A Method for Purification of *Clostridium perfringens* Phospholipase C from Recombinant *Bacillus subtilis* Cells

YOICHI HIRATA,¹ JUNZABURO MINAMI,² MICHIO KOYAMA,² OSAMU MATSUSHITA,²
SEI-ICHI KATAYAMA,² FU JIN,² HAJIME MAETA,¹ AND AKINOBU OKABE^{2*}

First Department of Surgery¹ and Department of Microbiology,² Kagawa Medical School,
Kita-gun, Kagawa 761-07, Japan

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We developed a method to purify *Clostridium perfringens* phospholipase C from a culture of recombinant *Bacillus subtilis* cells. This method consists of three purification steps, and it allowed us to obtain 6.2 mg of pure phospholipase C from 800 ml of culture.

α -Toxin is a phospholipase C (PLC) (EC 3.1.4.3) produced by *Clostridium perfringens*. Unlike many other bacterial phospholipases, this enzyme hydrolyzes both phosphatidylcholine and sphingomyelin, which are major components of the outer leaflet of eukaryotic cell membranes (14). It has been suggested that PLC exhibits various toxicities (6, 11, 12) and also that it stimulates the growth of certain cells (8). A number of toxins are produced by *C. perfringens* (12 toxins are known), and separation of PLC from the other toxins and enzymes is rather difficult. To understand the effect of PLC on cellular function and the underlying molecular mechanism, preparation of very pure PLC is very important. In this paper we describe a method that can be used to purify recombinant PLC (rPLC) in which *Bacillus subtilis* cells that are deficient in two major proteases are used.

B. subtilis DB104 (*his nprR2 nprE18 aprΔ3* [2]) was transformed with pMN α containing a 1.4-kb fragment of the PLC-encoding gene (5). When this strain was grown in Luria-Bertani broth containing 10 μ g of tetracycline per ml, fairly large amounts of PLC were produced. However, the amount of PLC decreased markedly during precipitation with ammonium sulfate and dialysis, probably because of degradation by residual protease activity in the organism (Fig. 1A). A medium containing glucose and a high concentration of sodium succinate has been reported to increase the yield of TEM β -lactamase 50- to 60-fold compared with the yield obtained under standard conditions (4), perhaps because of suppression of protease expression (10). Therefore, we examined PLC production by *B. subtilis* DB104 in SMT medium containing 0.5 M sodium succinate and 0.2% glucose (4) supplemented with 10 μ g of tetracycline per ml. When a culture was grown in 200 ml of SMT medium in a 500-ml flask at 35°C and 100 rpm on a reciprocal shaker, the growth rate decreased, but the level of PLC activity increased (data not shown). The culture supernatant prepared after 24 h of incubation exhibited PLC activity even after ammonium sulfate precipitation and dialysis (Fig. 1B).

Cultures of *B. subtilis*(pMN α) cells in SMT medium were collected from four flasks 24 h after incubation was begun. Proteins in the culture supernatant (760 ml) were precipitated with ammonium sulfate (60% saturation), and this was followed by affinity chromatography on a Sepharose 4B-linked

egg yolk lipoprotein column, as described by Takahashi et al. (13). After the column was washed with 140 ml of Tris-glycerol buffer (50 mM Tris-HCl, 30% [vol/vol] glycerol; pH 7.5), the enzyme was eluted with 250 ml of Tris-glycerol buffer containing 0.5 M NaCl and 0.02% (wt/vol) sodium deoxycholate (pH 7.5). The active fractions were collected and dialyzed against 1 liter of 50 mM Tris-CH₃COOH (pH 8) and then against 1 liter of the same buffer containing 1 M NaCl. Glycerol was added to the enzyme solution to a concentration of 30% (vol/vol), and then the preparation was subjected to chelating column chromatography. A chelating Sepharose Fast Flow gel column (1.6 by 2.5 cm; Pharmacia Biotech, Uppsala, Sweden) was washed and equilibrated with 50 mM Tris-CH₃COOH (pH 8) containing 1 M NaCl and 30% glycerol. After a sample was applied, the column was washed with the same buffer. The active enzyme fraction that was not adsorbed to the column was collected and then dialyzed against 1 liter of 50 mM Tris-HCl (pH 7.5) containing 0.1 mM ZnCl₂ with three changes. This final preparation was stored at -80°C until it was used.

The three purification steps yielded increasingly pure rPLC (Table 1 and Fig. 1B). The final step resulted in a homogeneous product that was purified approximately 130-fold, and 23% of the enzyme was recovered (Table 1). The apparent molecular mass of the rPLC was 43 kDa; this value was the same as the apparent molecular mass of the PLC from *C. perfringens* (cPLC). The N-terminal 11 amino acid residues were the same as the residues previously reported for cPLC (7), and the subsequent 39 amino acid residues were the same as the residues deduced from the nucleotide sequence of cPLC. An analysis in which we used peptide mapping and staphylococcal V8 protease revealed no significant differences in the relative positions or intensities of the peptide fragments of rPLC and cPLC (data not shown). In order to determine whether the two PLCs are functionally the same, we compared the specific activities and toxicities of the two enzymes. The specific activities of these enzymes, which were determined by using the turbidimetric method (1) and egg lecithin (Wako Pure Chemicals, Osaka, Japan), were almost identical; the levels of activity obtained for rPLC and cPLC were 22,800 $\pm$ 1,400 and 21,300 $\pm$ 500 U/mg (mean $\pm$ standard deviation), respectively. The hemolytic activities, as determined by the modified method of Takahashi et al. (13), differed slightly; the levels of activity obtained for rPLC and cPLC were 300 $\pm$ 19 and 380 $\pm$ 17 50% hemolytic units/mg (mean $\pm$ standard deviation), respectively. The difference in these activities may have been due to contamination of the cPLC sample by hemolysins, such as θ -toxin and non- $\alpha\delta\theta$ hemolysin. The dose

* Corresponding author. Mailing address: Department of Microbiology, Kagawa Medical School, Miki-cho, Kita-gun, Kagawa 761-07, Japan. Fax: 81 (878) 98-7109. Electronic mail address: microbio@kms.ac.jp.

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of rPLC preparations obtained at various purification steps. (A) Samples obtained from cultures of *B. subtilis*(pMN α) cells grown in Luria-Bertani broth containing 10 μ g of tetracycline per ml at 37°C for 7 h. Lane 1, molecular weight markers; lane 2, culture supernatant; lane 3, 60% (NH₄)₂SO₄ fraction; lane 4, preparation after dialysis of the 60% (NH₄)₂SO₄ fraction. (B) Samples obtained from cultures of *B. subtilis*(pMN α) cells grown in SMT medium at 35°C for 24 h. Lane 1, preparation after dialysis of the 60% (NH₄)₂SO₄ fraction; lane 2, fraction after affinity chromatography; lane 3, fraction after zinc chelate chromatography; lane 4, PLC purified from *C. perfringens*. A sample solution was mixed with a 4 $\times$ concentration of the sample buffer containing dithiothreitol and heated at 100°C for 5 min. Then 30 μ l of the resulting mixture was electrophoresed on a sodium dodecyl sulfate–12.5% polyacrylamide gel. Protein bands were stained with Coomassie blue. The values on the left are the molecular masses of the markers.

required to kill 50% of 6-week-old male ddY mice weighing 29 to 32 g that were injected intravenously was 90 ng per mouse for both enzymes; this value was similar to the previously reported value (15). Thus, we concluded that the rPLC was correctly processed and possessed biological properties characteristic of PLC.

B. subtilis cells can produce secretory proteins of gram-positive bacteria in the extracellular medium by using the promot-

ers and secretion signals present in their encoding genes (10). Unfortunately, PLC was very sensitive to a protease(s) produced by *B. subtilis*. This problem was not solved simply by using a *B. subtilis* strain deficient in the two major proteases. Increasing production of rPLC while minimizing protease production is critically important. Although the yield of rPLC obtained with our method was only threefold higher than the yield of cPLC obtained by the conventional method, the purified rPLC which we obtained was free of any clostridial toxins. Thus, our method should be very useful for determining the function of the enzyme (e.g., induction of an inflammatory response and stimulation of cellular growth through modulation of signal transduction) (9, 14).

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TABLE 1. Purification of rPLC^a

Fraction	Amt of protein (mg) ^b	Sp act (U/mg of protein) ^c	Purification (fold)	Yield (%)
Culture supernatant	3,600	2.0	1	100
(NH ₄) ₂ SO ₄ precipitation	75	92	47	97
Affinity chromatography	9.5	240	120	32
Chelate chromatography	6.2	260	130	23

^a All values are the values obtained with 760 ml of culture supernatant.

^b Averages of the values obtained in duplicate assays performed with the bicinchoninic acid protein assay reagent (Pierce Chemical Co., Rockford, Ill.); the values did not vary by more than 10%.

^c Averages of the values obtained in triplicate assays performed with *p*-nitrophenylphosphorylcholine as described by Kurioka and Matsuda (3); the values did not vary by more than 10%.