International Journal of Biological Macromolecules 95 (2017) 256–262

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Enhancement of solubility, purification and inclusion-bodies-refolding of an active pectin lyase from *Penicillium occitanis* expressed in *Escherichia coli*

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ARTICLE INFO

Article history:
Received 31 May 2016
Received in revised form
12 September 2016
Accepted 10 November 2016
Available online 11 November 2016

Keywords:
Pectin lyase
Heterologous expression
Inclusion bodies

ABSTRACT

Pectin lyase (pnl) is the only pectinase able to hydrolyze directly the highly methylated pectin without liberating the toxic methanol and without disturbing ester content responsible for specific aroma of juices. The cDNA of *Penicillium occitanis* pnl (mature form) was cloned into pET-21a as expression vector and over-expressed into *Esherichia coli*. Most of recombinant pnl was expressed as inclusion bodies. Pnl activity was confirmed by colorimetric assay. To enhance the solubility yield of the expressed pnl, the effects of induction temperature, host strain and expression level were optimized. Maximal production of functional pnl was obtained after induction by 0.4 mM IPTG at 30 °C and 150 rpm for 16 h. Interestingly, the use of Origami host strain, having an oxidized cytoplasm favoring disulfide bonds formation required for the active conformation of the enzyme, has significantly improved the yield of the soluble active form of recombinant pnl. This pnl was successfully purified through a single step purification using His-Trap affinity column chromatography. This work is the first to report pnl expression into Origami strain. Alternatively, the inclusion bodies were isolated, denatured by high concentration of urea and gradually refolded by successive dialysis, leading to their transformation into soluble and active form.

1. Introduction

Pectin is the most complex class of polysaccharides of the cell wall, very widespread in plant kingdom and essentially present in the primary cell walls and middle lamella of plants. It is composed of two regions "smooth region" and "hairy region" [1]. Given the complexity and the existence of this polymer in several forms in nature, various pectinolytic enzymes such as pectin lyase; pectat lyase; polygalacturonase and pectin methylesterase should intervene for their degradation.

Pectin lyases are the only pectinolytic enzymes known to catalyze directly the de-polymerization of a highly methylated pectin via β-elimination mechanism resulting in the formation of 4,5-unsaturated oligogalacturonates [2]. Contrarily to pectate lyases, this action doesn't need the previous action of pectinesterase that liberates methanol which is a toxic substance. Moreover, the ester group content in pectin responsible for specific aroma of fruit juices will not be disturbed during the action of pnl [1,2]. More importantly, being a lyase and not a hydrolase, pnl enzyme does not require water for its activity and, contrarily to pectate lyase, it is independent of calcium. These characteristics of pnl grant a great interest to this enzyme in food industry, essentially in fruit juice clarification and in oil extraction.

All pnl that have been reported in literature are endo-acting type and generally, their activity decreases with the decreasing of the methylation degree of pectins. According to the CAZY classification system, they belong to the family 1 of the polysaccharide lyases [3], and subclass of homogalacturonan degrading group of pectinolytic enzymes. Pnl are produced by various filamentous fungi, mainly by species of the genus *Aspergillus*, *Penicillium* and *Fusarium*, and rarely reported in bacteria and yeasts; moreover, gene encoding for pnl could be even found in many plants [4].

Among the techniques for improving the enzyme production is the selection of hyper-producing strains. Accordingly, our laboratory has selected the CT1 mutant from the wild strain of *Penicillium occitanis* (CL100) after a single round of mutagenesis using nitrous acid [5]. However, this mutant over-expresses several pectinases while some biotechnological applications require only one pectinolytic activity. So, the production of an individual pectinase enzyme in heterologous system becomes the best choice. Prokaryotic system is the most dominant host used for heterologous expression because bacteria are easy to manipulate, robust

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http://dx.doi.org/10.1016/j.ijbiomac.2016.11.036
0141-8130/

and able to grow rapidly and vigorously at high density with low cost [6]. Heterologous expression in *E. coli* of foreign gene, encoding a protein that exhibit complex structures, often leads to accumulation of most of expressed protein in insoluble and inactive form. About 70% of recombinant proteins are over-expressed in inclusion bodies [7]. To reduce the aggregation process of recombinant proteins, increasing by the way their solubility *in vivo*, certain parameters can be controlled such as concentration of inducer, time of incubation, host strains and induction temperature. On the other hand, inclusion bodies can also be exploited as a pre-purification step and can be thereafter solubilized and refolded into an active protein.

It was also shown that proteins with disulfide bonds are more prone to aggregation in the reducing cytoplasm of *E. coli.* Such problem can be overcome by either targeting the recombinant protein in the bacterial periplasm which is a more oxidant environment or by using specific host with a suitable oxidant cytoplasm. Indeed, Origami strain of *E. coli* have mutations in glutathione reductase (gor) and thioredoxin reductase (trxB), facilitating proper disulfide bond formation and better refolding and solubility of the recombinant protein [8].

The objective of this work was to express the gene encoding a pectin lyase from *P. occitanis* in the prokaryotic expression system *Escherichia coli*.

2. Materials and methods

2.1. Bacterial strains and plasmids

Escherichia coli Top10 strain and pGEMT Easy plasmid were used for DNA cloning. *E. coli* strains used for the expression: BL21 (DE3), Rosetta (BL21 derivative enriched in tRNAs genes for 7 rare codons (AGA, AGG, AUA, CUA, GGA, CCC, and CGG) on a chloramphenicol-resistant plasmid) and Origami (double mutated in glutathione and thioredoxin reductase genes). pET-21a (+) plasmid containing "T7*lac*" promoter was used for the expression. The bacterial strains were grown in Luria-Bertani medium LB (5 g/l yeast extract, 10 g/l tryptone, 5 g/l sodium chloride) supplemented with ampicillin (100 μg/ml) for transformants selection. 5 mM of DTT as exogenous reducing reagent was added to Origami strain culture to achieve a suitable growth rate.

2.2. Construction of recombinant pectin lyase

The pectin lyase cDNA used in this study was already cloned from *Penicillium occitanis* [9]. The mature pectin lyase coding sequence (without signal peptide) was amplified by PCR using oligonucleotides sequences primers: forward (5′ggATCCATCAgTCACTggCgCA3′) and reverse (5′AAgCTTTTAgTggTggTggTggTggTgAATAgTACCTTgTCCAgC3′). These two primers used were designed to introduce a *BamHI* site at the N-terminal and 6xHis codons followed by an ochre codon and a *HindIII* site at the C-terminal. PCR was performed with one cycle at 94 °C for 5 min, followed by 35 cycles of (denaturation for 30 s at 94 °C, annealing for 30 s at 55 °C, extension for 1 min 30 s at 72 °C) and a final extension step for 7 min at 72 °C. The fragment of 1200 pb was sub-cloned into pGEMT Easy vector and designed as pGEMT-pnl. Once verified by sequencing, this recombinant plasmid was digested with *BamHI* and *HindIII*, the insert was then introduced into the expression vector pET-21a (+) prealably linearized with the same restriction enzymes. The recombinant plasmid pET-pnl was confirmed by PCR, restriction enzyme analysis as well as by DNA sequencing, and finally transferred into the various *E. coli* expression strains.

2.3. Pnl expression in bacterial hosts

E. coli host strains transformed with recombinant plasmid pET21-pnl were used to inoculate 10 ml of LB medium supplemented with 100 μg/ml ampicillin and grown overnight at 37 °C with shaking at 220 rpm. 2 ml of such overnight pre-culture served to inoculate 200 ml of LB medium in 1 l erlenmeyer flasks. When the absorbance at 600 nm reached around 0.8, the expression of recombinant pnl was induced by adding various concentrations (0.1–0.5 mM) of IPTG (isopropyl-B-D-thiogalactoside) and shaking was continued at 150 rpm for 16 h at different temperatures (22, 30 and 37 °C). In order to analyze the expression levels and profiles, 100 μl of total proteins from post induction culture were analyzed by SDS-PAGE. A control culture of *E. coli* host harboring pET-21a (+) expression vector (devoid of pectin lyase sequence) was also analyzed in parallel.

2.4. Extraction and purification

2.4.1. Extraction of intracellular protein

Bacterial cells were spin down at 5000 rpm for 20 min and washed with 50 mM Tris-HCl pH8. 1 g of wet cells was washed twice in 50 mM Tris-HCl pH8 and re-suspended in 25 ml of lysis buffer (50 mM Tris-HCl pH8, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, and 0.1% Sarcosyl) and sonicated on ice at 40% amplitude for 30 min (10 cycles of 10 s on followed by 10 s off intervals). The lysate was centrifuged in the first time at 3000 rpm to remove unpopped bacteria and in the second time at 8000 rpm at 4 °C for 20 min to separate inclusion bodies from soluble fraction. The supernatant presenting the soluble fraction was collected to analyze the pnl activity, and the pellet presenting the insoluble inclusion bodies fraction was collected to perform the pnl enzyme refolding.

2.5. Purification of recombinant protein

The supernatant containing the soluble fraction of his-tagged-pnl was loaded onto 1 ml His-Trap affinity column chromatography (His-Trap™ FF crude, GE Healthcare Bio-sciences), equilibrated with wash buffer (20 mM phosphate buffer pH7.4, 500 mM NaCl and 10 mM imidazole). Adsorbed his-tagged-pnl recombinant protein was eluted by linear gradient of imidazole in the elution buffer (20 mM phosphate buffer pH7.4, 500 mM NaCl and 10–500 mM imidazole). The fractions containing pnl-his_6 fusion protein were collected to be further analyzed.

2.6. Protein refolding from inclusion bodies

The method of Chung for refolding protein from inclusion bodies was applied with slight modifications [10], as follows: Inclusion bodies, easily isolated by centrifugation from intracellular fraction, were washed two times with 50 mM Tris-HCl pH8, to remove contaminant soluble proteins, and then solubilized in denaturing buffer (20 mM Tris-HCl containing 3 M urea, 5 mM DTT, pH12), subsequently incubated for 2 h at room temperature with gentle stirring. After centrifugation at 15.000 rpm for 15 min, the supernatant representing the solubilized fraction was collected, diluted in renaturing buffer (50 mM Tris-HCl containing 0.2 mM oxidized glutathione and 2 mM reduced glutathione) and then, dialyzed over night against 20 mM Tris-HCl pH8 containing 1 M urea at 4 °C; and finally, dialyzed twice for four hours against 20 mM Tris-HCl pH8 at 4 °C, to remove gradually denaturant agent.

2.7. Pectin lyase activity assay

Pectin lyase activity was assayed by the method of Ramos [11] based on measuring the increase in optical density at 550 nm due to