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Comparison of the efficiency of concentrated soluble recombinant phospholipase D and natural phospholipase D enzymes

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Abstract: Phospholipase D (PLD) is a major virulence determinant of *Corynebacterium pseudotuberculosis*. Various studies have focused on the expression of recombinant PLD (rPLD) enzyme in different *Escherichia coli* strains, but generally a high yield of insoluble protein was reported. The aims of this study were to express soluble rPLD by different methods in *E. coli*. The rPLD and natural PLD (dPLD) enzymes were concentrated using an ultramembrane cassette system after the efficiencies of these concentrated enzymes were compared. The rPLD enzyme was expressed in One Shot®BL21(DE3) *E. coli* when induced by IPTG in TY medium. Soluble dPLD and rPLD enzyme hemolytic activities were determined using the reverse CAMP test. The nucleotide sequence of the rPLD gene was 99.7% similar to the PLD gene of *C. pseudotuberculosis* in the NCBI GenBank Database; these differences in nucleotides resulted in a difference in two amino acids. The rPLD protein concentration and the titer of hemolytic activity were 23.1 mg/mL and 1/256, respectively. Similarities in the enzyme characteristics were detected between rPLD and dPLD enzymes. These findings indicate that a protocol would be useful for the enhanced production of soluble rPLD in *E. coli* and a membrane cassette system for concentration the recombinant protein.

Key words: Enzyme, *Escherichia coli*, genome analysis, microbial protein synthesis, sheep

1. Introduction

Caseous lymphadenitis (CLA), caused by *Corynebacterium pseudotuberculosis*, is a chronic disease of sheep and goats. CLA is economically important in countries such as the United States, Canada, and Australia because it results in a loss of meat, milk, and wool production in livestock (1–4). *C. pseudotuberculosis* is a facultative, intracellular, gram-positive bacterium. Although CLA is generally characterized by the formation of suppurative abscesses in superficial and internal lymph nodes, in the visceral form of the disease, these abscesses can also be found in internal organs, such as the lungs, kidneys, liver, and spleen (5). The morbidity rate of this infection is higher than the mortality rate. Phospholipase D (PLD), a major virulence determinant of *C. pseudotuberculosis*, is known to play a critical role in infection by increasing vascular permeability, thereby causing the deterioration of the sphingomyelin structure of membranes and aiding the spread of *C. pseudotuberculosis* from internal organs to regional lymph nodes (6–8).

Recombinant proteins and peptides are generally expressed in *Escherichia coli* but this type of system can cause intracellular degradation of structural and/or functional properties of proteins (9–12). Therefore, novel strategies have been applied to enhance protein expression, especially for soluble proteins. The Structural Proteomics In Europe (SPINE) consortium comprises eight European laboratories (in Berlin, Marseille, Orsay, Oxford, Stockholm, Strasbourg, Utrecht, and Weizmann) that compare the protocols developed for expressing recombinant proteins in *E. coli* (13). The protocol established by the consortium has standards for parameters, including the *E. coli* strain, incubation temperature, optical density at induction, culture-vessel size and design, agitation level, type of media, lysis method, induction time, final concentration of isopropyl β-D-1-thiogalactopyranoside (IPTG), and incubation temperature during stimulation (13,14).

The aim of this study was to produce soluble recombinant PLD enzyme in BL21(DE3) *E. coli* using these recombinant protein protocol parameters, including an incubation temperature of 30 °C and a final concentration of 0.1 mM IPTG in TY medium. The efficiencies of recombinant PLD (rPLD) and natural PLD (dPLD) production were compared in a reverse CAMP test, and these enzymes were concentrated using a membrane cassette system.

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2. Materials and methods

2.1. Biochemical identification and culture

Colonies that were identified as *C. pseudotuberculosis* according to cultural and morphological examination were subjected to the biochemical tests described by Winn et al. (15).

2.2. Verification of PLD gene by PCR

C. pseudotuberculosis DNA extraction was performed using a Wizard Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instructions. The PCRs were performed with PLD-F and PLD-R1 primers according to Pacheco et al. (16) (Table). The PCR product was electrophoresed in a 1% (W/V) agarose gel in 1X Tris-acetic acid-EDTA buffer (81 mM Tris base, 18.87 mM acetic acid, 1 mM EDTA) and a 100-bp DNA ladder was used (Sigma-Aldrich, Germany).

2.3. Amplification of *PLD* gene for cloning

The CLON-F1 and CLON–R1 primers are specific to the *PLD* gene of *C. pseudotuberculosis* isolated from infected sheep based on the available genome sequence in the NCBI GenBank Database (accession number L16587.1) (Table). Each PCR reaction included 1X PCR buffer (with KCl), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.4 mM of each primer, 0.04 U of Taq DNA polymerase, and 10 ng/µL template DNA; ultrapure water was added to obtain a final volume of 50 µL. Thermal cycling was performed according to Pacheco et al. (16). The size of the PCR products was verified by comparison with a 100-bp DNA ladder (Sigma, Germany) and a 1-kb DNA ladder (Fermentas, USA) in agarose gel. PCR cycling was carried out according to Pacheco et al. (16). The PCR product was then purified using a GenElute Gel Extraction Kit (Sigma-Aldrich, Germany), and the pure PCR product was cloned using a Champion pET SUMO Protein Expression System (Invitrogen, USA).

2.4. Verification of ligated pET SUMO vector

Plasmids from randomly selected recombinant colonies on agar were isolated using a GenElute Plasmid Miniprep Kit (Sigma-Aldrich, Germany) according to the manufacturer's protocol. The recombinant plasmid was verified by PCR, restriction enzyme digestion (Thermo Scientific, USA), and sequence analysis using PLD CPF, PLD CPR, SUMO Forward, and T7 Reverse primers (Table). The sequencing was performed in another laboratory (BM Metabion, Turkey). The results were analyzed using the LALIGN EMBL-EBI program. The amino acid sequences were compared using the Clustal 2.1 Multiple Sequence Alignment program.

2.5. Expression of recombinant PLD in One Shot®BL21(DE3) *Escherichia coli*

The confirmed recombinant colony was incubated in LB medium with 50 µg/mL kanamycin at 37 °C for 16 h and the first culture was prepared. The first culture (1/10) was incubated in TY medium (17) with 50 µg/mL kanamycin at 37 °C and 200 rpm until optical density (OD) 550 = 0.6–0.8. Then medium was added so that the final concentration of IPTG (AppliChem, Germany) reached 0.1 mM, and it was incubated in a water bath for 4 h at 30 °C and 200 rpm (11,17). The control culture was treated by the same protocol without IPTG.

2.6. Determination of protein solubility

To determine whether the protein was soluble or insoluble, protein profiles were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the Laemmli protocol (18). Approximately 50 µg of protein was loaded into each lane, and the resolving gels were run for 2.5 h at 150 V. The gels were then dyed with Coomassie Brilliant Blue R-250 (AppliChem, Germany) using the protocol of Sambrook and Russell (19), and the proteins were verified with unstained protein marker (Thermo Scientific, USA).

2.7. Western blotting

The protein samples were electrophoretically separated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad, USA). Western blotting was performed using

Table. Specification of PCR primers.

Primer	Primer sequence (5' to 3')	PCR product
PLD-F	ATAAGCGTAAGCAGGGAGCA (16)	203 bp
PLD-R1	ATCAGCGGTGATTGTCTTCC (16)	203 bp
CLON-F1	ATGAGGGAGAAAGTTGTTTTATTC	924 bp
CLON-R1	TCACCACGGGTTATCCGCTACG	924 bp
PLD CPF	GTTCTATAAGACAGTCGGCGG	
PLD CPR	GTGATTGTCTTCCAGGCAGGT	
SUMO Forward	AGATTCTTGTACGACGGTATTAG (Invitrogen, USA)	
T7 Reverse	TAGTTATTGCTCAGCGGTGG (Invitrogen, USA)	

positive anti-PLD sera, antisheep IgG (whole molecule) antibody produced in donkey (Sigma-Aldrich, Germany), phosphate-citrate buffer with sodium perborate capsules (Sigma-Aldrich, Germany), and δ-phenylenediamine dihydrochloride (Sigma-Aldrich, Germany).

2.8. Determination of hemolytic activity

The culture supernatants of rPLD and dPLD were concentrated approximately 200-fold using a 0.45-µm (Hydrosart, Sartorius Stedim Slice 200, Germany) and 10-kDa (polyethersulfone, Sartorius Stedim Slice 200, Germany) membrane cassette system, respectively. The amount of protein was measured using a Pierce BCA Protein Assay Kit (Thermo Scientific, USA) according to the manufacturer's protocol.

A reverse CAMP test was used to measure the activities and titers of the concentrated rPLD and dPLD (20,21). For the synergistic hemolytic assay, 100-µL samples of the rPLD and dPLD were diluted 2-fold in sterile physiological saline (NaCl, 9 g in 1 L), and then 100 µL was inoculated into a hole on an agar base with 5% sheep blood agar plate and synergistic hemolytic efficiencies were determined with *Rhodococcus equi*.

3. Results

3.1. Verification and amplification of the *PLD* gene

The sections of the *PLD* gene amplified with PLD-F/PLD-R1 and CLON-F1/CLON-R1 primers appear as 203-bp and 924-bp fragments, respectively (Figures 1 and 2). The expected size of the pET SUMO vector ligated with the *PLD* gene is 6567 bp, as shown in Figure 3, lane 1. When the recombinant colonies were digested with SspI, samples 1 and 4–6 displayed the expected 4722-bp, 1278-bp, and 567-bp restriction products on agarose gel (Figure 4); the samples in lanes 2 and 3 were not the correct recombinant colonies, as indicated by the incorrectly sized restriction products.

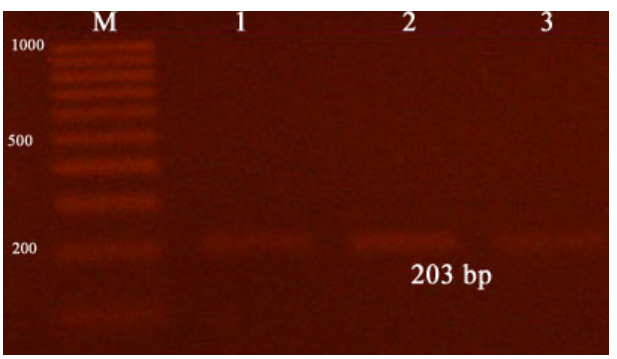

Figure 1. Visualization of the PLD amplification product obtained using PLF-F and PLD-R1 primers. M- Marker; PK- positive control; NK- negative control; lines 1 and 3- the verified *PLD* gene.

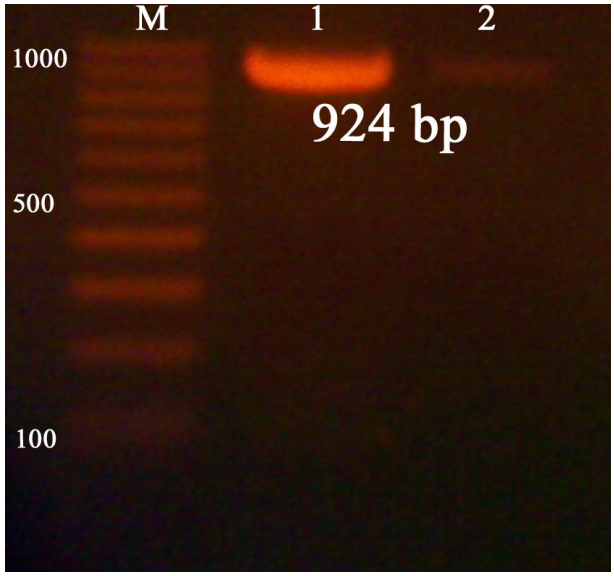

Figure 2. Visualization of the PLD amplification product obtained using CLON-F1 and CLONR1 primers. M- Marker; lanes 1 and 2- the amplified *PLD* gene by PCR.

3.2. Verification by bioinformatic methods for data analysis

The comparison of the *rPLD* gene with the gene in the NCBI GenBank database revealed 99.7% sequence similarity; differences were noted at the 448th and 449th

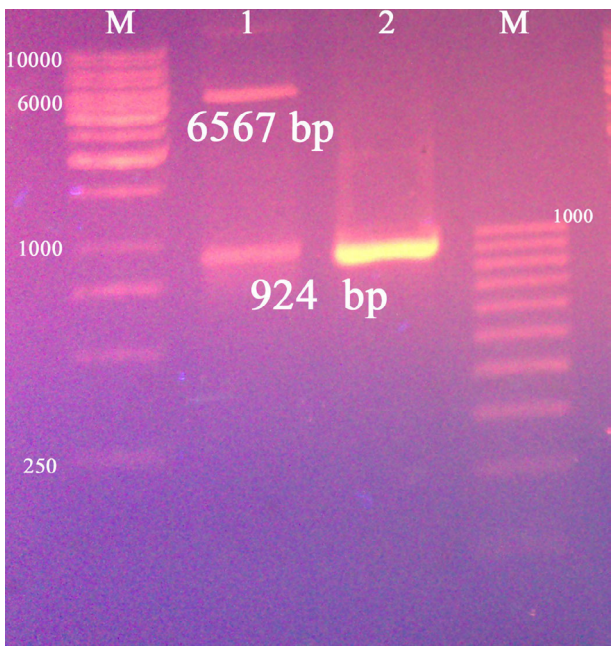

Figure 3. pET SUMO-PLD recombinant product. M- Marker; lane 1- pET SUMO vector ligated to *PLD* gene (6567 bp); lane 2- positive control.

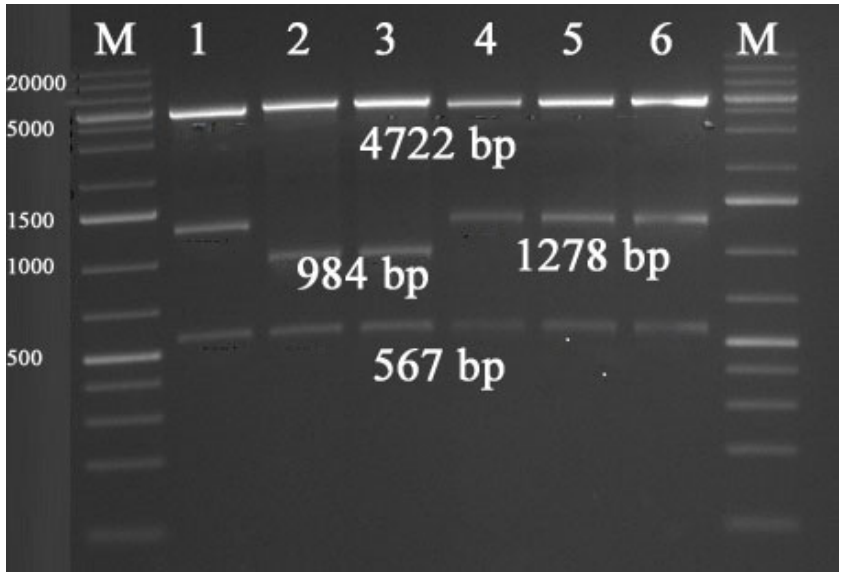

Figure 4. Verification of the pET-SUMO recombinant product by SspI digestion. M- Marker; lanes 1 and 4–6- correct recombinant colonies; lanes 2 and 3- incorrect recombinant colonies.

bases, corresponding to differences at the 181st and 307th amino acids in sequences of the NCBI *dPLD* and *rPLD* gene products.

3.3. Determination of the amount of soluble/insoluble protein

Soluble dPLD (33.8 kDa), soluble rPLD (45 kDa), pET SUMO fusion protein (11 kDa), and other intracellular and extracellular proteins were detected by SDS-PAGE and western blot. The amounts of concentrated and unconcentrated rPLD were compared with SDS-PAGE (Figures 5 and 6).

3.4. Determination of hemolytic activity

The amounts of soluble dPLD and rPLD concentrated using an ultramembrane cassette system were measured as 23.6 mg/mL and 23.1 mg/mL, respectively. The hemolytic activities of concentrated soluble dPLD and rPLD were 1/256 and 1/256 as measured by the reverse CAMP test (Figures 7 and 8).

4. Discussion

Exotoxin samples belonging to different *C. pseudotuberculosis* isolates are reported to be similar

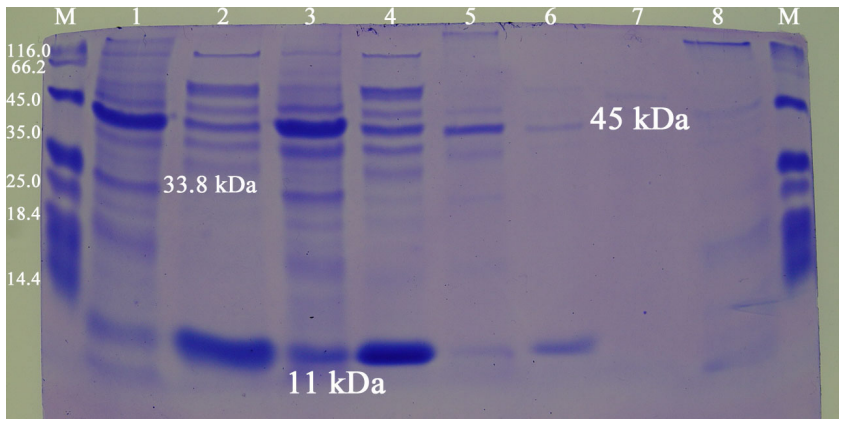

Figure 5. dPLD and rPLD detected by SDS-PAGE.: M- Marker; lane 1- dPLD; lanes 2 and 4- other intracellular protein belonging to *E. coli*; lane 3- other soluble protein belonging to *C. pseudotuberculosis*; lane 5- concentrated rPLD; 6- unconcentrated rPLD; lane 7- supernatant of control; lane 8- protein profile of BHI medium not including 5% sheep blood.

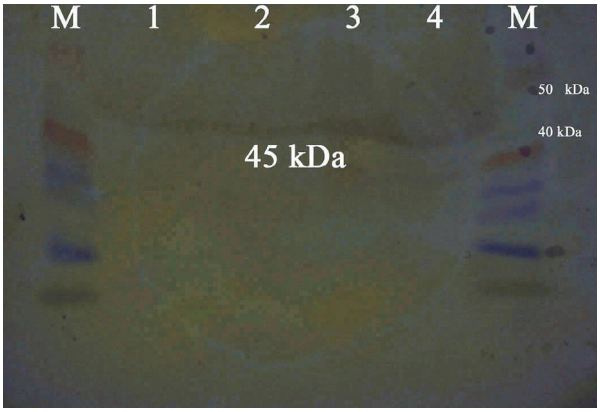

Figure 6. dPLD and rPLD detected by western blot. M- Marker; lanes 1–4- soluble rPLD.

antigenically (22,23). Differences have been noted between strains with respect to the amount of toxin and the size of the lesions (24). Various studies have characterized and examined the structure of natural and recombinant PLD exotoxins (7,21,25).

In this study, rPLD was produced using a pET SUMO vector, a TA cloning system, One Shot®BL21(DE3) *E. coli*, TY medium, and an ultramembrane cassette system. This protocol differs from those previously used to produce rPLD (25–27). Soluble rPLD was expressed using this protocol, in contrast with some other studies (25,27) but similar to the results reported by Songer et al. (26). However, Songer et al. found that the enzyme activity of the *C. pseudotuberculosis* culture supernatant was approximately 2.5-fold more effective than that of *E. coli* (pCpO5O). These results differ from our study; we found similar protein concentrations and hemolytic activities

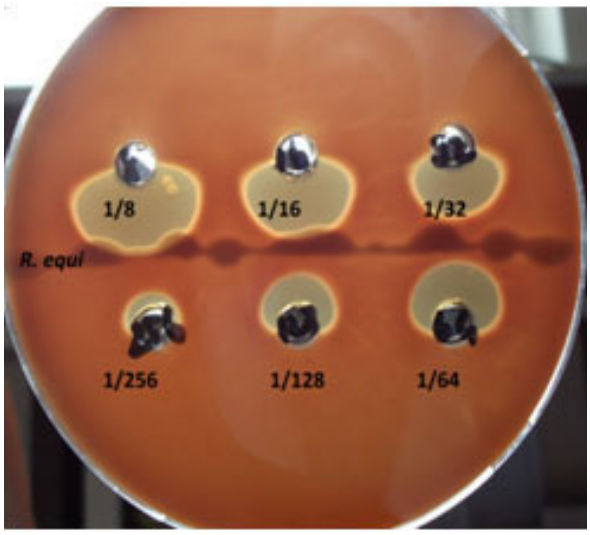

Figure 7. Enzyme titer of concentrated dPLD.

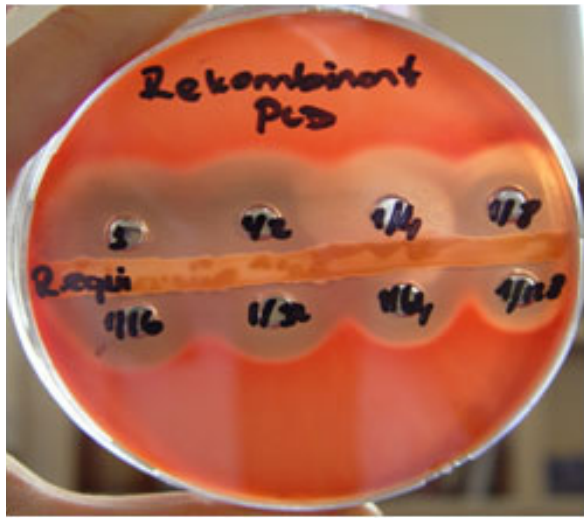

Figure 8. Enzyme titer of concentrated rPLD.

for soluble rPLD and dPLD as measured by a BCA protein assay and a reverse CAMP test, respectively. This may be due to the specific medium, incubation temperature, and final concentration of IPTG we chose as well as the use of a SUMO fusion protein system for the production of soluble recombinant protein. These findings are consistent with those of other researchers (17,28,29).

The amino acid sequence differences between dPLD and rPLD produced in this study and the protein in the NCBI database (a valine instead of a tyrosine at position 181 and the lack of a tryptophan at position 307) are likely due to differences in the *PLD* gene of *C. pseudotuberculosis* isolated from the field or were introduced by Taq polymerase during cloning. The hemolytic titers and the amount of concentrated rPLD and dPLD suggest that these sequence changes do not affect the functionality of the enzymes. Previous studies have not provided a similar characterization of rPLD and dPLD.

Our results suggest that the composition of medium, incubation temperature, final IPTG concentration, cloning method, and the type of promotor, fusion protein, and vector affect the amount of soluble rPLD that can be produced and that the ultramembrane cassette system may be useful for concentration of recombinant proteins under laboratory conditions.

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