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N. N. Khabipova

L. R. Valeeva

I. B. Chastukhina

M. R. Sharipova

Eugene V. Shakirov
*Marshall University*, shakirov@marshall.edu

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**Research Article**

# Heterologous Expression of Pantoea Agglomerans Phytase Gene Optimized for Plant-Host Expression

**N.N. Khabipova[1], L. R. Valeeva[1], I.B. Chastukhina[1], M. R. Sharipova[1] and E.V. Shakirov[1,2,*]**

[1]Kazan Federal University, Kazan, Russia
[2]Department of Integrative Biology,
University of Texas at Austin, Austin, TX, USA
*Corresponding author: E.V. Shakirov; e-mail: eugene-shakirov@utexas.edu

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## ABSTRACT

Here we report expression and characterization of recombinant bacterial phytase PaPhyC from *Pantoea* sp. Codon-optimized phytase gene was expressed *E.coli* BL21 pLysS and protein expression was confirmed by Western blotting. Recombinant protein expressed in *E.coli* has high phytase activity. We show that PaPhyC recombinant phytase has different molecular masses when expressed in bacteria and plants, suggesting that possible protein glycosylation in plants may influence its overall size.

**Keywords***:* phytase *Pantoea sp.*, recombinant protein expression, codon-optimization, transgenic plants, phosphorus deficiency.

## 1 INTRODUCTION

Phosphorus is one of the most important macronutrients necessary for all living organisms. Phosphorus is essential for many key cell molecules, such as ATP, nucleic acids and phospholipids; it is also required for regulation of metabolism, energy transfer, protein activation, and carbon metabolic processes [Chevalier et al., 2011]. Plants and animals typically obtain phosphorus in the form of phosphate from soil and food, respectively.

However, the bioavailable phosphate content in the soil worldwide is in sharp decline [Plaxton et al., 2011]. As the problem of phosphorus deficiency in plant nutrition continues to grow, severe implications arise for both animal and human nutrition. The addition of inorganic phosphate fertilizers to soil does not substantially increase phosphorus content in soil solution; rather, much of the added Pi is rapidly transformed into other compounds with limited bioavailability to plants [Bogri *et al.*, 2015]. At the same time, a large proportion of soil phosphorus is present in organic forms, with the most abundant form being phytate [George et al 2004; Lung et al., 2005]. Phytate is known to have negative effects on plant and animal nutrition, as it binds phosphates and immobilizes metal ions (Ca, Fe, Zn, Mg) [Torres et al., 2005]. Nevertheless, phytate can be a rich source of phosphorus for plants and animals if it is metabolized through the action of specific enzymes - phytases. This can be achieved by application of microbial phytases. Phytases are myo-inositol hexakis phosphatases that degrade phytate and release phosphates. Microorganisms, such as bacteria and fungi, are a rich source of highly active phytases. Bacterial phytases are used as feed additives or expressed

directly in crops as recombinant proteins. However, structural and catalytic characteristics of phytases can be modified upon expression in plants. Therefore, it is important to evaluate properties of recombinant phytases prior to performing transgenic expression in plants. The goal of this work was to characterize expression of recombinant PaPhyC phytase in E. coli.

## 2 MATERIALS AND METHODS

### Bacterial strains and plasmids

E. coli DH5α and BL21 (DE3) strains (Invitrogen) were used as cloning and expression hosts, respectively. Plasmid vector pET28b was used for cloning and expression studies.

### Cloning of the *PaPhyC* phytase gene

Nucleotide sequence of *Pantoea sp. paPhyC* phytase (KJ783401) gene was codon-optimized to improve protein expression in *Arabidopsis thaliana* using the Codon Adaptation Tool software (http://www.jcat.de). The codon-optimized sequence of *paPhyC* gene from pCEV04 plasmid (Valeeva et al., 2015) was amplified using primers 5'-ccatgggccaagaaactcctgaaggatatc-3' and 5'-ctcgagcttttcaaattgaggatgagaccacttagcagcattattc-3' with the following PCR conditions: one cycle at 98°C for 2 min, followed by 37 cycles of 98°C for 25 s, 57°C for 25 s, and 72°C for 1 min 30 s, with a final extension step at 72°C for 10 min. The amplified *paPhyC* product (1293 bp) was digested by NcoI and XhoI restriction enzymes and inserted into the pET28b expression vector (Novagen) as a fusion with an N-terminal six-histidine (6-His) tag. A ligation reaction was performed using T4 ligase (Thermo Scientific). *E.coli* DH5*α* and *E.coli* BL21(DE3)/pLysS cells were transformed by standard $CaCl_2$ protocol [Sambrook et al., 1989].

### Bacterial growth and preparation of cellular lysate.

For recombinant phytase expression in *E. coli* BL21 pLysS strain and the following examination of its activity, cells were grown in LB medium aerobically at 30 or 37°C for 18 h with 0.1 mM or 0.5 mM IPTG. Cells were harvested at 4°C by centrifugation for 10 min at 6,000 *g*. Cells were then washed three times with 20 mM sodium acetate buffer, pH 5.5, and lysed three times by repeated freezing at -80°C for 15 min and thawing at room temperature for 20 min, as described previously [Greiner, 2004]. After being resuspended in 20 mM sodium acetate buffer, pH 4.5, cells were sonicated on ice 10 times for 10 s with 30-s intervals. Cellular debris were removed by centrifugation at 4°C for 30 min at 18,000 rpm.

### Western-blot analysis.

30 µl of cellular lysate was mixed with Protein Sample dye, incubated for 5 min at 85º C and subjected to SDS-PAGE (12.5% acrylamide gel). Separated proteins were transferred onto a PVDF membrane for 50 min at 90 V. The membrane was blocked with 5% Skim Milk (Sigma Aldrich, Germany) in PBS-T buffer (Phosphate buffer, 0.1 % Tween-20) for 1 h at RT with shaking. PVDF membrane was then incubated with primary antibodies (6x-His Epitope Tag Monoclonal Antibody (HIS.H8), Thermo Scientific) at a dilution of 1:3,000 for 1 h at RT with shaking. The membrane was washed for 10 min three times in PBS-T buffer and incubated with secondary antibodies (Pierce Goat Anti-Mouse IgG, (H+L), Peroxidase conjugated, Thermo Scientific) at a dilution 1:10,000 for 30 min. After washing in PBS-T and PBS three and two times, respectively, the signal was visualized using a chromogenic substrate SuperSignal West Pico Stable Peroxidase Solution and SuperSignal West Pico Luminol/ Enhancer Solution (Thermo Scientific).

### Phytase activity assays.

Protein concentration was analyzed by a Dc Protein Assay (Bio-Rad) using bovine serum albumin as a standard. Phytase activity was quantified using a modified ammonium molybdate method [Heinonen, Lahti, 1981] by measuring the amount of released inorganic phosphorous. The enzyme solution (10 to 50 µl) was added to 350 µl of 10 mM sodium phytate (Aldrich, Germany) in 100 mM sodium acetate buffer, pH 4.5, and incubated at 37°C for 30 min. The reaction was stopped by the addition of 1.5 ml of freshly prepared 2:1:1 AAM solution

(acetone–5N $H_2SO_4$–10 mM ammonium molybdate), incubated for 2 min and 100 µl of 1M citric acid was added. Blank controls were prepared by adding AAM solution prior to the addition of enzyme. Optical density was measured at 355 nm (OD355) on a model 2550 Microplate Reader (Bio-Rad, USA). A calibration curve was built using the concentrations of inorganic phosphate in the range of 5 to 600 nmol. One unit (U) of phytase activity was defined as the amount of enzyme necessary to produce 1 µmol of inorganic phosphorous per min at 37°C. Statistical significance was determined using a Student's two-tailed *t* test with significance set at a *P* value of 0.05.

## 3 RESULTS AND DISCUSSION

### Cloning of codon optimized *Pantoea sp.* phytase gene *(paPhyC)*

For protein expression, multiple codons encoding the same amino acid can be utilized with different frequencies by different organisms. Since codon utilization varies between plants and many microbes, successful expression of recombinant bacterial proteins in plants is often stymied by this phenomenon also called codon usage bias [Camiolo *et al*., 2012]. To potentially improve bacterial protein expression efficiency in *Arabidopsis thaliana* plants, we initially conducted codon-optimization for phytase gene *paPhyC* from *Pantoea* sp (Valeeva *et al*., 2015). Following codon optimization, transgenic *A. thaliana* plants with integrated *paPhyC* gene were obtained and analyzed. Although phytase expression in plant cell wall protein fraction was detected by Western-blot analysis (Valeeva *et al*., 2015), the large-scale extraction and purification of this protein from plants proved to be difficult.

To nevertheless study this enzyme's properties, the codon-optimized *paPhyC* gene was further sub-cloned to pET28b plasmid for expression in bacterial cells. The modified *paPhyC* gene was first PCR-amplified from the pCEV04 plasmid (Valeeva *et al*., 2015) using gene-specific primers (Figure 1). Both the amplified *paPhyC* PCR product and pET28b vector were digested by XhoI and NcoI, ligated and transformed into *E. coli* DH5α cells. Transformants were selected on the LA medium with kanamycin (50 µg/ml). The presence of *paPhyC* sequence in pET28b was confirmed by PCR, restriction analysis and sequencing (Figure 2). pET28b plasmid with correct insert was then introduced into *E. coli* BL21 pLysS cells and re-confirmed by PCR analysis and sequencing (Figure 3). The map of the resulting plasmid is shown in Figure 4.

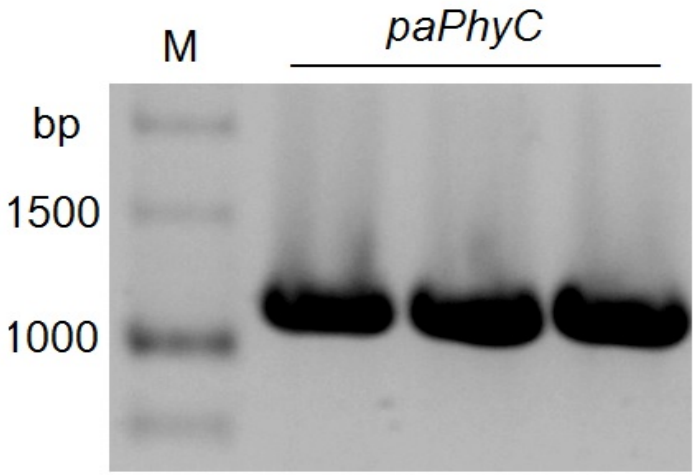


**Figure 1.** PCR amplification of codon-optimized *paPhyC* gene. The expected size for *paPhyC* PCR product is 1143 bp. M – molecular size markers (100-10000 bp).

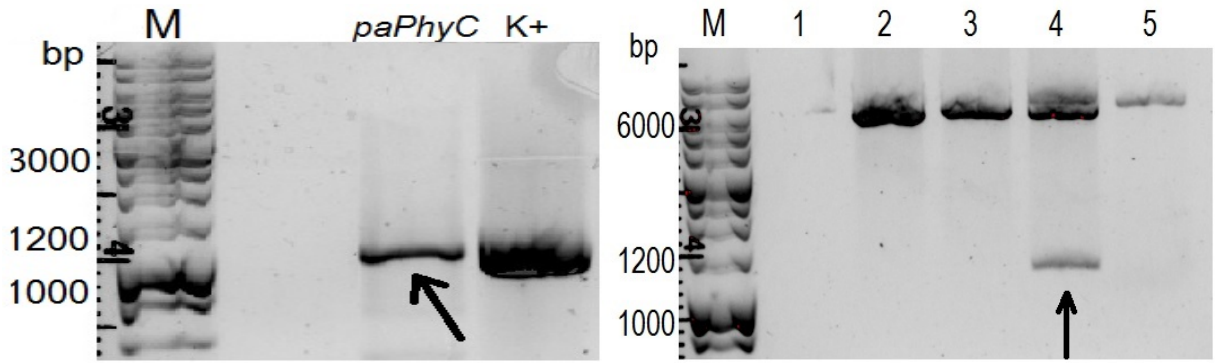


**Figure 2.** PCR amplification of *paPhyC* insert (left) and restriction analysis (right) of pET28b plasmid containing the *paPhyC* insert. M – molecular size markers (100-10000 bp). K+ - *paPhyC* amplification from control plasmid pCEV04. Arrow indicates the correct clone containing the expected PCR band (left) or insert (right).

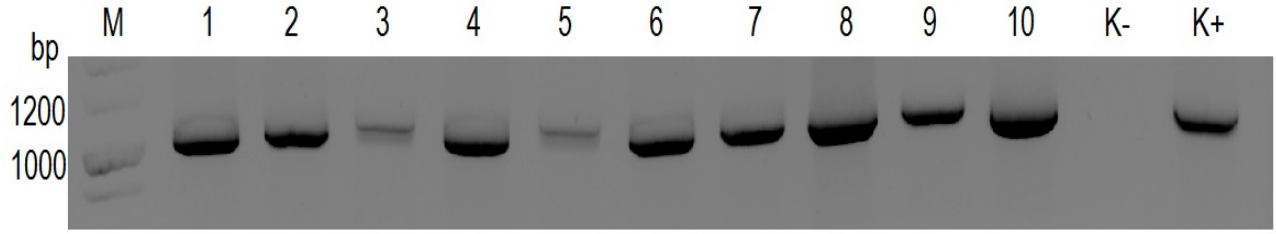


**Figure 3.** PCR analysis of *E. coli* BL21 pLysS cells from colonies transformed with pET28b vector containing the *paPhyC* insert. K+ - *paPhyC* amplification from control plasmid pCEV04. K- no DNA control.

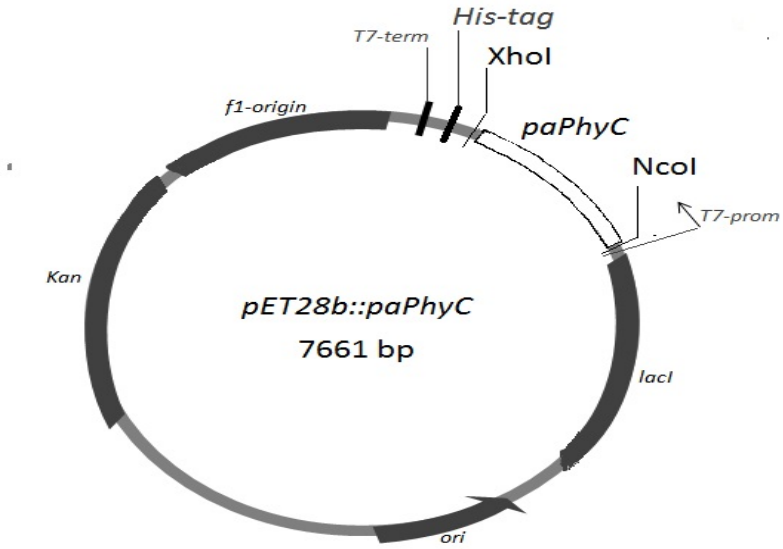


**Figure 4.** Construction of pET28b vector containing the *paPhyC* insert. Abbreviations: *paPhyC* – *Pantoea sp.* phytase gene; XhoI, NcoI – restriction sites; T7-prom – T7 bacteriophage promoter; T7-term – T7 bacteriophage terminator; His-tag - 6xHis-sequence; f1-origin – bacteriophage origin, ori – bacterial origine; Kan – kanamycine reistance; *lacI* – lacI regulator gene sequence.

**Expression of the recombinant phytase PaPhyC in bacterial cells**

Western analysis using an antibody specific for the His-tag revealed the presence of a strong band corresponding to the PaPhyC phytase in IPTG-induced cells (Figure 5). The optimal temperature of induction was 30° C, whereas IPTG concentration did not have any influence on induction level. The size of recombinant PaPhyC protein detected in *E. coli* BL21 pLysS cells (42 kDa) correlates well with the size of phytase protein expressed in the native *Pantoea* sp. strain [Greiner, 2004]. Interestingly, however, the molecular weight of the recombinant PaPhyC phytase expressed in *A. thaliana* plants was somewhat higher (~50 kDa)

[Valeeva et al., 2015], suggesting that additional post-transcriptional modifications of PaPhyC protein in plants could be responsible for the observed size differences. Specifically, glycosilation of recombinant microbial phytases expressed in plants was previously observed in several different studies [Lung *et al.*, 2005; Sylvain *et al.*, 2005]. The activity of recombinant phytase was investigated using lysate from *E. coli* BL21 pLysS cells. Phytase activity in cells treated with IPTG was 140 mU/mg, while control cells whithout IPTG showed no activity.

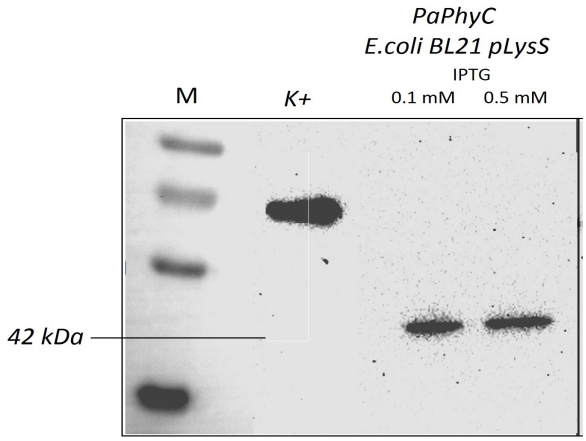


**Figure 5.** Western-blot analysis of PaPhyC phytase expressed in *E. coli* BL21 pLysS cells. BL21 pLysS cells expressing PaPhyC protein were grown at 30 C with 0.1 mM or 0.5 mM IPTG. The apparent molecular weight of recombinant *PaPhyC* protein is 42 kDa.

## 4.CONCLUSION

We conclude that the codon-optimized *paPhyC* phytase gene is effectively expressed in a bacterial expression system. The apparent molecular weight of recombinant phytase is 42 kDa, which correlates well with the size of phytase expressed in native bacteria *Pantoea* sp [Greiner, 2004]. Interestingly, PaPhyC expressed in transgenic plants shows a higher molecular weight of ~50 kDa, suggesting a potential plant-specific post-translation modification.

## 5. ACKNOWLEDGEMENTS

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