High-Level Secretory Production of Phospholipase A$_1$ by *Saccharomyces cerevisiae* and *Aspergillus oryzae*

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Phospholipase A$_1$ (PLA$_1$) is a hydrolytic enzyme that catalyzes the removal of the acyl group from position 1 of lecithin to form lyssolecithin. The PLA$_1$ gene, which had been cloned from *Aspergillus oryzae*, was expressed in *Saccharomyces cerevisiae* and *A. oryzae*. Through the modification of the medium composition and the feeding conditions of substrate, the production level of PLA$_1$ by *S. cerevisiae* was increased to a level fivefold higher than that indicated in a previous report. In the case of *A. oryzae*, introduction of multicopies of PLA$_1$ expression units, and the morphological change from the pellet form to the filamentous form were effective for the enhancement of PLA$_1$ production. We succeeded in producing 3,500 U/ml of PLA$_1$ using an industrial-scale fermentor.

Key words: phospholipase $A_1$; *Saccharomyces cerevisiae*; CPY; *Aspergillus oryzae*; mycelial morphology

Lyssolecithin is preferentially used in food technology, in animal feed products, and in cosmetics and pharmaceutical preparations as a surfactant.‡ Industrially, lyssolecithin is produced by enzymatic hydrolysis of lecithin. The most common enzyme used is pancreatin prepared from porcine pancreas. However, the use of pancreatin has certain disadvantages, such as its limited supply, its high cost and its handling difficulty. Therefore, a substitute enzyme in industrial lyssolecithin production has been sought.‡ Phospholipase A$_1$ (PLA$_1$) is the active component of pancreatin. Although secretory production of porcine and bovine pancreatic PLA$_1$ was done in *Saccharomyces cerevisiae* and *Aspergillus niger*, the secretion levels were only up to 10 mg/l.‡‡ To improve industrial lyssolecithin production, we considered the application of phospholipase A$_1$ (PLA$_1$), which is an enzyme catalyzing the removal of the acyl group from position 1 of lecithin to form lyssolecithin. On the basis of this strategy, the genomic DNA and cDNA encoding PLA$_1$ from *Aspergillus oryzae* were cloned by Watanabe et al.‡ They also succeeded in the secretory production of PLA$_1$ by the *S. cerevisiae* ssl1 mutant KS58-2D, a soluble vacuolar proteases missorting mutant,* as the host and a prepro-sequence of carboxypeptidase Y (CPY) as the secretory signal. However, the production level was 7.7 U/ml, which corresponded to 3.9 mg-protein/l according to its specific activity. Secretion of a large amount of PLA$_1$ into the culture broth is highly desirable from an industrial point of view. In this paper, to increase the production level of PLA$_1$ by *S. cerevisiae*, the medium composition and the feeding conditions of substrate were investigated. We also confirmed that the prepro-sequence of CPY was functional as a secretory signal in strain KS58-2D. Furthermore, we described the expression of the PLA$_1$ gene in *A. oryzae*. Due to the gene dosage effect and the improvement of mycelial morphology, we succeeded in enhancing the production of PLA$_1$ on an industrial scale.

Materials and Methods

*Strains and plasmids.* *S. cerevisiae* KS58-2D (MA-Ta ssl1 leu2 his3 ura3 gal80) was used for the gene expression and secretion of PLA$_1$. KS58-2D is a soluble vacuolar proteases missorting mutant derived from a

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Abbreviations: PLA$_1$, phospholipase $A_1$; CPY, carboxypeptidase Y; ssl1, super-secretion of lysozyme; CS, completely synthetic; vvm, volume per volume per minute; PCV, packed cell volume

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cross between KS51-3C and KSM-169. A. oryzae strains used in this study were M-2-3 (argB') and niaD300 (niaD'), which were isolated by Gomi et al. E. coli JM109 was used as the host for the propagation and manipulation of plasmids. Multicopy plasmid pCY339 (Fig. 1) was described previously. Multicopy plasmid pRAV601 (Fig. 1), in which PLA, cDNA was inserted between the GAL10 promoter and the 2-μm plasmid FLP terminator, was constructed from pCY303 and pRAV885. The plasmids for PLA, expression in A. oryzae, pGAP, pGAP, and pAAP (Fig. 2), were constructed from pUNG, pMARG, and pTaex3, respectively, which were constructed by Gomi et al. and pRAV885. In plasmids pGAP and pGAP, PLA, cDNA was inserted between the glaA promoter and the amyB terminator. In plasmid pAAP, PLA, cDNA was inserted between the amyB promoter and the amyB terminator.

Medium and cultivation. For the cultivation of yeast, a completely synthetic (CS) medium containing a vitamin mixture (2–10,000 μg/l), amino acid mixture (20–400 mg/l), and trace elements (40–500 μg/l) was prepared in medium composition described previously, and was further supplemented with 2% glucose, 2% galactose, 0.5% (NH₄)₂SO₄, 0.1% MgSO₄·7H₂O, 0.01% NaCl, 0.01% CaCl₂·2H₂O, 0.19% KH₂PO₄, 0.75% K₂HPO₄, and 0.5% BSA. Preculture in a 200-ml Erlenmeyer flask containing 20 ml of CS medium was done at 26°C for 3 days on a rotary shaker (210 rpm). After 1 ml of preculture broth was inoculated into 100 ml of fresh CS medium in a 500-ml Erlenmeyer flask, yeast cells were cultivated at 28°C for 7–11 days on a rotary shaker (210 rpm). The minimal medium for A. oryzae was Czapek-Dox (CD) medium consisting of 0.3% NaNO₃, 0.2% KCl, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, 0.002% FeSO₄·7H₂O, and 2% sucrose. DPY medium [2% dextrin, 1% polyethylene (Nihon Seiyaku), 0.5% yeast extract (Difco), 0.5% KH₂PO₄, 0.05% MgSO₄·7H₂O] was used for the production culture. After a loop of conidia was inoculated into 80 ml of DPY medium in a 500-ml Erlenmeyer flask, A. oryzae was cultivated at 26°C for 6–12 days on a rotary shaker (210 rpm). In a scale-up study, the first
and second precultures were carried out at 26°C for 4 days. The production culture with 5% of the inoculum in a 30-liter fermentor or a 500-liter fermentor containing 18 or 300 liters of production medium, respectively, was conducted at 26°C, 0.5 vvm of aeration, and 0.1 MPa of internal pressure for 5 days. Dissolved oxygen was maintained at about 5 ppm by changing the agitation speed.

**Genetic methods.** Standard procedures were followed for general DNA manipulations. Yeast transformation was carried out using the lithium-acetate method. The transformation of *A. oryzae* was done by the method of Gomi et al.

**Preparation of cell-free extract.** To measure intracellular enzymatic activity, cell-free extracts were prepared as follows. After washing with water and 100 mM Tris-HCl buffer (pH 7.5), the cells were resuspended in the same buffer and disrupted for 3 min with glass beads by a cell homogenizer (B. Braun Melsungen AG). The suspension was centrifuged at 15,000 rpm for 10 min at 4°C, and the resulting supernatant was used for the enzyme assays.

**Measurement of PLA activity.** The activity of PLA was measured photometrically as follows. A 0.1-ml portion of the assay sample was added to a mixture [0.5 ml of 4% Triton X-100 containing 2% SLP-white (Tsuru-licethin), 0.05 ml of 0.1 M CaCl₂ and 0.25 ml of 0.2 M sodium acetate buffer (pH 4.0)]. The reaction mixture was incubated at 37°C for 10 min followed by the addition of 0.1 ml of 1 N HCl to stop the reaction. The concentration of released fatty acid in 20 μl of the reaction mixture was measured by a Detaminar kit (Kyowa Medix) according to the suppliers' instructions. A quantity of 1 U was defined as the amount of activity that produces 1 μmole of fatty acid in 1 min.

**Purification of PLA from the culture broth of S. cerevisiae KS58-2D/pCY339.** Culture supernatant of 300 ml was concentrated to 20 ml using Centriprep-10 (Amicon). The concentrated sample was chromatographed on POROS2-HQ (Perceptive Biosystems) equilibrated with 20 mM Tris-HCl buffer (pH 7.5). PLA was eluted with a 0-500 mM NaCl gradient in the same buffer. The fractions in which PLA activity was detected were collected and concentrated to 0.5 ml. The partially purified sample by HPLC was then electrophoresed on sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE).

**N-terminal amino acid sequencing.** Proteins separated by SDS-PAGE were electroblotted onto a PVDF membrane. The proteins were stained with Coomassie Brilliant Blue R250 (Fluka), and the relevant band was excised. The filter sample was subjected to automatic Edman degradation using an Applied Biosystems 475A protein sequencer.

**Deglycosylation.** Proteins were deglycosylated with endoglycosidase H (Boehringer Mannheim) under denaturing conditions according to the suppliers' instructions.

**Western blot analysis.** Proteins separated by SDS-PAGE were electroblotted onto a PVDF membrane and developed with a rabbit anti-PLA₁ and horseradish peroxidase-conjugated goat anti (rabbit IgG) antibody (Bio-Rad), according to the suppliers' instructions.

**Southern blot analysis.** Genomic DNA of *A. oryzae* was purified according to the method of Yelton et al. The genomic DNA was digested with restriction endonuclease and the fragments were separated by 1% agarose gel electrophoresis. Following transfer onto a Hybond-N+ nylon membrane (Amer sham) by capillary action, the blot was hybridized with the 0.42-kbp HindII fragment encoding a part of PLA₁. The probe was labeled with digoxigenin-11-dUTP and a DIG nucleic acid detection kit was used for the detection according to the suppliers' instructions (Boehringer Mannheim).

**Results**

**Effects of signal sequence on the secretory production of PLA₁.**

Watanabe et al., previously reported that S. cerevisiae KS58-2D/pCY339 secreted PLA₁ into the culture medium. In the multicopy plasmid pCY339, the mature PLA₁ cDNA was fused to the gene encoding the prepro-sequence of CPY with two additional amino acids in the C-terminus (Fig. 1). To confirm that the prepro-sequence of CPY was functional as a secretory signal in the ssfI mutant KS58-2D, the N-terminal amino acid sequence of PLA₁ secreted by KS58-2D/pCY339 was analyzed. After 9 days of cultivation of KS58-2D/pCY339 in CS medium, the PLA₁ secreted in the culture supernatant was purified. A sample of the portion partially purified by HPLC was put on SDS-PAGE (Fig. 3), and the band corresponding to PLA₁ was excised and analyzed. The N-terminus of extracellular PLA₁ had the sequence Lys-Ile-Asp-Val-Ser-Ser-Leu-Leu-Asn, which coincided with the original N-terminus of the mature PLA₁ except for two additional amino acids in the N-terminus. As shown in Table 1, PLA₁ was not detected in the culture supernatant of KS58-2D/pAV601, in which the original prepro-sequence of PLA₁ was used as the secretory signal. These results demonstrate that the prepro-sequence of CPY is effective for secretory production of PLA₁ by ssfI mutant KS58-2D.
High-Level Secretory Production of Phospholipase A₁

Table 1. Comparison of PLÅ₁ Production in Various Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>PLÅ₁ (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Extracellular</td>
</tr>
<tr>
<td>KS58-2D</td>
<td>ND</td>
</tr>
<tr>
<td>KS58-2D/pCY339</td>
<td>10.0</td>
</tr>
<tr>
<td>KS58-2D/pRAV601</td>
<td>ND</td>
</tr>
</tbody>
</table>

Yeast cells were cultivated for 9 days in CS medium. The culture supernatants and cell extracts were used for measuring PLÅ₁ activity. ND, not detected.

Table 2. Effects of Medium Composition on PLÅ₁ Production

<table>
<thead>
<tr>
<th>K₂HPO₄ (%)</th>
<th>BSA (%)</th>
<th>Histidine (mg/l)</th>
<th>PLÅ₁ (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.75</td>
<td>0.5</td>
<td>20</td>
<td>5.9</td>
</tr>
<tr>
<td>0</td>
<td>0.5</td>
<td>20</td>
<td>14.2</td>
</tr>
<tr>
<td>0</td>
<td>2.0</td>
<td>20</td>
<td>20.9</td>
</tr>
<tr>
<td>0</td>
<td>2.0</td>
<td>60</td>
<td>29.4</td>
</tr>
</tbody>
</table>

Strain KS58-2D/pCY339 was cultivated for 7 days in CS medium. The culture supernatants were used for measuring PLÅ₁ activity.

Fig. 3. SDS-PAGE of Secreted PLÅ₁ from Strain KS58-2D/pCY339.

Strain KS58-2D/pCY339 was cultivated for 9 days in CS medium. The secreted PLÅ₁ in the culture supernatant was purified by ion-exchange chromatography. The fractions containing PLÅ₁ activity were electrophoresed. The proteins were stained with CBB. Lane 1, standard proteins with sizes indicated (in kDa); lane 2, the partially purified sample by HPLC. The arrow indicates PLÅ₁ which was confirmed by Western blot analysis using rabbit anti-PLÅ₁ antibodies.

**Improvement of the productivity of PLÅ₁ in S. cerevisiae**

When grown in CS medium, KS58-2D/pCY339 produced about 7.7 U/ml of extracellular PLÅ₁. To increase the PLÅ₁ production, we investigated the effects of media composition (potassium phosphate, BSA, and histidine). CS medium contains 0.75% dipotassium hydrogenphosphate. The omission of dipotassium hydrogenphosphate caused an increase of the PLÅ₁ production level (Table 2). We added BSA as a carrier protein to the culture medium to protect the secreted PLÅ₁ against extracellular proteases as described for the secretory production of CPY. When we did not add BSA to the culture medium, the amount of secreted PLÅ₁ was significantly decreased (data not shown). As shown in Table 2, the increase of BSA concentration was effective for the production of PLÅ₁. Furthermore, since strain KS58-2D is an auxotroph of histidine, the addition of histidine activated cell growth, resulting in increased secretion of PLÅ₁. On the basis of these results, we decided on the composition of the production medium (CSP medium). CSP medium contains the constituents of CS medium, except for dipotassium hydrogenphosphate, in addition to BSA (2%) and histidine (60 mg/l).

We previously described that the use of an inducible promoter, such as the GAL10 promoter, was preferred to a constitutive promoter since the expression phase was separated from the growth phase. CPY production using the GAL10 promoter was higher than that of the constitutive ENO1 or ADH1 promoter. Also, the highest PLÅ₁ production level was obtained when it was expressed under the control of the GAL10 promoter (data not shown). Strain KS58-2D is a gal80 mutant derived from strain K4. We previously reported that the expression level of the gene, which is under the control of the GAL10 promoter, increased in an S. cerevisiae gal80 mutant grown in a medium using ethanol as the sole carbon source. We also showed that ethanol feeding was effective for the increase of productivity in the gal80 mutant KS58-2D using the GAL10 promoter system. On the basis of these results, we investigated the effect of the addition of ethanol on the cultivation of KS58-2D/pCY339. The results are shown in Table 3. When KS58-2D/pCY339 was cultivated for 8 days in CSP medium, the production level of PLÅ₁ was about 30 U/ml. When ethanol was added to the 8-day culture broth, and cultivation was extended for three more days, the cells consumed ethanol for growth and the production level of PLÅ₁ increased 1.3-fold higher than that of the control culture (no addition). These results indicate that ethanol feeding was also effective for the improvement of PLÅ₁.
production. By modifying the medium composition and employing ethanol feeding, we succeeded in increasing the secretion of PLA₁ to about 39 U/ml.

**Overproduction of PLA₁ by A. oryzae**

The gene encoding PLA₁ was cloned from A. oryzae. 21 A. oryzae has been known to secrete a vast quantity of proteins. 21 To overproduce PLA₁ by A. oryzae, we constructed the PLA₁ expression plasmids (Fig. 2) and introduced them into A. oryzae M-2-3 or niaD300. The resultant transformants, designated as niaD300/pGNP, M-2-3/pGAP, and M-2-3/pAAP-4, secreted PLA₁ in the culture medium. When grown on DPY medium, the transformants produced 34.8-747 U/ml extracellular PLA₁, which was 60- to 7,500-fold higher than that of the host strain (Table 4). Figure 4(A) shows the analysis by SDS-PAGE of the culture supernatant of M-2-3/pAAP-11. The level of PLA₁ was almost the same as that of α-amylase, which is known to be secreted in large quantities. There were two kinds of PLA₁ (36 and 37 kDa). These had the same N-terminal sequence, Asp-Val-Ser-Ser-Leu-Leu-Asn-Asn-Leu, which coincided with the original N-terminus of the mature PLA₁. 21 Deglycosylation and Western blot analysis [Fig. 4(B)] also showed that they had the same molecular weight, suggesting that the difference in molecular weight was due to the degree of glycosylation.

**Effects of the copy number of the PLA₁ gene on the production level**

To estimate the copy number of the PLA₁ gene in the transformants, Southern blot analysis was done. As shown in Fig. 5, strong signals at 0.42-kbp corresponding to the 0.42-kbp fragment of the PLA₁ cDNA were observed in transformants M-2-3/pGAP, M-2-3/pAAP-4, and M-2-3/pAAP-11. About 15 or 20 copies of the PLA₁ gene were integrated into the chromosome in M-2-3/pGAP or M-2-3/pAAP, respectively. On the other hand, niaD300/pGNP showed the same intensity of 0.42-kbp and 0.54-kbp signals, which originated from the PLA₁ cDNA and the chromosome, respectively. This indicates that a single copy of plasmid pGNP was integrated into the chromosome. Figure 5 also shows that the production level of PLA₁ is proportionate to the copy number of the PLA₁ gene in the transformant.

**PLA₁ production in pilot scale and industrial-scale fermentation**

In the cultivation using DPY medium, M-2-3/
Fig. 5. Southern Blot Analysis of A. oryzae Transformants. Genomic DNA of 10 μg was digested by HindII and put onto a 1% agarose gel. Southern blot analysis was done, as described in Materials and Methods. Lane 1, standard DNAs with sizes indicated (in kb); lane 2, M-2-3, host strain; lane 3, M-2-3/pAAP-4; lane 4, M-2-3/pAAP-11; lane 5, M-2-3/pGAP; lane 6, niaD300, host strain; lane 7, niaD300/pGNP.

pGAP, M-2-3/pAAP-4, and M-2-3/pAAP-11 formed rigid pellets. As the cultivation proceeded, these pellets aggregated to each other and developed into a huge pellet (3-5 cm in diameter). Since we considered that the huge pellet was not suitable for PLAI production or the scale-up, we tried to reduce the size of the pellet or change to a filamentous form. Though Metz et al. have reported that several factors affect mycelial morphology,28 increase of concentration of nitrogen source and carbon source was sufficiently effective for the morphological change of the transformants. When 2% soybean meal and 6% dextrin was added to DYP medium (DS medium in Table 4), filamentous growth was observed in the cultivation of M-2-3/pGAP, M-2-3/pAAP-4, and M-2-3/pAAP-11. The increase of the nitrogen source and carbon source also enhanced the production level of PLAI, and the secretion of PLAI reached about 4,000 U/ml in M-2-3/pAAP-4 and M-2-3/pAAP-11. Figure 6 shows the course of PLAI production in a 30-l fermentor. Cells grew utilizing dextrin and secreted PLAI into the medium. When the concentration of dextrin in the medium decreased to about 13 g/l, cell growth and PLAI production remained constant. The final concentration of secreted PLAI reached about 3,500 U/ml, which corresponded to 1.8 g-protein/l according to its specific activity. In several runs in a 30-l fermentor, about 3,500 U/ml of PLAI was secreted constantly. Therefore, the cultivation method could be scaled up using an industrial-scale fermentor.

Discussion

Through the modification of the medium composition in the PLAI-production culture by S. cerevisiae, the production level increased from 5.9 to 29.4 U/ml (Table 2). We also applied ethanol feeding on the basis of the characteristics of the gal80 mutant KS58-2D and the GAL10 promoter system, as previously reported.20 The production level of PLAI reached 38.7 U/ml (Table 3). This is approximately fivefold higher than that in a previous report.25 We also showed that the prepro-sequence of CPY was functional as a secretory signal in stll mutant KS58-2D. Since the prepro-sequence was entirely cleaved off, we speculate that protease B, which is involved in the processing of proCPY to mature CPY,21 is concerned with the maturation of PLAI. We consider that this secretory production and the method shown in this study, such as the modification of the medium composition and ethanol feeding, are effective and can be applied for high-level production of other important heterologous proteins.

Since the gene encoding PLAI was cloned from A. oryzae,21 we consider that A. oryzae is suitable as a host to express the PLAI gene. We obtained 4 transformants, niaD300/pGNP, M-2-3/pGAP, M-2-3/pAAP-4, and M-2-3/pAAP-11. These transformants showed a considerably higher production level than that of the host strain (Table 4). It has been reported that the transformation system based on the nitrate reductase gene (niaD) has highly frequent homologous integration events and introduces single heterologous genes into the niaD locus.24,25 We consider that single-copy and homologous integration events...
occurred and generated niaD300/pGNP, as reported. On the other hand, we speculate that the transformation of M-2-3 with the plasmid carrying the argB gene gave random and multicopy integrants, because multiple bands were observed as shown in Fig. 5. Compared with the PLA₁ production between the transformants derived from M-2-3 and that from niaD300, introduction of multicopies of the expression units resulted in an increase in the production level.

We used the amyB and glaA promoters, which are strong promoters in A. oryzae, for the expression of the PLA₁ gene. The expression of the amyB and glaA genes is regulated by a similar mechanism at the transcriptional level, and the expression levels of these genes are almost the same. However, even taking into consideration the number of copies of the PLA₁ expression unit, the PLA₁ production level with the amyB promoter system was considerably higher than that with the glaA promoter system. We consider that the site of integration may affect the expression of the genes introduced.

In a PLA₁-producing culture, mycelial morphology affected the production level. When M-2-3/pGAP, M-2-3/pAAP-4, or M-2-3/pAAP-11 were cultivated in DPY medium, huge pellets (3–5 cm in diameter) were formed. It has been reported that when the pellet exceeds a critical radius, reduced growth occurs due to substrate limitation in the dense core of the pellet, resulting in reduction in productivity. Hosobuchi et al. reported that the use of a high-concentration medium caused the morphology of Penicillium citrinum to change from the pellet form to the filamentous form. We were also able to improve the morphology of A. oryzae M-2-3/pGAP, M-2-3/pAAP-4, and M-2-3/pAAP-11 by increasing the nitrogen source and carbon source. Due to the morphological change and increase in the nitrogen source and carbon source, we succeeded in the production of PLA₁ to about 4,000 U/ml, which corresponded to 2 g-protein/l according to its specific activity. This is a higher level of protein production when filamentous fungi are used as the host organism. We were also able to produce 3,500 U/ml of PLA₁ using an industrial-scale fermentor. We consider that investigation of the carbon source that can induce the amyB promoter, such as maltose, and subsequent feeding of the carbon source will lead to a further increase of PLA₁. We believe this work will contribute to the improvement of the industrial lyssolecithin production.

References