Biotechnology Letters **26:** 1475–1479, 2004.

High-level expression of recombinant phospholipase C from *Bacillus cereus* in *Pichia pastoris* and its characterization

Kook-Hwa Seo & Jong Il Rhee*
Faculty of Applied Chemical Engineering, BioProcess Technology Laboratory, Chonnam National University, YongBong-dong 300, GwangJu 500-757, Republic of Korea
**Author for correspondence (Fax: +82-62-530-0846; E-mail: jirhee@chonnam.ac.kr)*

Received 16 March 2004; Revisions requested 16 April 2004/2 June 2004; Revisions received 27 May 2004/13 July 2001; Accepted 21 July 2004

Key words: Bacillus cereus, characterization, expression, phospholipase C, *Pichia pastoris*

Abstract

The phospholipase c (*plc*) gene from *Bacillus cereus* was cloned into the pPICZC vector and integrated into the genome of *Pichia pastoris*. The phospholipase C (PLC) when expressed in *P. pastoris* was fused to the α-factor secretion signal peptide of *Saccharomyces cerevisiae* and secreted into a culture medium. Recombinant *P. pastoris* X-33 had a clear PLC band at 28.5 kDa and produced an extracellular PLC with an activity of 678 U mg^{-1} protein which was more than a recombinant *P. pastoris* GS115 (552 U mg^{-1} protein) or KM71H (539 U mg^{-1} protein). The PLCs were purified using a HiTrap affinity column with a specific activity of 1335 U mg^{-1} protein by *P. pastoris* GS115, 1176 U mg^{-1} protein by *P. pastoris* KM71H and 1522 U mg^{-1} protein by *P. pastoris* X-33. The three recombinant PLCs had high PLC activity in the low pH range of 4–5 and higher thermal stability (e.g. stable at 75 °C) than the wild-type PLC from *B. cereus*. Some organic solvents, surfactants and metal ions, e.g. methanol, acetone, Co^{2+} and Mn^{2+} etc., also influenced the activity of the recombinant PLCs.

Introduction

Phospholipases C (PLCs) are hydrolytic enzymes that catalyze the hydrolysis of phospholipids to diacylglycerol (DAG) and the original phosphorylated head group. These enzymes have been isolated from various microorganisms including *Clostridium* and *Bacillus* (Levine *et al.* 1998). The PLCs originating from *Bacillus cereus* have a broad substrate specificity (Martin *et al.* 2000) and have been extensively studied for regulatory functions related to the cellular signal transduction pathways in mammals. Recently, these enzymes have received considerable attention and have been used to obtain enantiomerically pure DAG and organic phosphates through their immobilization (Anthonsen *et al.* 1999).

The *plc* gene from *B. cereus* has been cloned and expressed in *E. coli* under the control of the T7 promoter (Tan *et al.* 1997). To purify the recombinant PLC produced in *E. coli*, a multitude of purification steps were required, and its productivity and stability were not much better than those from *B. cereus*. For industrial applications of PLC it is necessary to produce PLC as an extracellular protein. The enzyme should also be stable under industrial conditions. Some lipases or phospholipases have been over-expressed as an extracellular protein in the yeast *Pichia pastoris* (Minning *et al.* 1998, Quyen *et al.* 2003).

However, to our knowledge no PLC has been previously over-expressed in *P. pastoris*. In this study, we describe the cloning of the *plc* gene in *P. pastoris* using three strains, GS115, KM71H and X-33. The PLC produced by each recombinant strain was purified and characterized, its physicochemical properties determined and compared to the wild-type PLC obtained from *B. cereus*.

Materials and methods

Microorganisms and plasmids

The plasmid pMR1 containing the *plc* gene isolated from *Bacillus cereus* ATCC10987 was kindly provided by Prof M.F. Roberts (Tan *et al.* 1997). The plasmid pPICZαC and yeast strains *Pichia pastoris* GS115(his$^-$, Mut$^+$), KM71H(Mut^s, Arg$^+$), and X-33(Mut$^+$) were purchased from Invitrogen Co. *E. coli* DH5α (*supE*44 Δ*lacU*169 [Φ80*lacZ*ΔM15] *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) was used for subcloning the *plc* gene.

The plasmid pMR1 containing a *plc* gene was first linearized with *Eco*RI, and the *plc* gene was amplified by PCR with two primers, each containing a *Cla*I site and a *Kpn*I site. After digestion with *Cla*I and *Kpn*I, the PCR fragment was ligated into the pPICZαC, an *E. coli*-yeast shuttle vector containing the zeocin resistance gene. The plasmid pPICZαC containing the *plc* gene, referred to as plasmid pBPT44, was cloned in *E. coli* DH5α for amplification (Sambrook *et al.* 2001). The plasmid pBPT44 linearized by *Sac*I was introduced into the *P. pastoris* GS115, KM71H, and X-33 by the EasyComp transformation protocol following the manufacturer's instructions (Invitrogen).

Culture media and conditions

E. coli was grown in the low salt LB medium (1% w/v Tryptone, 0.5% w/v yeast extract, and 0.5% w/v NaCl) supplemented with 25 μg zeocin ml^{-1} in order to select for the transformant of the plasmid pBPT44. YPD medium (1% w/v yeast extract, 2% w/v peptone, and 2% w/v dextrose) with or without 100 μg zeocin ml^{-1} was used to grow *P. pastoris* wild type as well as the pre-culture of recombinant *P. pastoris*. Main cultures of recombinant *P. pastoris* were grown in BMGY medium (1% w/v yeast extract, 2% w/v peptone, 100 mM potassium phosphate buffer, pH 6, 1.34% w/v YNB, 4×10^{-5}% biotin, and 1% v/v glycerol) and the BMMY medium, which contains the same components as the BMGY medium, except for having 0.5% (v/v) methanol instead of 1% (v/v) glycerol.

Recombinant *P. pastoris* was cultured in a 500 ml baffled shake-flask containing 100 ml BMGY for 24 h giving an $OD_{600} = 2 \sim 6$. Cells were then harvested by centrifugation (5000 $\times$ *g*, 5 min) and cell pellets were resuspended in a 500 ml baffled shake-flask containing 100 ml BMMY medium for the induction of gene expression. During the cultivation at 30 °C and 200 rpm, methanol was added to give 0.5% (v/v) every 24 h and 2 ml culture was taken to determine cell growth. Culture supernatants were collected after centrifugation for the analysis of PLC activity.

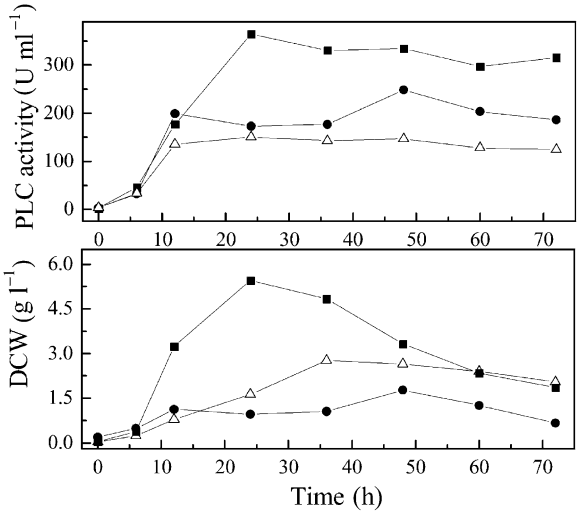

Fig. 1. Time courses of cell growth and PLC production during cultivation of three recombinant *P. pastoris* strains harbouring the plasmid pBPT44 (●, GS115[pBPT44]; Δ, KM71H[pBPT44]; ■, X-33[pBPT44]). Dry cell weight (DCW) (g l^{-1}) = 0.213 * OD_{600}.

Purification of PLCs

A HiTrap affinity column (1 ml, Amersham Pharmacia Biotech Co.) was used to purify PLCs from the culture supernatants: 2 ml crude enzyme solution was loaded on the column, washed with 5 ml loading buffer (20 mM sodium phosphate buffer, pH 7.4, containing 0.5 M NaCl) at 1 ml min^{-1} and the PLCs bound were then eluted with 1 ml loading buffer containing 0.5 M imidazole. Fractions of 0.2 ml were collected.

Assay of PLC activity

The activity of PLC was measured on a microtiter plate with 300 μl round wells using *p*-NPPC as a substrate in 100 mM borax buffer (pH 7.5) at room temperature according to the modified method of Kurioka *et al.* (1976). The absorbancy was measured at 410 nm. One unit (U) of PLC was defined as the amount of enzyme that cleaved to 1 μmol *p*-NPPC to *p*-nitrophenol and phosphorylcholine per min under the assay conditions.

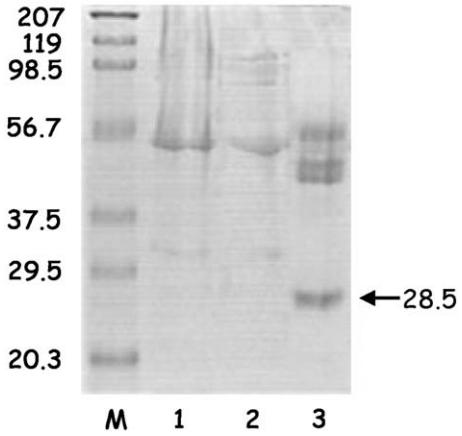

Fig. 2. SDS-PAGE after purification of PLCs using a HT column. Lane M, molecular mass standard indicated in kDa: lane 1, elution fractions of supernatants of GS115 from a HiTrap Chelating column. Lane 2, elution fractions of supernatants of KM71H from a HiTrap Chelating column. Lane 3, elution fractions of supernatants of X-33 from a HiTrap Chelating column.

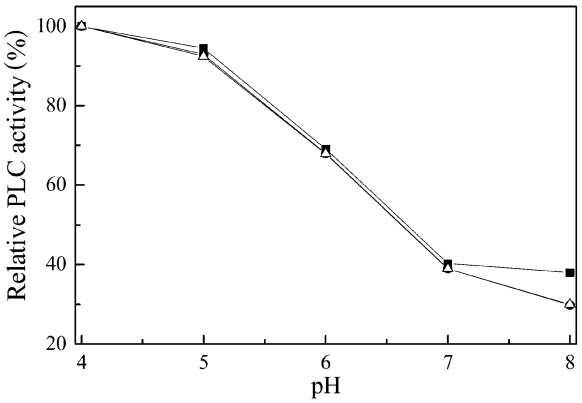

Fig. 3. Effect of pH on PLC activity. Maximal activity of PLC was set at 100% (52 U ml^{-1} for GS115, 49 U ml^{-1} for KM71H and 79 U ml^{-1} for X-33) and the data (•, GS115[pBPT44]; Δ, KM71H[pBPT44]; ■, X-33[pBPT44]) represent the arithmetic mean of a least three determinations. Buffer solutions (100 mM) used to adjust pH values: citrate buffer for pH 4 and 5, sodium phosphate buffer for pH 6 and 7, and borax buffer for pH 8.

Results

Plasmid construction

The cDNAs for the plc gene were placed under the control of the methanol-inducible AOX1 promoter. The size of PCR product purified by electrophoresis was 851 bp. The plasmid was designed so that the mature PLC was left with an N-terminal His^6-tag after cleavage of the α-factor secretion signal peptide. The catalytic properties of some lipases were not influenced by the presence of the His^6-tag extension (Bornscheuer *et al.* 1994). Integration of the linearized plasmid pBPT44 into *P. pastoris* genome resulted in some transformants with efficiently expressed and secreted PLCs. Transformation of colonies on the YPD agar plate containing 100 μg zeocin ml^{-1} was confirmed by the PCR.

Expression of PLC in P. pastoris

Recombinant yeast *P. pastoris* strains harbouring the plasmid pBPT44 were induced in 100 ml BMMY medium containing 100 μg zeocin ml^{-1} at 30 °C for 72 h. Recombinant *P. pastoris* X-33[pBPT44] reached a maximum dry cell mass (DCW) of 5.4 g l^{-1}, but *P. pastoris* GS115[pBPT44] and KM71H[pBPT44] reached only 1.82 g l^{-1} and 2.6 g l^{-1}, respectively (Figure 1). The PLC activity of culture supernatants reached a maximum of 248 U ml^{-1} for *P. pastoris* GS115[pBPT44] after 48 h induction, 150 U ml^{-1} for *P. pastoris* KM71H[pBPT44], and 365 U ml^{-1} for *P. pastoris* X-33[pBPT44] after 24 h of induction. The SDS-PAGE with the culture supernatants of *P. pastoris* X-33[pBPT44] showed a main band of 28.5 kDa which roughly corresponds to the molecular size of the wild-type PLC from *B. cereus* (Sigma).

PLC purification and SDS-PAGE

The crude PLC produced by recombinant *P. pastoris* strains was purified by using the HiTrap Chelating column. More than 95% of the total PLC activity was eluted from the column in the third fraction (i.e. 0.4 to 0.6 ml). The crude PLCs were purified about 2.2-fold with specific activities of up to 1300 U mg^{-1} protein (Table 1).

The purified PLCs were concentrated to about 8.3-fold (from 500 μl to 60 μl) by precipitation using triacetic acid and subjected to SDS-PAGE. Some protein bands of the purified PLCs on SDS-PAGE corresponded to the molecular weight of the PLC from Sigma (Figure 2): that is the recombinant PLC from *P. pastoris* X-33 had a main band of 28.5 kDa, with some other faint bands between 45 kDa and 70 kDa. The recombinant PLC from *P. pastoris* GS115 had one band on 56 kDa and that of KM71H had three bands of 56 kDa, 85 kDa and 100 kDa.

Table 1. Purification of PLCs from the culture medium of recombinant *P. pastoris*.

	Culture supernatants			HT column[c]		
	GS115	KM71H	X-33	GS115	KM71H	X-33
Total protein (μg)[a]	607	517	537	16	17	21
Total PLC activity (U)[b]	335	279	364	21	20	31
Specific PLC activity (U mg^{-1})[a]	552	539	677	1313	1176	1476
Purification (fold)	1	1	1	2.4	2.2	2.2

[a]Total protein masured using the Bradford assay, and the specific PLC activity is based on the amount of total protein amounts.
[b]Measured by the *p*-NPPC assay. One unit (U) is 1 μmol of *p*-NPPC converted in 1 min.
[c]Enzyme was purified through a a HiTrap Chelating column (1 ml).

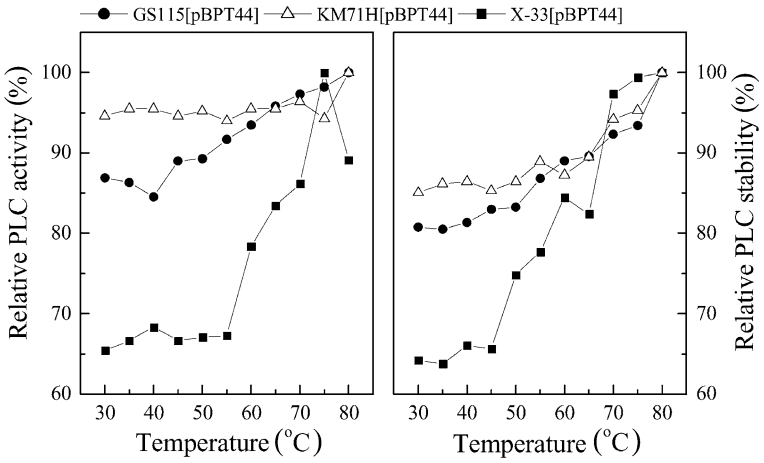

Fig. 4. Effect of incubation temperature on the activity and stability of recombinant PLCs. Maximum PLC activity was set at 100% (24 U ml^{-1} for GS115, 22 U ml^{-1} for KM71H and 35 U ml^{-1} for X-33) and the data (●, GS115[pBPT44]; Δ, KM71H[pBPT44]; ■, X-33[pBPT44]) represent the arithmetic mean of at least three determinations. Thermal stability of each recombinant PLC was determined by incubating the PLC solution for 12 h in a 100 mM borax buffer (pH 7.5) at various temperatures.

Effects of pH and temperature on the activity and stability of the recombinant PLCs

All recombinant PLCs showed maximum activity at pH 4 (see Figure 3).

The relative activity and stability of the PLC from *P. pastoris* GS115[pBPT44] were maximal at 80 °C (see Figure 4). The relative activity of the PLC from *P. pastoris* KM71H[pBPT44] was almost constant from 30 to 80 °C whilst that from *P. pastoris* X-33[pBPT44] peaked at 75 °C (see Figure 4). The stability of the latter, however, increased gradually up to 80 °C. These increases in PLC activity to 80 °C were unusual but were achieved repeatedly.

The activity of the PLC from *P. pastoris* X-33[pBPT44] in 100 mM borax buffer (pH 5 and 7) did not change over 24 h at 60 °C and 70 °C (data not shown).

Effects of organic solvents, surfactants, and metal ions on PLC activity

Thirty % (v/v) methanol and DMSO stimulated the activity of PLC from *P. pastoris* X-33[pBPT44] from 18 U ml^{-1} to 28 and 23 U ml^{-1}, respectively. Thirty % (v/v) 2-propanol and acetone increased the activity of PLC from *P. pastoris* GS115[pBPT44] at 15 U ml^{-1} by more than 30%, but reduced the activity of PLC from *P. pastoris* KM71H[pBPT44] at 13 U ml^{-1} by about 15%.

Surfactants such as Triton X-100, SDS, CTAB, or glycerol at 1% (w/v) had no significant effects on the PLC activity of three *P. pastoris*.

The activity of PLC from *P. pastoris* GS115[pBPT44] was increased in the presence of 5 mM Co^{2+}, 5 mM Mn^{2+}, and 5 mM Zn^{2+} by more than 10%, whereas 5 mM Ca^{2+} and 5 mM Zn^{2+} reduced the PLC activity of *P. pastoris* X-33[pBPT44]

by about 10%, although 5 mM Co^{2+} increased the activity of PLC from *P. pastoris* X-33[pBPT44]. The activity of PLC from *P. pastoris* KM71H[pBPT44] was not significantly affected by the addition of metal ions.

Discussion and conclusions

PLC recognizes phospholipids with polar phosphate head groups and stereospecifically cleaves the phospholipids into *sn*-1,2-diacylglycerol and phosphate monoesters (Hergenrother *et al.* 2000). A non-specific PLC is usually produced by *B. cereus*. A *plc* gene from *B. cereus* has been cloned in *E. coli* and recombinant *E. coli* produced PLC up to ca. 30–40 μg active PLC ml^{-1} of culture (Tan *et al.* 1997).

In this study recombinant *P. pastoris* produced PLC up to 360 U ml^{-1} in culture supernatants. The PLCs over-expressed in *P. pastoris* were characterized and their physicochemical properties were compared to those of the wild-type PLC from *B. cereus* ATCC 10987 (data not shown). There was no significant difference in pH stability between the recombinant PLC and the wild type, i.e. high PLC activity in the low pH range of 4–5. Thermal stability of the wild-type PLC from *B. cereus* was low at high temperatures (>50 °C), but the recombinant PLCs retained good thermal stability up to 80 °C, the reason for which must be explored in future studies.

Surfactants did not affect the activity of the recombinant PLC significantly, although 1% (w/v) Triton X-100 and CTAB increased the activity of the wild-type PLC by about 10%. Some organic solvents, such as methanol, 2-propanol, acetone, and DMSO at 30% (v/v), increased the activity of three recombinant PLCs by more than 10%. However, only DMSO activated the PLCs from *B. cereus* to any great degree, by more than 60%. The addition of 5 mM Zn^{2+}, Mn^{2+}, and Co^{2+} increased the activity of the wild-type PLC and two recombinant PLCs (except *P. pastoris* KM71H) significantly.

In conclusion, the *plc* gene from *B. cereus* was cloned in three strains of *P. pastoris*. Among the three *P. pastoris* strains, the *P. pastoris* X-33 had a high expression of PLC and showed a high PLC activity at pH 4 as well as good thermal stability up to 80 °C. Therefore, the recombinant PLC from *P. pastoris* X-33 would be preferable when hydrolyzing phosphatidylcholine in a biological process at low pH (<5) and high temperatures (>60 °C).

References

Anthonsen T, D'Arrigo P, Pedrocchi-Fantoni G, Secundo F, Servi S, Sundby E (1999) Phospholipids hydrolysis in organic solvents catalysed by immobilized phospholipase C. *J. Mol. Cat. B; Enzym.* **6**: 125–132.

Bornscheuer U, Reif O-W, Lausch R, Freitag R, Scheper T, Kolisis EN, Menge U (1994) Lipase of *Pseudomonas cepacia* for biotechnological purposes: purification, crystallization and characterization. *Biochim. Biophys. Acta* **1201**: 55–60.

Hergenrother PJ, Martin SF (2000) Phosphatidylcholine-preferring phospholipase C from *B. cereus*: function, structure, and mechanism. *Topics Curr. Chem.* **211**: 131–167.

Kurioka S, Matsuda M (1976) Phospholipase C assay using p-nitrophenyl-phosphorylcholine together with sorbital and its application to studying the metal and detergent requirement of the enzyme. *Anal. Biochem.* **75**: 281–289.

Levine L, Xiao D, Little C (1998) Increased arachidonic acid metabolites from cells in culture after treatment with the phosphatidylcholine-hydrolyzing phospholipase C from *Bacillus cereus. Prostaglandins* **34**: 633–642.

Martin SF, Follows BC, Hergenrother PJ, Trotter BK (2000) The choline binding site of phospholipase C (*Bacillus cereus*): insights into substrate specificity. *Biochemistry* **39**: 3410–3415.

Minning S, Schmidt-Dannert C, Schmid RD (1998) Functional expression of *Rhizopus oryzae* lipase in *Pichia pastoris*: high-level production and some properties. *J. Biotechnol.* **66**: 147–156.

Quyen DT, Claudia SD, Schmid RD (2003) High-level expression of a lipase from *Bacillus thermocatenulatus* BTL2 in *Pichia pastoris* and some properties of the recombinant lipase. *Protein Expr. Purif.* **28**: 102–110.

Sambrook J, Russell DW (2001) *Molecular Cloning: A Laboratory Manual*, 3rd edn. New York: Cold Spring Harbor Laboratory Press.

Tan CA, Hehir MJ, Roberts MF (1997) Cloning, overexpression, refolding, and purification of the nonspecific phospholipase C from *Bacillus cereus. Protein Expr. Purif.* **10**: 365–372.