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Extracellular expression of a thermostable phytase (phyA) in *Kluyveromyces lactis*

Mrudula Vasudevan Ushasree, Jalaja Vidya, Ashok Pandey*

Biotechnology Division, CSIR-National Institute for Interdisciplinary Science and Technology, Trivandrum 695019, India

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ABSTRACT

Functional expression of a thermostable phytase from *A. niger* was achieved in *Kluyveromyces lactis* GG799 cells. Effective secretion of recombinant enzyme (198 U ml^{-1}) in the fermentation broth at 72 h incubation at 22 °C was obtained. Purified enzyme showed a specific activity of 72 U mg^{-1}) and was detected on SDS-PAGE as a heavily glycosylated protein with a molecular weight of ≥140 kDa. Optimum temperature of the enzyme was at 55 °C and it showed a characteristic bi-hump pH profile with two pH optima (at pH 2.5 and 5.5). Enzyme showed considerable pepsin resistance with 60% activity retention after incubation with pepsin at the ratio of 1:1000. Enzyme was thermostable retaining 69 and 37% activity at 90 and 100 °C for 10 min respectively and remained active at these temperatures till 1 h. Deglycosylation studies demonstrated negligible effect of N-linked glycans on thermal properties. Multiple sequence alignment data revealed a conserved Asn at position 345 of this phytase which might contribute to its thermal properties. This thermostable phytase coupled with its noticeable protease resistance could be a better alternative to current commercial phytases.

1. Introduction

Phytases are feed enzymes, which have been the foremost, if not solely, marketed animal feed additive in the diets of monogastrics. Phytase hydrolyse phytic acid present in the plant based feed sequentially to release lower phosphorylated myo-inositol derivatives and inorganic phosphates, thereby reducing its anti-nutritional properties. Moreover, it helps to prevent eutrophication caused by the excretion of undigested phytic acid by monogastric animals [1,2]. Potential health benefits of phytate and lower myoinositol phosphates have recently gained special interest [3]. At present, bacterial and fungal phytases are dominating the global feed enzyme market. The first commercial phytase, Natuphos was produced from *Aspergillus niger*, which has been predominant in the feed enzymes market with almost 70% volume share.

The right choice of exogenous phytases to be added in the feed is a complex issue as almost all the naturally occurring phytases become inactivated at feed pelleting temperature (70–90 °C for 5–10 min). One of the ways to resolve this issue could be to increase the dosages of the enzyme in liquid formulations but that would increase the capital investments and costs. In spite of its high specific activity and pH profile in the acidic range, its lower thermostability is the main constraint for the wide application of *A. niger* phytase. At temperatures between 50 and 55 °C, this enzyme undergoes an irreversible conformational rearrangement that is associated with losses in enzymatic activity of 70–80% [4]. Protein engineering strategies have been employed to improve the thermal stability properties of phytases [5].

In order to reduce the cost of the enzyme, different recombinant systems have been used for the heterologous production of phytases. Yeast expression systems such as *Saccharomyces cerevisiae* [6,7], *Pichia pastoris* [8,9] and *Heninsula polymorpha* [10] have been efficiently used for the over-expression of *A. niger* phytases. In the present study, we describe the cloning and expression of a thermostable phytase from *A. niger* NII 08121. Here, a non-saccharomyces yeast, *Kluyveromyces lactis* was used for heterologous production of phytase. *K. lactis* has received substantial attention as a eukaryotic cell factory for heterologous protein production due to physiological properties that distinguish it from other yeasts [11]. Recombinant enzyme was purified and study on *in vitro* properties such as temperature, pH and pepsin stabilities were carried out. Effect of N-linked glycans on the thermostability of recombinant phytase was also determined. Multiple alignments of the amino acid sequences of this phytase with other thermostable and commercial enzyme was performed to find out the presence of conserved residues, which might contribute to the stability of this protein.

* Corresponding author. Tel.: +91 471 25 15 279; fax: +91 471 24 91 712.
E-mail addresses: ushusbt@gmail.com (M.V. Ushasree), vidyabt@gmail.com (J. Vidya), pandey@niist.res.in, ashokpandey56@yahoo.co.in (A. Pandey).

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2. Materials and methods

2.1. Chemicals and enzymes

Phytic acid sodium salt, 4-methyl umbelliferyl phosphate, *p*-nitrophenyl phosphate, α-naphthyl phosphate and Fast Blue salt were procured from Sigma Aldrich, USA. Restriction enzymes, T4 DNA ligases and Pfu DNA polymerases were purchased from MBI Fermentas (Opelstrasse, Germany). Endoglycosidase Hf (EndoHf) was purchased from NEB, USA.

2.2. Strains, plasmid and culture conditions

A. niger NII 08121 used for the isolation of phytase gene was maintained on potato-dextrose broth or agar at 30 °C. *K. lactis* GG799 cells were used as the expression host, which were grown in YPD (1% yeast extract, 2% peptone and 2% glucose) broth or agar for propagation, YCB medium (30 mM Tris–HCl, pH 7.0, 11.7 g l^{-1} of yeast carbon base and 5 mM acetamide) for the selection and YPCG medium (1% yeast extract, 2% peptone, 2% galactose and 2% casamino acids) was used for the expression studies. *E. coli* JM107strain was grown in Luria-Bertani (LB) medium at 37 °C. When required, ampicillin was added at 100 μg ml^{-1} in the medium as specified. Plasmid pKLAC1 (NEB, Berkly, USA) was used as intermediate and principal vector for cloning and expression of phyA gene into *E. coli* and *K. lactis* cells.

2.3. Gene cloning of fungal phytase

The genomic DNA from *A. niger* NII 08121 isolated by adopting CTAB extraction method [12] was used as the template for the PCR. Forward and reverse oligonucleotide primers KphyF 5′-ATT**CCTAGG**CTGGCAGTCCCCGCCTCGAGA-3′ and KphyR 5' – AT**AGGCCT**CTAAGCGGAACACTCCGC-3' with restriction sites AvrII and StuI (underlined respectively) were designed based on the previously reported GenBank entries. Nucleotide sequence encoding phytase, excluding the signal and intron sequences was isolated by the PCR. The reaction was performed as one cycle at 95 °C for 5 min, followed by 35 cycles at 95 °C for 40 s, 52.8 °C for 40 s, 72 °C for 1.3 min and final extension step at 72 °C for 10 min. The PCR amplicon was recovered from agarose gel by Qiaquick DNA gel extraction kit (Qiagen), double digested with *Avr* II and *Stu* I and ligated into the multiple cloning site of pKLAC1 vector. To construct the secreted form of recombinant protein, phytase gene was fused in the frame with the α-mating factor domain to generate the construct *phyA*::pKLAC1. Ligated products were transformed into *E. coli*–JM107and the clones were confirmed by restriction digestion and insert release from the recombinant construct.

2.4. Electroporation of K. lactis cells

The recombinant construct *phyA*::pKLAC1was linearized with SacII, recovered using the QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA) and electroporated into *K. lactis* competent cells according to the protocol described by Lin-Cereghino et al. [13]. Cells were grown in 50 ml YPD broth aerated at 30 °C until mid-log phase (OD_{600} 0.6–1.0) and harvested by centrifugation (8000 g, at 4 °C for 5 min) and re-suspended in 10 ml YPD broth containing 25 mM dithiothreitol (DTT) and 20 mM HEPES (pH8.0) and incubated at 30 °C for 15 min. Cells were recovered by centrifugation as above and were washed three times with sterile distilled water, followed by a wash by 4.0 ml 1 M sorbitol. The cells were finally suspended in sorbitol. Competent cell was mixed with 0.2 μg of linearized plasmid and electroporated at 1700 V. Immediately after applying the pulse, 1.0 ml of YPD broth was added and the transformed cells were grown at 30 °C for 1 h. The cells were pelleted, washed in sterile distilled water and plated on YCB agar plates supplemented with 5 mM acetamide. The selection plates were incubated at 30 °C for 4 days.

2.5. Phytase assay

Phytase assay was performed according to the protocol of Heinonen and Lathi [14]. Enzyme assay was done in 40 μl volume at 37 °C for 30 min in 100 mM sodium acetate buffer containing 1 mM sodium phytate. The reaction was interrupted by adding 160 μl of freshly prepared acetone/5 N H_2SO_4/10 mM ammonium molybdate (2:1:1, v/v). Released inorganic phosphate was quantified spectrophotometrically by measuring the absorbance at 355 nm. Phytase activity unit is defined as the amount of enzyme that catalyzes the release of 1 μM of inorganic phosphorus from sodium phytate per minute. Concentration of protein was estimated by Lowry's method [15].

2.6. Selection of recombinant yeast strains

Transformants appeared on selection plates and *K. lactis* cells were streaked on to YCB agar supplemented with 5 mM acetamide and 2% galactose. Plates were incubated at 30 °C for 4 days, followed by a second step of incubation at 37 °C for 10 min after over-layering the plate with 0.2 M sodium acetate buffer (pH 5.0) containing 4-methylumbelliferyl phosphate. Plates were then observed under UV illuminator for fluorescence.

Multi-copy genomic DNA integration of the linearized expression cassette was confirmed by the PCR using the specific primers provided by NEB. Transformed cells were re-suspended in 20 μl of 1 M sorbitol containing 2.0 mg ml^{-1} lyticase and kept at 37 °C for 1 h, followed by incubation at 96 °C for 10 min. Lyticase-treated cells were used for whole cell PCR.

2.7. Protein expression and purification

A single colony of multiple integrant was grown in 4.0 ml YPD medium at 30 °C for overnight. One millilitre of this overnight grown culture was pelleted and inoculated into 100 ml YPCG medium (pH 6.0) and grown at 22 °C at 200 rpm up to 144 h. At different time points, culture supernatant was collected and subjected to phytase assay. Purification was carried out by ion exchange and hydrophobic interaction chromatography. Lyophilized culture supernatant was dialysed against 50 mM Tris acetate buffer (pH 6.3), loaded on to Q sepharose column and eluted in a 0.1–1.0 M NaCl gradient in 50 mM Tris-acetate (pH 6.3). Pooled fractions containing phytase activity were passed over phenyl sepharose column and enzyme was eluted with a linear 2.0–0.5 M $(NH_4)_2SO_4$ gradient in 10 mM Tris acetate buffer (pH 6.3). Fractions containing phytase were resolved by SDS-PAGE and analyzed by zymogram analysis after incubating the gel in 2% triton X-100 at 37 °C for 1 h. The gel was further treated in 100 mM sodium acetate buffer (pH 5.5) containing 2.5 mg ml^{-1} α-naphthyl phosphate and 1.25 mg ml^{-1} Fast Blue salt at 37 °C for 1 h for developing the activity band.

2.8. In vitro properties of the recombinant phytase

2.8.1. Temperature and pH dependence

The effect of pH on enzyme activities of the purified phytase was studied in the presence of following (100 mM) buffers: glycine HCl, pH 1.5–3.5; Na acetate–acetic acid, pH 3.5–5.5; Tris acetic acid, pH 6.0–6.5 and Tris–HCl, pH 7.0–8.0.

Optimal temperature was determined in the range of 35–75 °C in acetate buffer of pH 5.5. pH stability was determined by incubating the purified phytase in the buffers of acidic pH (pH 1.5–5.0) at