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Full Length Research Paper

Improved purification and some properties of a novel phospholipase C from *Bacillus mycoides* strain 970

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A novel microbial phospholipase C from *Bacillus mycoides* strain 970 was purified to apparent homogeneity by an improved purification process, in which DEAE-Cellulose adsorption was first used to remove bulk of dark brown impurities and then a combination of DEAE-Cellulose ion-exchange, Phenyl Sepharose hydrophobic interaction and Sephacryl S-100 gel filtration chromatography was used to separate the enzyme from remained contaminants. The enzyme appeared to be a single peptide of 75.1 kDa on SDS-PAGE. The optimum temperature and pH of the enzyme were 60°C and 7.0 - 7.5, respectively. The enzyme was stable at temperatures lower than 50°C and pH 5 - 9.5. This purified phospholipase C was characterized as a metallophospholipase C and Ca^{2+}, Mg^{2+}, Zn^{2+}, Ba^{2+} were essential for its PLC activity. The enzyme showed no lecithinase activity on egg yolk agar and also no hemolytic activity on blood agar.

Key words: Phospholipase C, purification, metalloenzyme, nonhemolytic activity, *Bacillus mycoides*.

INTRODCTION

Phospholipases C (PLCs) [EC 3.1.4.3] hydrolyze phospholipids to generate 1, 2-diacylglycerol (DAG) and original phosphorylated head group (MacFarlane et al., 1941). Microorganisms can secrete PLCs in culture broth and their PLCs can be detected and purified more easily than that of plants and animals.

Microbial PLCs have been considered as major virulence factors of pathogens and the mechanisms of pathogenesis have been studied during the past years (Sakurai, 2006; Flores-Diaz et al., 2003). Additionally, they have also been used as a probe to explore the cell membrane structure and as useful tools to investigate the regulatory functions of eukaryotic PLCs because of their catalytic domain showing the same architecture as that of certain mammalian PLCs (Heinz et al., 1995; Essen et al., 1996). Currently, microbial PLCs have received considerable attention to their industrial applications in food processing, degumming process of vegetable oil, cosmetic manufacturing and pharmacy (Mo et al., 2009; Ciofalo et al., 2006; Zhang et al., 2005; Anthonsen et al., 1999). However, the industrial applications of PLCs have been somewhat limited because of relatively low activities and high costs. In order to exploit industrial applications of microbial PLCs, we screened four bacterial isolates producing extracellular PLCs from more than one thousand soil samples in the previous work (Wang et al., 2003). One was identified as *Bacillus cereus* strain 754-1, the others were identified as *B. mycoides* strain 970, 779 and 1107, respectively (Wang et al., 2004). *B. mycoides* strain 970, which has the highest PLC activities of four strains, was used to produce PLCs. To our knowledge, no PLCs from *B. mycoides* have been studied. In this study, we purified a novel extracellular PLC from *B. mycoides* strain 970 culture broth by an improved purification process and some properties of this purified enzyme were studied.

MATERIALS AND METHODS

B. mycoides strain 970 was isolated from 1268 natural soil samples

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and identified according to its morphologic, physiological-biochemical characteristics and 16S rDNA sequence. The strain was activated for 14 h at 30℃ on fresh LB slope agar and then subcultured in the modified LB medium (1% peptone and 0.5% beef extract, pH 7.0) for 10 h at 30℃ on shaker set at 200 rpm. The fermentation was carried out in a 5 L stirred tank bioreactor containing 3 L medium (1% peptone and 0.5% beef extract, pH 7.0). After addition of the inoculum (30 ml), the cultures were stirred at 600 rpm. The temperature and pH were controlled at 30℃ and 7.0. The bacterium was grown under aerobic condition (1.5 vvm) for 20 h.

Cells were removed from 3 L culture broth by centrifugation at 10,000 g for 30 min at 4℃. The supernatant was loaded on a DEAD-Cellulose column (4.5 × 20 cm) previously equilibrated with 20 mM Tris-HCl (pH 7.2) and then eluted with the same buffer. The yellow break-through fractions, which contain PLC activities, were collected and concentrated to about 200 ml by hollow fiber ultra-filtration (MWCO 10,000) and then diafiltrated with 20 mM Tris-HCl (pH 8.5) buffer by Centricon Plus-70 centrifugal ultra-filter at 3,500g for 20 min. The final volume of sample was kept 100 ml. The active sample was then applied to a DEAD-Cellulose column (1.6×20 cm) previously equilibrated with 20 mM Tris-HCl (pH 8.5) buffer and then eluted with a linear gradient of 0 - 0.2 M NaCl (400×400 ml). Protein concentration of every fraction (4 ml) was monitored continuously by absorbance at 280 nm. PLC activity of every fraction was measured with NPPC assay. The active fractions were pooled and diafiltrated with 20 mM Tris-HCl (pH 7.2) buffer. Solid $(NH_4)_2SO_4$ was added slowly to the active sample to 1 M. The sample was loaded onto a Phenyl Sepharose 6 Fast Flow column (1.1×10 cm) previously equilibrated with 20 mM Tris-HCl (pH 7.2) buffer containing 1 M $(NH_4)_2SO_4$ and then eluted with a linear gradient of 1.0 - 0 M $(NH_4)_2SO_4$ (200 × 200 ml). Fractions containing PLC activity were pooled and concentrated to about 15 ml. The concentrated sample was loaded onto a Sephacryl S-100 column (1.6 × 100 cm) equilibrated with 20 mM Tris-HCl (pH 7.2) buffer and eluted with the same buffer at 8 ml/h. The active fractions were pooled and concentrated to about 10 ml and stored at -20℃.

PLC activity was determined by NPPC method (Kurioka et al., 1976). Protein concentration was measured using the Bradford method (Bradford, 1976). Purity and molecular weight of the purified PLC was assayed by SDS-PAGE.

The optimal temperature and pH of the purified PLC activity were determined by measuring the enzyme activity in NPPC reaction mixture at different temperatures and pH. For the determination of heat and pH stability, the purified enzyme was pre-incubated at different temperatures and pH for 2 h, and then the residual activities were measured immediately.

To assay the metal ion requirement, the purified enzyme was pre-incubated in the presence of 1 mM EDTA at 37℃ for 10 min, then diafiltrated by Amicon Ultra-15 centrifugal filter with 20 mM Tris-HCl (pH 7.2) to remove EDTA and its cation chelats. Different metal ions (1 mM) were added into the inactivated enzyme solution respectively. The recovered PLC activity was determined immediately after incubation at 37℃ for 10 min.

The hemolytic activity of purified PLC was tested by blood agar (5% washed human erythrocytes in 2% agar) and lecithinase activity was checked by egg yolk agar (2% egg yolk in 2% agar). 100 ul of sample was added into Oxford cubs punched on the blood agar or the egg yolk agar and then incubated for 8 h at 37℃.

RESULTS AND DISCUSSION

The purification steps were summarized in Table 1. The PLC produced in culture broth was purified 40.8 fold to a specific activity of 15,370 U/mg and the recovery was 25%. In the first purification step of ion-exchange adsorption, the bulk of dark brown impurities were adsorbed on DEAE-Cellulose. PLC and a few yellow contaminants broke through the column. The remained colored contaminants were removed completely by the following ultra-filtration and diafiltration. This step is key to purify the PLC to homogeneity, because PLC is easy to polymerize with other impurities (Yamakawa et al., 1977). Traditional $(NH_4)_2SO_4$ salting out was often used as the first purification step, but it could promote the polymerization of PLC with other impurities. This was the reason why the salting out was cancelled and substituted by ion-exchange adsorption in our study.

As shown in Figure 1, a single protein band (lane 5) was seen on the SDS-PAGE, indicating the PLC in *B. mycoides* strain 970 culture supernatant had been purified to apparent homogeneity by this improved purification process. The molecular mass of the purified PLC was estimated to be 75.1 kDa, much higher than that of other *Bacillus* species PLCs, but similar to that of *Pseudomonas* species PLCs (Titball, 1993).

Figure 2 and 3 showed the effect of temperature and pH on the purified enzyme activity. The maximum activity was found at 60℃ and pH 7.0 - 7.5. The enzyme was stable at the temperature below 50℃ and over a broad pH range of pH 5 - 9.5. These features were not significantly distinct from other PLCs (Mo et al., 2009; Sugimori et al., 2006).

The result of metal ion requirement assay showed this purified PLC from *B. mycoides* strain 970 culture supernatant belonged to the group of metallophospholipase C (Table 2). Ca^{2+}, Ba^{2+} besides Zn^{2+}, Mg^{2+} was demonstrated to be essential for its PLC activity. Mn^{2+} could also recover part of the activity. This showed the differences of this purified PLC from other bacterial PLCs (Mo et al., 2009; Seo et al., 2004).

Hemolytic and lecithinase activity assay were illustrated in Figure 4. The purified PLC showed nonhemolytic activity (sample 2 in Figure 4b). Hemolytic activities were often considered as the important toxic origin of many microbial PLCs. Nonhemolytic activity of this purified PLC decreased its risk in the future application. It was surprising that this purified PLC appeared no lecithinase activity on egg yolk agar, yet it exhibited very high hydrolytic activity (15,370 U/mg) to NPPC. ρ-nitrophenylphosphorylcholine (ρ-NPPC), a structural derivative of phosphorylcholine (PC), was often used as an artifial substrate to examine PLCs activity. This result of lecithinase activity assay indicated PC was not specific substrate of this purified PLC and further demonstrated PLC did not recognize phospholipids only by polar phosphate head groups, the hydrocarbon tail was also important for correct substrate recognition (Flieger et al., 2000). The culture supernatant had weak hemolytic activity and weak lecithinase activity. It indicated *B. mycoides* strain 970 could synthesize two different PLC

Table 1. Purification of PLC from *Bacillus mycoides* strain 970 culture supernatant.

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Fold purification	Recovery (%)
culture supernatant	218.1	82215	377	1	100
DEAE-Cellulose (pH7.2)	25.5	54060	2,120	5.6	65.8
EAE-Cellulose (pH8.5)	3.6	33462	9,295	24.5	40.7
Phenyl Sepharose	2.04	25209	12,358	32.8	30.7
Sephacryl S-100	1.32	20553	15,370	40.8	25

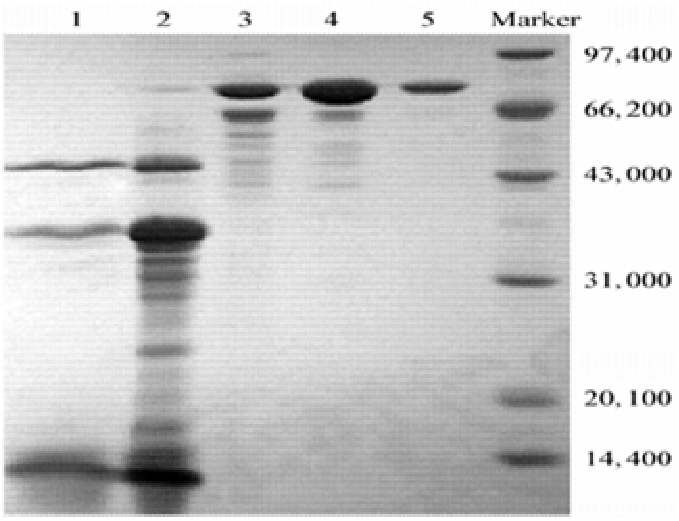

Figure 1. SDS-PAGE. Stacking gel is 3% polyacrylamide in pH 6.8 Tris-HCl buffer. Separation gel is 10% polyacrylamide in pH 8.9 Tris-HCl buffer. All samples were reduced with 2-mercaptoethanol and denatured by a boiling water bath for 5 min before electrophoresis. Protein bands were stained by Coomassie bright blue R-250. Lane 1: culture supernatant; Lane 2: concentrated fraction from DEAE-Cellulose adsorption; Lane 3: active fraction from DEAE-Cellulose chromatography; Lane 4: active fraction from Phenyl Sepharose chromatography; Lane 5: active fraction from Sephacryl S-100 gel chromatography.

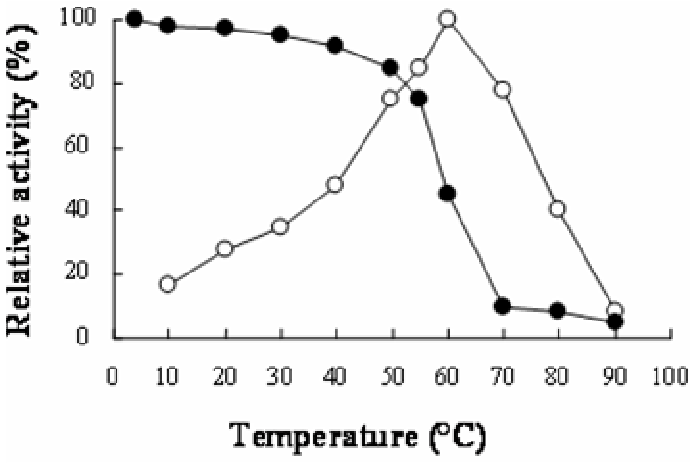

Figure 2. Effect of temperature on the activity and stability of the purified enzyme. ○— activity; ●— stability.

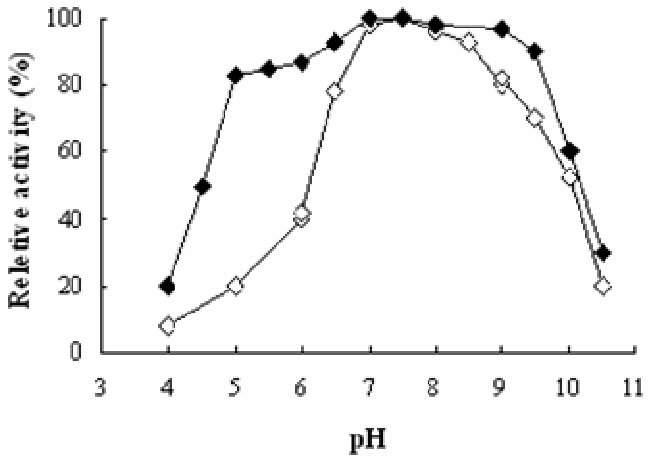

Figure 3. Effect of pH on the activity and stability of the purified enzyme. ∩— activity; ∅—stability.

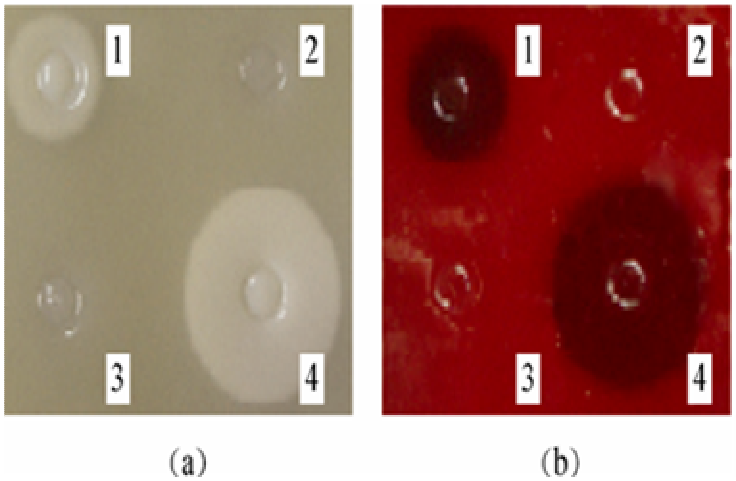

Figure 4. Hemolytic and lecithinase activity assay. 100 ul of samples were added into Oxford cub punched on egg yolk agar or blood agar. (a) is a egg yolk agar (2% egg yolk in 2% agar). (b) is a blood agar (5% washed human erythrocytes in 2% agar). Opal zone in the egg yolk agar indicated the lecithinase activity. Transparent zone in the blood agar indicated hemolytic activity. The samples were as followings: 1. culture supernatant; 2. purified PLC; 3. physiological saline; 4. control PLC Type 4 from *Bacillus cereus* (Sigma).

Table 2. Ion requirement of PLC from *Bacillus mycoides* strain 970 culture supernatant.

Treatment of PLC	Restored activity (%)
control	100
EDTA	0
$CaCl_2$	77.7
$MgCl_2$	79.5
$ZnCl_2$	81.5
$FeCl_3$	3.8
$MnSO_4$	46.4
$SnCl_2$	1.4
$BaCl_2$	80.3
$CuSO_4$	4.5
$AlCl_3$	2.4

at least. One is lecithin hydrolase which has hemolytic activity, the other is a nonhemolytic PLC which has no lecithin hydrolase activity and this purified PLC is the later. PLC type 4 from *B. cereus* (Sigma) was used as a control. It appeared apparent lecithinase activity and hemolytic activity. The results also demonstrated the correlation between the hemolytic activity and lecithinase activity of PLC.

Conclusion

An efficient and available purification process was established upon the improvement of other traditional purification process. A novel PLC was purified to homogeneity from *B. mycoides* strain 970 culture supernatant by this improved purification process. It appeared many different properties from other microbial PLCs. Although many microbial and mammalian PLCs were commercially available at present, it was difficult for PLCs to be applied in quantity just because of its high cost. New microorganisms screening, culture conditions optimization, gene modification and high-level expression are the future woks to develop industrial applications of microbial PLCs.

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