Purification and Characterization of Phospholipase B from Candida utilis

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Phospholipase B (PLB) from the asporogenous yeast Candida utilis was purified to homogeneity from a culture broth. The apparent molecular mass was 90–110 kDa by SDS–PAGE. The enzyme had two pH optima, one acidic (pH 3.0) and the other alkaline (pH 7.5). At acidic pH the enzyme hydrolyzed all phospholipids tested without metal ions. On the other hand, the PLB showed substrate specificity and required metal ions for alkaline activity.

The cDNA sequence of the PLB was analyzed by a combination of several PCR procedures. The PLB encoded a protein consisting of 643 amino acids. The amino acid sequence contained a lipase consensus sequence (GxSxG) and catalytic arginine and aspartic acid motifs which were identified as the catalytic triad in the PLB from Kluyveromyces lactis, suggesting that the catalytic mechanism of the PLB is similar to that of cytosolic phospholipase A2 (cPLA2), found in mammalian tissues.

Key words: phospholipase B; Candida utilis; phospholipid deacylating enzyme

The enzymes that catalyze the deacylation of phospholipids include phospholipase A1, A2, B, and lyso phospholipase. PLB catalyzes hydrolytic cleavage of both the sn-1 and the sn-2 acylester bonds of glycerophospholipids, and has been found in microorganisms, plants, and animal tissues. The phospholipid deacylating enzymes characterized so far from yeasts at the molecular level belong to the category of the PLB group. But, the enzymatic properties, such as molecular weight, optimum pH, effects of metal ions, substrate specificity, and biological significance vary widely depending on the species of yeast.

Candida utilis is an important food yeast and has been widely used in industry in fodder for domestic cattle. Therefore, it is important to keep the cell conditions fresh or intact during storage of the yeast. The durability of yeast cells is considered to be related closely to the activity of phospholipid deacylating enzymes in yeast, because they are suspected to play key roles in phospholipid turnover in the cell membrane.

Recently, three PLB genes (PLB1, PLB2, and PLB3) from Saccharomyces cerevisiae have been characterized. Deletion or overexpression of the PLB1 gene, which encodes a major phospholipid-deacylating activity in S. cerevisiae, did not result in any detectable phenotypic effects. On the other hand, Torulaspora delbrueckii showed shortness of growth period in the lag phase and an increased amount of cells at the final stage with disruption of PLB gene (PLB), and the cells of a PLB-overexpressed strain died rapidly even during the logarithmic growth phase, indicating that PLB activity must be involved in the determination of the cell durability of this yeast. A pathogenic yeast, Candida albicans, contained two PLB genes (caPLB1 and caPLB2). The virulence of the yeast was significantly attenuated by the disruption of caPLB1, compared with the isogenic wild-type parental strain. Accordingly, PLB activity has been identified as a potential virulence factor in C. albicans. These results suggest that the enzymatic properties and biological significance of the PLB differ depending on the species of yeast. Further studies on this enzyme with more varieties of yeast are important to understand the enzymatic properties and biological significance of the enzyme. In this study, we purified and characterized PLB from C. utilis better to understand the enzymatic properties and biological significance of this yeast.

Materials and Methods

Chemicals. All the phospholipids were from Sigma (St. Louis, Mo, USA). Molecular weight marker proteins were from Daiichi Pure Chemicals (Tokyo, Japan). Phenyl-Sepharose CL-4B, a Hiload 16/60 Superdex 200 pg column, and a Mono Q HR5/5 column were from Pharmacia Biotech (Uppsala, Sweden). A column of TSK gel G3000SW was obtained from Tosoh (Tokyo, Japan). The enzymes that catalyze the deacylation of phospholipids include phospholipase A1, A2, B, and lyso phospholipase. PLB catalyzes hydrolytic cleavage of both the sn-1 and the sn-2 acylester bonds of glycerophospholipids, and has been found in microorganisms, plants, and animal tissues. The phospholipid deacylating enzymes characterized so far from yeasts at the molecular level belong to the category of the PLB group. But, the enzymatic properties, such as molecular weight, optimum pH, effects of metal ions, substrate specificity, and biological significance vary widely depending on the species of yeast.

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Purification of PLB. Candida utilis was cultured aerobically in YEMP medium at 24°C for 72 h. After cultivation, the culture broth was centrifuged at 15,000 × g for 10 min at 4°C and the supernatant (one liter) was concentrated 10-fold by ultrafiltration using an Amicon YM-10 ultrafiltration membrane (Millipore, Bedford, MA, USA). Solid ammonium sulfate was added to 80% saturation (W/V) at 0°C. The pH of the solution was adjusted with 1 N aqueous solution of ammonia to 7.5. The solution was left overnight at 4°C, and then the precipitate was removed by centrifugation at 20,000 × g for 90 min at 4°C. The supernatant was put on a phenyl-Sepharose CL-4B column (2.5 × 44 cm) equilibrated with 50 mM Tris–HCl buffer (pH 7.5) containing 80% saturated ammonium sulfate. After the unretained materials were eluted out with the same buffer, elution was performed with 50 mM Tris–HCl buffer (pH 7.5) containing no ammonium sulfate. The flow rate was 0.4 ml per min. The fractions (6.5 ml each) were collected with a fraction collector, and their PLB activity was measured as described below. PLB activity was eluted only with the buffer containing no ammonium sulfate. Active fractions were concentrated to 4 ml with an Ultrafree-15 Centrifugal Filter Device (Millipore, Bedford, MA, USA).

The concentrated sample was put on a Hiloald 16/60 Superdex 200 pg column equilibrated with 50 mM Tris–HCl buffer (pH 7.5) containing 0.1 mM NaCl. Elution was performed with the same buffer at a flow rate of 1 ml per min at 3°C, and fractions (2 ml each) were collected. Active fractions were pooled, dialyzed against distilled water at 0°C, and then lyophilized.

The lyophilized sample was dissolved in 50 mM Tris–HCl buffer (pH 7.5) and put on a Mono Q column (HR5/5) equilibrated with the same buffer. The column was eluted at a flow rate of 1 ml per min with a linear gradient of NaCl (0–0.5 m) for 60 min. Active fractions were dialyzed against distilled water at 0°C and lyophilized. The lyophilized sample was stored at −20°C and used as purified enzyme.

Assay of PLB activity. A standard reaction mixture for alkaline pH consisted of 0.8 ml of 50 mM Tris–HCl buffer (pH 8.0) containing 1.6 mM phosphatidylcholine (PC), 0.25% (W/V) Triton X-100, and 0.1 ml of enzyme solution in the presence of metal ions. For standard assay of acidic pH, the reaction mixture consisted of 0.8 ml of 50 mM glycine–HCl buffer (pH 3.0) containing 1.6 mM PC, 0.25% Triton X-100, and 0.1 ml of enzyme solution. Total lipids in the reaction solution were extracted by the method of Bligh and Dyer.5) The amount of free fatty acid was measured with Iatroscan TH-10 as previously reported.5) One unit of enzyme activity was defined as the amount of enzyme needed to produce 1 μmol of free fatty acid per min at 30°C.

DNA sequencing of PLB from C. utilis. Reverse-transcriptional PCR analysis was used for DNA cloning of PLB. Total RNA was extracted from exponential growth phase cells according to the methods of Carlson and Botstein.16) One μg of total RNA was reverse-transcribed into cDNA using SuperScript II and oligo(dT) promoter. PCR amplification was performed using degenerate oligonucleotide primers designed on the basis of the highly conserved amino acid sequences found in S. cerevisiae PLB1,10) T. delbrueckii PLB,17) C. albicans PLB1,13) and C. albicans PLB2.14) The following primers were synthesized: forward primer, 5’-GGIGGIGGITYMNGCIGT-3’ (I = inosine, Y = C/T, M = A/C), based on amino acid sequence of GOGYRAM and reverse primer, 5’-TGRTRTCYTCIC-CICCRCT-3’ (R = A/G), based on DGGEDNQ. An approximately a 900 bp band of PCR product, corresponding to the expected size, was purified from agarose gel, cloned into pT7Blue T-vector (Novagen) and sequenced. The sequence of the DNA fragments was determined using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA).

Other analytical methods. The protein concentration of each purification step was estimated by the A280 nm and/or measured with a Micro BCA Protein Assay.
Reagent Kit (Pierce, Rockford, USA) with bovine serum albumin as a standard. SDS–polyacrylamide slab gel electrophoresis (SDS–PAGE) was performed by the method of Laemmli with 7.5% running gel. Gels were stained with Coomassie Brilliant Blue R-250 for proteins and periodic acid Schiff (PAS)-reagent for carbohydrates. The apparent molecular mass was estimated by SDS–PAGE.

The N-terminal amino acid sequence of purified PLB was determined using a PSQ-2 amino acid sequencer (Shimadzu, Kyoto, Japan).

Results

Purification of PLB

*C. utilis* was cultured aerobically in YEMPD medium at 24°C. The cell density of the culture medium reached a maximum at 72 h of culture. PLB activity released into the culture broth was measured at various growth stages. PLB activity increased in proportion to the cell density during incubation for 72 h, and further incubation did not result in an increase of PLB activity in the culture broth, so we attempted to purify the enzyme from 72 h culture broth. The culture broth was concentrated and fractionated with ammonium sulfate. The PLB was purified further by four column chromatography steps. The purification procedure is summarized in Table 1.

Molecular mass

On SDS–PAGE, the final sample migrated as a broad single band at a position corresponding to about 90–110 kDa. This protein was also stained strongly with PAS-reagent, indicating that PLB is a glycoprotein (Fig. 1).

N-Terminal amino acid sequence

The purified PLB was blotted onto PVDF membrane and subjected to N-terminal amino acid sequence analysis. The N-terminal amino acid sequence of this purified PLB was NH$_2$-X-X-P-S-G-S-Y-A-P-A-X-V-S-X-P-E-D-A-S-F-V-R-P-A-S-G-I-S-E (X represents amino acids which could not be specified during degradation cycles). The N-terminal of the matured PLB from *T. delbrueckii* was also undetected. 17)

Effects of pH and temperature

Figure 2 illustrates the pH optima for fatty acid release by PLB. The amount of fatty acid released by the enzyme reaction at pH 1.5 was determined by subtract-
ing the amount of fatty acid produced in the absence of the enzyme. Non-enzymatic production of fatty acid was observed at pHs from 2.0 to 9.0. PLB showed two pH optima, at pH 3.0 and pH 7.5. The enzyme was inactive at acidic pH levels from 5.0 to 6.5. The optimum temperature for PLB activity was 40°C when assayed at pH 3.0, but the activity was lost completely with incubation above 80°C (data not shown).

**Kinetics of enzyme reaction**

Figure 3 shows the reaction progress curves on hydrolysis of substrate. The hydrolysis reaction at pH 3.0 showed an almost linear rate up to 30 min incubation. The reaction progress curve at pH 8.0 was examined in the presence of Al³⁺, because PLB was activated most strongly by Al³⁺ at alkaline pH. The hydrolysis reaction at pH 8.0 increased after a certain lag time and showed a typical sigmoidal curve. The existence of a lag time and sigmoidal curve of hydrolysis at pH 8.0 have been observed also with PLBs from *T. delbrueckii* and *K. lactis*. During hydrolysis, the substrate was hydrolyzed without intermediate accumulation of lysophospholipid (Fig. 4). Therefore, this enzyme was classified as PLB according to the definition of McMurry and Magee and the proposal of Doi and Nojima.

**Substrate specificity**

Table 2 shows hydrolysis activity against various phospholipids. PLB hydrolyzed all phospholipids tested at acidic pH. Lyso-PC and PC were hydrolyzed more rapidly than other substrates. In contrast, hydrolysis at alkaline pH was specific to the phospholipids depending on metal ions. PLB hydrolyzed only lyso-PC and...
phosphatidic acid in the presence of Ca\textsuperscript{2+}, and PC was hydrolyzed in the presence of Al\textsuperscript{3+}. The enzyme from *K. lactis* also hydrolyzed PC most strongly in the presence of Al\textsuperscript{3+}.\textsuperscript{18} 14-kDa secretory PLA\textsubscript{2} and cPLA\textsubscript{2} have been reported to have activity only at pH 7.0–8.0 and usually to require Ca\textsuperscript{2+} for activity,\textsuperscript{21} but there is no report describing the effect of Al\textsuperscript{3+} on PLA\textsubscript{2} from animal cells.

**Effect of metal ions**

Table 3 shows the effects of metal ions on hydrolysis of PC. The activity at pH 3.0 was independent on metal ions and on added EDTA. Fe\textsuperscript{3+} and Hg\textsuperscript{2+} inhibited the enzyme activity. Other metal ions slightly stimulated the enzyme activity. On the other hand, the enzyme required the metal ions for activity at alkaline pH. The highest activation was observed with Al\textsuperscript{3+}. The optimum concentration of Al\textsuperscript{3+} for the activation of enzyme was 20 mM (Fig. 5).

**Acyltransferase activity**

When purified PLB from *C. utilis* was incubated with lyso-PC, a small but significant amount of concomitant PC was generated at pH 3.0, but not at pH 8.0 (Fig. 6). PLBs from microbes,\textsuperscript{22–24} plant cells,\textsuperscript{25} and animal cells\textsuperscript{26} have also been reported to convert lyso-PC to PC as a minor activity.

**Sequencing of the PLB gene**

The major PCR product with cDNA from *C. utilis* was approximately 900 bp. The fragment was subcloned to pT7Blue T-vector, and the nucleotide sequences of 10 inserts were determined. All 10 clones showed the same nucleotide sequence, and the amino acid sequence deduced from the nucleotide sequence of the fragment was highly similar to those encoded by the PLB genes from *S. cerevisiae*\textsuperscript{9} and *T. delbrueckii*,\textsuperscript{17} suggesting that the cloned fragment encoded part of the PLB gene of *C. utilis*. The sequence was thus extended downstream by the 3'RACE method and upstream by the 5'RACE method. The following gene-specific primers were synthesized for the RACE method: 3′RACE primer, 5′-CTGGATGAGGACTCGGACGAC-3′, and 5′RACE primer, 5′-CTGTAGCAGCCCACCTAGTCC-3′, based on the nucleotide sequence determined by the degenerate oligonucleotide primers. The amplified PCR products were purified from agarose gel, cloned into pT7 Blue T-vector, and sequenced. Finally we obtained 2,134 bp DNA and a protein consisting of 643 amino acids (Fig. 7). The molecular mass calculated from the deduced amino acid sequence was 70.73 kDa. The deduced amino acid sequence of the N-terminal region of the protein coincided thoroughly with the N-terminal sequence of purified PLB determined with a protein sequencer, except for some unspecified amino acids. We designated it *plb* of *C. utilis* (accession no., AB 114901).

**Table 3. Effects of Metal Ions on Phospholipase B**

<table>
<thead>
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<th>Metals (10 mM)</th>
<th>pH 3.0</th>
<th>pH 8.0</th>
</tr>
</thead>
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<tr>
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<tr>
<td>CaCl\textsubscript{2}</td>
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<tr>
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</tr>
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<tr>
<td>EDTA</td>
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</tr>
<tr>
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<td>20.0</td>
<td>0.0</td>
</tr>
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</table>

**Fig. 5. Effect of Aluminum Chloride (AlCl\textsubscript{3}) Concentration on Enzyme Activity.**

Phospholipase B activity was measured at pH 8.0 by the standard assay method as described in “Materials and Methods,” in the presence of various concentrations of AlCl\textsubscript{3}.
showed 55.2%, 53.0%, 52.5%, 43.5% similarity with those encoded by *T. delbrueckii* PLB, *S. cerevisiae* PLB1, *K. lactis* PLB, and *C. albicans* PLB1, respectively. The amino acid sequence had 16 possible N-glycosylation sites and 8 possible O-glycosylation sites. The 17 N-terminal stretches of amino acids must be a signal peptide sequence with a predicted cleavage site between amino acids 17 and 18, because the cDNA sequence encodes 17 amino acid residues that are absent from the mature protein. The sequence similarity in the signal peptide regions among the PLBs was lower than in other regions. The amino acid sequence encoded by *plb* of *C. utilis* contained one conserved serine (Ser-138) in the lipase consensus sequence (G-X-S-X-G) characteristic of the subtilisin family. Moreover, two conserved catalytic arginine and aspartic acid containing-regions (Arg-100 and Asp-386), which were confirmed by site-directed mutagenesis to be catalytically essential amino acids in the PLB from *K. lactis*, were also found in PLB from *C. utilis*. The C-terminal consisted of 16 hydrophobic amino acids, but did not appear to contain the typical motifs for glycosylphosphatidylinositol (GPI) anchor attachment. The C-terminal region of PLB was rich in serine residue. A serine rich region at the C-terminal moiety has been found in other PLBs from various species of yeast, but the significance of this serine rich region has not been clarified yet.

**Discussion**

In the present study, PLB was purified to homogeneity from the culture filtrate of *C. utilis*. The purified protein was a protein with an apparent molecular mass of 90–110 kDa by SDS–PAGE. The molecular mass calculated from the deduced amino acid sequence was about 70.73 kDa, and 16 potential N-glycosylation sites and 8 potential O-glycosylation sites were found. Therefore, *C. utilis* PLB is a glycoprotein. The carbohydrate moiety was calculated to occupy about 27–56% of the molecular mass. The broad protein band on SDS–PAGE is thought to be due to heterogeneity of the carbohydrate moiety linked covalently to the protein moiety of the enzyme (glycoform). The PLBs characterized in other species of yeast are also high molecular weight proteins and all known forms are highly glycosylated. Accordingly, high molecular weight and high glycosylation appear to be common characteristics of PLB from yeast, and a high carbohydrate content of PLB in yeast is probably important in the protection of enzyme activity against proteolytic degradation by proteolytic enzymes during the secretory process into the medium, as suggested in the PLB from *T. delbrueckii*.

The purified PLB from *C. utilis* catalyzed the hydrolytic cleavage of both acylester bonds of glycerophospholipids (PLB activity), lysophospholipids (lysophospholipase activity), and synthesis of phospholipids from

![Fig. 6. Thin-Layer Chromatography of the Reaction Products from Lysophosphatidylcholine.](image)
Phospholipase B from *Candida utilis*
lyso phospholipids (lyso phospholipase/transacylase activity), showing that these activities reside on the same protein. The same biochemical activities on a single enzyme protein were also found in PLBs from *Penicillium notatum,*2,22) *Cryptococcus neoformans,*28) *S. cerevisiae,*3,4) *Schizosaccharomyces pombe,*24) and *C. albicans.*23) The result that three enzymatic activities reside on a single protein appears to be a common property of PLB from microbes.

The most prominent characteristics of PLB from yeast were their pH optimum and the effects of metal ions on enzyme activities. The PLB activities in microorganisms such as *S. cerevisiae,* *S. pombe,* and *C. albicans* in plants, and in animal tissue, release fatty acids in a calcium-independent manner. Accordingly, PLB is considered to be a calcium-independent enzyme. Contrary to this, we found for the first time that PLB from yeasts, *T. delbrueckii* and *K. lactis,* showed activity in two pH regions, pH 2.5–3.5 and pH 7.0–8.0, but the activity at pH 7.0–8.0 required metal ions in the PLBs from both yeasts. The PLB purified in this study from *C. utilis* was also active in two pH regions, pH 2.0–4.0 and pH 7.0–8.5, and metal ions were essential for the activity at neutral to alkaline pH. Moreover, among cell homogenates prepared from 25 strains in 8 genera of yeast, 13 strains in 7 genera showed phospholipid deacylating activity at pH 8.0 in the presence of calcium ion.29) *S. cerevisiae* showed one pH optimum at acidic pH, as reported by Ichimasa et al.3) and Witt et al.4) But the commercial yeast *S. cerevisiae* is a strain with improved properties due to selection of colonies with higher durability or higher fermentation activity. Selection over a prolonged period might lead to a loss of certain inherent properties of phospholipid deacylating activity that are unfavorable to the durability of yeast cells. Therefore, some strains of *S. cerevisiae* are...
thought to be an improved yeast. This idea appears to be supported by our result that the experimental strains *S. cerevisiae* X2180 and *S. cerevisiae* S288C showed two pHs optima at acidic and alkaline pHs.\(^{20}\) Moreover, disruption of the PLB gene of *T. delbrueckii*, which had two pH optima, increased durability to the same level as *S. cerevisiae*.\(^{12}\) These results suggest that the property of hydrolyzing the fatty acid ester bond of phospholipids at two pH regions is not specific to PLBs from *T. delbrueckii* and *K. lactis*, but is rather a general property of PLB in wild strains of yeast. The effects of metal ions on enzyme activities at alkaline pH and acidic pH were similar as between *C. utilis* and *K. lactis*. Both PLBs were activated most strongly by \(\text{Al}^{3+}\) and \(\text{Fe}^{3+}\) at alkaline pH. Divalent metal ions such as \(\text{Mn}^{2+}, \text{Cu}^{2+}, \text{Fe}^{2+}, \text{Co}^{2+}\), and \(\text{Zn}^{2+}\) were less effective for the activation of both enzymes. The purified PLB from a yeast, *Rhodotorula minuta*, was also activated by \(\text{Fe}^{3+}\) and \(\text{Al}^{3+}\) most strongly (unpublished data). These results suggest that trivalent \(\text{Fe}^{3+}\) and \(\text{Al}^{3+}\) are the most preferential metal ions for yeast PLB activity at pH from neutral to alkaline. But the concentration of \(\text{Fe}^{3+}\) and \(\text{Al}^{3+}\) necessary for the activation of PLB is unphysiological. Therefore, it is now unknown whether the alkaline activity of *C. utilis* PLB has a significant function in cell physiology. Contrary to this, \(\text{Fe}^{3+}\) inhibited activity of both PLBs from *C. utilis* and *K. lactis* at acidic pH. This inhibition by \(\text{Fe}^{3+}\) was also observed for PLBs from two other yeasts, *S. cerevisiae* and *S. Pombe*. The inhibition by \(\text{Fe}^{3+}\) in acidic pH might be due to the change in micelle structure of the substrate owing to high concentrations of \(\text{Fe}^{3+}\), because phospholipase activity depends greatly on the state of the substrate.

Two forms of PLB, a soluble form and a membrane-bound form, have been found and isolated from two species of yeasts, *S. cerevisiae*\(^{6,4}\) and *T. delbrueckii*.\(^{5,30}\) The soluble form was isolated from the culture broth, and the membrane-bound form from the cell membrane fraction after solubilization with detergent. The two forms showed similar enzymatic properties. In genetic studies, PLB1 from *S. cerevisiae* consisted of 664 amino acids\(^{8}\) and PLB from *T. delbrueckii* consisted of 649 amino acids.\(^{17}\) Both PLBs contained the sequence KKNAG or KKNAA in the C-terminal region of the enzyme, to which a GPI anchor was attached. The membrane-bound form then is an enzyme to which a GPI anchor is attached, and the soluble form might be a secreted enzyme that does not attach a GPI anchor during processing. The PLB from *C. utilis* was a little shorter in the C-terminal region than those of *S. cerevisiae* or *T. delbrueckii*, and contained no signal of GPI anchor attachment. In fact, purified PLB from *C. albi cans* and *K. lactis* are all soluble PLB, and no membrane-bound form has been found in either yeast. The biological significance of the fact that *S. cerevisiae* and *T. delbrueckii* have a signal to which a GPI anchor is attached has not yet been made clear.

Yeast PLB appears to be a rare enzyme, because a single protein contains three catalytic activities, phospholipase B activity, lysophospholipase activity, and lysophospholipase/transacylase activity, and two optimum pH ranges. The unique enzymatic properties will become more clear by analyzing the three-dimensional structure of the protein.

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**References**


