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Secretory expression of a phospholipase A_2 from *Lactobacillus casei* DSM20011 in *Kluyveromyces lactis*

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The *pla2* gene encoding a phospholipase A_2 (EC 3.1.1.4) of *Lactobacillus casei* DSM20011 was cloned and expressed in the yeast *Kluyveromyces lactis* GG799 successfully for the first time. The structural *pla2* gene fused in frame with the *K. lactis* secretion signal α-mating factor was integrated into the *LAC4* locus and expressed under the control of the *LAC4* promoter. $sPLA_2$ activity was detected in the culture supernatant during shake flask culture of *K. lactis*/pKLAC1-*pla2*. In comparison with the control strain *K. lactis*/pKLAC1, SDS-PAGE analysis revealed a 17-kDa recombinant protein band in *K. lactis*/pKLAC1-*pla2*, which was consistent with the predicted molecular weight of the mature protein. Real-time quantitative PCR analysis indicated that the copy number of the integrated *pla2* gene ranged from 2 to 6 and positively correlated with $sPLA_2$ activity. When the inducer galactose was used as the carbon source, the $sPLA_2$ activity in the culture supernatant of the recombinant that harbored six *pla2* gene copies reached 1.96 ± 0.15 U/mL. The influence of the culture composition and conditions on the recombinant $sPLA_2$ activity in shake flask culture were also studied. When the recombinant was cultured at 30°C in a YPD medium culture volume of 70 mL in a 250-mL shake flask with an initial pH of 7.0, the $sPLA_2$ activity reached 2.16 ± 0.18 U/mL.

[**Key words:** Secretory phospholipase A_2; *Lactobacillus casei*; Heterologous expression; Real-time quantitative PCR; Copy number; *Kluyveromyces lactis*]

Phospholipase A_2s (PLA_2s, EC 3.1.1.4) are lipolytic enzymes that catalyze the hydrolysis of the 2-acyl ester bond of 1,2-diacyl-3-*sn*-phospholipids, yielding lysophosphatidylcholine and free fatty acids (1). PLA_2s are widely studied and can be broadly divided into three categories: Ca^{2+}-dependent secreted PLA_2s ($sPLA_2$s), Ca^{2+}-dependent cytosolic PLA_2s ($cPLA_2$s), and Ca^{2+}-independent cytosolic PLA_2s ($iPLA_2$s) (2). They can be used extensively in many industrial processes, such as the production of lysolecithin, and even play key roles in bread making, the egg yolk industry, and enzymatic degumming of edible oils (3).

The most extensively studied PLA_2s are the eukaryotic secretory PLA_2s that are found abundantly in pancreatic tissues and various animal species including snakes and insects. The secreted PLA_2s are characterized by their low molecular mass (14–19 kDa) and millimolar calcium requirement (4).

The heterologous expression of PLA_2 is of considerable interest for its pharmacological functions and the enzymatic activity of hydrolysis of the phospholipids. To date, the most extensive studies on PLA_2 have been employed in the area of cloning and expressing the eukaryotic secretory PLA_2 genes (5–7). Mammalian PLA_2s, such as porcine and bovine, have been expressed in the host *Saccharomyces cerevisiae* (8,9). Attempts have been made to express the eukaryotic PLA_2 gene in *Aspergillus* by mutating the cysteines to increase the secretion (10). Researchers have also tried to express the eukaryotic PLA_2 in *Escherichia coli* with a fusion protein, but this approach requires the processing of protease to obtain a mature PLA_2 (11). Although yeast, fungal and bacteria expression systems can be used to produce the eukaryotic PLA_2 by fermentation, there are some difficulties in these systems, such as the structure of eukaryotic PLA_2, which has many disulfide bonds that may influence the correct folding of the protein in *E. coli*, resulting in the formation of an inclusion body (12). Some studies have succeeded in generating soluble expression of the eukaryotic PLA_2 as a fusion protein, but attempts to cleave the fusion protein were unsuccessful (11). Additionally, the recombinant PLA_2 may be toxic to the *E. coli* and yeast because of its lipolytic activity (13,14). However, few studies have investigated the microbial secretory PLA_2. The first microbial secreted Ca^{2+}-dependent PLA_2 was identified in the mycorrhizal ascomycete *Tuber borchii*, and then some homologous PLA_2s were found in fungi (15). $sPLA_2$s gene from the filamentous ascomycete *Aspergillus oryzae* were cloned and expressed in *E. coli* and extracted from inclusion bodies (16). The first phospholipase A_2 identified in prokaryotes was from *Streptomyces violaceoruber*. After identification, the PLA_2 gene was artificially synthesized with

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TABLE 1. Oligonucleotide primers used in this study.

Primers[a]	Nucleotide sequence (5′ to 3′)
pla2-F	CCG**CTCGAG**AAAAGAACGACTAAGACTGAGTTTAAT
pla2-R	GGA**AGATCT**TCAACCACCAAACAACTTATATG
P1-F	TACCGACGTATATCAAGCCCA
P2-R	ATCATCCTTGTCAGCGAAAGC
P3-F	CAGTGATTACATGCATATTGT
GAPDH-F	CCG**CTCGAG**GGTTTCGGTAGAATCGGTAG
GAPDH-R	GGA**AGATCT**ACTCTTGAAGAGTAACCG
RT-GAPDH-F	GAAGCTTTGATGACTACCGTTC
RT-GAPDH-R	CTTTACCGACAGCCTTAGCAG
RT-pla2-F	ACAATGTTGATCCCGTTGTCC
RT-pla2-R	CCGCAATAATTCCCATACACCAG

Sequences in bold are restriction enzyme sites.
[a] F and R indicate the forward and reverse primers, respectively. P1, P2 and P3 refer to the manufacturer's instruction (24).

codon optimization and was expressed extracellularly in a recombinant strain of *E. coli* (17,18).

A variety of prokaryotic and eukaryotic organisms have been employed as heterologous hosts in attempts to express $sPLA_2$. Compared with the *E. coli* expression system, the *Kluyveromyces lactis* system provides generous quantities of soluble protein and avoids renaturation from inclusion bodies. Production of a foreign protein via the eukaryotic secretory pathway ensures high fidelity of folding, assembly and modification processes required for biological activity. The yeast *K. lactis* has been studied for decades and has a well-established track record of safe use in various food industry applications. Enzymes from *K. lactis* have GRAS (generally regarded as safe) status, permitting their use in various food and feed applications (19). In the food industry, *K. lactis* is used for the production of prochymosin (20). Additionally, high-level secretion of recombinant serum albumin and interleukin-1β can be achieved using this expression system in pharmaceutical research (21,22).

In this study, with a view toward applications in the food and pharmaceutical industries, we cloned and expressed the gene encoding $sPLA_2$ from *Lactobacillus casei* DSM20011 in *K. lactis* GG799. To our knowledge, this is the first report on the heterologous expression of prokaryotic microbial $sPLA_2$ in *K. lactis*. Then, we analyzed the correlation between the $sPLA_2$ activity and the integrated *pla2* gene copy number. Different cultivation conditions were also investigated to study the effects on the production of heterologous $sPLA_2$ in *K. lactis*.

MATERIALS AND METHODS

Strains, vectors, and culture media The *L. casei* DSM20011 strain was used as the source of the *pla2* gene and was maintained on Man Rogosa Sharpe (MRS) medium at 30°C. The *E. coli* strain JM109 was used as the cloning host and was grown in Luria–Bertani (LB) medium containing 100 μg mL^{-1} ampicillin at 37°C. The pMD19-T Simple Vector (Takara Biotechnology Co., Dalian, China) was used as the cloning vector. A wild-type haploid *K. lactis* strain GG799 (New England Biolabs Inc., MA, USA) was used as the host for the heterologous expression and was grown in YPD medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose) at 30°C. YP medium containing 20 g/L galactose (YPG), YP medium containing 20 g/L lactose (YPL), YP medium containing 10 g/L glucose and 10 g/L lactose (YPDL), YP medium containing 10 g/L glucose and 10 g/L galactose (YPDG), and YPD medium containing 10 g/L casamino acids (YPDC) were used to cultivate the recombinant yeast *K. lactis*. The plasmid used for this study was the *E. coli-K. lactis* shuttle vector pKLAC1 (New England Biolabs Inc.). Nitrogen-free yeast carbon base (YCB, Sigma Inc.) was used to screen the *K. lactis* transformants. Media were solidified with 2% agar when needed.

Chemicals L-alpha-Lecithin was purchased from Acros. DNA restriction enzymes, *Taq* DNA polymerase, T4DNA ligase, and RNase A were supplied by Fermentas and used according to the manufacturer's recommendations. SYBR Premix ExTaqII was purchased from TaKaRa Biotechnology Co. All other chemicals were analytical grade. All aqueous solutions were prepared with deionized and distilled water.

Construction of the expression plasmid and yeast transformation Genomic DNA was isolated from *L. casei* DSM20011 and used as the template for the amplification of the gene encoding PLA_2 (GenBank accession No. 9458037). The *pla2* gene was amplified with exclusion of its signal peptide by PCR using a forward primer and reverse primer (Table 1). Amplification resulted in an XhoI restriction site at the 5′ end and a BglIIsite at the 3′ end. The resultant PCR product was digested with XhoI and BglII and then ligated to the plasmid pKLAC1 that had been digested with the same restriction enzymes to create the expression vector pKLAC1-*pla2* in which the PLA_2 expression cassette was under the direction of the *K. lactis* signal α-mating factor and under the control of the *LAC4* promoter and terminator sequences. The clones contained sequences for reconstitution of the Kex protease site immediately upstream of the start codon of the *pla2* gene. The plasmid pKLAC1-*pla2* was digested with SacII and the linear products were transformed into *K. lactis* GG799 competent cells by electroporation (23). Transformants were selected on YCB agar medium containing 5 mM acetamide and grown at 30°C for 3–4 days. Then, the transformants with the correct integration of the expression DNA at the *LAC4* locus in the *K. lactis* GG799 genome were confirmed by PCR using an integration primer (Table 1) to amplify a 1.9-kb product. Furthermore, multicopy integration resulted in the amplification of a 2.3-kb fragment using integration primers (Table 1) according to the manufacturer's instruction (24). With the same procedure, the transformants that were integrated with the linearized vector pKLAC1 were used as the control strain.

Secretory expression of recombinant PLA_2 and the PLA_2 enzymatic activity assay Several positive transformed clones were cultured in liquid YPD medium with shaking (200 rpm) at 30°C for 24 h. Next, the seed culture was inoculated into a flask containing 50 mL of YPD medium and shaken (200 rpm) for 72 h at 30°C. The supernatant culture was obtained by centrifugation, and the production of the recombinant $sPLA_2$ was first tested by assaying the enzymatic activity on agar plates containing 5% egg yolk as the substrate. Then, the supernatant was analyzed for protein expression by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The $sPLA_2$ activity was determined using the pH-stat titration assay. $sPLA_2$ assays were performed at 37°C in a 10-mL solution consisting of 4% L-alpha-Lecithin, 50 mM Tris-HCl (pH 8.0), 30 mM $CaCl_2$, and 1% Triton X-100. The reaction was stopped by anhydrous ethanol, and the liberated fatty acid released from the phosphatidylcholine was titrated with 20 mM NaOH. One unit of $sPLA_2$ activity was defined as the amount of enzyme that liberates 1 μmol of fatty acid from the phospholipid per minute at 37°C.

Real-time quantitative PCR The quantitative PCR was performed in a fluorometric thermal cycler (CFX 96 Real-Time PCR Detection System, Bio-Rad). The total volume of the PCR was 10 μL, and each reaction mixture contained SYBR Premix ExTaqII PCR buffer and 0.2 μM of each forward and reverse primer (Table 1). Thermocycling was conducted with the following protocol: 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and at 60°C for 30 s with a single fluorescent reading taken at the end of each cycle. Each run was completed with a melting curve analysis to confirm the specificity of the amplifications. The Ct value was determined using the instrument's software. A negative amplification control of a sample containing no DNA was used. The PCR amplifications were performed in triplicate. To calculate the recombinant *pla2* gene copy number, a standard-curve quantitative method was used. We compared the quantified results of the *pla2* gene with the *GAPDH* gene, and the amount of the *pla2* gene was divided by the amount of the endogenous gene.

Shake flask fermentation conditions The seeds of the recombinant were cultivated in a 250-mL Erlenmeyer flask containing 50 mL YPD medium for 24 h at 30°C and shaken at 200 rpm before inoculation. Samples were withdrawn for determination of the cell density and extracellular $sPLA_2$ activity after 72 h unless indicated otherwise. Biomass was determined by measuring optical density (OD) using a spectrophotometer by obtaining absorbance at 600 nm. The biomass concentration was measured gravimetrically as the dry cell mass (g l^{-1}) by centrifuging 1 mL of the original culture broth at 12,000 rpm followed by drying the cells at 80°C overnight. One OD unit corresponded to a dry weight (DW) of 0.374 mg/mL.