[BIO37] Cloning and expression of the *Glomerella cingulata* cutinase recombinant gene in the yeast, *Pichia pastoris*

Norhatiah Md. Lias, Farah Diba Abu Bakar, Roohaida Othman, Zulkeflie Zamrod, Sheila Nathan, Abdul Munir Abdul Murad and Nor Muhammad Mahadi

School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi 43600 Selangor, Malaysia.

Introduction

Glomerella cingulata (anamorph. *Colletotrichum gloeosporioides*) is a phytophatogenic fungus produces cutinase enzyme causing anthracnose disease of a wide range of plants in the tropics and subtropics. Cutinases are hydrolytic enzymes that are able to hydrolyse ester bonds in the cutin polymer of higher plants which is a polyester composed of hydroxyl and epoxy fatty acids. It has been presented as a versatile lypolytic enzyme, showing interesting properties for industrial products and processes, such as household detergents, in the dairy industry for the hydrolysis of milk fat, in the oleochemical industry, in the synthesis of ingredients for personal-care products and pharmaceuticals, and in the biodegradation of plastics (e.g. polycaprolactone) (Carlvaho et al. 1999).

Majority of cutinase studies have been done with a fungal pathogen, *Fusarium solani* f. sp. *pisi* to elucidate its physiological and biochemical properties from molecular level to bioprocess development (Carlvaho et al. 1999). However, studies on *G. cingulata* cutinase has not been extensively investigated (Abu Bakar et al. 2001). In order to elucidate *G. cingulata* cutinase molecular properties and to obtain the knowledge of the cutinase structure, we proposed to express the recombinant cutinase in the methylotrophic yeast, *Pichia pastoris*.

This methylothropic yeast has been developed as an expression system for high-level production of recombinant protein and high-yield expression of various proteins by secretion into the culture supernatant or by intracellular localization (Cregg et al. 2000). *Pichia* offers the features of (i) methanol-induced expression of heterologous genes integrated into the genome adjacent to the alcohol oxidase 1 (*AOX1*) gene promoter, (ii) high cell density in inexpensive, chemically defined media, and (iii) the capacity to carry out post-translational modifications resembling those of mammalian cells, correctly folded and glycosilated (Fidler et al. 1998). Because of these characteristics, *P. pastoris* might be a suitable expression system for the production of biologically active recombinant cutinase. In this study we describe the cloning and expression of the *G. cingulata* cutinase recombinant gene in *P. pastoris.*

Materials and methods

Plasmid construction and yeast transformation

An intronless cutinase gene was amplified from *G. cingulata* genomic DNA by SOE-PCR (Newton and Graham, 1997) and was cloned into an intermediate cloning vector, pCR 2.1 TOPO™ (Invitrogen) and designated as pNML1 plasmid. To construct the cutinase gene for the yeast expression, the intronless cutinase gene from the plasmid pNML1 was subcloned into the *P.pastoris* expression vector, pPICZB (Invitrogen) at the *Kpn*1 and *Xba*1 sites (Figure 1). The construct was designed for amplification via PCR of a 624 bp cutinase gene fragment that lacks the cutinase signal peptide. The resulting plsmid, pPICGly, was linearized by the restriction enzyme *Pme*1 followed by transformation into *P. pastoris* wild type strain X33 by electroporation at 1500V voltage, 25μF capacitance and 200Ω resistance. *Pichia* transformants were selected on YPD (1% yeast extract, 2% peptone and 2% dextrose) plates containing 100 μg/ml zeocin at 30°C for 72h followed by isolation of putative multicopy clones by replica plating on YPD 500 μg/ml zeocin, YPD 1000 μg/ml zeocin and YPD 1500 μg/ml zeocin. The zeocin hyperresistant transformants (1500μg/ml) were analyzed for the presence of the cutinase insert by PCR and Mut (Methanol utilization) phenotype that performed according to the

method as described in the manual of the *Pichia* expression kit (Invitrogen).

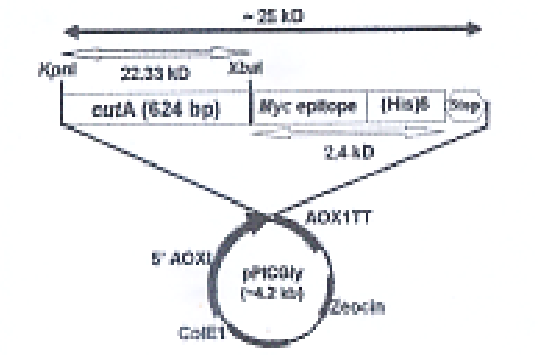

FIGURE 1 The 624 bp intronless cutinase gene cloned into pPICZB, *Pichia* intracellular expression vector fused to myc-epitope and polyhistadine-tag (His_6) at the C-terminus resulted the recombinant plasmid pPICGly for cutinase expression.

Expression of recombinant cutinase

The *P. pastoris* hyperresistant transformants were tested for cutinase expression. The *P. pastoris* clone was inoculated into 100 ml BMGY medium (100 mM potassium phosphate, pH 6.0, 10 g/L yeast extract, 10 g/L casamino acid, 20 g/L peptone, 13.4 g/L yeast nitrogen base, 0.4 mg/L biotin and 1% glycerol) and incubated on a shaker with aeration at 30°C for 18h to generate cell biomass before induction. Cells were harvested by centrifugation at 5000g for 10 min at room temperature. The cells was subsequently resuspended in 100 ml BMMY medium (BMGY containing 1% methanol but without 1% glycerol) with started OD_{600} = 1 and incubated with vigorous shaking at 30°C for four days to induce expression. Methanol was added every 24h to a final concentration of 1% to maintain the induction medium from evaporative loss.

The expression samples were separated by centrifugation (10 000g for 10 min at 4°C). The cells were lysed by vortex for 8 min with the same volume of breaking buffer (50 mM sodium phosphate, pH 7.4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA and 5% glycerol) and glass beads (50 µm; Sigma). The cell debris was further separated from the soluble supernatant by centrifugation (12 000g for 10 min at 4°C). The total protein of samples was determined by the method of Bradford (Bradford, 1974). The soluble supernatants were subjected to purification and protein analyses.

Purification of recombinant cutinase

The protein extract was purified on the AKTA*prime* Purification System (Amersham) using HiTrap Chelating HP 1 ml column loaded with 0.1 M $NiSO_4$ with binding capacity at 12 mg/column. Purification of the protein sample (His_6 fusion protein) was carried out by immobilized metal-affinity chromatography (IMAC) equilibrated in binding buffer (20 mM sodium phosphate, 0.5 M NaCl, pH 7.4) and fraction eluted in elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 0.5 M imidazole pH 7.4) performed according to the pre-programmed template for automatic column preparation and purification of His_6 fusion protein on a single HiTrap Chelating HP column method as described in the AKTA*prime* manual.

SDS-PAGE and western blot

The proteins were analyzed by SDS-PAGE, using 5% stacking gel and 15% separating gel followed by staining with Coomassie brilliant blue. For Western blot analysis, the proteins were separated by SDS-PAGE and the gel was electrophoretically transferred to nitrocellulase membrane. The membrane was blocked for 1 hour at room temperature with 5% skimmed milk powder in PBS pH 7.5 followed by incubation with the primary antibody (monoclonal mouse anti-myc antibody, Invitrogen) at 4°C overnight and with the secondary antibody (anti-mouse IgG horseradish peroxidase conjugate, Promega) for 1 hour incubation at room tempearture. The immunodetection of the myc-tagged protein was subsequently performed as the manufacturer's instructions using ECL Western blotting analysis system (Amersham).

Cutinase/esterase assay

Cutinase/esterase activity was determined by monitoring the hydrolysis of p-nitrophenylbutyrate (PNPB) by method of Van der Vlugt-Bergmans et al. (1997). A total of 263 µl of sample was added to a reaction mixture composed of 25 mM potassium phosphate buffer pH 7.0, 0.05% Triton X-100 and 30 µl of PNB resuspended at a

concentration of 10 mM in 0.25 mM phosphate buffer pH 7.0 and 0.5% Triton X-100 in a final volume of 300 µl. Aliquots of 80 µl of this reaction was transferred into the wells of a microtitre plate and incubated at 30 °C for 30 min. PNB hydrolysis subsequently was measured spectrophotometrically at 405 nm. Background caused by nonenzymatic substrate degradation was determined in a parallel assay containing protein sample that had been treated for 5 min at 100 °C, and subtracted from the values of nontreated protein sample. One unit of estereolytic activity was defined as the amount of enzyme convert 1 nmole of PNPB to *p*-nitrophenol for 1 min under the specified condition. The extinction coefficient of *p*-nitrophenol was considered to be 1.84 x 10^4 (Mcm), as indicated by the supplier, Sigma.

Results and discussion

Construction and cloning of expression gene

A cutinase gene construct of 624 bp was amplified by SOE-PCR. This gene construct lacks the 52 bp intron and the 69 bp sequence that encodes the putative signal peptide. The presence of the insert within the recombinant plasmid, pPICGly was confirmed by enzyme digestion with *Kpn*1 and *Xba*1 restriction enzymes (Figure 2). Sequencing was carried out for both strands of this plasmid using *Kpn*1 adapter primer and *Xba*1 adapter primer that flank the cutinase gene insert. Sequencing has confirmed the insertion of the cutinase fragment in the cloning and expression vector. More than 100 colonies of *Pichia* transformants were obtained on YPD plate supplemented with 100 µg/ml zeocin. Putative multicopy clones by replica plating on YPD plate at low, medium and high concentration of zeocin (100, 500, 1000 and 1500 µg/ml) showed that fifteen transformants were zeocin hyperresistant transformants (1500 µg/ml). The hyperresistant transformants were gave positive amplified products by PCR screening and Mut^+ phenotype.

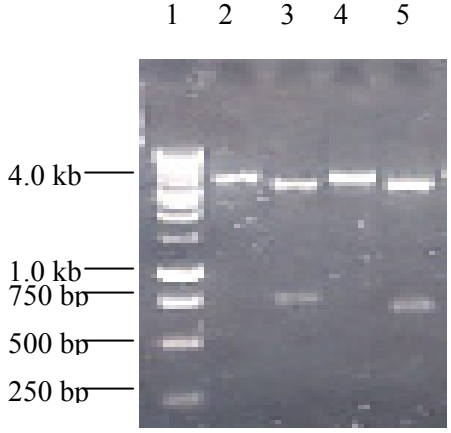

FIGURE 2 The recombinant plasmid, pPICGly digested with Kpn1 and Xba1 (lane 5), single digested with Kpn1 (lane 4) and DNA marker (lane 1).

The Mut phenotype is dependant on the points of plasmid recombination into the yeast genome. Clones which have had one of their alcohol oxidase (*AOX1*) genes disrupted will not grow as quickly on methanol and are termed "methanol utilization slow" (Mut^S) whereas Mut^+ (methanol utilization) clones are able to grow in medium containing methanol. Mut^+ strain can produced highest amount of the recombinant protein since methanol is the carbon source used by *P. pastoris* for induction in protein expression.

Cutinase expression and purification

The hyperresistant transformants were selected for cutinase expression due to correlation of higher resistance level of zeocin with higher copy number of the inserted gene integrated in the genome, which tend to produce more protein of interest and high level of expression. The targeted protein size was identified as ~ 25kD band and was confirmed by western blotting.

TABLE 1 Purification table of *G. cingulata* recombinant cutinase.

Fraction	Volume (ml)	Total protein (mg)	Specific activity (U/mg)	Total activity (U)	Recovery (%)	Purification fold
Crude	3.0	0.75	5.0	3.75	100	1
Affinity chromatography	2.0	0.08	21.1	1.7	45.3	4.2

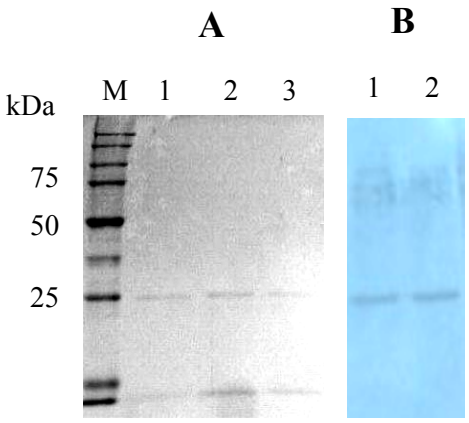

FIGURE 3 SDS-PAGE of protein purified from *Pichia* recombinant clone (A; lane 1, 2 and 3) and western blot of the purified protein (B; lane 1and 2). Lane M, protein marker.

The enzyme activity of the protein production performed by *p*-nitrophenylbutyrate (PNPB) hidrolysis was obtained three fold higher than the wild type yeast strain, *P. pastoris* X33. Protein extracted from cells lysate were purified by immobilized metal-affinity chromatography on the AKTA*prime* Purification System (Amersham) using HiTrap Chelating HP 1 ml column. The eluted fractions were analyzed by SDS-PAGE, western blot (Figure 3) and tested for enzyme activity. The specific activity increased four-fold with less than half of the protein in the crude sample recovered upon purification (Table 1). The total protein yields of pure cutinase about 0.008 mg/L culture volume.

Conclusion

The cutinase recombinant protein was successfully produced in the yeast *P. pastoris* by intracellular heterologous protein expression. The cutinase enzyme has been purified in small quantity and work is underway to improve protein purification in larger volume to produce sufficient purified protein for crystallisation.

Acknowledgments

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