Journal of Biotechnology 147 (2010) 1–6

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

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

Antimo Di Maro[a,1], Irma Terracciano[b,1], Lucia Sticco[a], Luisa Fiandra[c], Michelina Ruocco[d], Giandomenico Corrado[b], Augusto Parente[a], Rosa Rao[b,*]

[a] Dipartimento di Scienze della Vita, Seconda Università di Napoli, Via Vivaldi 43, I-81100 Caserta, Italy
[b] Dipartimento di Scienze del Suolo, della Pianta, dell'Ambiente e delle Produzioni Animali, Università degli Studi di Napoli "Federico II", Via Università 100, Portici, Napoli 80055, Italy
[c] Dipartimento di Biologia, Università degli Studi di Milano, Via Celoria 26, Milano 20133, Italy
[d] Dipartimento di Arboricoltura, Botanica e Patologia Vegetale, Università degli Studi di Napoli "Federico II", CNR-IPP, Via Università 100, Portici, Napoli 80055, Italy

ARTICLE INFO

Article history:
Received 25 October 2009
Received in revised form 26 February 2010
Accepted 10 March 2010

Keywords:
Biopesticides
Enzymatic activity
Nicotiana tabacum
Protein purification
Recombinant protein
Transgenic plant

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^{-1} fresh leaf weight, which represents 0.2% of total soluble proteins. The yield of the purification was about 14% (2 mg kg^{-1} fresh leaf weight). The comparison between the biochemical and kinetic properties of the rChiA with those of a commercial *Serratia marcescens* chitinase A 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 phytopatogenic 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.

Published by Elsevier B.V.

1. Introduction

Reducing the use of synthetic compounds to control biotic stress is a major objective of sustainable agriculture (Epstein and Bassein, 2003). Biopesticides, 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).

* Corresponding author. Tel.: +39 081 2539204; fax: +39 081 2539481.
E-mail address: rao@unina.it (R. Rao).
[1] These authors contributed equally to this work.

0168-1656/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.jbiotec.2010.03.005

The expression and purification of enzymes for industrial applications in transgenic plants has been achieved for several proteins, including xylanase (Patel 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 Na_2HPO_4, 2.7 mM KCl, 1.4 mM KH_2PO_4), 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 were separated by SDS-PAGE on a Mini-Protean II mini-gel apparatus (Bio-Rad, Milan, Italy), using 6% (w/v) stacking polyacrylamide gel and 12% (w/v) separation gel (Laemmli, 1970). The Positope™ (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, Milan, 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-methyl umbelliferyl-β-D-N-N′-N″-acetyl-chitotriose (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 nmol of substrate in 20 μl final volume of McIlvaine 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-methylumbelliferone 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* (Centrifuge Avanti J-25, Beckman Coulter, CA, USA), at 4 °C for 60 min. The supernatant, at 4 °C, was brought to 40% $(NH_4)_2SO_4$, stirred for 2 h and centrifuged at 30,000 × *g* for 40 min. The supernatant, which contained the chitinase activity, was saturated with $(NH_4)_2SO_4$, stirred overnight and centrifuged for 45 min at 30,000 × *g*. The pellet was solubilised and dialyzed twice against water and once against 10 mM Tris·Cl, pH 7.3 at 4 °C. The insoluble material was removed by centrifugation at 30,000 × *g* for 20 min at 4 °C and the supernatant was loaded onto a Q-Sepharose Fast Flow column (20 cm × 3 cm i.d.; GE Healthcare, Milan, Italy), equilibrated in 10 mM Tris·Cl, 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 N_2 pressure (4 bars) and magnetic stirring. This concentrated acid fraction was gel-filtered on a Hi-Load 26/60 Superdex™ 75 (separation range 70-3 kDa; GE Healthcare, Milan, Italy), equilibrated and eluted with 10 mM Tris·Cl, pH 7.3, containing 0.35 M NaCl, at a flow rate of 2.5 ml/min, using the AKTA Purifier 100 FPLC (GE Healthcare, Milan, Italy). 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 Source™ 15Q PE 4.6/100 (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 10 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 Superdex™ 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 Brillant 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 Na_2CO_3 (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 Ca^{2+} and Mg^{2+} (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 chitinase 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 20 °C after addition of a suitable enzyme aliquot.