HETEROLOGOUS EXPRESSION OF A CHITINASE FROM *CUCUMIS SATIVUS* IN A RECOMBINANT YEAST

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Abstract

Many plants infected by pathogens develop resistance mechanisms, including the production of pathogenesis-related (PR) proteins. Among the PR proteins, several have been identified as chitinases. The PR-8 protein from *Cucumis sativus* is an extracellular acidic chitinase belonging to the class III which includes bifunctional lysozyme/chitinase enzymes. Here we report the cloning and the expression of a cDNA encoding the cucumber PR-8/chitinase in the methylotrophic yeast, *Pichia pastoris*. The recombinant chitinase secreted into the medium was purified by metal-affinity chromatography and analysed. Site-directed mutagenesis was also carried out to investigate the role of some highly conserved residues.

Introduction

Many plants infected by pathogens develop resistance mechanisms including the production and accumulation of proteins well known as pathogenesis-related (PR) proteins [1-3]. Among the PR proteins, several were found to display chitinase activity [4-5]. Chitinases are hydrolytic enzymes that degrade chitin, a β-1,4-linked homopolymer of N-acetyl-D-glucosamine. Plant chitinases have been reported to play a role in defense against pathogenic fungi that contain chitin in their cell wall [6].

Seven classes of plant chitinases have been described based on amino acid sequence features [7-9] and they are grouped into two families of glycosyl hydrolases, families 18 and 19 [10]. Class III chitinases belong to the family 18 and include bifunctional enzymes with a lysozyme activity [11]. They do not share sequence identity with the other classes of plant chitinases. This type of chitinase comprises the hevamine isolated from rubber tree [12], the plant concanavalin B that seems to have lost its catalytic activity [13] and a chitinase from *Cucumis sativus* [14].

Here we describe the expression of active cucumber PR-8/chitinase in *Pichia pastoris* and the purification of the recombinant protein. Site-directed mutagenesis was also carried out to investigate the role of three acidic amino acids in a very highly conserved region.

Material and Methods

Strains, plasmids and media

Cucumber PR-8 cDNA was expressed in *Pichia pastoris* X-33 (Invitrogen, The Netherlands) using pPICZαA vector. *P. pastoris* cells were grown in YPD for biomass production. To induce the PR-8 expression, the cells were grown in BSM medium complemented with methanol as sole carbon source and incubated during 96 hours.

DNA procedures

The cDNA encoding cucumber PR-8 protein was cloned in pPICZαA vector in fusion with a 6(His) tag either at the C-terminus or at the N-terminus. *P. pastoris* was transformed by electroporation according to the manufacturer's protocol. The transformed clones were selected on medium containing 0.01% zeocin.

The Quick Change II kit (Stratagene, USA) was used for site-directed mutagenesis. Three primers were used for generating D122N, D124N and E126Q mutations.

Enzyme assays

Chitinase activity was estimated by using radiolabelled glycolchitin (home-made preparation) or CM-Chitin RBV (Loewe, Germany). Endo- and exochitinase activity was measured using PNP-(GlcNAc), PNP-(GlcNAc)$_2$ and PNP-(GlcNAc)$_3$ (Sigma). Lysozyme activity was determined using a suspension of *Micrococcus luteus* cells (Sigma) [19].

Purification of recombinant PR-8

The recombinant protein was secreted in the culture medium. After centrifugation, the supernatant was collected and loaded onto a Ni^{2+}-affinity column (HisTrap HP column, GE Healthcare). The purification was performed in denaturing conditions and the purified protein was renatured by dialysis against citrate buffer pH 5.2.

Protein analysis

Electrophoresis in SDS-polyacrylamide gels were performed according to the method of Laemmli [15]. Detection of chitinase activity was carried out on glycolchitin gels as described by Trudel and Asselin [16]. Immunodetection of the (His)-tag was performed by western blot technique using monoclonal Penta-His antibodies (Qiagen, Germany) and the BM Chemiluminescence WB kit (Roche, Germany). Protein concentration was determined by the method of Lowry et al [17] using bovine serum albumine as standard.

Results and discussion

Cucumber PR-8 cDNA was cloned into pPICZαA vector in fusion with a C- or N-terminal polyhistidine tag and it was used to transform *P. pastoris* X33 strain. After selection on 0.01% zeocin and verification by PCR, transformants were screened for chitinase expression and secretion into the growth medium. After 96 hours induction, the culture supernatant was collected and chitinase activity was assayed using CM-Chitin RBV. A *P. pastoris* strain transformed with the vector pPICZαA was used as control.

Recombinant PR-8 (rPR-8) was purified from the supernatant of the best chitinase producing transformant. The presence of polyhistidine tag in rPR-8 was confirmed by immunoblotting with anti-His antibodies. The purification was performed onto a Ni^{2+}-affinity column in denaturing conditions. Before loading onto the column, the supernatant was dialyzed against buffer containing 8M urea. After washing, the column was then eluted with buffer containing 500mM imidazole. Flowthrough and eluate were assayed for chitinase activity using radiolabelled glycolchitin as substrate. Chitin activity is recovered in the first elution peak (fractions 34-36) as shown in fig. 1. The chitinase-active fractions were pooled and concentrated for further analysis.
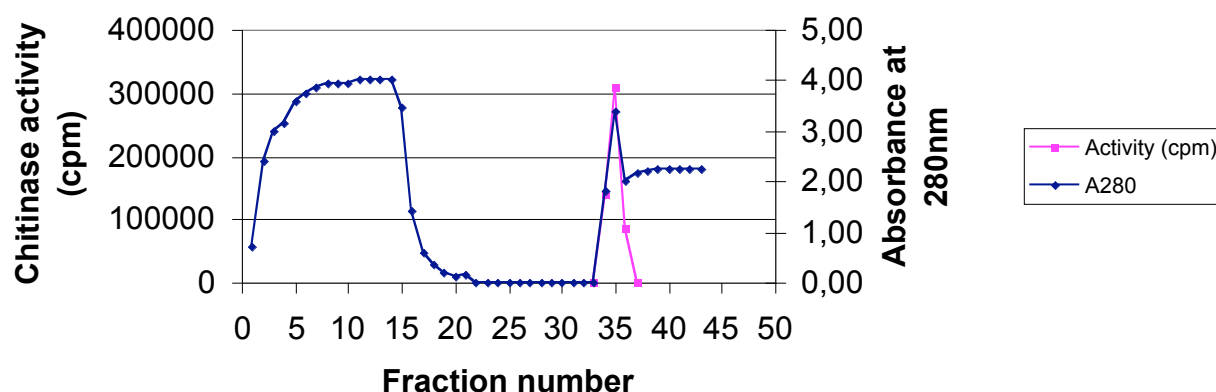

Figure 1 : Chromatography of recombinant PR-8 on a Ni^{2+}-HisTrap HP column.

Purification of rPR-8 by IMAC yielded pure protein as judged by SDS-PAGE. The molecular mass of the protein was estimated to be about 30 kDa, which is nearly identical to the mass of the native protein [14]. rPR-8 showed hydrolytic activity towards chitin polymers like glycolchitin and carboxymethyl-chitin. The enzyme activity was also assayed with PNP-chitooligosaccharides. Our results suggested that rPR-8 exhibited endochitinase activity but no exochitinase activity. In addition, we tested rPR-8 for lysozyme activity with *Micrococcus luteus* cells. Interestingly, the recombinant enzyme displayed a weak lysozyme activity proving thus the bifunctional character of the enzyme like in other class III chitinases as hevamine for example [12]. This observation supports the hypothesis that the bifunctional chitinase/lysozyme property would be specifically associated with the class III chitinases.

The catalytic role of three acidic residues in the DXDXE conserved region was investigated by site-directed mutagenesis. The mutated enzymes were expressed and purified by the same methods as the wild-type enzyme. Our results showed that replacement of the glutamate residue with another amino acid (E126Q mutant) resulted in loss of enzymatic activity. The D122N and D124N mutants displayed lower catalytic activity than the wild-type: respectively 80% and 90% residual activity. In conclusion, our study confirmed that the glutamate in the conserved motif is essential for catalysis. As aspartate mutants retained significant activity, our data could suggest that these residues are less critical for catalysis. However other studies showed that the mutation of Asp140 and/or Asp142 had drastic reducing effects on the enzyme activity, as for example in ChiB from *Serratia marcescens* [18] and in hevamine from *Hevea brasiliensis* [19]. In the case of cucumber PR-8 protein, it will thus be interesting to continue this preliminary study in order to better understand the molecular catalytic mechanism of this enzyme.

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