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Crystallisation of Pectate Lyase 10A Catalytic Module From Cellvibrio japonicas

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Abstract

Pectate lyase enzymes degrade pectate and its mechanism is deducible from its structure. pectate lyase 10A (Pel10Acm) from *Cellvibrio japonicus* NCIMB 10462 was cloned and expressed in *E.coli* BL21(DE3), purified and characterised, and its crystals produced using a cocktail of precipitants. The Rod cluster crystals of Pel10Acm produced in this study were grown from the precipitating mixture of 0.2M $MgCl_2$, 25% PEG (polyethylene glycol), 2K MME (Monomethyl ether), $MgCl_2$; PEG; MME; 2K, 2000 Daltons).

Keywords: Rod cluster crystals, Pectata lysae enzymes, IMAC, Crystallisation Screening

1. Introduction

Pectate lyases (Pel) (EC 4.2.2.2) are enzymes that break down pectate by cleaving its polymeric α-1,4-linked galacturonic acids via *β*-elimination mechanism (Li *et al*., 2014). In addition, they belong to the class of polysaccharide lyases, which are categorised in 5 out of 12 families (Kluskens *et al*., 2003). Pectate lyases have been structurally characterized in *Pseudomonas cellulose* (Charnock *et al*., 2001 & Brown *et al*., 2001), *Erwinia chrysanthemi* (Jenkins *et al*., 2004) and *Phytophthora capsici* (Wang, *et al*., 2011). They affect various plant species cause tissue rotting (Marin-Rodriguez *et al*., 2002). *Cellvibrio japonicus* had been experimentally indicated to degrade the main plant cell wall polysaccharides, involving crystalline cellulose, xylan and mannan (DeBoy *et al*., 2008). The degrading enzymes have a complicated molecular architecture comprising of catalytic modules and non-catalytic carbohydrate binding modules (Zhang *et al*., 2014).

X-ray crystallography remains the most frequent technique that utilises to determine protein structures due to its ability to resolve large biomolecules (DeLucas *et al*., 2003). Furthermore, the X-ray crystallography has allowed explaining exceptions to common structural motif in Pels (Charnock *et al*., 2001, Abbott and Bornston, 2007). An essential element of X-ray crystallography is acquiring well-arranged crystals of the target protein (DeLucas *et al*., 2003). Furthermore, high quality crystals of appropriate size are a significant requirement for utilizing X-ray crystallography to define the 3-dimensional structure of proteins (Guo *et al*., 2014). The objective of the present work is to acquire high quality crystals of the catalytic module of the recombinant enzyme Pectate lyase 10A (Pel10A) *Cellvibrio japonicus* NCIMB 10462. Pel10Acm was cloned and expressed in *E. coli* BL21 (DE3), purified, characterized and its crystals generated using a cocktail of precipitants.

2. Materials and Method

Production of chemically competent BL21(DE3) *cells*

BL21(DE3) cells was centrifuged for 1 min and the supernatant was poured off. The pellet was then resuspended in 0.9 ml of chilled $MgCl_2$-$CaCl_2$ solution by vortexing and return to ice for 3 sec after every 3 sec of vortexing. The solution was incubated on ice for 10 min. The cells was centrifugeed again for 1 min and the pellet was resuspended in 50µl of 0.1 M $CaCl_2$.

Transformation of competent BL21(DE3) *cells with the catalytic module of the pectate lyase gene sequence (pel10acm)-containing* pET-28a

A 5 µl of the *pel10acm*-containing pET-plasmid DNA were added to the cells (previous section), and mixed by swirling with the pipette tip. The microcentrifuge tube was incubated on ice firstly for 20 min at 42^{o}C and incubated futherly for 90 sec at the same temperature. 200 µl of LB medium were added to the microcentrifuge tube and incubated in a water bath set at 37^{o}C for 45 min. The entire contents of the microcentrifuge tube were spreaded onto LB agar plate containing 50 µg/ml kanamycin and incubated at 37^{o}C overnight.

Inoculation of auto-induction medium with BL21(DE3) *cells carrying the catalytic module of the pectate lyase gene sequence (pel10acm)-containing* pET-28a.

Kanamycin was added to a final concentration of 100 µg/ml and inoculated with a colony from LB agar plate for a week. The inoculated glass conical flask was incubateed at 30^{o}C with shaking at 200 rpm, then colonies LB agar plate were counted and recorded the number. The culture was spun down in 50 ml Falcon tube at 4000 x g for 15 min, poured off the supernatant and frozen.

Purification and preparation of Pel10Acm for crystallisation screening

The culture were spun down after overnight incubation and the pellet was frozen.
The cell pellet was resuspended in 500 µl of Lysis buffer by vortexing, and the resuspended pellet was then transferred to a 1.5 ml microcentrifuge tube. The cell suspension was incubated on a rotating mixer at a slow setting for 15 min at room temperature. The supernatant of centrifuged lysed cell suspension was poured into a fresh 1.5 ml microtube and kept on ice until required.

Purification of Pel10Acm using IMAC

A 10 ml syringe was filled with 5 ml of elution buffer and inserted in the connector. The elution buffer was applied to the column and the eluant was then collected in the 100 ml beaker. The syringe was removed and refilled with 5 ml of 18.2 MΩ/cm water and inserted again into the connector. The charging buffer followed by the binding buffer were applied to the column and the eluant (from each) was collected in a 100 ml beaker. A 10 ml syringe was filled with 5 ml of washing buffer and applied to the column and the eluant was collected in the 100 ml beaker. The 10 ml syringe was removed, refilled with 2 ml elution buffer, applied to the column and then the eluant was collected in a fresh empty tube.

Desalting of Pel10Acm

A 10 ml syringe was filled with 10 ml desalting buffer and inserted into the connector. The desalting buffer was applied to the column and the eluant was collected in the 100 ml beaker. The syringe was remove and refilled with 1.5 ml of the eluant that contains purified Spy1600 protein that kept from the HisTrap column. The purified Spy1600 protein was applied to the column and the eluant was collected in the 100 ml beaker. The syringe was again refilled with 2 ml of desalting buffer and applied to the column and the eluant was then collected in a fresh empty tube.

Concentrating Pel10Acm using ultrafiltration

Pippette 500 µl of purified desalted Pel10Acm into the filter device of a 10 kDa molecular weight was cut off (MWCO) ultrafiltration spun column. The concentrated Pel10Acm volume made up to 200 µl of 18.2 MΩ/cm water and inverted the filter device. Therefore, centrifuged for 1 min (at 14,000 x g) in the collection tube to collect the concentrated Pel10Acm and then the sample was left on the ice.

Crystallisation screening of Pel10Acm

Determination of enzyme concentration.

The entire desalted purified concentrated protein sample was applied to a plastic UV-microcuvette and its absorbance was measured at 280 nm. Then the sample was transferd into a fresh 1.5 ml microcentrifuge tube and kept on ice. The concentration of the undiluted enzyme solution (in mg/ml) was determined using Beer-Lambert law (A=εcl, where A= absorbance, ε = extinction coefficient, c = concentration and l = path length).

Setting up crystallisation trays.

Using the 10 ml syringe containing 1 ml of vacuum grease (the grease was applied to the rims of the wells of a 24-well crystallisation tray), a 2 µl of enzyme were carefully added to the centre of a single cover-slip. Another 2 µl of well solution were added to the previouse 2 µl drop and well mixed by repeated pipetting. The cover-slip was carefully inverted and sealed the drops above the well solution by applying gentle pressure with the forceps. Immediately, the crystallisation trays were placed in the constant temperature crystallisation room.

Analysis of Pel10Acm

***Determination of protein purity by denaturing polyacrylamide gel electrophoresis* (SDS-PAGE)**

A 5 µg of concentrated enzyme solution were made up to 20 µl with 18.2 MΩ/cm water. 5 µl of loading buffer was added and the solution was incubated for 3 min a boiling water bath. The solution was then centrifuged for 1 min. 20 µl of the sample was loaded onto a SDS-PAGE gels. The gel contained one lane loaded with 5 µl (~ 1 µg of protein per band) of low molecular weight markers (relative molecular weights: 66000, 45000, 36000, 29000, 24000, 20000, 14200, and 6500).

Biochemical assay of enzymatic activity

It is very important to establish that enzymes are stable during the crystallisation process. Therefore, the activity of enzyme would assay to establish whether it is stable as a concentrated solution or it is gradually/totally inactivated. A 1.0 µg of the enzyme was added to a mixture made of 80 µl (1.625 mg/mL polygalacturonic acid), 10 µl (500 mM CAPS buffer, pH 10.0), and 10 µl (1 mM $CaCl_2$) in a plastic UV-microcuvette. The cuvette was placed in a UV-vis spectrophotometer and the absorbance of the mixture was monitored (at λ_{max}= 232 nm) every 5s over a 1 min period.

Analysis of crystallisation trays

Using the binocular microscope, each drop was analyzed and the result was record using an observation sheet (provided). Typical observations and photographs of the best crystal will be described and shown, respectively.

3. Results

***Transformation of E. coli* BL21(DE3) *with the catalytic module of Pectate lyase gene sequence (Pel10Acm)-containing* pET-28a.**

The assessment of the transformed *E. coli* BL21(DE3) cells cultured overnight at 37°C produced 34 colony forming units (cfus).

Determination of Pel10Acm concentration

The absorbance of the desalted purified concentrated pectate lyase for crystallisation was measured and its concentration was calculated following Beer-Lambert law (where c= A/εI, A at 280nm = 0.323, ε of Pel10Acm = 73800 mol^{-1} L cm^{-1} and I =1cm). Accordingly, the concentration of Pel10Acm was calculated and founded equal to 4.38×10^{-6} mol/L = 4.38 µM**.**

Determination of the volume of the enzyme solution required for SDS-PAGE

SDS-PAGE was utilised to determine the pectate lyase 10A (Pel10A) sample purity and 5µg of Pel10A required. Pel10A sample volume required was calculated as follows:

- Amount of enzyme needed = 5 µg.
- Concentration of Pel10Acm solution = 4.38×10^{-6} mol/L**.**
- Molecular weight of Pel10Acm = 37634.7g/mol.
- The concentration (g/L) = molar concentration × relative molecular mass.
- Pel10Acm (g/L) = $4.38\times10^{-6}\times 37634.7$ = 0.1648 g/L = 0.1648 µg/µL.
- Volume required = 5/0.1648 = 30.33 µL.

Determination of Pel10Acm purity by denaturing (sodium dodecyl sulphate) polyacrylamide gel electrophoresis (SDS-PAGE)

Analysis of the Pel10Acm sample by SDS-PAGE (Figure 1) shows the successful purification of Pel10Acm as the matching position of the desired Pel10Acm band (red arrow) with the reference band (36000) in the standard lane (M), represents the obtaining enzyme was pure as its position matched to its molecular weight range.

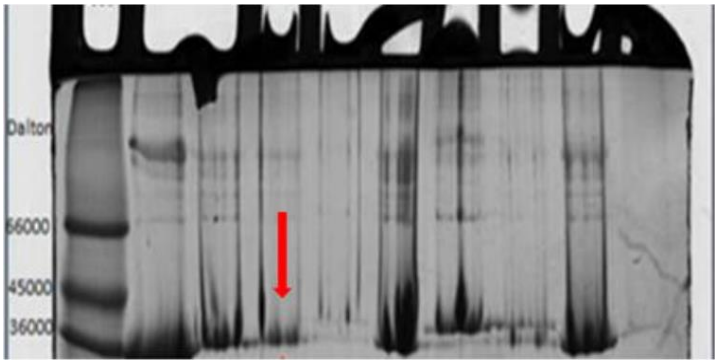

Figure 1: SDS-PAGE gel of Pel10Acm (M lane, low molecular weight markers; red arrow refers to purified band of Pel10Acm.

Biochemical assay of enzymatic activity of Pel10Acm

The Pel10Acm was enzymatically active through the increasing absorbance measurements achieved using a time series observation on addition of polygalacturonic acid to the enzyme (Table 1, Figure 2).

Table 1: Volues of absorbance (λ_{max}= 232nm) of the Pel10Acm activity on polygalacturonic acid. Data was recorded every 5 sec over 1 min period.

Time (sec)	Absorbance	Time (sec)	Absorbance
00.0	0.000	35.0	0.759
05.0	0.650	40.0	0.766
10.0	0.667	45.0	0.790
15.0	0.679	50.0	0.840
20.0	0.694	55.0	0.830
25.0	0.723	60.0	0.872
30.0	0.728		

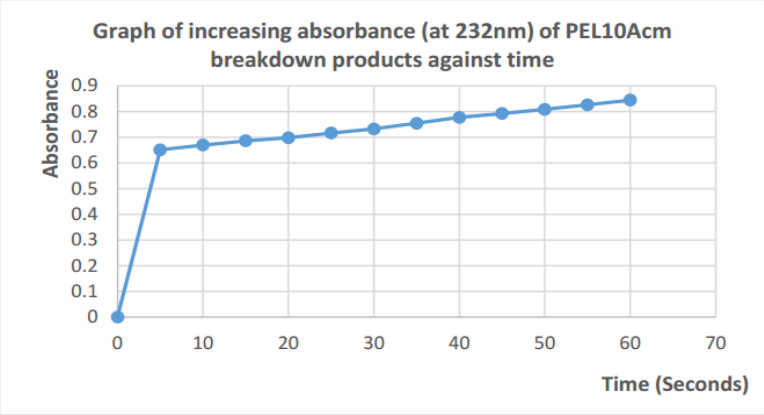

Figure 2: The enzymatic activity of Pel10Acm on polygalacturonic acid breakdown, using UV-vis spectrophotometer at 232 nm. The absorbance was recorded at every 5sec over a 1 min.

Analysis of Pel10Acm crystals

The crystals obtained from precipitant screen were rod cluster crystals (Figure 3).-

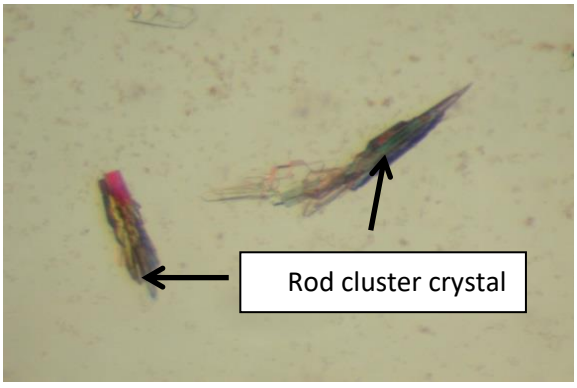

Figure 3. Rod cluster crystals of Pel10Acm, grown from the precipitating mixture of 0.2M $MgCl_2$, 25% PEG, 2K MME ($MgCl_2$, PEG, MME, 2K, 2000 Daltons).

4. Discussion

Pectate lyases (Pel) (EC 4.2.2.2) are enzymes that break down pectate by cleaving its polymeric α-1,4-linked galacturonic acids via β-elimination mechanism (Li *et al*., 2014). They belong to the class of polysaccharide lyases, which are categorized in 5 out of 12 families (Kluskens *et al*., 2003). Pectate lyases have been structurally characterized in *Pseudomonas* cellulose (Charnock *et al*., 2001) *Erwinia chrysanthemi* (Jenkins *et al*., 2004), Phytophthora capsici (Wang, *et al*., 2011). They affect various plant species causing tissue rotting (Marin-Rodriguez *et al*., 2002). In addition, *Cellvibrio japonicus* had been experimentally indicated to degrade whole the main plant cell wall polysaccharides, involving crystalline cellulose, xylan and mannan (DeBoy *et al.*, 2008). The degrading enzymes have a complicated molecular architecture composing of catalytic modules and non-catalytic carbohydrate binding modules (Zhang *et al*., 2014).

The catalytic module of recombinant pectate lyase 10A was expressed in *E. coli* BL21(DE3), purified and crystallised. It had a final concentration of 0.165 µg/µL and its molecular weight ascertained giving credence to the purity (Figure 1). The number of cfus post-transformation showed the efficiency in plasmid uptake by *E. coli* BL21(DE3) (Tu *et al*., 2005). Applying an auto-induction medium for Pel10Acm expression enabled a switch in energy substrate utilization in the *E. coli* culture matching its late log growth phase appropriate for recombinant protein expression. IMAC purification enabled by the histidine tag (Carson *et al*., 2007) was appropriate as the enzyme's biochemical activity was not compromised (Figure 2) and had a high level of purity shown with its molecular weight via SDS-PAGE, about 36KDa (Jayani *et al*., 2005 and Rath *et al*., 2009). Attaining high levels of protein purity is a critical factor for success in protein crystallisation (DeLucas *et al*., 2003). The enzymatic activity indicates the competence of the protein (Payasi *et al*., 2009) and prior insight to its structural integrity before crystallisation (Charnock *et al*., 2001 and Soriano, *et al.*, 2006). Several studies revealed a temperature, cofactor e.g. Ca^{2+} and pH had an essential impact on pectate lyase activity (Zhang *et al*, 2013 and Ouattara *et al*., 2010).

This observation in addition to the adequate purification technique are important factors for producing large quantities of homogenous and quality crystals for X-ray crystallography (Smatanova, 2002). An essential element of X-ray crystallography is acquiring well-arranged crystals of the target protein (DeLucas *et al*., 2003). Furthermore, high quality crystals of appropriate size are a significant requirement for utilizing X-ray crystallography to define the 3-dimensional structure of proteins (Guo *et al.*, 2014). The crux of this study is to acquire high quality crystals of the catalytic module of the recombinant enzyme pectate lyase 10A (Pel10A) *Cellvibrio japonicus* NCIMB 10462. The aim is achieved by producing rod cluster of Pel10Acm

crystals. Utilizing of adsorption and desorption method for simultaneously improving a protein crystallisation rate and the crystals quality (Guo *et al*., 2014).

In conclusion, The combination of precipitants 0.2 M $MgCl_2$, 25% PEG and 2K MME has been identified as appropriate. This study is recommended for systematic optimization to enable the production of larger crystals of Pel10Acm for X-ray diffraction studies.

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