Cloning of the phytase gene *phyA* from *Aspergillus ficuum* 3.4322 and its expression in yeast

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A *phyA* gene was cloned from *Aspergillus ficuum* 3.4322 by reverse transcription polymerase chain reaction. The amplified fragment was cloned into the pMD18 T-vector and sequenced. Nucleotide sequence analysis of the *phyA* gene showed that it comprised 1347bp without the signal peptide sequence and encodes a polypeptide of 448 amino acids. The *phyA* sequence has been deposited in GenBank (accession number: AF537344). Expression vectors pYPA1 and pYPA2 were constructed by cloning the *phy*A gene with and without the signal peptide sequence into the yeast expression vector pYES2. The recombinant plasmids were transformed into *Saccharomyces cerevisiae* INVSc1 by the method of LiAc. Phytase activity was found in pYPA2 (about 11.55IU/ml) endocellular fluid and in pYPA1 supernatant (about 11.60IU/mL) by galactose inducing. The results demonstrated that the *phyA* gene had been expressed in *Saccharomyces cerevisiae* and the signal sequence of *Aspergillu ficuum*3.4322 could facilitate the phytase secretion from *S. cerevisiae* efficiently.

Key words: *Aspergillus ficuum* 3.4322, expression, *phyA*, phytase, secretion, *Saccharomyces cerevisiae* INVSc1.

Introduction

Phytate, *myo*-inositol hexaphosphate, is the major storage form of phosphorus in plants. It accounts for 1%-3% in the seeds of cereals and legumes and 60%-80% of the total phosphorus (P) in plants. Because monogastric animals lack endogenous phytase in their tracts, they can only metabolize 40% of phosphorus in the form of phytic acid. Most of the phytic acid phosphorus excreted by the animals without absorption, causes not only the waste of phosphorus but also the environmental pollution (Nelson, 1967).

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Phytate is also considered as an anti-nutritional factor by chelating various metal ions, such as calcium, zinc, iron, potassium as well as protein, thereby decreasing their bio-availability (Sharma, 1978).

Phytase is capable of hydrolyzing phytate into inositol and phosphorus, therefore the mineral elements and protein utility efficient and the energy balance of the foodstuff can be improved. Plants and most microorganisms such as yeasts, *Aspergillus*, and bacteria can produce phytase (Wodzinski, and Ullah, 1996; Li *et al*., 1997). Phytase from microorganisms has been mostly studied, and is used widely as an additive in foodstuff. However, the production of phytase in native microorganisms is low, so the application of phytase is limited because of high cost. With the development of molecular biology, constructing transgenic microorganisms capable of producing phytase has become fashionable because it can improve the production and industrialization of phytase.

In this paper we cloned the phytase gene from *Aspergillus ficuum* 3.4322 by RT-PCR and then transformed it into *Saccharomyces cerevisiae* INVSc after sequencing. It expressed and produced normal phytase activity.

Materials and methods

Strains and plasmids

Aspergillus ficuum 3.4322 was purchased from China General Microbiological Culture Collection Center. *Saccharomyces cerevisiae* INVSc1 (MAT α *his3 Δ1 leu2 trp1-289 ura3-52*/MAT α *his3Δ1 leu2 trp1-289 ura3-52*) and its expression vector pYES2 were kindly provided by professor Xing Laijun, Department of Microbiology, Nankai University; pMD18-T Vector was purchased from TakaRa Biotechnology (Dalian) Co., Ltd.

Total RNA preparation

The hyphae of *Aspergillus ficuum* 3.4322 was inoculated into PDA medium and shaken at 100 rev/min for 2-3 days at 28ºC and mycelia was collected. The total RNA was extracted by method of Guamidine Thiocyanate (Sambrook *et al*., 1989).

Cloning phyA gene

In order to amplify the phytase gene without its signal sequence from *A. ficuum* 3.4322, an upstream primer and a downstream primer were designed according to the *A. ficuum phyA* sequence that had been published (GenBank

Accession:AY013315). The *Xba*I site at 5' end and a *Kpn*I site at 3' end were added to the primers with the purpose of determining the direction of gene insertion and subsequent cloning.

Upstream primer: 5'ATGTCTAGACTGGCAGTCCCCGCCTCGAGA-3'
Downstream primer:
5'-CTAGGTACCCTAAGCAAAACACTCCGCCCAATC-3'.

The reverse transcription reaction mixture included (in 10 μl volume) 200 μmol/L of dNTP, 10U of reverse transcriptase RAV-2, 1 μmol/L of downstream primer, 6 μl RNA template (2 μg) and ddH_2O as a supplement to the system. The mixture was incubated for 45 minutes at 42ºC, then 5 minutes at 95ºC to damage RAV-2. PCR reaction (in 20 μl volume) mixture was prepared by adding 2 μl RT product, 200 μmol/L of dNTP, 1 μmol/L of Upstream and Downstream primer, 3U of pfu DNA polymerase. The reaction was carried by pre-denaturing for 5 minutes at 94ºC denaturing for 45 seconds at 94ºC, annealing for 45 seconds at 55ºC, extension for 75seconds at 72ºC, 30 cycles, last extension for 10 minutes at 72ºC.

The DNA fragment obtained by RT-PCR was purified by the PCR Fragment Recovery Kit purchased from TakaRa Biotechnology (Dalian) Co, Ltd, then cloned into the pMD18-T vector. The methods for *E. coli* competent cell preparation and transformation were according to the methods of Sambrook *et al*. (1989) The transformants were screened according to white-to-blue phenomena. Then positive transfomants were determined by digestion of their plasmids using the restriction enzymes *Xba*I and *Kpn*I. The positive transfomants containing the *phyA* gene was sequenced in the Sangon Biotechnology (Shanghai) Co., Ltd.

Sample preparation and the assay of phytase activity

Transformation of *S. cerevisiae* INVSc1 was carried out according to method of LiAC (Adams *et al*., 2000). The pYPA1, pYPA2 transformants and the yeast containing pYES2 plasmid were initially grown at 30ºC in YEPD medium. When the optical density culture at 600 nm (OD_{600}) reached 1.5, 2% galactose was added to induce phytase expression for 60 hours. The extracellular enzyme samples were prepared by collecting the culture supernatant after centrifugation at 5000 rpm for 5 minutes at 4ºC. The endocellular enzyme samples was prepared as follows: the cells were harvested by centrifugation (7,000 g for 2 minutes at 4ºC), washed with NaAc buffer (0.1mol/L, pH 5.0) and transferred into a mortar after being diluted by NaAc buffer (0.1mol/L, pH 5.0) again. The samples were frozen at -20ºC, grinded

and frozen and ground again. The supernatants were collected after centrifuging.

Extracellular and endocellular enzyme activity were determined by AMES method (Ames, 1966). Briefly, 50 μl of supernatants were transferred to a 1.5 ml Eppendorf tube containing 500 μl of NaAc buffer and 100 μl of 0.1mol/l calcium phytate to start the reaction, which was carried at 37ºC for 1 hour and stopped by adding 300 μl of 10% trichloroacetic acid. The mixture was mixed with 500 μl of sulfuric acid, ammonium molybdate, and ascorbic acid (1 volume of 10% ascorbic acid, 6 volumes of 0.42% Molybdenum-1N H_2SO_4) for the determination of free phosphorus concentration. The control was run by adding 300 μl of 10% trichloroacetic acid before adding the enzyme sample. One phytase unit (1U) is defined as the activity that releases 1 μmol of inorganic phosphorus from calcium phytate at pH 5.0 and 37ºC (1U = 1×10^3 IU).

Results

Cloning of phytase gene

The desired 1.4Kb fragment of *phyA* gene was cloned from *A. ficuum* 3.4322 by RT-PCR, ligated with pMD18-T vector and transformed into *E. coli* JM103. Transformants were screened and the positive transformant phyA6 were selected. Then the plasmid of transformant phyA6 was sequenced. The result showed that the fragment contained 1347 base pairs without the signal peptide sequence, and encoded a polypeptide of 448 amino acids (Fig. 1). The active-site sequence of histidinol acid phosphatase, CQVTFAQVLSRHGARYPTDSKGK, was located at the position +52 - +74 of the amino acid sequence. RHGARYPT was the most conservative sequence of the phytase active site from microorganism (Kostrewa *et al*., 1997) and there were ten potential glycosylation sites (Asn-X-Ser/Thr) in the amino acid sequence (Yanming *et al*., 1999. Rodriguez *et al*., 1999).

Comparison of the sequence obtained with the cDNA sequence of *phyA* from *A. niger* NRRL3135 (GenBank Accession no. M94550) showed that the nucleotide homology was as high as 92% and there were 99 different base pairs between the two stains. The amino acid homology between them was as high as 95%. The sequence and location of the active-sites of phytase in *A. ficuum* 3.4322 were the same as those of *A. niger* NRRL3135.

The sequencing results showed that the *phyA* gene was inserted into the multiple clone site of pMD18-T vector inversely. The pMD18-T *phy*A plasmid

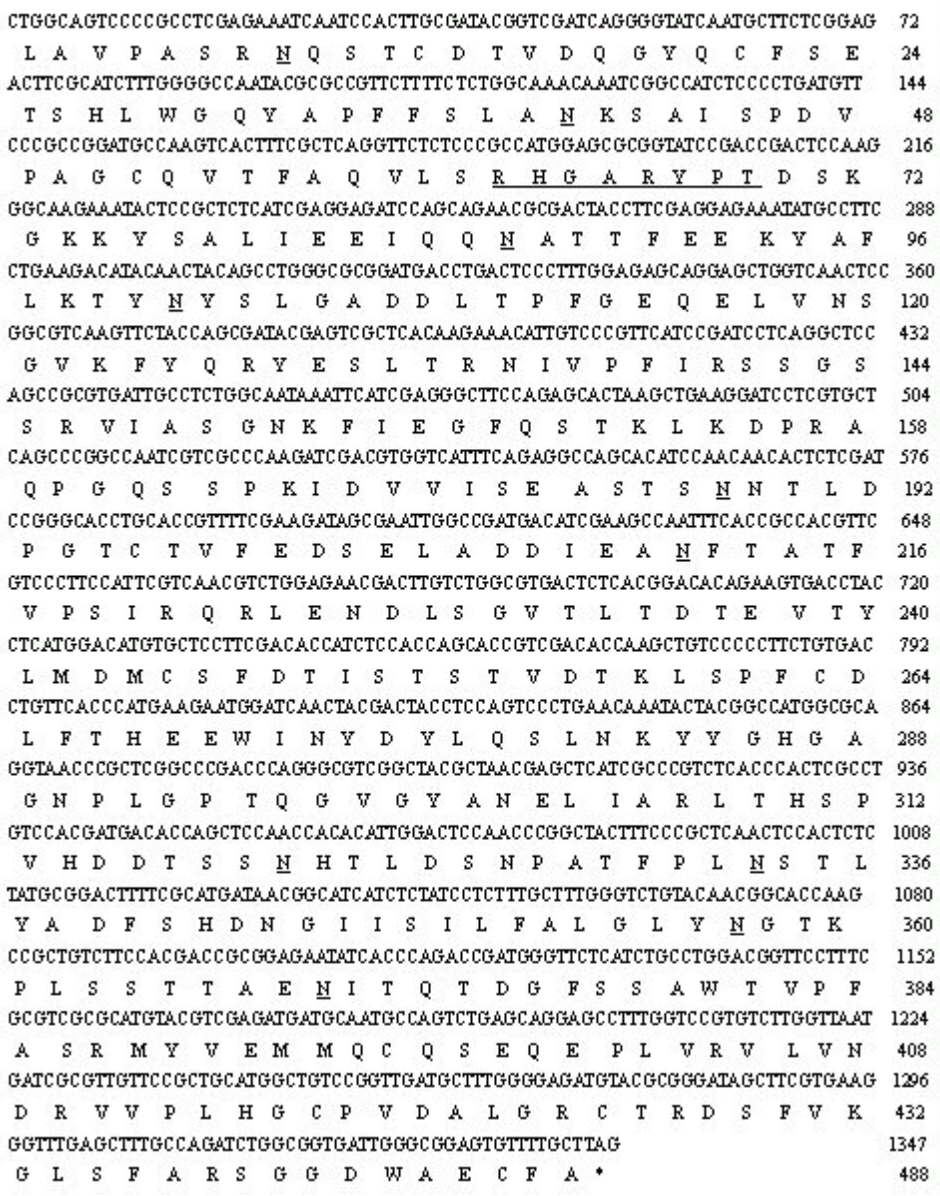

Fig. 1. The cDNA sequence of *phyA* gene from *A. ficuum* 4.4322 and deduced amino sequence. The active-site amino acids sequence and the potential N-glycosylation were underlinded.

was digested by *Hind*III and *Eco*RI, then the *phyA* fragment was inserted into the yeast expression vector pYES2 .The following vector named pYPA2 (Fig. 2) containing the correct ORF of *phyA* gene was constructed. The start codon

ATG and termination codon TAG were added at the ends of *phy*A gene respectively by PCR primers.

Another pair of primers was designed in order to obtain extracellular phytase. The primers contained an upstream *Xho*I site and a downstream *Apa*I site:

Upstream primer: 5'-CTCGAGATGGGCGTCTCTGCTGTTCTACTTC-3'
Downstream primer: 5'-GGGCCCCTAAGCAAAACACTCCGCCCAATCA-3'

The *phy*A gene with its signal sequence was obtained by RT-PCR and then cloned into a pMD 18-T vector. The recombinant plasmids were digested by enzymes *Xho*I and *Apa*I. The *phyA* gene fragment was cloned into pYES2 to construct the yeast expression vector pYPA1 (Fig. 3).

Expression of phyA gene in yeast

pYES2 is a shuttle plasmid containing the URA3 gene which codes uridine synthetase. The *S. cerevisiae* INVSc1 strain is auxotrophic of uridine because of the mutation of URA3 gene, so transformants can be screened with this marker. pYES2 has an inducible GAL1 promoter, which could be suppressed by glucose and induced by galactose. Therefore, the expression of *phyA* gene in the pYPA1 and pYPA2 could be regulated by glucose or galactose in the medium.

The plasmids pYPA1 and pYPA2 were transformed into *S. cerevisiae* INVScl (Ura^-)by the LiAC method. The positive tansformants were selected and induced to express phytase in the medium with galactose.

Phytase activity was determined in the culture supernatants of pYPA1 transfomants and in the endocellular fluid of pYPA2 transfomants as described in the materials and methods. In this study, the extracellular phytase activity from pYPA1 transfomants was about 11.60 IU/ml (Table 1). The expression products cannot be secreted from the yeast cell containing pYPA2 because both pYES2 and *phyA* gene in pYPA2 have no signal peptide. The activity of phytase was about 11.55 IU/ml in the pYPA2 endocellular fluid (Table 1).

Table 1. Phytase activities (IU/mL) in pYPA1 medium supernatants and in pYPA2 endocellular fluid

Sample P	content (mmol/L)	Enzyme unit (IU)	Phytase activity (IU/ml)
PYPA2	0.0365	0.577	11.55
PYPA1	-	-	11.60

*The OD_{820} values of control were modulated to zero during the measurement of samples.

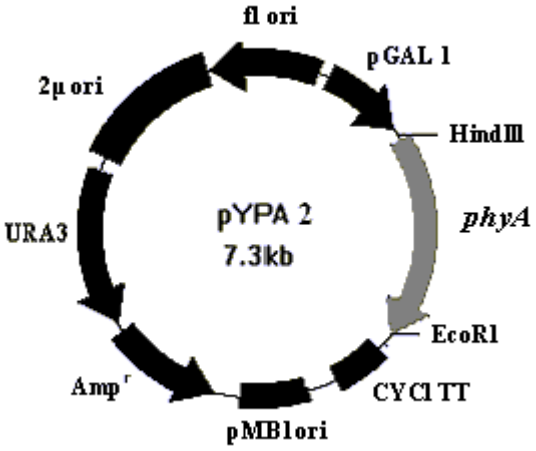

Fig. 2. Construction of the expression vectors pYPA1 and pYPA2. The recombinant expression vector of pYPA2.

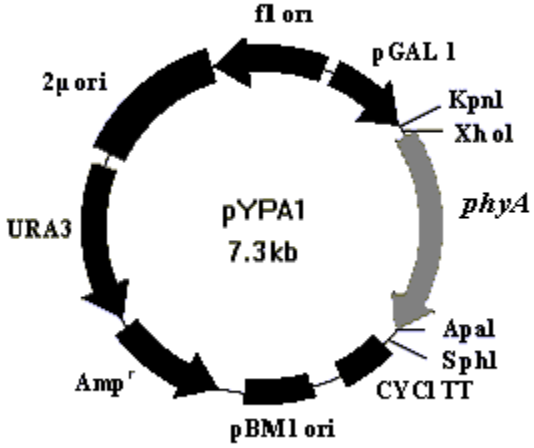

Fig. 3. The recombinant expression vector of pYPA1.

Discussion

In this paper the *phyA* gene encoding phytase was cloned from *Aspergillus ficuum* 3.4322 by reverse transcription polymerase chain reaction (RT-PCR). It comprised 1347 bp without the signal peptide sequence and coded a polypeptide of 448 amino acids. The conservative amino acid

sequence of histidinol acid phosphatase, CQVTFAQVLSRHGARYPTDSKGK, was located at +52-+74, which was the active-site sequence (Fig. 1). The DNA sequence showed 92% identity with those of *A. niger* NRRL3135.

The expression of extracellular and endocellular phytase activities in *S. cerevisiae* showed that the *phyA* gene from *A. ficuum* 3.4322 could be transcripted, translated and processed correctly in *S. cerevisiae.* The results also demonstrated that the signal peptide from *A. ficuum* was also able to facilitate the phytase secreted from *S. cerevisiae*. Such result was similar to that reported by Yanming *et al*. (1999). This indicated that there might be a similar signal-processing system in *A. ficuum* and in *S. cerevisiae*. The difference of phytase activity between endocellular and extracellular was not significant (11.55 IU/ml and 11.60 IU/ml respectively) (Table 1), which indicated that the signal peptide from *A. ficuum* was highly efficient for phytase secretion in *S. cerevisiae.*

Although the functional phytase was successfully expressed, the activity and the quantity of expressed phytase were not so high as expected. There might be two reasons. Firstly, it might relate with the strength of the promoter GAL1. Liu and Wang (1998) and Liu *et al.* (1998) established that when the medium contained 0.1% glucose, the promoting intensity of GAL1 was half that when there was no glucose in medium. The promoter was suppressed completely with 0.5% glucose in medium. In the study, the medium contained 2% glucose, so the GAL1 promoter was suppressed to some extent and resulted in the low phytase activity. Secondly, there was codon bias in *S. cerevisiae.* Sharp *et al* (1986) investigated the codon bias in *S. cerevisiae* of about 110 genes. The results showed that the arginine codons codons (CGT, CGA, CGC, CGG, AGA and AGG) were preferential utilization, in which AGA was used by 86.6%, but CGA and CGG were never used. Yao *et al*. (1998) mentioned that when the 4 Arg codons (3 CGG and 1 CGA) of *phy*A2 from *A. niger* 963 were mutated, the expression of phytase was increased by 37 times. The *phy*A gene from *A. ficuum* 3.4322 had 17 codons for Arg, three of which were CGA and one was CGG. The codon bias might also cause the low expression of phytase in *S. cerevisiae.*

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