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Development of Recombinant Phosphatidylcholine-specific Phospholipase C from *Bacillus thuringiensis*

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Abstract. Microbial enzymes have a leading position among industrial enzymes due to their enormous variety and valuable technological properties. Phospholipases that catalyze the cleavage of phospholipids are the most widely used lipolytic enzymes. In this article, we present development and comparative study of 12 recombinant variants of Phosphatidylcholine-specific phospholipase C which we previously selected from the natural collection of *Bacillus thuringiensis* as a promising to create industrial producers of enzymes used in the process of plant oil degumming and in other biotechnological areas.

INTRODUCTION

The phospholipase C (PLC) is phosphodiesterase that cleaves the phospholipids releasing diacyl glycerol and the phosphorylated head-group [1]. PLCs were found in wide range of organisms and are essential for its growth, development, and virulence [2]. PLCs in living systems are divided into the inositol-specific PLC (PI-PLC) cleaves only phosphatidylinositol-4,5-bisphosphate and the phosphatidylcholine-preferring phospholipase C (PC-PLC) prefers phosphatidylcholine, but able to hydrolyze other phospholipids as well [3]. Bacterial PC-PLCs are used in industry for plant oils degumming and to increase the yield of cheese, in addition, the use of the enzyme in the biodiesel production is considered [4-7].

Phospholipase C from *Bacillus* is the optimal enzyme for industrial oil degumming, since it does not exhibit hemolytic activity, has the most suitable spectrum of substrate specificity, thermal stability, high specific activity and safety [8].

The enzyme is an extracellular, monomeric tri-zinc protein. It is synthesized as a 283-amino acid (a.a.) long pre-proenzyme from which the 24-a.a. long presequence and the 14-a.a. long prosequence are cleaved to form the 245 a.a. mature enzyme [9]. Although PC-PLC from *B. cereus* and *B. anthracis* are successfully used in industry, the creation of improved enzymes is the subject of interest [8]. Various strategies are used for this purpose, including screening of natural variants of the enzyme, engineering of the molecule, improvement of the expression system and cultivation process optimization.

In this work, we developed 12 variants of recombinant PC-PLC selected from natural collection of *B. thuringiensis* due to its genetic similarity to *B. cereus*, one of the optimal sources of PLCs. We used *Bacillus subtilis*, a genetically closed bacterium, as an expressing producer of PC-PLC to be transformed with recombinant constructs. *B. subtilis* is a well-studied Gram-positive bacterium which has many outstanding features. It is non-pathogenic, has superior protein secretory capability, and has excellent biochemical and physiological characteristics. Downstream purification of secreted heterologous proteins has to be relatively standard because the proteins are to be harvested from the culture medium.

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MATERIALS AND METHODS

Cloning of the *plc* Genes

The *B. thuringiensis* gDNA was isolated with a commercially available DNA isolation kit («Dia-m», Russia). The PLC_EagI_F (3'-CGGCCGGTCATGAAAACGATGGGG-5') with EagI restriction site at the 5'-end, Bac_PLC_1505R (3'-CCTTTTAGCAATTTACCTTTCACGT-5') and PLC_GNR_6H (3'-GGTTTGATACGTACGGAAATCGTGGAGGATCTCATCATCACCATCACCACTAAGCTAGCTT-5') contained His-tag at the 3'-end primers were designed.

In this study, we used *plc* DNA sequences encoding the 12 mature PC-PLC enzymes. Here we amplified DNA fragments by standard PCR protocol and primers mentioned above in a way to have specific restriction sites at their ends. First, 12 variants of *plc* gene were cloned into intermediate carrier pJet1.2/blunt vector («CloneJET PCR Cloning Kit», «Thermo Scientific», USA). Then, plasmid DNA was digested with EagI and XbaI and subcloned in a directed way into a binary vector pHTE. Structure of all resulting plasmids was confirmed by Sanger sequencing. All 12 plasmids were purified and transformed into *B. subtilis* WB800N by electroporation to express correspondent PC-PLC variants from *B. thuringiensis*.

Cultivation of *B. subtilis* WB800N and Expression of the PC-PLC

The *B. subtilis* WB800N strains transformed with recombinant pDNA were prepared from a 5-ml overnight culture and grown on 2xYT liquid medium at 37 °C, with a rotation of 170 rpm. Expression of the PC-PLC was induced at an OD_{600}=0.6 by adding Isopropyl β-d-1-thiogalactopyranoside (IPTG) to a final concentration 1 mM. After 12 h, cells were separated from the media by centrifugation (5 min, 4 °C, 6000×g), the presence of the target protein (28 kDa) in the supernatant was determined by SDS-PAGE electrophoresis.

Enzyme Purification

His-tagged PC-PLC was purified by Ni-affinity chromatography under native conditions [10]. Target protein was eluted from metal ions column (Sepharose 6 fast flow (GE Healthcare, USA) by 100–500 mM imidazole buffer (30 mM NaH_2PO_4, 500 mM NaCl, 20 mM imidazole, pH 7.4). The protein was detected by immunoblot analysis using anti His-tag antibodies. The fraction containing the target protein was dialyzed against Tris-HCl buffer (10 мМ Tris-HCl, 150 мМ NaCl, pH 8.0).

p-Nitrophenylphosphorylcholine Assay

10 μl of bacterial culture supernatant and 90 μl of a 10-mM solution of p-Nitrophenylphosphorylcholine (p-NPPC) in borax-HCl buffer (10 mM, pH 8.0) containing 0.1 M $ZnCl_2$ and 60% (w/v) sorbitol were mixed in wells of a microtiter plate. The plate was incubated at 37 °C for 15 min. The released p-nitrophenol was quantified by measurement of its absorbance at 405 nm. The control contained 2xYT medium.

Thermostability was determined by heating of the enzymes at 80 °C for 1.5 hours and subsequent p-NPPC-assay as described above.

RESULTS AND DISCUSSION

Recombinant PC-PLCs Production

Nucleotide sequences encoding 12 different variants of PC-PLC propeptide from *B. thuringiensis* were cloned into the binary vector pHTE providing high yield of pDNA in *E. coli* as well as high level of expression of target genes in *B. subtilis*. The plasmid vector contains the *Epr* signal sequence for high yield protein secretion into the culture medium and chloramphenicol-resistant factor (*cat*) for the selection of positive clones of producers.

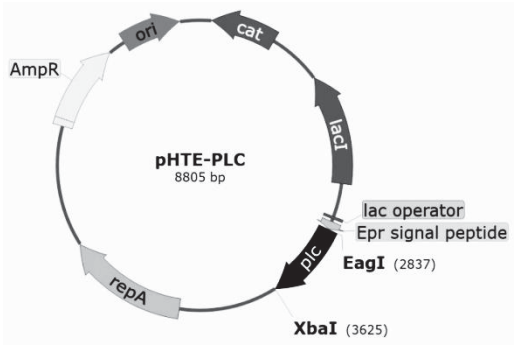

FIGURE 1. Map of the pHTE-PC-PLC plasmid vector for expression of recombinant PC-PLCs.

The *B. subtilis* WB800N strains transformed with pHTE-PC-PLC were grown in 2xYT medium, and then protein expression was induced by adding 1 mM IPTG. Samples were taken 2, 4, 6 hours after induction. Bacterial cells were separated by centrifugation and the presence of the target protein (28 kDa) in the culture medium was determined by SDS-PAGE. The activity of enzymes was determined in p-NPPC assay as described above (Fig. 2). Next, the expression vector for His-tagged PC-PLC production and purification was created (Fig. 3).

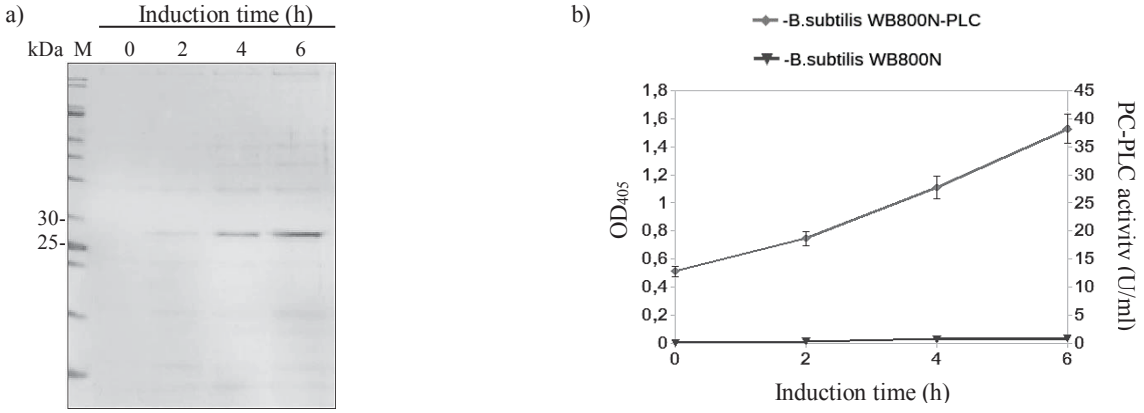

FIGURE 2. Analysis of gene expression and specific activity of pHTE-based recombinant PC-PLC in *B. subtilis* WB800N: a) SDS-PAGE of cell culture medium at 0, 2, 4 and 6 hours after IPTG induction. The 28kDa protein bands correspond to the M.W. of PC-PLC; b) hydrolytic activity of secretory recombinant PC-PLC from the culture medium at 0, 2, 4 and 6 hours after IPTG induction. The data represent the mean ± SD from three independent experiments.

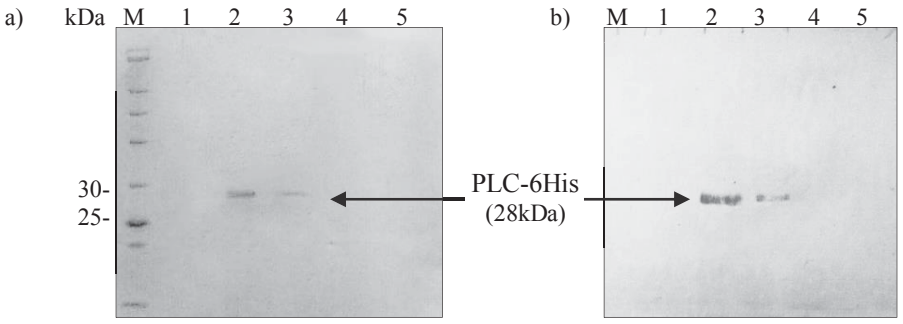

FIGURE 3. SDS-PAGE (a) and Western blot analysis (b) of eluted fractions of affinity purified PLC-6His protein. Lanes 1 to 5 contain fractions eluted with 100, 150, 200, 250 and 500 mM of imidazole.

The study of Thermostability of Recombinant Enzymes

Enzymatic degumming of oils is usually carried out at a temperature of about 80 °C for a few minutes to 1–2 hours. Therefore, we determined the activity of enzymes in the same time interval.

It was shown that all variants of recombinant phospholipases C retained their enzymatic activity. As for PC-PLC-1, -4, -6, -9, -10, -11, -12 variants, we noticed a slight decrease of enzyme activity. At the same time, activity reduction of PC-PLC-2 and -8 variants obtained from *B. thuringiensis* isolated from the geyser field on the Kamchatka Peninsula was not observed (Fig. 4).

The recombinant variant of truncated 238 a.a. enzyme (PC-PLC-3) also exhibited phospholipase activity. It seems that the deletion of the C-terminal amino acid residues of PC-PLC does not affect the activity of the enzyme, which is consistent with published data [11].

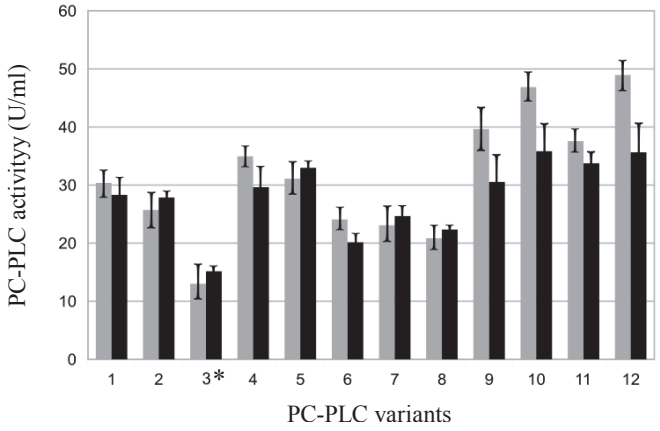

FIGURE 4. Evaluation of activity of recombinant PC-PLC variants under standard conditions (gray bars) and after heating at 80 °C for 1.5 hours (black bars): 3*, truncated protein. The data represent the mean ± SD from three independent experiments.

CONCLUSIONS

The *B. subtilis* strain producers of 12 *B. thuringiensis* phosphatidylcholine-specific phospholipase C recombinant variants were developed. All obtained enzymes showed phospholipase activity and withstand temperature treatment at 80 °C for 1.5 hours without significant decrease of activity. Thus, these enzymes demonstrated some essential qualities for industrial plant oils degumming and will be further studied in more detail as candidates for use in various fields of biotechnology.

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