Catalysis Communications 107 (2018) 39–42

Contents lists available at ScienceDirect

Catalysis Communications

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

Short communication

Preparation of low-molecular-weight citrus pectin by recombinant *Bacillus subtilis* pectate lyase and promotion of growth of *Bifidobacterium longum*

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ARTICLE INFO

Keywords:
Pectate lyase
Pectin
Hydrolysis
Low-molecular-weight citrus pectin (LCP)
Bifidobacterium longum

ABSTRACT

pel gene (GenBank: KM244046.1), which encodes *Bacillus subtilis* pectate lyase (PelB), was high-level expressed in *Escherichia coli* BL21 (DE3) and the recombinant enzyme was named rePelB. After induction by IPTG at 15 °C for 24 h, specific activity of rePelB in culture supernatant of pCpel-6 reached 197.4 ± 5.20 U/mg. The optima of rePelB were 60 °C and pH 5.0, respectively. Hydrolysis of citrus pectin by rePelB in 50 L grass reactor was optimized by implementing RSM. HPLC analysis revealed molecular mass of citrus pectin and obtained LCP was 290 and 5.8 kDa, respectively. LCP can significantly promote growth of *Bifidobacterium longum*.

1. Introduction

Degradation of pectin requires synergistic actions of several enzymes, including *endo*-pectate lyase (EC. 4.2.2.2), *exo*-pectate lyase (EC. 4.2.2.9), pectin lyase (EC. 4.2.2.10), exo-polygalacturonase (EC. 3.2.1.67), and endo-polygalacturonase (EC. 3.2.2.15) [1,2]. Pectate lyase is responsible for the eliminative cleavage of α-1, 4-glycosidic bond of pectate, producing oligosaccharides with 4-deoxy-α-D-mann-4-enuronosyl groups at non-reducing ends *via* β-elimination mechanism [3,4]. Endo-pectate lyase hydrolyzes pectin and generates multiple products of various sizes; such products include both small and large oligomers, such as galacturonic acid, oligo-galacturonates, and low-molecular-weight citrus pectin (LCP) [5]. LCP can be obtained by modified pectin with enzymes, high temperature, pH treatment, or a combination of these treatments [6]. Various studies revealed that LCP effectively countered colon carcinoma, breast carcinoma, and gastrointestinal carcinoma by decreasing the expression level of Galectin-3 (Gal-3) in cancer cells. LCP with the correct molecular structure has been extensively investigated as Gal-3 inhibitor [7–9].

In our previous study, the activities of polygalacturonase and pectate lyase from *Bacillus subtilis* JL-13 were 37.6 and 7.8 U/mg, respectively [10]. Activities of these enzymes are low for industrial purposes, and their purification is difficult and tedious. In the present study, the *Bacillus subtilis* pectate lyase gene, *pel* (GenBank: KM244046.1), was cloned and heteologously expressed in *Escherichia coli* fused with the Trigger factor (TF) chaperone. LCP was obtained from the hydrolysates of citrus pectin through ultra-filtration. Growth effect of prepared LCP on *Bifidobacterium longum* was investigated.

2. Materials and methods

2.1. Materials

Bacillus subtilis JL-13 strain, which produces pectate lyase, was isolated from the soil of Hangzhou Botanical Garden in our previous study [10]. The fusion cold shock expression vector, pCold TF, was purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Medium components were obtained from Oxoid. Citrus pectin, *D*-(+)-galacturonic acid, polygalacturonate, and bovine serum albumin (BSA) were acquired from Sigma-Aldrich (Shanghai) Trading Co., Ltd. (Shanghai, China). High-affinity Ni-charged resin was provided by GenScript Biotechnology Co., Ltd. (Nanjing, China). All other chemicals used in this study were of analytical grade.

2.2. Cloning of pel and its expression

pel gene was amplified from genome of *Bacillus subtilis* JL-13 with the following specific primers: 5′- ACAGGTACCATGAAAAAAGTTATG TTAG -3′ for Pel1 and 5′ - CTAAGCTTATTCAATTTACCCGCACCC -3′ for Pel2, containing *Kpn* I and *Hin*d III restriction sites (underlined),

Abbreviations: PelB, *Bacillus subtilis* pectate lyase; *pel*, the gene encoding PelB; rePelB, recombinant PelB expressed in *E. coli* BL 21; PCR, polymerase chain reaction; LCP, low-molecular-weight citrus pectin; DP, degree of polymerization; HPLC, high-performance liquid chromatography; RSM, response surface methodology; K_m, Michaelis-Menten constants; V_{max}, maximal activity

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https://doi.org/10.1016/j.catcom.2018.01.017
Received 14 November 2017; Received in revised form 29 December 2017; Accepted 14 January 2018
Available online 17 January 2018
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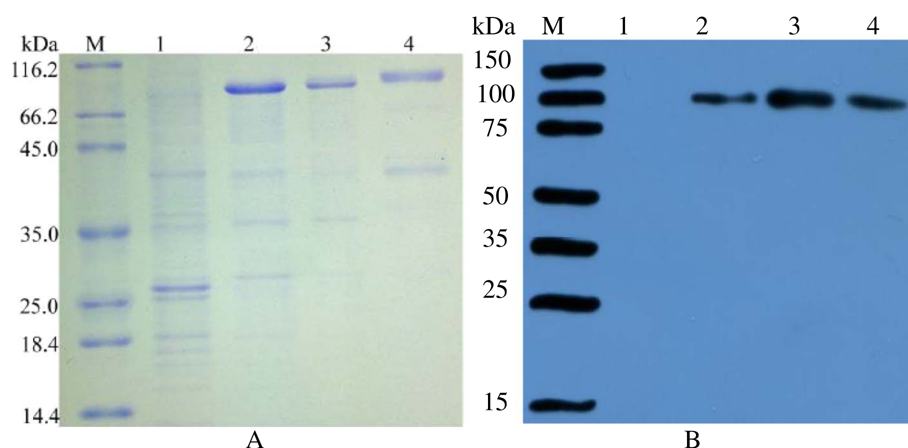

Fig. 1. SDS-PAGE (A) and Western blot (B) analysis of rePelB.
Note: Lane M: standard protein marker; Lane 1: *E. coli* BL21 harboring the pCold TF vector induced by IPTG at 15 °C for 24 h used as control; Lane 2: ultrasonication supernatant of pCpel-6 induced by IPTG at 15 °C; Lane 3: purified rePelB by Ni-chelating affinity chromatography; Lane 4, fermentation supernatant of pCpel-6 induced by IPTG at 15 °C.

respectively. The recombinant pCold-pel plasmid was transformed into *E. coli* BL21 (DE3). The positive transformant, i.e., pCpel-6, with the highest enzyme activity in small-culture analysis was selected for scale-up expression. Scale-up expression of rePelB at 15 °C by pCpel-6 was implemented according to Xu et al. [11].

2.3. Purification and activity assay

The culture supernatant of pCpel-6 was concentrated through vacuum freeze-drying. The concentrated sample was purified by using high-affinity Ni^{2+}-charged resin. The pectate lyase activity was assayed by measuring changes in absorbance at 235 nm with polygalacturonate as substrate [12,13]. One unit of activity was defined as the amount of the pectate lyase that released 1.0 μmol of unsaturated product from polygalacturonate per 1 min under the optimal conditions. K_m and V_{max} were determined from the initial velocities by using polygalacturonate as substrate (1–10 mg/mL). Each assay was performed in triplicates, and mean values were obtained.

2.4. SDS-PAGE and western blot analysis

All of the samples (purified rePelB, fermentation supernatant, control, ultrasonic supernatant of pCpel-6 cell induced by IPTG) were subjected to Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In the Laemmli system, stacking and separating gels consisted of 5% and 12% polyacrylamide, respectively [14]. The mouse monoclonal His tag antibody and horseradish peroxidase-labeled goat anti-mouse IgG antibody were used in the Western blot assay.

2.5. Optimum temperature and thermostability of rePelB and PelB

The effect of temperature on pectate lyase (rePelB and PelB) activity was determined from 30 °C to 90 °C. The highest activity was 100%. For the thermal stability assay, the enzyme was treated with heat from 30 °C to 90 °C for 5 min and then placed in an ice-water bath for 5 min. Residual activity was determined under optimal conditions. The activity of untreated enzyme under optimal conditions was 100%.

2.6. Optimum pH and pH stability of rePelB and PelB

The effect of pH on pectate lyase (rePelB and PelB) activity was assayed from pH 3.0 to pH 9.0 at 60 °C. The highest activity was 100%. For the pH stability, rePelB was incubated in buffer solutions (pH 3.0–9.0) at 25 °C for 1 h. Residual activity was determined under optimal conditions. The activity of untreated enzyme under optimal conditions was 100%.

2.7. Effect of metal ions on activity of rePelB and PelB

Various metal ions (Zn^{2+}, Cu^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, Al^{3+}, Fe^{3+}, Pb^{2+}, Ag^{+} and K^{+}) solutions with the final concentration of 0.5 mM and 1 mM were added to the rePelB and PelB, respectively. The residual activity was determined under optimal conditions. The activity that observed in the absence of metal ion was taken as 100%.

2.8. Optimal hydrolysis of citrus pectin through response surface methodology

To obtain the LCP from hydrolysates, hydrolysis of citrus pectin by rePelB in a 50 L grass reactor system was optimized by implementing response surface methodology (RSM) [15]. Optimization was targeted at the enzyme/pectin ratio (rePelB:citrus pectin = U:mg), reaction time, stirring speed, and their interactions. On Basis of central composite design (CCD) (three variables and 5 levels), 20 experiments were performed to fit the polynomial model: $Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{11}X_1^2 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{22}X_2^2 + \beta_{23}X_2X_3 + \beta_{33}X_3^2$, Optimum values of the selected variables were determined by solving the regression equation and analyzing the response surface plots. Concentration of substrate and rePelB was 1.5% (*w/v*) and 0.1 mg/mL. Hydrolysis was conducted at 45 °C and pH 5.0.

2.9. Determination of molecular weights of hydrolytic products

The 5.0 L hydrolysate, which was from citrus pectin hydrolyzed by rePelB and PelB under the RSM optimal conditions, respectively, was separated by using an ultra-filtration module with a polyethersulfone membrane (Millipore Biomax, 10 and 3 kDa cut-off) and trans-membrane pressure of 0.8 kg/cm^{-2}. The permeating liquid (10 kDa cut-off) was concentrated by the 3 kDa membrane. Citrus pectin and the obtained sample (by rePelB and PelB, respectively) were analyzed by high-performance liquid chromatography (HPLC) with a PW-M gel column maintained at 30 °C, pure water as the mobile phase (0.6 mL/min) and an injection volume of 20 μL. Sugar peaks were screened with a Shimadzu RID-10A refractive index detector. Molecular weights of citrus pectin and obtained samples were calculated based on the standard curves of beta glucans [16].

2.10. Effect of LCP on growth of Bifidobacterium longum in vitro

Different concentration of LCP from citrus pectin by rePelB was added the glucose-free de Man, Rogosa and Sharpe (MRS) broth and their growth stimulation effect on *Bifidobacterium longum* was evaluated. The *Bifidobacterium longum* was cultured in the medium at 37 °C for 24 h in an anaerobic incubator. Microbial OD 600 nm was assayed [17].

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