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Continuous production of phospholipase D using immobilized recombinant *Streptomyces lividans*

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Abstract

Using biomass support particles (BSPs) as a cell immobilized matrix, immobilized recombinant *Streptomyces lividans* continuously produced phospholipase D (PLD) in a yield of about 1.5×10^4 U/L in each of eight batches. In contrast to the original strain *Streptoverticillium cinnamoneum*, this heterologous expression system with an immobilization method is capable of producing secretory PLD with an 8-fold greater efficiency. The presence of both glucose and tryptone in the initial culture medium also promoted secretory production, and PLD activity around 3.0×10^4 U/L were achieved. In addition, the promoter region of PLD ORF was deduced, and three types of plasmid having different lengths of promoter sequence were constructed. The deduced sequence had same effect on either of PLD production or mycelium immobilization, and the transformants harboring each of three plasmids showed the similar cultivation profiles (3.0×10^4 U/L). A combination of the immobilization method with BSPs and *S. lividans* transformant harboring the deduced plasmid has the potential for producing secretory PLD in the culture supernatant continuously.

Keywords: Phospholipase D; Biomass supports particles (BSPs); Immobilization; Promoter region; *Streptomyces lividans*

1. Introduction

Biomass support particles (BSPs) have been used for the immobilization of yeast [1], fungi [2], and mammalian cells [3] (see review Ref. [4]). BSPs, a porous matrix, can be prepared from a variety of materials, including polyurethane, silicon, stainless steel, polyester, and polyvinyl. Suspension cultures of organism with BSP can promoted the adhesion of cells to the porous matrix surface, and subsequently the cells become immobilized during cultivation. This method for cell immobilization has great potential for enhancing the production of proteins or chemicals in culture supernatants [1–5]. Compared with other immobilization methods, this immobilization method has several advantages as follows: (i) no requirement for the addition of chemicals; (ii) no need for the pre-production of cells; (iii) the aseptic handling of particles is unnecessary; (iv) large mass transfer rate of substrate and production within particles; (v) the reuse of particles; (vi) durability against mechanical shear; (vii) ease of bioreactor scale-up; (viii) low costs in comparison to many active techniques.

Phospholipase D (PLD) (EC 3.1.4.4) is a known as a phospholipid metabolizing enzyme [6]. A major substrate of PLD, phosphatidylcholine (PC), is hydrolyzed to phosphatidic acid (PA) and choline. PLD also catalyzes the interconversion of polar head groups by virtue of its transphosphatidylation activity; for example, PC can be converted into phosphatidylethanolamine (PE) in the presence of monoethanolamine. Therefore, PLD can be used to synthesize various phospholipids containing different polar head groups, showing versatile physiological functions [7,8]. Therefore, the construction of a PLD enzyme production system for use in phospholipid modification in plant scale would be highly desirable from an industrial standpoint.

We previously reported that an actinomycete, *Streptoverticillium cinnamoneum*, secretes the highest activity of PLD into a culture medium among the actinomycetes examined [9], and the possibility of immobilizing *Stv. cinnamoneum* on polyurethane foam has been reported [5]. We also determined the gene that encodes PLD from same strain's chromosomal DNA [10], and

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established an over-expression system for the secretion of PLD of *Stv. cinnamoneum* using *Streptomyces lividans* as a host [11]. Then, it was also investigated that the putative promoter region sequence, consisting from 1.4 kbp, promotes the secretory protein expression in culture medium. In this paper, for the continuous production of recombinant PLD, the condition required for the immobilization of recombinant *S. lividans* on polyurethane foam BSPs and its repeated cultivation were investigated. In addition, the effect of the length of the promoter region of the plasmid, pUC702-proPLD1 used in study, on the production of PLD and immobilization of mycelium were also investigated.

2. Materials and methods

2.1. Strains, plasmid, and chemicals

Stv. cinnamoneum (NBRC 12852) secreting the highest activity of PLD in medium among the strains examined [9] was used as a control strain. *S. lividans* 1326, which secretes no PLD activity into the culture medium, was used as a host cell for the transformation. A shuttle vector between *Escherichia coli* and *S. lividans*, pUC702-proPLD1–3 (Fig. 1B) [11], which are derived from the pIJ702 vector for *S. lividans* [12], were used for production of secretory PLD. All chemicals used in this study were of analytical grade.

2.2. PLD assay

PLD activity was typically measured by a spectrophotometric assay using PC (Nacalai Tesque, Kyoto, Japan) as the substrate [10]. The reaction mixture (total volume, 100 μL) consisted of 0.5% (w/v) egg-yolk PC, 0.1% (v/v) Triton X-100, 40 mM Tris–HCl (pH 7.4), and 40 μL of an enzyme sample. After incubation at 37 °C for 20 min, the reaction was terminated by the addition of 50 μL of a solution containing 50 mM EDTA and 100 mM Tris–HCl (pH 7.4), and the PLD enzyme was immediately denatured by heating at 95 °C for 5 min. After cooling the reaction mixture to room temperature for 5 min, 500 μL of 20 mM potassium phosphate buffer (pH 7.4) containing 21 mM phenol, 0.60 mM 4-aminoantipyrine, 3000 U/L of *Arthrobacter* choline oxidase (Toyobo, Osaka, Japan), and 720 U/L of horseradish peroxidase (Toyobo, Osaka, Japan) was added. After incubation at 37 °C for 5 min, the absorbance of the reaction mixture was measured at 505 nm. A calibration curve was obtained by using a standard solution of choline chloride instead of the enzyme solution. One unit (U) of hydrolytic activity of PLD was defined as the amount of enzyme that produced 1 μmol choline min^{-1}.

2.3. Immobilized cultivation of recombinant S. lividans

One spore of the pUC702-proPLD1 transformed *S. lividans* (pUC702-proPLD1/*S. lividans*) was inoculated in a test tube containing 5 mL of TSB medium [17 g/L pancreatic digest of casein, 3 g/L papaic digest of soybean meal, 2.5 g/L glucose, 5.0 g/L sodium chloride, 2.5 g/L dipotassium phosphate (BD Diagnostic Systems, Sparks, MD, USA)] supplemented with 5 μg/mL thiostrepton (Sigma, St. Louis, MO), followed by incubation at 28 °C for 3 days with

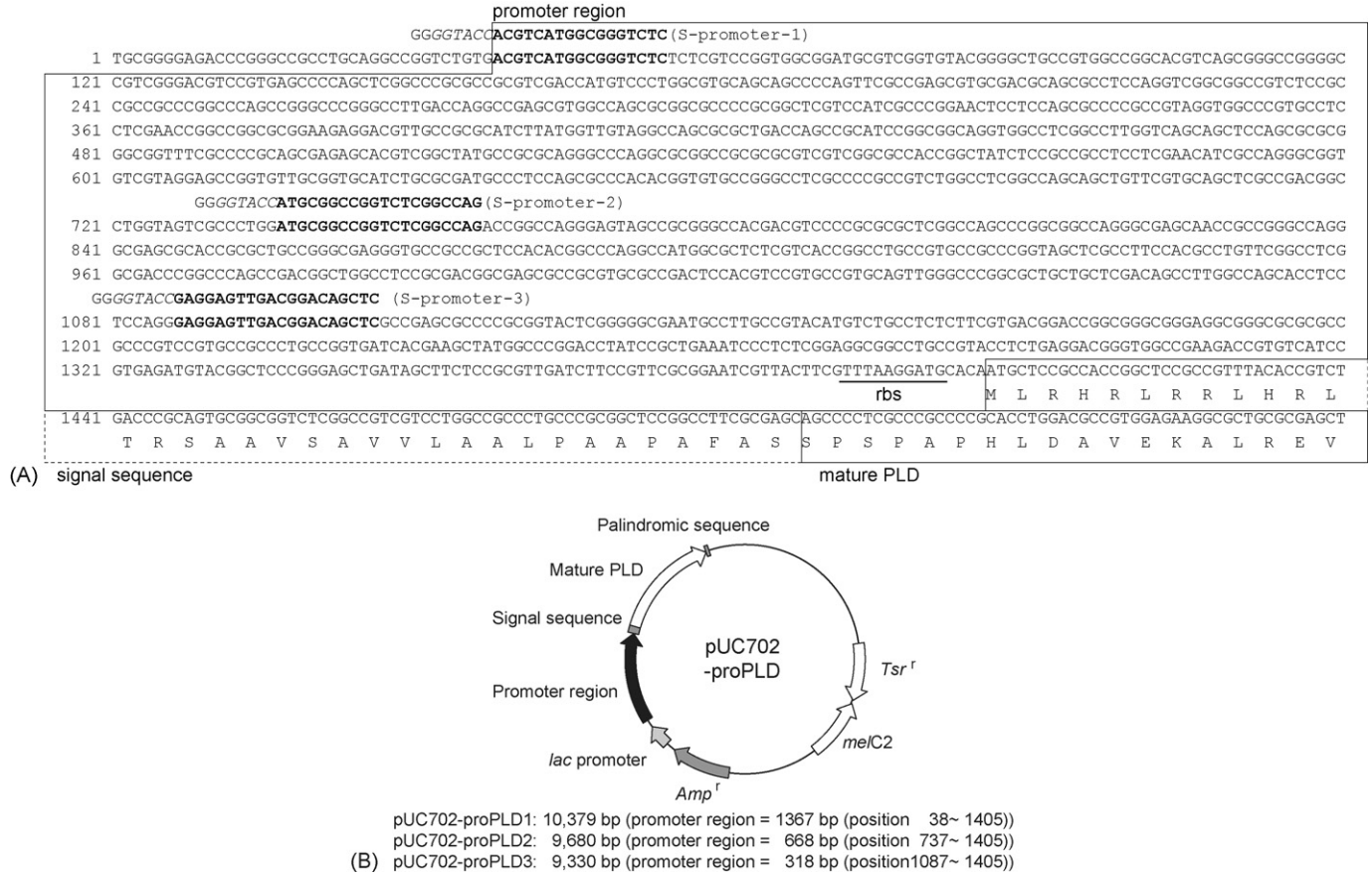

Fig. 1. Construction of promoter region deleted plasmid. (A) The promoter region sequence of *Stv. cinnamoneum* PLD. For amplifying of three types of promoter regions introduced upstream of the PLD ORF, three primers (S-promoter1–3) were used in each PCR, respectively. In each primer, the bold sequence and the italic sequence represent the annealed position with the template sequence and the restriction enzyme *Kpn*I site, respectively. The underlined sequence indicates a ribosome binding site (rbs). (B) Illustration of the three constructed plasmids. These plasmids have the same sequence, except for the promoter region length. The length of each of the plasmid was indicated 1367, 668, and 318 bp, respectively.