

Full Length Research Paper

Characteristics of the secretory expression of pectate lyase A from *Aspergillus nidulans* in *Escherichia coli*

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Pectate lyases from *Aspergillus* spp. are a major kind of industrial pectinases, can improve the surface properties of natural fibers and have promising applications in medicine, food, textile and other industries. Pectate lyases catalyze the eliminative cleavage of de-esterified pectin, which is a major component of the primary cell walls of many higher plants. The Pectate lyase A (PelA) gene without an N-terminal signal peptide sequence from *Aspergillus nidulans* was recombinantly expressed using *Escherichia coli* as the host strain and pET-20b(+) as expression vector with a pelB N-terminal signal peptide. PelA biosynthesis reached the maximum production field (450 U ml⁻¹ medium) at 0.5 mM IPTG, 37°C, 200 rpm, for 2 h and the expressed PelA primarily appeared in extracellular medium. Calcium ion had a more obvious promotion than glycine and SDS to the extracellular enzyme fields, and the C-terminal sequence of Pel A might have an important effect on the transportation through the outer-membrane of *E. coli*. The time (2h) of reaching the maximum enzyme at 37°C implied that the PelA expression was very significant to pectate lyase industrial production.

Key words: Characteristics, extracellular expression, pectate lyase A, *Aspergillus nidulans*, *Escherichia coli*.

INTRODUCTION

Pectin is a major component of the plant primary cell wall and its biological function is to cross-link cellulose and hemicellulose fibers, providing rigidity to the cell wall (Carpita and Gibeaut, 1993; Zhao et al., 2008; Lagaert et al., 2009). Although the precise composition of pectin varies among species, all pectin is composed primarily of galacturonans (Wolf et al., 2009). Pectinases are inducible extracellular enzymes secreted by microorganisms that are capable of degrading pectin of plant cell walls, such as pectate lyase (EC 4.2.2.2), pectin lyase (EC 4.2.2.10), polygalacturonase (EC 3.2.1.15) and pectin methylesterase (EC 3.1.1.11) (Gummadi and Kumar, 2005; Jayani et al., 2005). Pectate lyases, otherwise known as pectate transeliminases, catalyse the eliminative cleavage of de-esterified pectin, which is a major

component of the primary cell walls of many higher plants (Carpita and Gibeaut, 1993). Pectate lyases have been found in bacteria (Lei et al., 1987; Nasser et al., 1993), fungi (Debing et al., 2006), and plant (Zhao et al., 2008); however, studies have focused on fungal pectate lyases, including *Aspergillus niger* (Benen et al., 2000; Debing et al., 2006), and *Aspergillus nidulans* (Ho et al., 1995). Pectate lyases from microorganisms had been extensively investigated by biochemical and structural approaches. Crystal structure analysis of several pectate Lyases from *Erwinia chrysanthemi* and *Bacillus subtilis* had been researched (Nakaniwa et al., 2003; Creze et al., 2008).

Pectate lyases from *Aspergillus* spp are a major kind of industrial pectinases, can improve the surface properties of natural fibers and have promising applications in medicine, food, textile and other industries (Pilnik and Rombouts, 1985; Kashyap et al., 2001; Semenova et al., 2006; Liu et al., 2009). Previous research showed that pectate lyase A (PelA) of *A. nidulans* exhibited its optimum level of activity over the range of pH 7.5-10 at

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50°C, which has industrial promising applications (Zhao et al., 2007). In order to improve the field of PelA of *A. nidulans*, we previously cloned and expressed *A. nidulans* pectate lyase A in *Escherichia coli* (Zhao et al., 2007) and *Bacillus subtilis* (Zhao et al., 2008).

In the present research, we found that the extracellular secretion of PelA from *A. nidulans* in *E. coli* with a high efficiency and PelA maybe be transported through the outer membrane by type 2 secretion system (Tyss2). In order to research both the mechanism of transporting PelA through the outer membrane in bacteria and the industrial production of pectate lyase A, the effects of C-terminal end and other factors on the extracellular secretion of PelA were researched.

MATERIALS AND METHODS

Strain and plasmids

The Pel A gene used in this study was cloned from pelA-pET-28a (Zhao et al., 2007). The *E. coli* strain DH5 α was used as the host for gene manipulation and the pMD 18-T plasmid (TaKaRa, Dalian, People's Republic of China) as the clone plasmid. *E. coli* BL21 (DE3) (Novagen, Madison, WI, USA) was used as the expression host and pET-20b(+) plasmid (Novagen) as expression vector with a pelB signal peptide from *Erwinia carotovora* (Lei et al., 1987).

Cloning and expression of pelA and pelA free of C-terminal 30 aa sequence

This pelA gene (without signal peptide) was amplified with PfuUltraTM High-Fidelity DNA Polymerase (Stratagene, La Jolla, CA, USA), sequence-specific primers (p1: sense 5'-CCATGTCACCTGCGCCGGACCTC-3' and p2 antisense 5'-GCGGCCGATTACAAT TTCTGTCCGGCTGTACC-3') and pelA-pET-28a (Zhao, 2007) as template; then a clone plasmid of pelA/pMD 18-T and expression plasmid of (pelA/pET-20b (+)-) were constructed. The pelA gene free of c-terminal end 30AA (named pelAc30) was amplified with PfuUltraTM High-Fidelity DNA Polymerase (Stratagene, La Jolla, CA, USA) and sequence-specific primers (p1: sense 5'-CCATGTCACCTGCGCCGGACCTC-3' and p3 antisense 5'-GCGGCCGAGGTTCCCTCG GGCACCGAGTTACTCG-3'); then a clone plasmid of pelAc30/pMD 18-T and expression plasmid of (pelAc30/pET-20b(+)) were constructed. For expression, pelAc30/pET-20b (+) or pelA /pET-20b(+) was transformed into *E. coli* BL21 (DE3) (Novagen). In LB-1% (w/v) Glc with 50g Amp ml⁻¹, the transformants were cultivated at 37°C and 200 rpm min⁻¹ to an absorbance (at 600 nm) of 0.8. Then culture broth was added with IPTG at 0.1-1.0 mM final concentration (except as indicated elsewhere and incubated) at 15-37°C and 200 rpm min⁻¹ for another 0 to 24 h for induction of recombinant protein synthesis.

The effect of Ca²⁺ or Gly or SDS on pel A expression

Some substances (0 to 30 mM calcium chloride or 0.2-0.8% Glycine (Gly) or 0-0.1% sodium dodecyl sulfate (SDS)) were added to culture broth at 0.5 mM IPTG concentration, at 37°C and 200 rpm min⁻¹ for 2 to 8 h for induction of recombinant protein synthesis.

Extraction and purification

After expression, the culture was centrifuged at 10,000 g at 4°C and the resulting supernatant was collected for subsequent assay. For periplasmic proteins, the cells were twice washed with 10 mM Tris/HCl, pH 7.0. and treated with 1 mg/ml lysozyme, 10 mM Tris/HCl, pH 8.0 and centrifuged 10,000 g at 4°C. The resulting supernatant was loaded on to a Ni²⁺-nitrilotriacetate-agarose column (Novagen) for purification of recombinant expressed PelA according to the system manual. Eluted PelA was dialysed against 10 mM Tris/HCl (pH 7.0) with dialysis membrane (dialysis tubing cellulose membrane, size: 25×16 mm, Sigma-Aldrich, St. Louis, MO, USA) for subsequent assay.

SDS-PAGE

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was carried out using 11.0% polyacrylamide gel stained with Coomassie brilliant blue (CBB) R-250. A sample solution containing 2 g of protein was applied to each lane Figure 1.

Enzyme activity assay

To assay PelA activity, 0.5 ml of the reaction mixture containing 50 mM Tris/HCl (pH 8.5) 1 mM calcium chloride, 1 mg PGA from Sigma (P 1879), and appropriate enzyme solution was incubated at 50°C for 10 min (Macmillan and Vaughn, 1964) except as indicated elsewhere. The reaction was stopped by adding 1 ml of 0.02 M HCl and then measured for the increase in absorbance at 235 nm (Macmillan and Vaughn, 1964). One enzyme unit of PelA was defined as the amount of enzyme that forms 1 mol of 4, 5-unsaturated product in 1 min under the conditions of assay. The molar extinction coefficient for the unsaturated product at 235 nm is 4,600 M⁻¹cm⁻¹mm (Collmer et al., 1988).

RESULTS

Expression and the c-terminal sequence of PelA affecting the extra cellular expression

We successfully expressed the pelA gene without a signal peptide sequence from *A. nidulans* using *E. coli* as the host strain. The pelA encoding the mature region was fused with the hexahistidine tag placed behind the MCS in the pET-20b (+) with *pelB* coding sequence, which is a periplasmic signal peptide of Sec A path. Under the induction of 0.5 mM IPTG, the cells of pelA/ pET-20b(+)/*E. coli* BL21 (DE3) could express PelA at 37°C 2 rpm, for 2 to 8 h; surprisingly, the expressed PelA primarily appeared in extra cellular medium and not in periplasmic space. Interestingly, if PelA was free of c-terminal sequence (30 Aa), the expressed PelA primarily appeared in periplasmic space, not in extra cellular medium.

The effects of IPTG and temperature on the extra cellular expression

For researching PelA extra cellular expression in *E. coli*,

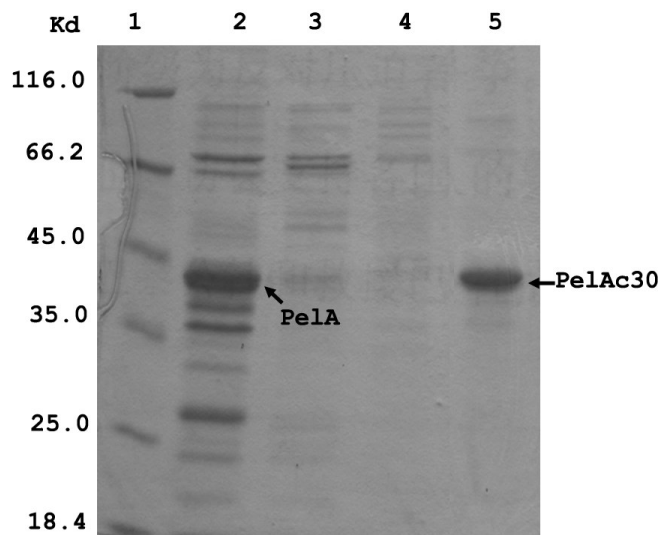


Figure 1. Assay of PelA by SDS-PAGE.

1: marker; 2: extra cellular fraction from *pelA/pET-20b (+)-E. coli* BL21 (DE3) induced by 0.5 mM IPTG, 37°C, 2 h; 3: Soluble fraction of in periplasm extract from *pelA/pET-20b (+)-E. coli* BL21 (DE3) induced by 0.5 mM IPTG, 37°C, 2 h; 4: extra cellular fraction from *pelAc30/pET-20b(+)/E. coli* BL21(DE3) induced by 0.5 mM IPTG, 37°C, 2 h; 5: Soluble fraction of in periplasm extract from *pelAc30/pET-20b(+)/E. coli* BL21(DE3) induced by 0.5 mM IPTG, 37°C, 2 h.

we examined the conditions of expressing PelA in *E. coli*. First, the effect of IPTG (0.1-1.0 mM) on PelA extra cellular expression was researched (Figure 2a). *E. coli* cells with *pelA/pET-20b (+)* was cultivated at 0.1-1.0 mM IPTG, pH7.0, 37°C, 200 rpm for 8 h for expressing PelA. The result showed that maximal enzyme production (450 U/ml) was reached at 1.0 mM and enzyme production was enhanced with IPTG concentration increase, but when IPTG concentration was higher than 1.0 mM, no obvious enhancement of enzyme production (data not published). Second, the effect of temperature on PelA extra cellular expression was researched (Figure 2b) and it was observed that from 15 to 35°C, PelA biosynthesis reached about 100 Uml⁻¹ medium, and at 37°C got to the maximum production, 450 U ml⁻¹ medium. PelA biosynthesis reduced when temperature was lower than 37°C; enzyme production was reduced by 45% at 15 to 35°C compared to that at 37°C (Figure 2b). At last, we researched the effect of time on the expression and found that both the time before the enzyme got to the maximum activity (Figure 2c) and the lag time of enzyme biosynthesis were shortened (not published) at 37°C.

The effect of Ca²⁺ on the extra cellular expression

Calcium causes a significant promotion of the PelA extra-secretion of *pelB/pET-20b (+)/E. coli* BL21 (DE3). Figure 3 shows that PelA production got to 625 U/ml at 20 mM

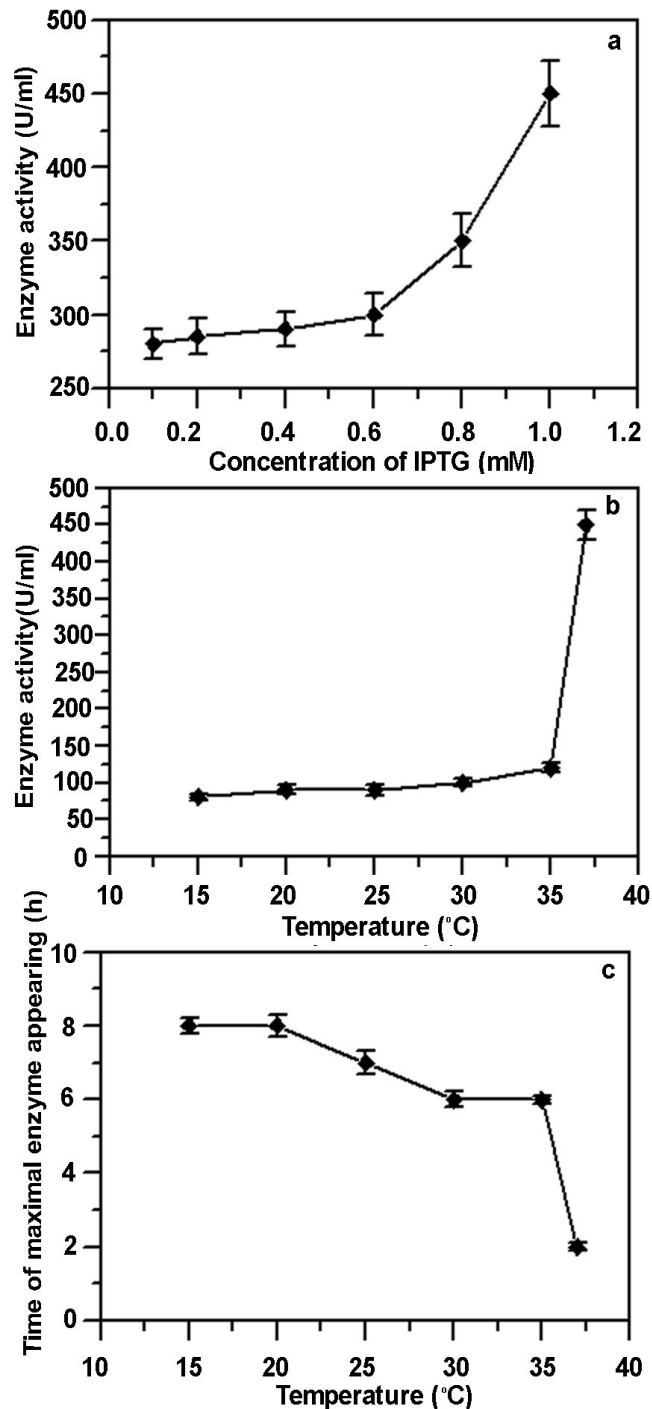


Figure 2 Effects of IPTG, temperature and time on PelA extra cellular expression.

a: The expression was applied at 0.1-1.0 mM IPTG, 37°C, 200 rpm, for 2 h; b: The expression was applied at 1.0 mM IPTG, pH7.0, 15-37°C, 200 rpm, for 2-8 h; c: The expression was applied at 1.0 mM IPTG, pH 7.0, 15-37°C, 200 rpm, for 2-8 h.

calcium and this was higher than that of the control (450 U/ml).

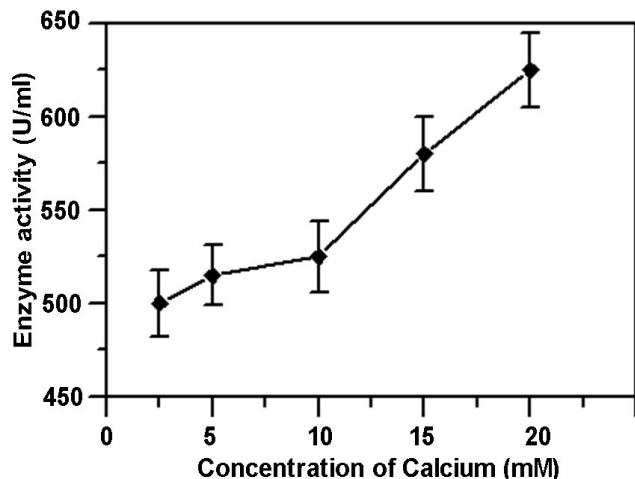


Figure 3. The effect of Ca^{2+} on the PelA extral cellular expression. The expression was applied at 1.0 mM IPTG, pH 7.0, 37°C, 200 rpm, 2.5-20 mM calcium, for 2 h.

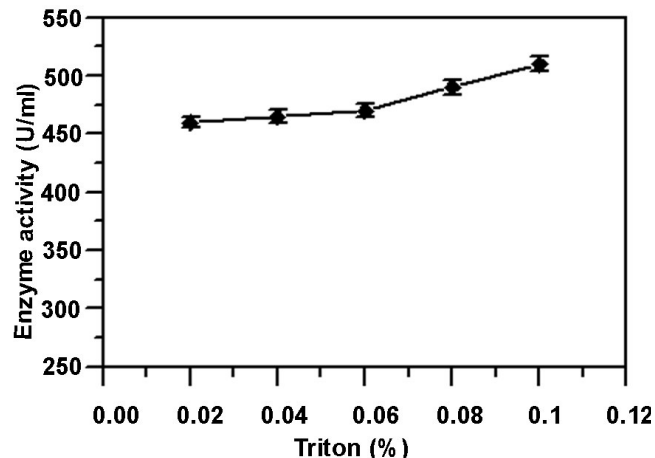


Figure 5. The effect of SDS 100 on the PelA expression. The expression was applied at 1.0 mM IPTG, pH7.0, 37°C, 200 rpm, 0.02-0.12% glycine, for 2 h.

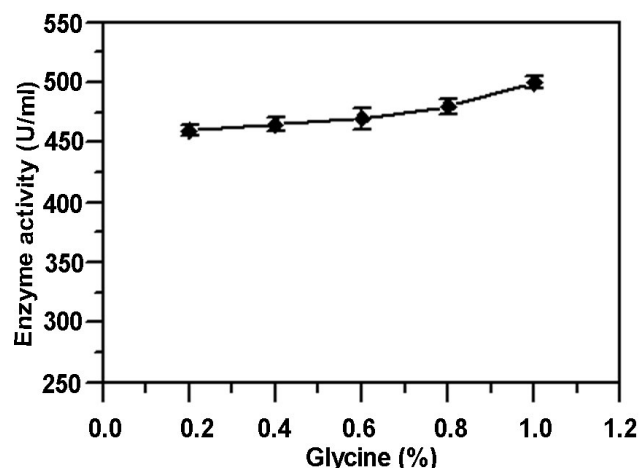


Figure 4. The effect of glycine on the PelA expression. The expression was applied at 1.0 mM IPTG, pH 7.0, 37°C, 200 rpm, 0.2-1.2% glycine, for 2 h.

The effect of glycine on the extra cellular expression

Glycine brings a little promotion for the PelA extra-secretion of *pelA/pET-20b (+)/E. coli* BL21 (DE3). Figure 4 shows that the PelA production got to 475 U/ml at 1% glycine concentration and this was a little higher than that of the control (450 U/ml).

The effect of SDS on the extra cellular expression

SDS brings a little promotion for the PelA extra-secretion of *pelA/pET-20b (+)/E. coli* BL21 (DE3). Figure 5 shows the that PelA production got to 500 U/ml at 0.1% SDS concentration and this was a little higher than that of the control (450 U/ml).

DISCUSSION

The effect of temperature on the PelA extracellular expression

PelA biosynthesis got to about 450 Uml⁻¹ medium at 37°C, reaching the maximum production, which increases 4.5 folds than 100 U/ml medium at 15 to 35°C. Both the time of reaching the maximum enzyme activity and the lag time of enzyme biosynthesis were shortened at 37°C. At 37°C, some protein transport paths were constructed and opened. Precious research suggested that the TYSS2 path genes cluster was regulated by a σ_{70} and σ_{70} transcripts is opened only at 37°C (Yang et al., 2007). At 37°C, PelA might be transported through outer membrane by some special path, but at 15 to 35°C, a little of PelA was secreted into medium by membrane mechanical leakage.

The time (2 h) of the maximum enzyme at 37°C implied that the PelA expression was very important to pectate lyase industrial production.

The effects of calcium, glycine and SDS

Some chemical and enzymatic strategies were employed to increase the permeability of the outer membrane such as addition of magnesium, calcium, EDTA, glycine, SDS and Triton (X-100) and enzymatic (lysozyme) treatments (Shokri et al., 2003; Choi and Lee, 2004). In this research, the effects of calcium, glycine and SDS were investigated and the results obtained showed that calcium had a more obvious promotion than glycine and SDS. This might be because calcium has a direct action on the PelA transport path or the related transport path is dependent on calcium ions for activity (Korotkov et al., 2009). In the PelA expression system, major protein was

transported through the outer membrane by a special path and a little recombinant protein was reserved in periplasmic space, so, glycine and SDS did not have an effective action on the PelA extracellular secretion.

The c-terminal sequence of PelA affecting the extra cellular expression

In the present report, Pel A from *Aspergillus nidulans* was expressed with recombinant plasmid in *E. coli*. The used plasmid pET-20b (+) has a signal peptide PelB, which can be resigned by the SecA path of *Erwinia carotovora* or *Escherichia coli* (Rusch and Kendall, 2007; Zimmer et al., 2008). The N-terminal signal peptide can secrete the protein into periplasmic space by SecA path. In the strain of pelA/pET-20b(+)/*E. coli* BL21(DE3), a great deal of PelA was found in medium when the expression was applied at 37°C for 2 h; and in a short time, the PelA medium could not result from outer membrane damnification. If the pelA gene was free of C-terminal 30 aa sequence and the strain of pelAc30/pET-20b (+)/*E. coli* BL21 (DE3) was constructed to express PelA protein; it was found that the recombinant protein (free of C-terminal 30 aa sequence) majorly appeared in periplasmic space, not in medium. The C-terminal 30 aa sequence might have an extracellular signal peptide (Qian et al., 2008; Zimmer et al., 2008) or a significant conformational change promoted by the C-terminal deletion which may also hinder translocation through the outer membrane.

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