hamatum* [12]. 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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**References**


