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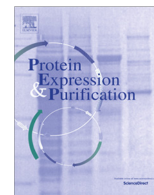

Characterization of recombinant pectate lyase refolded from inclusion bodies generated in *E. coli* BL21(DE3)

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ABSTRACT

Pectate lyase (EC 4.2.2.2) gene from *Bacillus subtilis* RCK was cloned and expressed in *Escherichia coli* to maximize its production. In addition to soluble fraction, bioactive pectate lyase was also obtained from inclusion body aggregates by urea solubilization and refolding under *in vitro* conditions. Enzyme with specific activity ~3194 IU/mg and ~1493 IU/mg were obtained from soluble and inclusion bodies (IBs) fraction with recovery of 56% and 74% in terms of activity, respectively. The recombinant enzyme was moderately thermostable ($t_{1/2}$ 60 min at 50 °C) and optimally active in wider alkaline pH range (7.0–10.5). Interaction of protein with its cofactor $CaCl_2$ was found to stimulate the change in tertiary structure as revealed by near UV CD spectra. Intrinsic tryptophan fluorescence spectra indicated that tryptophan is involved in substrate binding and there might be independent binding of Ca^{2+} and polygalacturonic acid to the active site. The recombinant enzyme was found to be capable of degrading pectin and polygalacturonic acid. The work reports novel conditions for refolding to obtain active recombinant pectate lyase from inclusion bodies and elucidates the effect of ligand and substrate binding on protein conformation by circular dichroism (CD) and fluorescence spectrofluorometry.

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Introduction

Pectinesterases and depolymerases are two major groups of enzymes responsible for pectin degradation. Pectinesterases catalyze removal of methoxyl groups, whereas depolymerases, which include hydrolases and lyases, cause degradation of polymer backbone [1]. Pectate lyase facilitates breaking of glycosidic bonds in unmethylated pectates by β-elimination reaction. Generally, pectate lyases are considered to be more specific for the degradation of unmethylated polygalacturonate, however, they have also been observed to act on esterified pectin [1]. Pectate lyases have varied applications due to variation of their pH optima. Pectate lyases having pH optima in acidic range are used in food and juice industries, whereas, alkaline pectate lyases are used in beverages fermentation, oil extraction, plant fiber processing and pulp and paper industries [2–4].

There are several reports on cloning, expression and purification of pectate lyase from various microorganisms [5–12]. However, recovery of the recombinant protein has been poor due to losses during purification. Moreover, high-level expression of recombinant proteins in *Escherichia coli* is known to lead to aggregation and formation of inclusion bodies (IBs)[1] [13]. Pectate lyase from *Bacillus subtilis* possesses 15 proline residues. It has been reported that slow *cis–trans* isomerization around X-Pro bonds play a critical role in protein refolding process that subsequently affects the recovery of bioactive protein [14]. Recovery of active protein from IBs by *in vitro* refolding is a complex process and requires optimization at various steps [15]. The formation of IBs can be minimized if low temperature conditions are provided to the microorganism during incubation after induction. The formation of IBs also provides some advantages such as resistance of protein to proteolytic degradation, retaining of the native secondary structures of the protein and finally, aggregation makes the protein purification process more convenient and efficient. If the protein from IBs can be refolded into active form, this would provide a viable process for large-scale production of active enzymes [16,17].

In our laboratory, we have been studying various parameters for optimization of pectinase production from the wild type *B. subtilis* strain RCK [18]. Invariably, these studies have shown that pectate lyase is produced along with other pectin degrading and cellulolytic enzymes. However, specific industrial applications of pectate

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[1] *Abbreviations used:* IBs, inclusion bodies; CD, circular dichroism; EDTA, ethylenediaminetetracetic acid; PMSF, phenylmethanesulfonylfluoride; OFAT, one factor at a time.

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lyase require the sole action of the enzyme on its substrates, and therefore, it is imperative to produce this enzyme under regulated conditions. Homologous recombinant expression of pectate lyase in *B. subtilis* has been reported earlier [6]. However, secretion of a range of cellulolytic enzymes is undesirable for certain applications which make the process slightly unfavorable [9]. Keeping in view the potential application of alkaline and thermostable pectate lyase, an attempt was made to clone and express pectate lyase gene from *B. subtilis* RCK in *E. coli* BL21(DE3). The recombinant protein expressed as a bioactive soluble fraction and as an insoluble inclusion bodies (IBs) fraction. To maximize the recovery of active enzyme, it is necessary to optimize the conditions for refolding of pectate lyase from IBs. *In vitro* refolding of this class of enzymes is generally unexplored. In the present study, we have attempted to optimize conditions for obtaining active pectate lyase from insoluble aggregates (IBs). The recombinant protein obtained though this process was purified and its secondary and tertiary structure was characterized after binding with its substrate and cofactor using circular dichroism (CD) and fluorescence spectrometry. Moreover, action of the enzyme on polygalacturonic acid and citrus pectin was analyzed by HPLC by observing substrate degradation products.

Materials and methods

Bacterial strains, plasmids, enzymes, antibiotics and other chemicals

B. subtilis RCK was maintained on agar plate containing 0.5% glucose, 0.5% peptone, 0.5% yeast extract, 0.15% KH_2PO_4, 0.01% $MgSO_4{\cdot}7H_2O$ and 2.0% agar, pH 9.0. The gene construct in pET28a vector was maintained in *E. coli* DH5α and the expression was performed in *E. coli* BL21(DE3) [*F*− *ompT hsdSB* (*r*−$_B$ *m-B*) *gal dcm* (DE3)] (Novagen, USA). Stocks of ampicillin (100 mg/ml) (HiMedia) and kanamycin (100 mg/ml) (HiMedia) were prepared in MilliQ water and stored at 4 °C. Working concentrations of ampicillin and kanamycin were used as 100 μg/ml and 50 μg/ml, respectively. Other chemicals were used as Tris base (Sigma–Aldrich, USA) for maintaining specific pH, ethylenediaminetetracetic acid (EDTA) (Sigma–Aldrich, USA) as chelating agent, trypsin (Sigma–Aldrich, USA) for protein digestion, deoxycholic acid (DOC) (Merck, USA) as detergent, $CaCl_2$ (Merck, USA) as catalyst ligand and Phenylmethanesulfonylfluoride (PMSF) (Sigma–Aldrich, USA) as protease inhibitor.

Cloning of pectate lyase

Genomic DNA from *B. subtilis* RCK was isolated following Murmur's method [19]. Pectate lyase gene was amplified using genomic DNA as template and the primers: forward_ 5′CAGCACATATGAAAAAAGTGATGTTAGCTACGGC3′ and, reverse_ 5′CATACTCGAGTTAATTTAATTTACCCGCACCCGC3′. The gene was amplified using PCR reaction composed of initial denaturation at 95 °C for 5 min followed by 30 cycles of amplification containing denaturation at 95 °C for 1 min, primer annealing at 52 °C for 30 s and elongation at 72 °C for 30 s. A final elongation time of 7 min at 72 °C was provided for end filling. The amplified fragment and pET28a vector were digested with *Nde*I and *Xho*I restriction enzymes and both insert and vector were ligated by T_4 DNA ligase (NEB, UK) at 4 °C. *E. coli* DH5α competent cells were transformed with ligation mixture. Initial screening of positive transformants was carried out by providing 50 μg/ml kanamycin selections on Luria–Bertani (LB) agar plate. Final authenticity of the recombinant construct was confirmed by nucleotide sequencing analysis and the gene sequence was submitted to NCBI (accession number AFH66771.1).

Expression of pectate lyase

E. coli BL21(DE3) competent cells were transformed with recombinant pectate lyase plasmid construct and transformed BL21(DE3) cells were cultivated in 100 ml Erlenmeyer flask containing 15 ml LB medium at 37 °C up to absorbance 0.8 at 600 nm. The culture was induced with 0.7 mM IPTG and post-induction growth was monitored by sampling at regular intervals. OD was measured by diluting the culture 10 times after attaining OD beyond 1.0. Uninduced culture was used as control. The expression of recombinant pectate lyase was confirmed by SDS–PAGE and western blotting (Semidry transfer, Bio-Rad system). The cell mass from 50 ml induced culture was spin down and resuspended in 50 mM Tris HCl buffer (pH 8.5). This cell suspension was sonicated by 10 cycles of sonication programmed as 10 s pulse on and 20 s pulse off at 4 °C. Sonicated culture was centrifuged at 17,000×*g*, 4 °C for 20 min and the supernatant was evaluated for pectate lyase activity. Enzyme activity was determined by measuring the change in absorption at 235 nm in 100 mM glycine NaOH buffer (pH 9.0) containing 0.2% polygalacturonic acid (w/v) ($\varepsilon = 4600\ M^{-1}cm^{-1}$) and 0.44 mM $CaCl_2$. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of unsaturated oligogalacturonide per minute at 235 nm [5]. The amount of protein was measured by Bradford's method using BSA as standard.

Enzyme production

Recombinant pectate lyase was purified from cells harvested from 1 L culture media. Transformed *E. coli* BL21(DE3) cells were cultivated in 2 L Erlenmeyer flasks, each containing 500 ml LB medium, at 37 °C up to absorbance 0.8 at 600 nm. The culture was induced with 0.7 mM IPTG and grown at 25 °C. Induced cell mass was harvested at $OD_{600\ nm}$ 3.27 after 6 h of induction by centrifugation at 8000×*g* for 15 min at 4 °C. Cell mass having total wet weight of 3.017 g (from 1 L culture broth) was washed with 50 mM Tris buffer at pH 8.5 and resuspended in 40 ml of buffer A (50 mM Tris buffer, pH 8.5, 1 mM PMSF and 5 mM EDTA). The cells were sonicated as described earlier and sonicated culture was centrifuged at 17,000×*g*, 4 °C for 20 min. Culture supernatant was analyzed for enzyme activity, while the remaining pellet was resonicated in the same buffer except replacing 5 mM EDTA with 1% (w/v) sodium deoxycholate. Thereafter, the supernatant was analyzed for enzyme activity and IBs in the form of pellet were washed twice with 25 mM Tris HCl, pH 8.5. Finally, pellet was resuspended in 2 ml of MilliQ water and quantified for protein concentration. The soluble fraction from crude lysate was directly purified using Ni–NTA affinity column chromatography. The insoluble fraction in the form of IBs was solubilized and refolded as described in refolding of inclusion bodies further.

Purification of pectate lyase from soluble fraction

Soluble fraction of the enzyme in the lysate supernatant was purified by Ni–NTA affinity column chromatography. Empty spin column (Bio-Rad, USA) of 5 cm length having 1.2 ml bed volume was packed with Ni–NTA resin. Column was equilibrated with 4 column volume of 50 mM Tris HCl buffer, pH 8.5, containing 1 mM PMSF and 100 mM NaCl. The elution buffer containing different concentrations of imidazole were used to optimize the efficient elution of the pure pectate lyase. Different elution fractions were assayed for enzyme activity and quantification of protein content. The homogenous fraction was concentrated by 10 kDa cutoff Amicon tube (Pall Life Sci., USA) and dialyzed at 4 °C against 25 mM Tris HCl buffer, pH 8.5 to remove imidazole.

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