Protein Expression and Purification xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Protein Expression and Purification

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

# Chitinase III in *Euphorbia characias* latex: Purification and characterization

Delia Spanò [a], Kristyna Pospiskova [b], Ivo Safarik [b,c], Maria Barbara Pisano [d], Francesca Pintus [a], Giovanni Floris [a], Rosaria Medda [a,*]

[a] Department of Sciences of Life and Environment, University of Cagliari, I-09042 Monserrato (CA), Italy
[b] Regional Centre of Advanced Technologies and Materials, Palacky University, Slechtitelu 27, 783 71 Olomouc, Czech Republic
[c] Department of Nanobiotechnology, Institute of Nanobiology and Structural Biology of GCRC, Na Sadkach 7, 370 05 Ceske Budejovice, Czech Republic
[d] Department of Public Health, Clinical and Molecular Medicine, University of Cagliari, I-09042 Monserrato (CA), Italy

ARTICLE INFO

*Article history:*
Received 31 July 2015
Accepted 25 August 2015
Available online xxxx

*Keywords:*
*Euphorbia characias*
Latex
Magnetic particles
Chitinase
Chitin

ABSTRACT

This paper deals with the purification of a class III endochitinase from *Euphorbia characias* latex. Described purification method includes an effective novel separation step using magnetic chitin particles. Application of magnetic affinity adsorbent noticeably simplifies and shortens the purification procedure. This step and the subsequently DEAE-cellulose chromatography enable to obtain the chitinase in homogeneous form. One protein band is present on PAGE in non-denaturing conditions and SDS–PAGE profile reveals a unique protein band of 36.5 ± 2 kDa. The optimal chitinase activity is observed at 50 °C, pH 5.0. *E. characias* latex chitinase is able to hydrolyze colloidal chitin giving, as reaction products, N-acetyl-D-glucosamine, chitobiose and chitotriose. Moreover, we observed that calcium and magnesium ions enhance chitinase activity. Finally, we cloned the cDNA encoding the *E. characias* latex chitinase. The partial cDNA nucleotide sequence contains 762 bp, and the deduced amino acid sequence (254 amino acids) is homologous to the sequence of several plant class III endochitinases.



## 1. Introduction

*Euphorbia characias* (Fig. 1) is a shrub growing abundantly in vast Mediterranean areas. In Sardinia island (Italy) this plant grows exuberantly in various habitats, near streams and in open woods, in mountains and near the sea. *E. characias*, as all Euphorbiaceae, is characterized by the presence of laticifers, highly specialized cells with a tubular or branched anastomosed structure. Laticifers contain a sticky milky sap called latex, with a diversified composition and different roles. Latex provides contributions to plant defence mechanisms through repelling insects, controlling the growth of microbial phytopathogens, and producing toxic effects on herbivores [1,2]. In *E. characias* latex several substances have been well identified: sugars, free radical-scavenging molecules, polyphenols, flavonoids, alkaloids, terpenoid compounds, tocopherols, fatty acids and sterols ([3,4] and references therein). Moreover, *E. characias* latex contains rubber [5,6] and a large number of proteins and enzymes [7–10] some of these interacting in a common metabolism [9,11].

Chitinases [E.C. 3.2.1.14], hydrolyze β-1,4-glycosidic bonds of chitin and have an ubiquitous distribution in animals, plants, insects, fungi, bacteria and viruses. These enzymes are involved in growth and development processes, participate in cell death and in stress response to heavy metals and counteract oxidative stress ([12] and references therein). Moreover, chitinases can act as calcium storage proteins that bind calcium ions with high capacity [13]. Plant chitinases are grouped in two glycosyl hydrolase families called 18 and 19, according to the sequence homology. The family 18 includes classes III and V chitinases, whereas the family 19 consists of classes I, II, IV and VII ([12] and references therein). Plant chitinases have an important role in plant defense against chitin-containing pathogens, but they also show antiviral, antibacterial, antifungal and insecticidal properties. Plant chitinases have been purified and characterized from seeds of *Astragalus membranaceus* [14], *Punica granatum* [13], *Vicia faba* [15], from fruits of *Diospyros kaki* [16], *P. granatum* [17], *Ananas comosus*

*Abbreviations:* DEAE, diethylaminoethyl; ELC, *E. characias* latex chitinase; GlcNAc, *N*-acetyl-D-glucosamine; KPi, potassium phosphate; NaOAc, sodium acetate; pNP, 4-nitrophenolate; pNPNAG, *p*-nitrophenyl N-acetyl-β-D-glucosaminide.

* Corresponding author.
*E-mail address:* rmedda@unica.it (R. Medda).

http://dx.doi.org/10.1016/j.pep.2015.08.026
1046-5928/

**Fig. 1.** *Euphorbia characias*, the Mediterranean spurge.

[18], *Ficus awkeotsang* [19], and from latex of *Ipomoa carnea* [20], *Morus alba* [21], *Ficus microcarpa* [22], and *Hevea brasiliensis* [23].

Several plant chitinases have been isolated by affinity chromatography on chitin columns [22,24]. Magnetic separation techniques have recently been widely used for the isolation and purification of proteins and peptides from various types of biological samples. Utilization of magnetic affinity materials enables protein separation even from raw biological samples without pretreatment. After incubation and binding of target compounds on magnetic sorbent, the whole magnetic complex can be easily, gently and rapidly removed from the sample using a magnetic separator [25,26]. Magnetic chitosan and chitin particles have already been used for one-step partial purification of *Solanum tuberosum* tuber lectin [27,28] and hen egg white lysozyme [29].

Due to the presence of defense proteins in the plant latex, we were interested in the determination of chitinase activity in *E. characias*. We have identified a chitinase in the latex of this plant (*E. characias* latex chitinase, ELC) and in this study we present novel purification method of the enzyme using magnetic chitin particles. We describe the development of magnetic separation procedure followed by DEAE-cellulose chromatography for easy, fast and highly reproducible isolation of the highly purified ELC. Some biochemical characteristics of the enzyme are also described. Moreover, we report the partial nucleotide and the deduced amino acid sequence encoding for *E. characias* chitinase, showing the conserved residues leading to identification of ELC as a class III endochitinase.

## 2. Materials and methods

### 2.1. Chemicals

All chemicals were obtained as pure commercial products and used without further purification. Powdered chitin from shrimp shells, ferrous sulfate heptahydrate, sodium hydroxide, 3,5-dinitrosalicylic acid, *p*-nitrophenyl N-acetyl-β-D-glucosaminide, Triton-X 100, calcofluor white M2R, N-acetyl-D-glucosamine, chitobiose and chitotriose were from Sigma–Aldrich Co (St. Louis, MO, USA). Diethylaminoethyl (DEAE) cellulose was from Whatman international Ltd. (England).

### 2.2. Preparation of magnetic chitin

Magnetic iron oxides nano- and microparticles were prepared as follows: 1 g iron(II) sulfate heptahydrate was dissolved in 100 mL water and pH of this solution was gradually increased up to the value 11–12 by the slow addition of sodium hydroxide solution (1 M) under stirring; during this process a precipitate of iron hydroxides was formed. Then, this suspension was diluted up to 200 mL with water, inserted into regular kitchen microwave oven (700 W, 2450 MHz) and treated for 10 min at maximum power. After cooling the particles formed were repeatedly washed with water until neutral pH of the magnetic suspension was reached.

Magnetic chitin was prepared by thorough mixing 10 g powdered chitin with 20 mL magnetic particles suspension (1 part of completely sedimented particles and 4 parts of water) in a small beaker or short test-tube with a spatula to distribute magnetic particles homogeneously within the modified material. The mixture was dried completely at 60 °C and then magnetic chitin was repeatedly washed with water and/or working buffer [30].

### 2.3. Purification of chitinase

*E. characias* latex, exuded from cut apical branches of several plants, was collected during the whole year at several locations in southern Sardinia (Italy) and centrifuged at 9000 rpm for 30 min.

The supernatant (20 mL) was diluted up to 100 mL with 10 mM potassium phosphate (KPi) buffer, pH 7.0, and incubated with magnetic chitin (10 g), previously equilibrated with the same buffer. The incubation was carried out for 1 h, at 4 °C, under continuous stirring. Afterwards, the complex enzyme-magnetic chitin was separated from latex using a strong magnetic separator (NdFeB permanent magnet). This complex was washed with 10 mM KPi buffer pH 7.0 to remove the ballast proteins. Then, the bounded proteins were separated from magnetic chitin with 100 mM acetic acid, pH 2.8. The solution was immediately adjusted to pH 7.0 with 1 M NaOH and dialyzed against 10 mM KPi buffer pH 7.0, for 12 h at 4 °C. This step was repeated 5 times (with fresh portions of latex) to obtain a sufficient amount of proteins to be subjected to further treatment. The dialyzed was loaded on DEAE cellulose column (1 × 5 cm) equilibrated with 10 mM KPi buffer pH 7.0, at 4 °C. Under these conditions the chitinase was not bound to the column and was collected, lyophilized, and stored at −20 °C.

### 2.4. Protein determination, ELC activity assays and analysis of the reaction products

Protein concentration was determined as previously described [6] using bovine serum albumin as a standard for the calibration curve.

ELC activity was calculated by measuring the amount of the reducing end groups of *N*-acetyl-D-glucosamine (GlcNAc) using colloidal chitin as substrate [17]. Colloidal chitin was prepared from crab shell chitin according to the method of Usharani and Gowda [31]. The standard reaction mixture contained the enzyme solution and 1% (w/v) colloidal chitin in 650 μL of 100 mM sodium acetate (NaOAc) buffer, pH 5.0. This mixture was incubated at 40 °C for 30 min. The reaction was stopped by the addition of 250 μL of 1% (w/v) 3,5-dinitrosalicylic acid reagent and 100 μL of 10 M NaOH to the reaction mixture. Afterwards, this mixture was heated in

Please cite this article in press as: D. Spanò et al., Chitinase III in *Euphorbia characias* latex: Purification and characterization, Protein Expr. Purif. (2015), http://dx.doi.org/10.1016/j.pep.2015.08.026