Purification and N-terminal sequencing of a 42 kDa Trichoderma viride chitinase isoform effective against Rhizoctonia solani


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ABSTRACT: Trichoderma viride extracellular chitinase of molecular weight 42 kDa was purified. Its N-terminal sequence was determined. N-terminal sequence comparison revealed 70-100% similarity to those of already characterised chitinases of similar molecular weight from other fungi. A chitin affinity column fraction that contained the enriched 42 kDa chitinase showed a higher chitinase specific activity. The 42 kDa chitinase purified to homogeneity by electroelution inhibited in vitro the growth of the rice sheath blight isolate of Rhizoctonia solani.

Key words: Biological control; Trichoderma viride; rice; Rhizoctonia solani; chitinases, mycoparasitsm

The biocontrol agents, Trichoderma viride and T. harzianum (Papavizas, 1985) have been shown to be mycoparasitic on Rhizoctonia solani (Wells et al., 1972) causing rice sheath blight, an important rice disease worldwide (Herdt, 1991). Trichoderma spp. are known to produce hydrolytic enzymes viz., chitinases, b-1, 3-glucanases, proteases and volatile and non-volatile antibiotics (Elad et al., 1982). The observation that chitin is one of the main structural components of fungal cell walls (Bartnicki-Garcia, 1968) led to the hypothesis that the Trichoderma chitinases are involved in their mycoparasitic activity (Elad et al., 1980, 1983). The lytic activity of several strains of Trichoderma spp. on cell walls of phytopathogenic fungi was correlated with the degree of biological control of these pathogens in vivo (Papavizas, 1985; Vidhyasekaran and Balasubramanian, 1995; Vidhyasekaran et al., 1996). T. harzianum, which produces an endochitinase was demonstrated to be a potent biocontrol agent against several phytopathogenic fungi including R. solani (Cook and Baker, 1983; Pe’er and Chet, 1990). The chitinolytic enzymes from T. harzianum were shown to inhibit spore germination and germ tube elongation of chitin containing fungi (Lorito et al., 1993; 1993a). Haran et al., (1996) showed differential and temporally-separated expression of T. harzianum chitinases during its mycoparasitism on different hosts and they hypothesised that the differential expression of T. harzianum chitinases may influence its antagonism against a specific host.

Haran et al., (1993) purified and characterized a 41 kDa endochitinase from the culture filtrates of T. harzianum. Likewise, Fekete et al., (1996) identified a chitinase sequence in Trichoderma hamatum which showed high levels of similarity to a 42 kDa chitinase gene of T. harzianum. Carsolio et al. (1994) cloned and characterized the 42 kDa endochitinase. Expression of the 42 kDa cDNA clone in E. coli conferred the bacterium with the ability to induce chitinase activity. This chitinase has been shown to have an in vitro lytic activity on Botrytis cinerea. Garcia et al. (1994) showed the chitin-inducibility and glucose-repressibility of the 42 kDa chitinase of T. harzianum. Lorito et al. (1996) demonstrated that the mycoparasitic interaction relieved the 42 kDa endochitinase-encoding gene (T. harzianum) from catabolite repression. Margolies-Clark et al. (1996 and 1996a) demonstrated the overexpression of the 42 kDa endochitinase gene of T. harzianum in T. harzianum and T. reesei when the gene was driven by the promoter of major cellulase gene. Our earlier studies (Krishnamurthy et al., 1999) identified a set of effective strains of T. viride including the most effective one T.
viride MNT7. This strain expressed a substrate (colloidal chitin or R. solani cell wall)-inducible and glucose-repressible chitinase of molecular weight 42 kDa, along with a 30 kDa and 64 kDa major chitinases and a set of minor ones. The present study was directed towards purifying and further characterising the 42 kDa MNT7 chitinase.

MATERIALS AND METHODS

Maintenance of cultures

Trichoderma viride strain MNT7 (Nakkeeran et al., 1996) and Rhizoctonia solani strain RS7 (Sriram et al., 1997) were used for the study. The cultures were maintained on potato dextrose agar (PDA) plates, by routine subculturing at regular intervals.

Purification of T. viride extracellular chitinases

a. Induction of extracellular chitinolytic enzymes and preparation of cell-free culture filtrates

For inducing extracellular chitinolytic enzymes, liquid medium (1% potassium nitrate, 0.5% potassium dihydrogen phosphate, 0.25% magnesium chloride heptahydrate, 0.2% iron (III) chloride hexahydrate, 1% colloidal crab shell chitin and 1% v/v V8 juice-Harman et al., 1993) was inoculated with PDA discs containing T. viride MNT7. The culture was grown on a rotary shaker (125 rpm) at 25±2° C for 5 d. The culture was filtered through Whatman 1 filter paper and the filtrate was cleared by centrifugation at 15,000 x g for 10 min at 4° C.

b. Ammonium sulphate fractionation

The cell-free culture filtrate was brought to 40% saturation of ammonium sulphate and incubated overnight at 4° C. The precipitated proteins (0-40% fraction) were pelleted down at 20,000 x g for 20 min at 4° C. The pellet was suspended in a minimal volume of 50mM sodium phosphate buffer (SPB) pH 7. This fraction was dialysed against excess of phosphate buffer. The dialysate was concentrated by lyophilisation. The proteins were quantified (Bradford, 1976) and analysed on a 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel.

c. Chitin affinity chromatography

The 0-40% ammonium sulphate fraction was resolved on a preparative native polyacrylamide gel in Tris-glycine buffer without SDS. The 42 kDa chitinase band was excised and the protein was electroeluted. The eluted proteins was dialysed against 25mM SPB and the dialysate was concentrated by lyophilisation.

d. Electroelution of 42 kDa chitinase

The chitin affinity column fraction was resolved on a preparative native polyacrylamide gel in Tris-glycine buffer without SDS. The 42 kDa chitinase band was excised and the protein was electroeluted. The eluted proteins was dialysed against 25mM SPB and the dialysate was concentrated by lyophilisation.

Western blotting analysis

After each step of purification, the fractions were subjected to Western blotting analysis (Winston et al., 1987), in order to confirm the identity of the 42 kDa chitinase. The primary antibody used for the study was an anti-barley chitinase antiserum, a polyclonal antibody raised in rabbit. It was a generous gift from Dr. M. Legrand, Institute de Biologie Moléculaire des Plantes (IBMP), Cedex, France.

Chitinase assays and bioassays

Chitinase activity was estimated following the procedure suggested by Boller et al. (1983) with the 0-40% ammonium sulphate fraction or chitin affinity column fraction using colloidal chitin (Roberts and Selitrennikoff, 1988) as the substrate.

Bioassays were performed in Petri plates (9 cm diam) containing 15 ml PDA. Sterile filter paper discs of 0.6 cm were placed 2 cm away from the centre. A mycelial disc of RS7 was placed at the centre of the plate. The plates were incubated at room temp until the mycelial growth from the centre reached near the filter paper disc. The 0-40% ammonium sulphate fraction or chitin affinity column fraction or electroeluted 42 kDa chitinase was added onto the paper discs. Suitable control with buffer alone was also maintained. Inhibition zone was visualized after 2 d.

N-terminal amino acid sequencing

The 0-40% ammonium sulphate fraction was resolved on a 10% SDS-polyacrylamide gel (Laemmli, 1970). The protein profiles were electroblotted on a polyvinylidene difluoride (PVDF) membrane and the membrane was stained with Ponceau S. The zone of the membrane carrying the intended band was cut out, washed in distilled water and dried. The excised bands were analysed by an automated Edman degradation using Applied Biosystems Sequencer (Matsudaira, 1987; Tempst and Riviera, 1989).
RESULTS AND DISCUSSION

The 42 kDa chitinase was found in 0-40% ammonium sulphate fraction (Fig. 1a). The detection of the 42 kDa protein by the anti-barley chitinase antiserum, in Western blotting analysis confirmed its identity. The subsequent chitin affinity chromatography of the 0-40% ammonium sulphate fraction enriched the 42 kDa chitinase (Fig. 1b). For bioassay, the 42 kDa chitinase was purified to homogeneity by electroeluting it from a native preparative polyacrylamide gel. Fig. 1c shows the SDS-PAGE profile of the electroeluted protein and its detection was confirmed by Western blotting analysis (data not shown).

Fig. 1. SDS-Page of (a) 0-40% ammonium sulphate fraction of extracellular proteins of T. viride MNT7; (b) Chitin affinity column chromatography fraction; (c) Electroeluted 42 kDa protein. M, standard protein markers; Arrow indicates 42 kDa chitinase

Specific chitinase activity of the fractions increased as the 42 kDa chitinase became enriched through successive steps of purification. The specific chitinase activity of the chitin affinity chromatography fraction was 10-fold higher when compared to that of the crude cell-free culture filtrate. The electroeluted 42 kDa chitinase showed more than a 2-fold increase in activity as compared to that of the crude cell-free culture filtrate (Table 1). In bioassays, while the 0-40% ammonium sulphate fraction was able to prevent completely the spread of R. solani mycelium towards the periphery of the plate, the chitin affinity column fraction exhibited an inhibition zone of around 1 cm diam. The purified chitinase was effective in inhibiting the mycelial growth of R. solani when 20 μg of the same was spotted onto the disc (Fig. 2). The apparent decrease in chitinase-specific activity and anti-fungal nature of the electroeluted 42 kDa chitinase when compared to those of ammonium sulphate and chitinase affinity column fractions could be attributed to the exclusion (during purification) of other antifungal factors in the culture filtrate (Table 1).

The 42 kDa protein had an N-terminal sequence of ASGYANAVYF. Comparison of this sequence with those of already characterised chitinases revealed a high degree of homology (Table 2). While it exhibited a 70% homology with the N-terminal sequence of an extracellular chitinase from Trichoderma virens (Baek and Kenerley 1998), it showed a complete homology with that of the 42 kDa endochitinase from T. harzianum (Carsolio et al., 1994) and a PCR amplified chitinase from T. hamatum (Fekete et al., 1996) and an endochitinase gene from T. hamatum (Giczey et al., 1997). This sequence also showed 80% similarity to that of a chitinase from an entomopathogenic fungus Metarrhizium anisopliae (St-Leger et al., 1996). The data indicate the probability of a high degree of sequence conservation in this protein. This 42 kDa chitinase from MNT7 appears to be the first of its kind to be reported in T. viride.

Table 1. Estimation of specific activity of chitinases

<table>
<thead>
<tr>
<th>Step</th>
<th>Specific activity (μmol GlcNAc eq. h⁻¹mg⁻¹ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Culture filtrate</td>
<td>0.157</td>
</tr>
<tr>
<td>II. 0-40% ammonium sulphate fraction</td>
<td>1.010</td>
</tr>
<tr>
<td>III. 0-40% Chitin affinity chromatography fraction</td>
<td>1.569</td>
</tr>
<tr>
<td>IV. Electroeluted 42 kDa chitinase</td>
<td>0.330</td>
</tr>
</tbody>
</table>

Fig. 2. Antifungal activity of extracellular proteins of T. viride MNT7. 1. 0-40% ammonium sulphate fractions; 2. Chitin affinity column chromatography fraction of 0-40% ammonium sulphate fraction; 3. Control; Electroeluted 42 kDa chitinase at (a) 20 mg, (b) 40 mg and (c) 60 mg concentrations

An oligonucleotide probe has been synthesized based on the N-terminal sequence of the 42 kDa chitinase. Attempts are underway to isolate the corresponding clone from a cDNA library constructed using mRNAs isolated from T. viride grown on colloidal chitin. Our ultimate aim is to introgress the identified gene into elite indica rice lines so as to evolve sheath blight-resistant cultivars.
Table 2. Amino acid sequence homology of seven other chitinases compared with Trichoderma MNT7 42 kDa chitinase of the present study

<table>
<thead>
<tr>
<th>Source Reference</th>
<th>Homologous N-terminal sequences</th>
</tr>
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<tbody>
<tr>
<td>Trichoderma viride MNT7 42 kDa chitinase</td>
<td>A S G Y A N A V Y F</td>
</tr>
<tr>
<td>Trichoderma harzianum ech-42 chitinase</td>
<td>Carsolio et al. (1994)</td>
</tr>
<tr>
<td>T. harzianum CHIT42</td>
<td>Garcia et al. (1994)</td>
</tr>
<tr>
<td>T. hamatum chitinase</td>
<td>Fekete et al. (1996)</td>
</tr>
<tr>
<td>T. virens chitinase</td>
<td>Baek and Kenerley (1998)</td>
</tr>
<tr>
<td>T. hamatum chitinase secreted by Saccharomyces cerevisiae</td>
<td>Giczey et al. (1997)</td>
</tr>
<tr>
<td>Aphanocladium album chitinase</td>
<td>Blaiseau et al. (1992)</td>
</tr>
</tbody>
</table>

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