Protein Expression and Purification 80 (2011) 217–223

Contents lists available at SciVerse ScienceDirect

Protein Expression and Purification

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

Expression and purification of a biologically active *Phytophthora parasitica* cellulose binding elicitor lectin in *Pichia pastoris*

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ARTICLE INFO

Article history:
Received 30 May 2011
and in revised form 18 July 2011
Available online 26 July 2011

Keywords:
Elicitor
Oomycete
Pichia pastoris
Plant immunity
Cellulose
Lectin

ABSTRACT

The *Phytophthora parasitica* cellulose-binding elicitor lectin, (CBEL), is a cell wall-localized protein playing a key role in cell wall organization and adhesion of the mycelium to cellulosic substrates. CBEL is a potent elicitor of plant immune responses and this activity is linked to its ability to bind plant cell wall components. In order to scale up the production of active CBEL, we reported here the cloning and expression of a His-tagged version of CBEL in the yeast *Pichia pastoris*. Selection of a high-producing *P. pastoris* clone and optimization of the purification procedure allowed a yield of about 2 mg of pure protein per liter of culture filtrate. The identity of the recombinant protein was confirmed by western-blot analysis, N-terminal protein sequencing, and by peptide mass fingerprinting. The cellulose-binding affinity and the lectin activity of the recombinant protein were identical to the native CBEL. Its elicitor activity, tested on *Arabidopsis thaliana* leaves, was similar to the native CBEL protein as it displays a similar biological activity on plant immune responses inducing defense gene expression and localized necroses of the infiltrated leaf tissues. The present work suggests that *P. pastoris* can be a suitable host for the production of compounds active on plants or for the development of new agricultural products able to stimulate plant immunity.

Introduction

During their whole life, plants are exposed to pathogenic microorganisms and, similarly to animals, they have developed during evolution sophisticated defense mechanisms to avoid disease [1]. Besides being induced upon contact with pathogenic microorganisms, active defense reactions can also be triggered upon perception of microbial compounds, called elicitors, which are widespread in a group of organisms or limited to specific strains of a microbial species [2]. Microbial elicitors belong to various chemical classes as oligo- and polysaccharides, lipids, peptides and proteins have been identified [2]. Since elicitors are able to induce plant immunity against pathogenic microorganisms, their use for the development of products able to protect crops has been envisaged. Their application for crop protection requires the development of economically-reliable production processes. Abundant natural sources of elicitors have been identified, such as marine algae which produce active polysaccharides [3–8]. In the case of proteinaceous elicitors, an attractive strategy to obtain large amount of elicitors is to express them in heterologous hosts such as bacteria or yeasts. This strategy has been used to produce commercially the bacterial protein harpin, an elicitor identified in the phytopathogenic bacteria *Erwinia amylovora* [9,10].

Oomycetes form a group of fungi-like microorganisms which comprises major animal and plant pathogens such as *Phytophthora infestans* the Irish famine potato pathogen [11]. These organisms are evolutionary distinct from true fungi and produce original elicitors such as elicitins [12] and the cellulose binding elicitor lectin[1] (CBEL) [13,14]. CBEL, which was originally purified from the *Phytophthora parasitica* cell wall, induces strong defense reactions when infiltrated into leaf tissue of various plant species including tobacco and *Arabidopsis* [14,15]. CBEL is a modular non-enzymatic protein harboring a duplication of two types of domains, a cellulose-binding domain belonging to the family I (fungal) of carbohydrate binding module (CBM1) and an Apple/ PAN domain which is involved in carbohydrate or protein-protein interactions [16]. In tobacco, treatments with a solution containing CBEL protected plants against *P. parasitica* infection [14] suggesting that this protein could be used as the active ingredient of protective products. But the purification of native CBEL from *P. parasitica* cell wall

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[1] *Abbreviations used:* CBEL, cellulose binding elicitor lectin; YPD, yeast-extract-peptone dextrose; TCA, trichloroacetic acid; DOC, deoxycholic acid; FPLC, fast-protein liquid chromatography; MALDI-TOF MS,matrix assisted laser desorption ionisation-time of flight mass spectrometry; rCBEL, recombinant CBEL protein.

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doi:10.1016/j.pep.2011.07.007

results in low yield (0.5 μg/g mycelium fresh weight), strongly limiting the development of this strategy for agronomic use. To circumvent this problem, an alternative strategy resides on the production of CBEL in a heterologous host. Previous work using *E. coli* resulted in the formation of inclusion bodies which required the development of tedious procedures to obtain active and soluble CBEL [17]. Since CBEL is a glycosylated protein which harbors 24 cysteine residues engaged in at least 10 disulphide bridges [14], we decided to use the yeast *Pichia pastoris* as heterologous host system. Here, we report the optimization steps allowing the obtainment of several mg per liter of biologically active pure CBEL protein from culture medium of *P. pastoris*.

Material and methods

Strains, plant cultivars and culture media

The *P. pastoris* strain SMD1163, mutated on the two proteases PRB1 and PEP4, with His^- and Mut^+ phenotypes was obtained from Invitrogen (Carlsbad, CA, USA). Components of yeast growth media were purchased from Difco (Cockeysville, MD, USA) and Sigma (Saint-Quentin, France). *Arabidopsis thaliana* accession Col0 was used to test elicitor activity. BMGH medium (0.1 M KH_2PO_4 buffer pH 6.0, 1.34% (w/v) YNB without amino acids, 1% (v/v) glycerol, 4 $mg.L^{-1}$ histidine and 0.04 $mg.L^{-1}$ biotin) was used for yeast pre-culture. BMMH (BMGH with methanol instead of glycerol) for recombinant protein expression. *Phytophthora parasitica* race 0 was grown seven days on liquid 5% V8-juice liquid medium at 28 °C, before extraction of native CBEL as previously reported [14].

Plasmid construction

The CBEL coding region of *Phytophthora parasitica* (Genbank accession number X97205) was amplified by PCR from cDNA using oligonucleotides PpCBEL_Pichia_F (5′-gaattcgcctgctcgactccctcattcggcaactgtggc t -3) and PpCBEL_Pichia_R (5′-5′-tctagattgagagtccccgacacagcaccgatcttggtg 3′). Oligonucleotides were purchased from Eurogentec (Angers, France). The insert was cloned in pGEM-T and digested between 5′ *EcoRI* and 3′ *XbaI* sites. Digested-purified inserts were ligated into pPICZαA vector (Invitrogen) and introduced into *E. coli* cells. Positive clones were identified by PCR analysis and purified plasmids (pPICZαA-CBEL) were verified by sequencing using AOX3 and AOX5 primers (Invitrogen). This strategy placed *CBEL* construct (without native signal peptide) under the AOX promoter of *P. pastoris* in fusion with the *S. cerevisiae* α peptide for secretion of recombinant protein into the culture medium.

Transformation and selection of P. pastoris clones expressing CBEL

pPICZαA-CBEL was digested by SacI and introduced by electroporation (Biorad-Gene Pulser) into SMD1163 (pep4, prb1, his4) *P. pastoris* strain grown in yeast-extract-peptone dextrose (YPD) medium according to the manual of the Pichia Expression kit (Invitrogen). Electroporated cells (1500 V, 25 μF and 600 Ω) were resuspended in cold sorbitol (1 M) and placed 2 h at 28 °C before spreading on selective YPD plates containing 0.1 to 0.5 $mg.ml^{-1}$ of Zeocin. After successive spreading and growth at 28 °C, 5 colonies were randomly selected and the integration of the expression cassette into the genome of SMD1163 strains was verified by PCR using the 5′ and 3′ AOX primers and PpCBEL_Pichia_R, PpCBEL_Pichia_F oligonucleotides.

Optimisation of heterologous expression of CBEL elicitor in P. pastoris

P. pastoris transformants were grown overnight in 250 ml flasks at 28 °C in 25 ml of BMGH with constant shaking (250 rpm). Cells were harvested by centrifugation (5 min, 3000 g) and expression of the recombinant protein was induced by re-suspension of the cells in 1L flask in 50 ml of BMMH medium followed by incubation at 28 °C under continuous shaking (250 rpm). Cells densities of 1, 3, 6, 9 (OD600), methanol concentrations of 1%, 2%, 5% (v/v), and incubation times of 4 h, 24 h,48 h, were tested. Culture supernatants were collected after incubation by centrifugation (5 min, 4000 g).

SDS–PAGE, Western blotting

The *P. pastoris* extracellular medium was collected by centrifugation and proteins were concentrated using trichloroacetic acid (TCA)/deoxycholic acid (DOC) technique. Briefly, TCA was added to the culture medium (0.01% v/v), and the mixture was incubated on ice for 30 min. Deoxycholic acid was added to obtain a final concentration of 0.2% (v/v). After 30 min on ice, proteins were recovered by centrifugation (15 min, 15,000 g, 4 °C). The pellet was dried and washed with acetone at -20 °C. Proteins were solubilized in SDS–PAGE loading buffer, boiled and analyzed by SDS–PAGE. Proteins were stained with coomassie blue using the GelCode Blue Stain Reagent (ThermoFisherScientific) or by silver staining as described in [18]. Western Blot analysis was performed as described in [13] with polyclonal primary antibodies targeting the myc-epitope (1/1000) and secondary antibodies labelled with alkaline phosphatase (1/10 000) (Sigma). The revelation was done according to the detection kit using NBT/BCIP reagents as recommended by Invitrogen. For glycan structure analysis, native and purified CBEL were separated by SDS–PAGE and blotted onto Immobilon PVDF membranes (Millipore, Bedford, Mass). Revelation of digoxigenin-labeled lectins bound to specific carbohydrate structures was achieved using the DIG glycan differentiation kit (Roche Applied Science). As control, a 14-3-3 protein (BMH1) from *S. cerevisiae* expressed in *E. coli*, DH5α was used [19].

Total protein concentration was estimated by Bradford colorimetric assay, using the Biorad Protein Assay Kit.

Purification of recombinant protein

The culture filtrate was diluted 5 times in a buffer A (Na_2HPO_4 buffer 20 mM, NaCl 500 mM pH 7.45), and subjected to cation exchange chromatography (HisTrap™ HP Column) in a fast-protein liquid chromatography (FPLC) system (GE Healthcare Life Sciences). Briefly, the Ni^{2+}-exchange chromatography column was equilibrated by passing through, successively, 5 ml of buffer A, 5 ml of a buffer B (buffer A with 500 mM imidazole) and 5 ml of buffer A again. The culture filtrate was loaded onto the column at a flow of 1 $ml.min^{-1}$. Column-bound proteins were then eluted by an increasing level buffer B (8%, 40% and 100% corresponding to 40 mM, 200 mM and 500 mM of imidazole) at a flow of 1 ml/min, and 1 ml fractions were collected. Fractions were dialysed overnight at 4 °C against ultra high-quality water using GeBAflex-tube (molecular weight cut off of 3.5 kDa, Gene Bio-Application L.T.D.).

Mass spectrometry analysis and N-terminal sequencing

The protein band observed after SDS–PAGE analysis of purified fractions was excised from the gel, digested by trypsin and subjected to matrix assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) as previously described [20] with additional reduction/alkylation before trypsin treatment. Peptide mass fingerprinting data was analyzed by MS-FIT (Protein