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ABSTRACT

Alkaline phytase isolated from pollen grains of *Lilium longiflorum* (LIALP) possesses unique catalytic and thermal stability properties that suggest it has the potential to be used as a feed supplement. However, substantial amounts of active enzymes are needed for animal feed studies and endogenous levels of LIALP in lily pollen are too low to provide the required amounts. Active rLIALP2 (coded by *LIAlp2*, one of two isoforms of alkaline phytase cDNA identified in lily pollen) has been successfully expressed in intracellular compartments of *Pichia pastoris*, however enzyme yields have been modest (25–30 mg/L) and purification of the enzyme has been challenging. Expression of foreign proteins to the extracellular medium of *P. pastoris* greatly simplifies protein purification because low levels of endogenous proteins are secreted by the yeast. In this paper, we first describe the generation of *P. pastoris* strains that will secrete rLIALP2 to the extracellular medium. Data presented here indicates that deletion of native signal peptides at the N- and C-termini of rLIALP2 enhanced α -mating factor (α -MF)-driven secretion by four-fold; chicken egg white lysozyme signal peptide was ineffective in the extracellular secretion of rLIALP2. Second, we describe our efforts to increase expression levels by employing a constitutive promoter from the glyceraldehyde-3-phosphate dehydrogenase gene (P_{GAP}) in place of the strong, tightly controlled promoter of alcohol oxidase 1 gene (P_{AOX1}). P_{GAP} enhanced the extracellular expression levels of rLIALP2 compared to P_{AOX1} . Finally, we report on the optimization of the culture medium to enhance yields of rLIALP2. The strength of P_{GAP} varies depending on the carbon source available for cell growth; secreted expression of rLIALP2 was highest when glycerol was the carbon source. The addition of histidine and Triton X-100 also enhanced extracellular expression. Taken together, the employment of P_{GAP} under optimized culture conditions resulted in approximately eight-fold (75–80 mg/L) increase in extracellular activity compared to P_{AOX1} (8–10 mg/L). The *P. pastoris* expression system can be employed as a source of active alkaline phytase for animal feed studies.

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1. Introduction

The inability of non-ruminant animals to digest phytates in animal feed has contributed to phosphate pollution of soil and water bodies downstream of agriculturally intensive areas [27,32,35,38]. Elevated phosphorous levels in water bodies have resulted in eutrophication, harmful algal blooms and the sudden dying of large numbers of fish of all ages, often referred to as fish kill, all over the world [1,2]. Supplementation of animal feed with phytases has proven to be an effective strategy to alleviate phosphorous contamination and increase the bioavailability of phosphorous and essential minerals to animals [18,38]. The digestive tracts of animals such as pigs, chicken and fish vary

widely, as does the efficacy of phytases in these environments. Therefore, substantial amounts of an array of phytases with a range of catalytic and stability properties is needed for animal feed studies [5,20].

Alkaline phytase isolated from the pollen grains of Easter lily, *Lilium longiflorum* (LIALP) has unique catalytic and thermal stability properties suggesting that it has the potential to be used as a feed supplement [15,4]. Two isoforms of alkaline phytase cDNA, *LIAlp1* and *LIAlp2*, which code for proteins LIALP1 and LIALP2 containing 487 and 511 amino acids, respectively, were identified in our laboratory [10,26]. LIALP2 contains -Arg-His-Gly-Thr-Arg-Ala-Pro-, the signature peptide in the active site of acid phytases (maximum activity between pH 2 and 6), yet exhibits maximum enzymatic activity at pH 8.0 (the pH in the small intestines) and shows little amino acid sequence similar to acid phytases (approximately 20%) or alkaline phytase from a *Bacillus amyloliquefaciens* strain (less than 25%) [41,31]. High-level

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expression of acid phytases from microorganisms is well documented (for example [23,33], however heterologous expression of an alkaline phytase from a plant source in amounts necessary for feed studies has been a challenge [45,16].

The rLIALP2 protein was expressed in a catalytically active form in *Pichia pastoris* under the control of a tightly-regulated strong promoter, the methanol-inducible promoter P_{AOX1} . However, the expression yield of intracellular rLIALP2 was modest (8–10 mg/L) [16]. Several factors influence the expression of foreign proteins, and the limiting step(s) varies with protein, promoter and host strain [3,7,22]. Efforts to relieve the rLIALP2 biosynthesis bottleneck (s) in *P. pastoris* included optimization of transgene copy number (one copy was best), addressing differences in codon bias by gene sequence optimization, and lowering the cultivation temperature to facilitate proper protein folding; these optimization strategies lead to a modest increase in intracellular expression (25–30 mg/L) [45]. The data suggested that protein-folding processes may be the most consequential limiting factor in rLIALP2 production [45].

Purification of rLIALP2 from intracellular extracts of *P. pastoris* has proven to be challenging [16]. A major advantage of extracellular expression of proteins in *P. pastoris* is the ease of purification of foreign proteins because *P. pastoris* secretes low levels of endogenous proteins and steps such as cell lysis and cellular debris removal are eliminated [7,12,13,22,25]. The secretion of properly folded proteins is influenced by the secretion signal in the expression host and any native signal peptide(s) that may be present in the transgene [36]. The most commonly used secretion signal in yeast is the *Saccharomyces cerevisiae* α -mating factor prepro leader sequence (α -MF), an 89 aa sequence containing a 19 aa pre-sequence and a 60 aa pro-region [7,22,40]. Chicken egg white lysozyme (CL, Accession No. NP_001001470) signal peptide has also been reported to successfully drive secretion of proteins to the extracellular medium [21,19]. However, determining the optimum secretion signal for maximum secretion of foreign proteins is protein specific and requires trial-and-error experiments [3,7,22].

The strong, tightly regulated P_{AOX1} is the most widely utilized promoter in *P. pastoris*. Although P_{AOX1} generally produces high levels of foreign proteins, there are cases where employing a constitutional promoter such as P_{GAP} has resulted in significantly higher expression levels [46,44]. The use of P_{GAP} has operational advantages over P_{AOX1} : P_{GAP} eliminates the use of methanol, a toxic flammable compound; the need for transferring cells from non-methanol carbon sources to methanol-containing medium is eliminated; and the cells do not need a high oxygen concentration, thus allowing for more growth medium in culture flasks [43,44].

In this paper we describe our efforts to: (1) generate *P. pastoris* strains which secrete alkaline phytase to the extracellular medium so that enzyme purification is simplified, (2) investigate if a constitutive promoter P_{GAP} will enhance rLIALP2 expression compared to a tightly controlled promoter P_{AOX1} , and (3) optimize culture conditions to enhance the extracellular expression of active rLIALP2 in *P. pastoris*.

2. Materials and methods

2.1. Materials

Escherichia coli TOP10F', *P. pastoris* strains X-33 and expression vector pPICZA were purchased from Invitrogen (Carlsbad, CA). Primers were purchased from Integrated DNA Technologies (IDT DNA, Coralville, IA). Fast SYBR Green Master Mix MicroAmp® 8-Tube Strip (0.1 mL) and MicroAmp® Optical 8-Cap Strip were purchased from Applied Biosystems (Carlsbad, CA). Casamino acids, peptone, tryptone, yeast extract, glycerol, biotin, methanol, Zeocin, phenol and isoamyl alcohol were purchased from Fisher (Pittsburgh, PA). Yeast nitrogen base was purchased from BD

Biosciences (Sparks, MD) and yeast lyticase, Triton™ X-100 and L-histidine from Sigma–Aldrich (St. Louis, MO).

LB medium (1.00% tryptone, 0.50% yeast extract and 1.00% NaCl) was used for propagation of *E. coli* (TOP10F'). LB plates (1.00% tryptone, 0.50% yeast extract, 1.00% NaCl and 1.50% agar) with kanamycin (0.025 mg/mL) were employed for selection of transformants of TOP10F'. Low salt LB plates (1.00% tryptone, 0.50% yeast extract and 0.50% NaCl) with Zeocin (0.025 mg/mL) were used for selection of *E. coli* transformed with pGAPZA, pPICZ α A and pGAPZA- α -MF vectors.

P. pastoris positive colonies were all selected on YPD plates (1.00% yeast extract, 2.00% peptone, 2.00% dextrose and 2.00% agar) with Zeocin (0.10 mg/mL). Positive clones transformed with different constructs were grown in YPD (1.00% yeast extract, 2.00% peptone and 2.00% dextrose), BMGY (2.00% peptone, 1.00% yeast extract, 100 mM potassium phosphate, 1.34% yeast nitrogen base, 4×10^{-5} % biotin and 1.00% glycerol, pH 4.0, 6.0 or 7.5), BMMY (2.00% peptone, 1.00% yeast extract, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5} % biotin and 1.00% methanol), YPD+G (1.00% yeast extract, 2.00% peptone, 2.00% dextrose and 1.00% glycerol), YPDS (1.00% yeast extract, 2.00% peptone, 2.00% dextrose and 1.00 M sorbitol), BMGHY (BMGY pH 6.0 supplemented with 0.004% histidine), BMGY Triton X-100 (BMGY pH 6.0 supplemented with 0.01% Triton X-100) and BMGHY Triton X-100 (BMGHY supplemented with 0.01% Triton X-100).

2.2. Construction of expression vector and transformation of *P. pastoris*

The expression constructs pPICZ α A-op-rLIAlp2 (I), pPICZ α A-op-rLIAlp2- Δ C (II), pPICZ α A-op-rLIAlp2- Δ N Δ C (III), pPICZA-CL-op-rLIAlp2- Δ N Δ C (IV), pGAPZA-op-rLIAlp2 (V) and pGAPZA- α -MF- Δ N Δ C (VI) were designed as follows: briefly, each specific gene insert was amplified with respect to primer sets as shown in Table S1 (Supplementary information). op-rLIAlp2 was obtained from previous constructs [45,16]. op-rLIAlp2 with and without N-terminus (Δ N) and C-terminus (Δ C) amino acid residues were inserted into various vectors to yield the recombinant plasmid. PCR amplification (40 cycles of 94 °C for 30 s, 58 °C for 50 s and 72 °C for 2 min and a final elongation of 5 min at 72 °C) were employed to yield the desired products. The amplified products were purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) per manufacturer's instructions. The method for inserting secretion signal CL into pPICZA and α -MF into pGAPZA using PCR amplification for construct IV and VI are illustrated in Figs. S3.1 and S3.2 (Supplementary information). Purified inserts and vectors were digested by restriction enzymes Not I/EcoRI (constructs I, II, III and IV) and BstBI/Not I (constructs V and VI). Digested inserts and vectors were ligated with T4 DNA ligase (14 h at 16 °C) per manufacturer's instructions. Competent TOP10F' were prepared and transformed with recombinant plasmids I, II, III, IV, V and VI according to Sambrook and Russell [45] and positive transformants were selected on low salt LB plates with Zeocin (0.025 mg/mL). Recombinant plasmids were isolated from TOP10F' cells with Qiagen Plasmid Maxi Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. The isolated plasmids were linearized with PmeI (I, II, III and IV) or BspHI (V and VI) at 37 °C for 16 h. Competent X-33 cells were prepared and transformed with the different recombinant plasmids as described previously [45].

The insertion of recombinant vectors into *P. pastoris* was confirmed by PCR amplification and separation by agarose gel electrophoresis; separation of products by agarose gel electrophoresis yielded the expected length corresponding to different primer sets. The recombinant plasmids were sequenced to confirm that insertions were in the correct reading frame (Nevada Genomics Center, Reno, NV).

2.3. Expression of alkaline phytase and cell lysis

The expression of rLIALP2 in *P. pastoris* and the extraction of the extracellular and intracellular enzyme were performed as follows: a single colony selected on YPD agar plates containing Zeocin (0.10 mg/mL) was inoculated in BMGY medium (5 mL) and grown at 30 °C overnight. Aliquots of 2 mL starter culture were added to various culture media (50 mL in 125 mL baffled flask covered with sterile cheese cloth, if secreted expression 1.00% casamino acid included) as indicated. In case of clones transformed with construct **I**, **II**, **III** and **IV**, cells were collected after 1 d by centrifugation at 3000 × g for 5 min and resuspended in BMMY (20 mL, with 1% casamino acid) and grown for 2 d at 20.5 ± 0.5 °C. Sterile distilled deionized H₂O (1.8 mL) and methanol (0.2 mL) were supplemented every 24 h (10% of the culture volume) to BMMY medium. Clones transformed with construct **V** and **VI** were continuously grown in indicated media (50 or 25 mL) for 2 d. Aliquots of 1 mL of growth medium containing secreted active enzyme were taken daily, centrifuged at 10,000 × g for 2 min at 4 °C and dialyzed against Tris–HCl buffer (10 mM, pH 7.4) for about 20 h and enzymatic activities determined. Cell pellets and supernatant were collected separately after 2 d growth by centrifugation at 10,000 × g for 5 min and wet cell mass was recorded.

The cell pellets were resuspended in lysis buffer (Tris–HCl, 50 mM, pH 7.4; PMSF, 1 mM; 2 mL per g). Aliquots of 1 mL of resuspended cell pellets were added to 0.5 mm zirconia/silica disruption beads (0.5 mL, Research Products International Corp., Mt. Prospect, IL) and lysed with Vortex Genie 2 for 30 s by vigorous mixing followed by 30 s incubation on ice. This mixing/freezing cycle was repeated for a total of 6 cycles. Portions containing intracellular enzyme were collected by centrifugation at 12,000 × g for 15 min at 4 °C and 1 mL aliquots of supernatant containing secreted active enzyme were dialyzed against Tris–HCl buffer (10 mM, pH 7.4) for 20 h to remove phosphates and salts before assaying for enzymatic activity.

2.4. Alkaline phytase activity assay

The alkaline phytase activities were determined by measuring the amount of inorganic phosphate released by the enzyme from sodium phytate [45]. The reaction mixture contained Tris–HCl buffer (100 mM, pH 8.0), sodium phytate (1 mM), KCl (0.5 M), CaCl₂

(1 mM), NaF (10 mM) and aliquots of dialyzed enzyme solution in a total volume of 125 μL. The reaction mixtures were incubated at 37 °C for 1 h and the reaction stopped by the addition of TCA (50% w/v, 25 μL). The incubated samples were centrifuged at 10,000 × g at room temperature for 2 min and 75 μL of the supernatant was added to 175 μL detection solution (6:1 ratio of 0.42% ammonium molybdate w/v in 0.5 M H₂SO₄ and 10% w/v ascorbic acid). The mixture was incubated at 37 °C for an additional 1 h. An aliquot of 200 μL (out of a total volume of 250 μL) was transferred to a 96-well microplate spectrophotometer (BioTek Epoch, Winooski, VT) and the absorbance at 820 nm was measured. The amount of inorganic phosphate was determined from the standard curve generated with KH₂PO₄. Controls were run in parallel with every assay. The assay conditions in control tubes were identical to the experimental assay except that TCA was added to the reaction mixture before the active enzyme so the enzyme was denatured on contact. One unit of alkaline phytase activity was defined as the release of 1 μmol of Pi from sodium phytate in 1 h at 37 °C. All experiments were conducted in duplicates.

To determine the concentration of rLIALP2 in extracellular medium, aliquots of dialyzed enzyme solution were separated on SDS gel and regions corresponding to rLIALP2 were cut with a sharp razor. The gel strip was cooled in liquid nitrogen, finely ground in a pre-cooled mortar and pestle and the protein extracted with 2 × 350 μL of Buffer (Tris–HCl, 10 mM; pH 7.4) followed by centrifugation at 5000 × g for 20 min. The supernatants were cooled and the protein content was determined by Bradford assay with BSA as the standard.

2.5. SDS-PAGE

Proteins were separated by gel electrophoresis under denaturing conditions on 13% resolving gel and 7% stacking gel and visualized by Coomassie Blue staining. Electrophoresis was carried out at a constant voltage (200 V) for 100 min at room temperature.

2.6. Protein assay

Protein concentrations were estimated by the Bradford dye-binding method using the Bio-Rad assay reagent (Bio-Rad Laboratories, Hercules, CA) according to manufacturer's instructions. Bovine serum albumin was used as the standard.

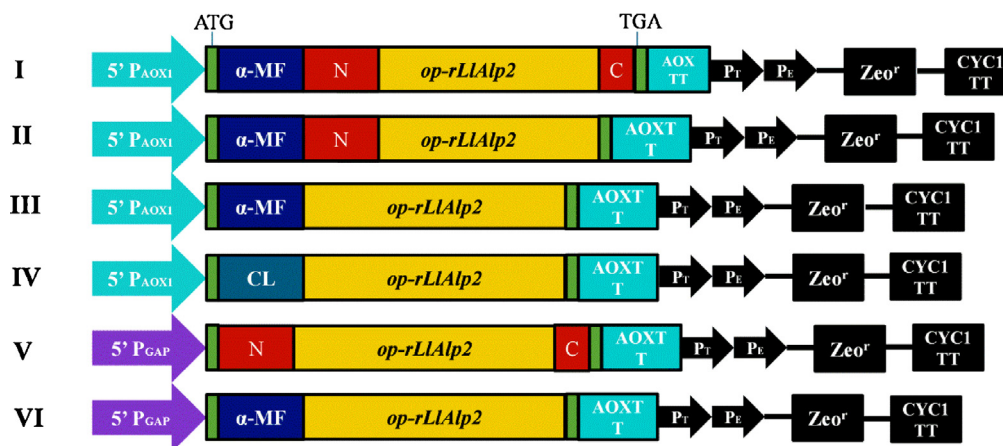


Fig. 1. Map of rLIALP2 expression constructs. P_{AOX1} and P_{GAP}, *P. pastoris* AOX1 and GAP promoter; α -MF, α -MF secretion signal peptide; N, 19 aa signal peptide at N-terminus; *op-rLIALp2*, cDNA of optimized alkaline phytase; C, five amino acid residues (QKTEL); AOX1T, AOX1 transcription termination region; P_T, *S. cerevisiae* transcription elongation factor 1 promoter that drives expression of *Sh ble* gene in *P. pastoris*; P_E, promoter that drives expression of *Sh ble* gene in *E. coli*; Zeo^r, Zeocin resistant gene from *Streptoaloteichus hindustanus* bleomycin gene (*Sh ble*) and CYC1TT, CYX1 transcription termination region. **I**, full length optimized *rLIALp2* sequence (*pPICZ α A-op-rLIALp2*); **II**, truncated *op-rLIALp2* without QKTEL at the C-terminus (*pPICZ α A-op-rLIALp2 Δ C*); **III**, truncated *op-rLIALp2* without the first 19 aa at the N-terminus and without QKTEL at the C-terminus (*pPICZ α A-op-rLIALp2 Δ N Δ C*); **IV**, *op-rLIALp2- Δ N Δ C* was introduced downstream of CL (*pPICZ α -CL-op-rLIALp2- Δ N Δ C*); **V**, *pGAPZA-op-rLIALp2* driven by P_{GAP}; and **VI**, extracellular expression directed by α -MF (*pGAPZA- α -MF-op-rLIALp2*).

2.7. Quantitative real time PCR (qPCR)

qPCR using Fast SYBR[®] Green dye was employed to determine the *rLlAlp2* gene copy number. qPCR experiments were conducted with the ABI 7300 instrument (Applied Biosystems, Carlsbad, CA). Genomic DNA was extracted and purified, and concentration determined using a Nanodrop spectrophotometer. 10 ng of genomic DNA was used as the template in all qPCR analysis. Primers were designed according to Fast SYBR[®] Green Master Mix protocol; amplified products were designed to be between 150 and 200 bp in length [45].

All qPCR experiments were performed in triplicate with the recommended program. Only samples displaying C_T values lower than 26 and standard deviations lower than 0.25 were used to determine the gene dosage. Absolute quantification was applied to calculate the copy number of *rLlAlp2*. The *Gpd* gene, which is the single copy in *P. pastoris* genome, was amplified in parallel with *rLlAlp2* in all qPCR reactions. C_T values were determined in triplicate and copies of genomic DNA were determined from the corresponding standard curve. The absolute copy number of *rLlAlp2* was calculated by dividing the genomic DNA copies of *rLlAlp2* by the genomic DNA copies of *Gpd* based on the generated *Gpd* standard curve. Primers and experimental details were described previously [45].

3. Results and discussion

3.1. Effect of native signal peptides on expression levels of rLlALP2 controlled by P_{AOX1}

In lily pollen, LlALP is localized on the surface of membrane-bound phytin granules [4]. To probe the presence of signal peptide(s) in rLlALP2, analysis of the deduced amino acid sequence of rLlALP2 (Accession No. ABD96176) with SignalP 4.0 [9,29] and TargetP 1.1 [34,8,29] was conducted. The analysis revealed the presence of a signal peptide with a cleavage site between aa 19 and 20 at the N-terminus. Additionally, the presence of a potential ER-membrane retention sequence at the C-terminus (QKTEL) was predicted by the PSORTII program [28].

The influence of native signal peptides at the N- and C-termini of rLlALP2 on α -MF-driven secretion in *P. pastoris* cannot be predicted *a priori*. Therefore, in an effort to delineate the influence of the native signal sequences on α -MF-directed secretion, three expression constructs were generated by inserting three forms of *rLlAlp2* downstream of the α -MF secretion signal in pPICZ α A vectors (Fig. 1; experimental details in Supplementary information): **I**, full length optimized *rLlAlp2* sequence (*pPICZ α A-op-rLlAlp2*); **II**, truncated *op-rLlAlp2* without QKTEL at the C-terminus (*pPICZ α A-op-rLlAlp2 Δ C*); and **III**, truncated *op-rLlAlp2* without the first 19 aa at the N-terminus and without QKTEL at the C-terminus (*pPICZ α A-op-rLlAlp2 Δ N Δ C*). Optimized sequences (*op-rLlAlp2*) with or without the N- or C-terminal signal peptides were amplified using primers (Supplementary information) and inserted into pPICZ α A vector downstream of the α -MF signal peptide. *P. pastoris* X-33 was transformed with linearized constructs **I**, **II** and **III** [45], and six clones from each transformation were randomly selected and expressed.

P. pastoris was first grown in glycerol-based medium (BMGY, 50 mL) for 24 h and then transferred to methanol-containing medium (BMMY, 1.00% casamino acid, 20 mL) to induce expression. After two days of induction, phytase activity in the extracellular medium and intracellular cell lysate was determined. All six clones transformed with construct **I** exhibited low phytase activity (2.5 ± 0.5 U/25 mL), with over 50% of the activities localized in intracellular compartments (Fig. 2, Panel A). Alkaline phytase expression levels in all six transformants with construct **II** were

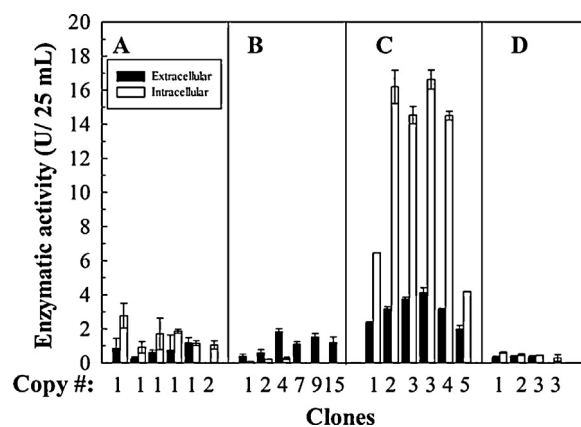


Fig. 2. Expression levels and copy numbers of *rLlAlp2* in *P. pastoris*. Clones (X-33) were transformed with expression construct **I** (Panel A), construct **II** (Panel B), construct **III** (Panel C) or construct **IV** (Panel D). All clones from each transformation were randomly selected and grown in BMGY (50 mL) at 29.5 ± 0.5 °C for 24 h and transferred to methanol-containing medium BMMY (25 mL, 1.00% casamino acid) (20.5 ± 0.5 °C) for 48 h. Alkaline phytase expression levels were determined by assaying for enzymatic activity in dialyzed supernatant containing extracellular active enzyme or cell lysate. Extracellular medium or cell lysate from non-induced cultures were used as controls. All experiments were conducted in duplicates, error bars represent \pm SEM, $n = 2$. Gene copy number was determined by qPCR. All qPCR amplifications were conducted in triplicates and average values are presented above.

barely detectable (2 ± 0.5 U/25 mL) in the expression medium or intracellular lysate (Fig. 2, Panel B). All six clones transformed with construct **III** showed significantly higher extracellular expression levels of rLlALP2 (8–21 U/25 mL), however 75% of enzyme activity was retained in intracellular compartments (Fig. 2, Panel C). The data suggests that the presence of native signal peptides at the N- and C-termini had a detrimental effect on α -MF-driven rLlALP2 secretion in *P. pastoris*. Deletion of native signal peptides at both N- and C-termini of rLlALP2 yielded the highest levels of active enzyme. Therefore, *op-rLlAlp2- Δ N Δ C* was employed in the subsequent secretion expression studies.

In an effort to investigate the effect of employing CL as the secretion signal, X-33 cells were transformed with construct **IV** (Fig. 1) and the expression levels were investigated. *op-rLlAlp2- Δ N Δ C* was introduced downstream of CL and inserted into the pPICZ α A vector to generate construct **IV**: *pPICZ α A-CL-op-rLlAlp2- Δ N Δ C* (Fig. 1, experimental details in Supplementary information). Four clones transformed with construct **IV** were randomly selected and rLlALP2 expressed by induction with methanol. The activity of rLlALP2 secreted to extracellular medium as well as that retained within intracellular compartments was significantly lower when directed by CL (1 ± 0.5 U/25 mL) (Fig. 2, Panel D) compared to α -MF (Fig. 2, Panel C). In CL-driven expression, both extracellular and intracellular activity levels were low irrespective of gene dosage (Fig. 2, Panel D).

Copy number of the transgene in transformed clones significantly influences intracellular expression levels of rLlALP2 in *P. pastoris*; increase in copy number of *rLlAlp2* led to decrease in expression yields, single copy clones produced the highest yields of enzyme activity [45]. The copy number of *rLlAlp2* in transformed clones was determined (according to [45]) so that expression levels of constructs with the same copy number could be compared. The data in Fig. 2, Panel C reveals that both intracellular and extracellular expression levels were highest when the copy number was two to four and decreased significantly when the copy number increased to five.

The time course of rLlALP2 expression after methanol induction was investigated in clones transformed with construct **III**. Extracellular medium and cell lysates were assayed for alkaline

phytase activity for six days post induction at 24 h intervals. rLIALP2 activity both in the extracellular medium and in the intracellular lysate reached maximum levels two days after induction and did not vary significantly for the next four days (data not shown).

3.2. Intracellular and extracellular expression levels of rLIALP2 under the control of P_{AOX1} and P_{GAP}

To gain a detailed understanding of the expression of rLIALP2 in *P. pastoris* under the control of P_{GAP} , both intracellular and extracellular expression were monitored. Expression constructs under the control of P_{GAP} were generated for intracellular expression (construct **V**) and extracellular expression (construct **VI**) (Fig. 1).

First, experiments were conducted to investigate the ability of P_{GAP} to drive intracellular expression of rLIALP2. *P. pastoris* was transformed by electroporation with linearized expression construct **V**. Ten clones transformed with construct **V** were randomly selected and intracellular rLIALP2 level was assayed; enzyme activity was detected in all cell lysates, and wide variability in total intracellular rLIALP2 activity was observed (Fig. 3A). When the copy number of *rLIALp2* in the 10 clones was determined, the data revealed that the five clones with one copy of *rLIALp2* yielded highest expression levels [9 ± 2 U]; four clones with two copies of *rLIALp2* produced 20–50% lower activity compared to clones with a single copy, and no alkaline phytase activity was detected in the clone with four copies (Fig. 3A). Levels of rLIALP2 expression under P_{GAP} decreased with increasing gene dosage (Fig. 3A), similar to observations with P_{AOX1} expression, suggesting that gene dosage is not the major bottleneck for rLIALP2 expression in *P. pastoris*.

3.3. Optimization of culture conditions for enhanced expression

The strength of P_{GAP} is greatly influenced by culture conditions such as nutrients, pH and temperature [43,46,44]. The carbon source available to the host organism has a significant impact on extracellular expression, so the transformed clone was grown on three carbon sources (1.00% casamino acid was present in all expression media): 1.00% glucose supplemented with 1.00%

glycerol (YPD+G at pH 6.0), glucose supplemented with 1 M sorbitol (YPDS at pH 6.0) and glycerol-based media (BMGY at pH 4.0, 6.0 and 7.5) (Fig. 3B); the highest-producing clone containing construct **VI** was used for this investigation. The data indicate that glycerol is the best carbon source for P_{GAP} -driven extracellular expression of rLIALP2 in *P. pastoris*.

Previous investigations have indicated that proteolytic stability of secreted proteins can be improved by altering the pH of the growth medium: a decrease of pH from 6.0 to pH 4.0 or 3.0 resulted in an increase in protein activity [17,6]. However, in the case of rLIALP2, total enzyme activity was similar ($\pm 10\%$) when grown in BMGY at pH 4.0, 6.0 or 7.5 (Fig. 3B). This suggests that total enzyme activity of rLIALP2 recovered from the extracellular medium is not dependent on the pH of the medium. The addition of the non-ionic detergent Triton X-100 significantly improved the extraction of rLIALP2 from lily pollen [37,4]. The addition of Triton X-100 also enhanced the recovery of a hydrophobic serine protease, urokinase-type plasminogen activator in *P. pastoris* [14,42]. When Triton X-100 [0.01%] was added to BMGY, total enzyme activity in extracellular media increased by three-fold (Fig. 3B) suggesting that Triton X-100 helped partition rLIALP2 to the soluble fraction. Nitrogen concentration also has a substantial impact on expression yields [43,22]. Supplementation of histidine (0.004%) to the growth medium resulted in approximately 30% increase in enzyme activity in the extracellular medium [39]. Optimized expression conditions that yielded maximum rLIALP2 activity were as follows: baffled flask (125 mL) containing BMGHY medium (50 mL) supplemented with 0.01% Triton X-100 and grown at $20.5 \pm 0.5^\circ\text{C}$ for a period of two days.

In order to compare the extracellular expression levels of rLIALP2 under the control of P_{GAP} to levels under the control of P_{AOX1} , seven clones transformed with construct **VI** (driven by P_{GAP}) and six clones transformed with construct **III** (driven by P_{AOX1}) were randomly selected and expressed under optimum conditions. Four out of seven clones under the control of P_{GAP} had a single copy of *rLIALp2* and these produced the highest yield of extracellular active enzyme (42 ± 6 U/50 mL). One clone with two copies produced 10% ($5\text{--}10$ U/50 mL) that of clones with a single copy, and no enzyme activity was detected in clones with three or four copies of *rLIALp2* (Fig. 3C). This indicates a strong negative

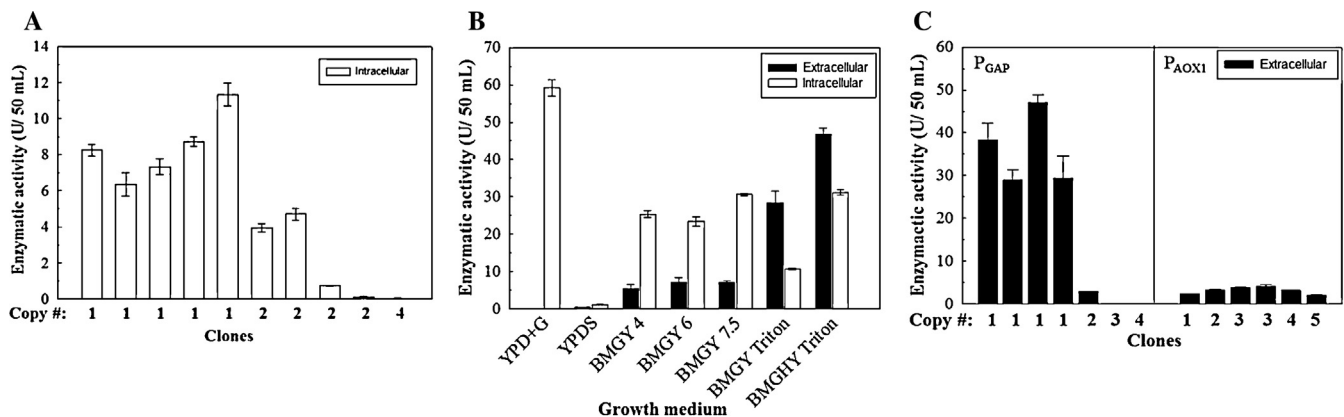


Fig. 3. Expression level of rLIALP2 driven by P_{GAP} . Panel A: intracellular expression levels and copy numbers of *rLIALp2* in clones transformed with construct **V**. A total of 10 clones were randomly selected and grown in YPD medium (50 mL) at $29 \pm 0.5^\circ\text{C}$ for 48 h and cell pellets were collected and lysed; Panel B: effect of growth medium on extracellular and intracellular expression levels of rLIALP2 in *P. pastoris* clones transformed with construct **VI**. The best producer (with copy number one) was selected and grown on various media (YPD+G: 1.00% glucose and 1.00% glycerol, YPDS: 1.00% glucose and 1 M sorbitol, BMGY 4, 6 and 7.5: 1.00% glycerol, pH 4.0, pH 6.0 and pH 7.5; BMGY Triton: 1.00% glycerol, 0.01% Triton X-100 and BMGHY: 1.00% glycerol, 0.01% Triton X-100 and 0.004% histidine) as indicated at $20.5 \pm 0.5^\circ\text{C}$ for 48 h; Panel C: effect of promoters and copy number of *rLIALp2* on extracellular expression level of rLIALP2 in *P. pastoris* clones, transformed with expression vector *pGAPZ α A-op-rLIALp2- Δ N Δ C* (construct **VI**) and *pPICZA- α -MF-op-rLIALp2- Δ N Δ C* (construct **III**). Seven clones transformed with construct **VI** were randomly selected and grown in BMGHY Triton (50 mL) at $20.5 \pm 0.5^\circ\text{C}$ for 48 h. Extracellular medium from time=0 cultures were used as controls. Six clones transformed with construct **III** were grown as described in Fig. 2. Extracellular medium from non-induced cultures were used as controls.

correlation between transgene copy number and extracellular expression level of rLIALP2 in *P. pastoris* driven by P_{GAP} . The extracellular expression levels of clones under the control of P_{AOX1} were uniformly low; irrespective of copy number, they produced 4–5 U/50 mL, approximately 10–12% of that produced by P_{GAP} (Fig. 3C). To determine the yield of rLIALP2, aliquots of extracellular medium were separated on SDS gel and the protein content determined by Bradford assay as described in Section 2. Clones under the control of P_{GAP} produced 75–80 mg/L of rLIALP2 compared to clones under the control of P_{AOX1} which produced approximately 8–10 mg/L.

4. Conclusion

In summary, α -MF-driven extracellular expression of rLIALP2 was achieved in *P. pastoris*. Data presented here suggest that the presence of native signal peptides at the N- and C-termini of rLIALP2 interfered with the secretion of rLIALP2 to the extracellular medium. Host cells suffer metabolic stress when the transcription level of the foreign transgene is high and this may lead to a reduced yield of foreign protein [3,11,22,24]. By switching from the strong, tightly regulated promoter P_{AOX1} to the weaker, constitutive promoter P_{GAP} and optimizing culture conditions, an eight- to ten-fold increase in extracellular expression of rLIALP2 was achieved. The *P. pastoris* expression system can be employed as a source of active alkaline phytase for animal feed studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.btre.2015.03.005>.

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