Purification and characterization of a viral chitinase active against plant pathogens and herbivores from transgenic tobacco

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\texttt{Abstract}

The Autographa californica nucleopolyhedrovirus chitinase A (AcMNPV ChiA) is a chitinolytic enzyme with fungicidal and insecticidal properties. Its expression in transgenic plants enhances resistance against pests and fungal pathogens. We exploited tobacco for the production of a biologically active recombinant AcMNPV ChiA (rChiA), as such species is an alternative to traditional biological systems for large-scale enzyme production. The protein was purified from leaves using ammonium sulfate precipitation followed by anion exchange and gel-filtration chromatography. Transgenic plants produced an estimated 14 mg kg\textsuperscript{−1} fresh leaf weight, which represents 0.2% of total soluble proteins. The yield of the purification was about 14% (2 mg kg\textsuperscript{−1} fresh leaf weight). The comparison between the biochemical and kinetic properties of the rChiA with those of a commercial Serratia marcescens chitinase indicated that the rChiA was thermostable and more resistant at basic pH, two positive features for agricultural and industrial applications. Finally, we showed that the purified rChiA enhanced the permeability of the peritrophic membrane of larvae of two Lepidoptera (Bombyx mori and Heliothis virescens) and inhibited spore germination and growth of the phytopathogenic fungus Alternaria alternata. The data indicated that tobacco represents a suitable platform for the production of rChiA, an enzyme with interesting features for future applications as “eco-friendly” control agent in agriculture.

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1. Introduction

Reducing the use of synthetic compounds to control biotic stress is a major objective of sustainable agriculture (Epstein and Bassein, 2003). Biocidal activities, being molecules derived from natural sources such as animals, plants and bacteria, are thought to pose lower risks than conventional pesticides. Both in developing and developed countries, there is a broad agreement in encouraging their use and in exploring new production methods (Byerlee et al., 2009). The replacement of chemical pesticides by bioinsecticides is expected to make a significant contribution for the protection of human health and the environment, with the consequent reduction of toxic residues on food crops as well as the preservation of biodiversity (Hynes and Boyetchko, 2006). However, a suitable compound should meet different requirements: to have a broad spectrum of activity, to have a low impact on the environment and to be cost effective (Hynes and Boyetchko, 2006).

Previously, we demonstrated that transgenic tobacco plants expressing the chitinase A from the Autographa californica nucleopolyhedrovirus virus (AcMNPV ChiA) showed reduced damages after fungal pathogens and lepidopteran larvae attack, without effects on a non-target insect population (Corrado et al., 2008). Those data indicated that the AcMNPV ChiA is an interesting candidate as a molecule of biological origin for crop protection. Besides agricultural applications, the enzymatic hydrolysis of chitin is a process of increasing interest for the medical and industrial sectors such as the production of chito-oligosaccharides and N-acetyl-D-glucosamine (Pichyangkura et al., 2002), the preparation of sphaeroplasts and protoplasts from yeast and fungal species (Mizuno et al., 1997; Balasubramanian et al., 2003) and the bioconversion of chitin waste (Vyas and Deshpande, 1991). Furthermore, chitinases also possess antibacterial, hypocholesterolemic and antihypertensive activities (Bhattacharya et al., 2007) and are also useful as food quality enhancer (Bhattacharya et al., 2007).
The expression and purification of enzymes for industrial applications in transgenic plants has been achieved for several proteins, including xylanase (Pate et al., 2000), transglutaminase (Claparols et al., 2004), endoglucanase (Ziegler et al., 2000) and laccase (de Wilde et al., 2008). Here we present data on the production, purification and characterization of a recombinant AcMNPV ChiA enzyme (hereinafter rChiA) from transgenic tobacco and the evaluation of its activity on fungal pathogens and insects.

2. Materials and methods

2.1. Quantification of ChiA by immunofluorescence analysis

Transgenic tobacco plants expressing the rChiA protein were as reported (Corrado et al., 2008). Leaves were homogenized in liquid nitrogen and total soluble proteins (TSP) extracted in 1× PBS (137 mM NaCl, 4.3 mM Na2HPO4, 2.7 mM KCl, 1.4 mM KH2PO4), pH 7.4 added with 1 mM PMSF. The homogenate was centrifuged at 14,000×g for 15 min at 4°C. Protein content was determined by the method of Bradford (1976) using Bio-Rad protein assay (Bio-Rad, Milano, Italy), with bovine serum albumin as standard. TSP extracted from transgenic plants or from untransformed controls was separated by SDS-PAGE on a Mini-Protein II mini-gel apparatus (Bio-Rad, Milano, Italy), using 6% (w/v) stacking polyacrylamide gel and 12% (w/v) separation gel (Laemmli, 1970). The PositopeTM (Invitrogen, Milan, Italy), a 53 kDa recombinant protein specifically engineered to contain different tags for the detection with different antibodies was used as quantitative standard. Separated proteins were transferred onto polyvinylidene difluoride (PVDF) membrane by electroblotting with Mini Trans-Blot Cell (Bio-Rad, Milano, Italy). The blot was probed with the anti-c-myc polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), as a primary antibody (dilution 1:500) and anti-rabbit IgG conjugated with Cy5 (GE Healthcare, Milan, Italy) as a secondary antibody (dilution 1:2500). Quantification of the fluorescent signal was performed with Typhoon 9410 Laser Scanner and data were read out and analysed with the Image Quant 5.2 software (GE Healthcare, Milan, Italy).

2.2. Enzymatic activity

The enzymatic activity of the rChiA was assessed using 4-methylumbelliferyl-β-d-N,N-acetylchitotriose (4MU-(GlcNAe)3; Sigma–Aldrich, Milan, Italy) as substrate for the detection of endo-chitinase activity as previously reported (McCreath and Gooday, 1992; Rao et al., 2004). Typically, each reaction used 25 ng of chitinase and 4 nM of substrate in 20 μl final volume of Mcllvaine buffer, for 30 min at 20°C. Hereafter, we refer to these conditions as “standard conditions”. Utilizing this substrate unit definition is: one unit of chitinase activity will release 1 μmol of 4-methylumbelliferyl from the substrate per minute. Fluorescence was monitored using Victor 31420 fluorimeter (Perkin Elmer, Monza, Italy). Aliquots of TSP from transgenic and untransformed plants and purified enzyme were assayed in standard conditions (Rao et al., 2004).

2.3. Protein purification

Leaves (100 g) were homogenized in 500 ml of 1× PBS buffer supplemented by 5 mM EDTA, 1 mM PMSF and 1.5% PVP-40, by 20-s bursts at full power, using a Waring Blender (Waring Products, CT, USA). The homogenate was filtered through Miracloth paper (Inalco, Milan, Italy) and centrifuged at 30,000 × g for 45 min at 30,000 × g. The supernatant was dialysed against 45 min at 0.35 M NaCl, pH 7.3. Bound acidic proteins were eluted with 10 mM Tris-Cl, pH 7.3, containing 1 M NaCl. Eluted acidic proteins were concentrated in a 50 ml Amicon concentrator equipped with a PM-10 membrane (Millipore Corporation, MA, USA) under N2 pressure (4 bars) and magnetic stirring. This concentrated acid fraction was gel-filtered on a Hi-Load 26/60 SuperdexTM 75 (pH final volume of 1 ml/min). Fractions containing proteins with molecular weight of about 60 kDa were pooled, dialyzed against 10 mM Tris-Cl, pH 7.3 and subjected to sequential chromatographies on the AKTA Purifier: (i) chromatography on the anionic column SourceTM 15Q PE 4/60 (GE Healthcare, Milan, Italy), equilibrated with 10 mM Tris-Cl (pH 7.3), run at a flow rate of 1 ml/min. Proteins eluted using a NaCl linear gradient made up by 100 mM Tris-Cl (pH 7.3) and same buffer, containing 0.3 M NaCl (total volume 60 ml; 1 ml min⁻¹ flow rate); (ii) analytical gel-filtration on SuperdexTM 75 10/300 GL column (separation range 70–3 kDa), equilibrated in 10 mM Tris-Cl (pH 7.3), containing 0.35 M NaCl. The purification was monitored after each step by SDS-PAGE gels stained with Coomassie Brilliant Blue G-250. The isolated rChiA was then detected by immunoblot.

2.4. Amino acid sequencing

rChiA, separated by SDS-PAGE, was transferred onto PVDF membrane and directly subjected to Edman degradation on a Procise Model 491 sequencer (Applied Biosystems, Monza, Italy) for N-terminal sequencing as previously described (Di Maro et al., 2001).

2.5. Optimal conditions for enzyme activity

The influence of pH on the chitinolytic activity of the recombinant purified rChiA and of commercial Serratia marcescens chitinase A (hereinafter cChiA; Sigma–Aldrich, Milan, Italy) was determined at 20°C in buffer solutions whose pH was adjusted to the desired value. The buffer systems used (25 mM final concentration) were as follows: Na-citrate (pH 3.0); Na-acetate (pH 4.0 and 5.0); Na-phosphate (pH 6.0 and 7.0); Tris-Cl (pH 8.0 and 9.0) and Na2CO3 (pH 10.0). Reactions were carried out for 30 min. Chitinase activity was also carried out by varying EDTA concentration (0–100 mM). The dependence of the chitinases on divalent cations was determined at different concentrations of Ca²⁺ and Mg²⁺ (0–32 mM). The ionic divalent optima for chitinolytic activity dependence was determined adding NaCl (0–600 mM). For these studies, rChiA protein and cChiA were used at a concentration of 0.25 ng μl⁻¹. Thermophilicity was evaluated in the temperature range 20–80°C by measuring chitinase activity for 30 min. Thermostability of both chitinases was estimated for 4 h at optimal temperature (50°C). For these studies, rChiA protein and cChiA were used at a concentration of 0.25 ng μl⁻¹ under standard conditions (Rao et al., 2004).

2.6. Enzyme kinetics

The kinetics of the two chitinases enzymes were estimated by using different concentrations of 4MU-(GlcNAe)3 (0.2–8 μM) in buffer 25 mM Na-phosphate, pH 6.0. The reaction was performed for 5 min at 30°C after addition of a suitable enzyme aliquot.
The kinetic data were calculated from double-reciprocal plots according to the method of Lineweaver and Burk (1934). The apparent Michaelis–Menten constant ($K_m(app)$) and apparent maximum velocity ($V_{max(app)}$) values (for the presence of substrate inhibition) were determined as the reciprocal absolute values of the intercepts on the $x$- and $y$-axes, respectively, of the linear regression curve.

2.7. Isolation of Bombyx mori and Heliothis virescens peritrophic membranes and permeability measurements

The isolation of B. mori and H. virescens peritrophic membrane, the incubation in the absence (control) or in the presence of 40 and 50 mg l$^{-1}$ of rChiA for B. mori and H. virescens PMs, respectively, and the flux measurements of the methylene blue dye (2 mg ml$^{-1}$) after 2 h of incubation at room temperature, were performed as described in Rao et al. (2004).

2.8. Bioassay on Alternaria alternata

Direct antifungal activity of the rChiA protein against A. alternata was determined using a spore germination test carried out as previously described (Lorito et al., 1994; Corrado et al., 2005). Effect on germ tube elongation was determined by measuring at least 50 germ tubes randomly chosen.

3. Results

3.1. Preparation of the recombinant chitinase enzyme

Tobacco transgenic lines expressing a chimeric ChiA gene were already available (Corrado et al., 2008). In these lines, the sequence coding the mature AcMNPV ChiA is fused, at the 5′ end, to a sequence coding a tobacco Signal Peptide, to target the propeptide to the secretory pathway, and at the 3′ end, to a sequence coding the HDEL, as endoplasmic reticulum retention signal, and a myc epitope (Fig. 1a). The transgene is under the control of the constitutive CaMV 35S RNA promoter. Transgenic lines did not display any obvious phenotypic abnormalities and we focused our attention on line 9, which accumulated the highest amount of recombinant protein (data not shown). An estimation of the amount of the rChiA protein produced in transgenic plants was carried out by a fluorimetric immuno-assay calibrated on a serial dilution of the PositopeTM, a recombinant tobacco plants (transgenic line 9). The blot was probed with the anti-c-myc polyclonal antibody as a primary antibody and anti-rabbit IgG conjugated with Cy5 as secondary antibody. Lanes: 1, total proteins extracted from untransformed tobacco plants (30 μg); lanes 2, 3 and 4: total proteins extracted from transgenic tobacco plants (30 μg); lanes 5, 6 and 7: 25, 50 and 100 ng of PositopeTM used as standard. (c) Chitinolitic activity of total proteins extracted from untransformed tobacco plants (N. tabacum) and the transgenic line 9.

3.2. Biochemical characterization of the purified enzyme

The biochemical and kinetic properties of the recombinant enzyme were determined and compared to those of cChiA (Figs. 3 and 4). The rationale of this choice was to evidence differences between the two proteins that show 59.4 sequence identity (Hawtin et al., 1995). Sequence alignment of the plant purified rChiA and cChiA is provided in Supplementary Fig. 1. Under standard conditions used for these experiments the specific activity of rChiA and cChiA were 5.7 and 7.1 mU mg$^{-1}$, respectively. Both chitinases were active in the pH range 5.0–7.0, with a pH optimum of 6.0 (Fig. 3a). However, the rChiA maintained 30% activity at pH 10.0, while the cChiA retained only about 15% of its optimum activity. As shown in Fig. 3b, both chitinases were active in a wide temperature range (30–80 °C), with the peak activity at 50 °C. Interestingly, after 4 h, the purified rChiA retained 86% of its chitinolytic activity while the cChiA lost almost completely the enzymatic activity (Fig. 3c). The presence of increased concentrations of EDTA in the assay mixture decreased the enzymatic activity of the purified rChiA (Fig. 4a), while they did not affect the cChiA. For this reason the enzyme activity was investigated in the presence of different concentration of Ca$^{2+}$ and Mg$^{2+}$. The presence of 5–30 mM Ca$^{2+}$ increased the chitinolytic activity of cChiA of about 10%, while Mg$^{2+}$ at concentration >10 mM decreased the enzymatic activity (data not shown). Similarly, the activity of rChiA was reduced in the presence of high concentration of NaCl (Fig. 4b).
3.3. Kinetic properties

Kinetic parameters for the chitinase activity of the two enzymes were determined at low substrate concentrations (μM) because both chitinases were inhibited at high substrate concentrations. At low substrate concentrations, both chitinases exhibited sigmoid kinetics. Moreover, the substrate inhibition of the rChiA was higher than that observed for cChiA. The $V_{\text{max(app)}}$ values for rChiA and cChiA were found to be $1.09 \times 10^{-8}$ and $1.18 \times 10^{-6}$ μmol min$^{-1}$, respectively. The $K_{M(app)}$ values were $3.5 \pm 0.15$ and $15 \pm 0.58$ μM for rChiA and cChiA, respectively. The $K_{M(app)}$ and $V_{\text{max(app)}}$ obtained from cChiA were in good agreement with those previously reported (Krokeide et al., 2007), while these parameters were obtained for the first time for AcMNPV recombinant chitinase. The lower value of $K_{M(app)}$ for rChiA likely shows the higher substrate inhibition detected for this enzyme (data not shown).

3.4. Activity of the purified enzyme on insect peritrophic membrane and fungal spores

The biological activity of the purified enzyme was evaluated analysing the permeability of the peritrophic membrane (PM) of two lepidopteran larvae, B. mori and H. virescens, and the effect on the spore germination and germ tube elongation of a phytotoxic fungus, A. alternata.

The effect of rChiA on the permeability of B. mori and H. virescens PMs was estimated by monitoring the transmembrane flux of the dye methylene blue (Mw of 319.9), a good tracer of PM permeability (Rao et al., 2004; Corrado et al., 2008; Fiandra et al., 2009), after incubation for 2 h in vitro with the purified recombinant enzyme. In B. mori PMs exposed to 10 or 50 μg ml$^{-1}$ of rChiA (Fig. 5a), a progressive increase of methylene blue flux was observed: the increase was highly significant at the highest concentration of enzyme tested. In the PMs of H. virescens larvae treated with 40 μg ml$^{-1}$ rChiA, the flux of methylene blue was also significantly higher than that of control (Fig. 5b).

In order to examine the effect of rChiA on phytotoxicity fungi, we determined the inhibitory effect on fungal germination and germ tube growth of A. alternata. As illustrated in Fig. 6a and b, both chitinases showed significant inhibition of germination and germ tube elongation. At 75 μg ml$^{-1}$ the inhibition effects of the two enzymes were similar.
Fig. 4. Graphical representation of the rChiA activity (□) in the presence of 0–100 mM EDTA (a) and 0–600 mM NaCl (b) determined by assaying the enzyme in the standard condition at the indicated concentrations. (□ – □), cChiA used as control.

4. Discussion

Chitinases are hydrolytic enzymes produced by a vast range of organisms, including insects, plants and animals. Because of their specific activity towards chitin, they are considered to be highly selective, being for instance non-toxic to higher vertebrates. For this reason, these enzymes have been long deemed promising candidates as biopesticides and as enhancers of plant protection (Kramer and Koga, 1986). In addition, chitinases are also induced in plants in response to biotic stress and, as implied by different studies, their heterologous expression should not have detrimental effects on plant growth and development (Ding et al., 1998; Howie et al., 1994). The recombinant chitinase was purified from tobacco leaves in which the enzyme was present at a concentration of about 0.2% of TSP, a value that falls into the 0.1–1% of TSP range typically observed for the proteins produced in nuclear transformants of tobacco plants (Twyman et al., 2003). This species was chosen as non-food and non-feed crop that can supply one of the most abundant leaf biomass per acre (Nikolov and Woodard, 2004). In a previous study, we reported the purification of a truncated AcMNPV Chi A from Escherichia coli with a yield of about 3 mg l\(^{-1}\). This low yield was likely the consequence of the accumulation of the recombinant protein in the bacterial inclusion bodies (Rao et al., 2004). The production of the recombinant enzyme in tobacco plants overcome some of the limitations of the enzyme production in bacteria.

The main factor that prompt us to test tobacco plants as a cost-effective production system is the downstream processing of the recombinant protein (Nikolov and Woodard, 2004). Our aim was to develop a general and potentially scalable downstream process that could be commercially feasible for extraction from large amounts of tobacco biomass. In our conditions, the recovery of the purified enzyme was 2 mg kg\(^{-1}\) FLW, corresponding to a yield of 14%. It is likely that alternatives downstream processing, which may include the use of a specific extraction buffer with protease inhibitors and an affinity tag chromatography, could achieve higher recovery.

We showed that the purified recombinant enzyme displays a number of interesting features. The rChiA, compared to the commercial enzyme, retains its activity at higher pH values (>9), confirming its suitability as biopesticide against herbivorous insects. The midgut environment of Lepidoptera larvae is usually at pH values higher than nine (Dow, 1992), explaining why, among the chitinases so far tested, only the insect moulting chitinase from Manduca sexta or the chitinase from AcMNPV significatively perturbed larval growth of lepidopteran species (Ding et al., 1998; Rao et al., 2004). The biological activity of the rChiA on herbivorous insects was experimentally confirmed by its ability to increase the permeability of the PM of both B. mori and H. virescens larvae. In addition, the enzyme showed antifungal activity towards A. alternata, a property that is shared by a number of microbial and plant chitinases. However, in comparison to the S. marcescens chitinase A, the rChiA was more effective in inhibiting germ tube elongation at lower doses. More interestingly, while the optimum temperature of the enzyme was not different from that of several chitinases, the rChiA outperformed the control enzyme in the stability assay at 50 °C. This feature strongly increases rChiA value for industrial processing of chitin, because current limitations on the use of chitinases are their instability and their activity within a narrow temperature and pH range.

In conclusion, our data indicated that the rChiA, purified with a reduced number of steps from transgenic tobacco plants, could be very useful for the chitin industry, as biopesticide, and for other biotechnological applications.
both enzymes were 24 h old and data represent means ± S.E. of 3 replicates per treatment, respectively. Asterisks indicate significant differences between the two chitinases (*P<0.05).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiotec.2010.03.005.

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