

RECOMBINANT THERMOTOLERANT PHYTASE PRODUCED IN E.COLI

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Abstract: Phytic acid (myo-inositol hexakisphosphate) and its salts (phytates) are the major storage form of phosphorus in plants. Monogastric animals including hogs, poultry, and fish cannot utilize phytates as a source of phosphorus unless they are enzymatically destroyed with exogenous enzyme—phytase. Phytases are added to fodder in ever increasing dosage to improve utilization of plant-derived phosphorus because this reduces dependence of farms on inorganic fodder phosphates. Because of technological considerations, feed phytases have to withstand elevated temperatures (60-80°C), which are used during preparation of fodder. Enzymatic feed additives are becoming of high demand in Kazakhstan, and development of domestic technologies for production of agricultural enzymes is an ongoing challenge to the country's biotechnology.

Objectives: To develop a system for recombinant expression of industrially important thermotolerant phytase and confirm activity and thermal stability of the recombinantly expressed enzyme.

Methods: De novo gene synthesis, expression of 6xHis-tagged protein in E.coli, immobilized metal affinity chromatography, biochemical tests for activities of phosphatase and phytase.

Results: Thermotolerant phytase was produced in E.coli using recombinant expression system. The obtained enzyme had phosphatase activity (hydrolyzed p-nitrophenyl phosphate) and phytase activity (hydrolyzed sodium phytate). The recombinant phytase tolerated increase of incubation temperature up to 70°C and demonstrated increase in activity towards phytate with increase in the reaction temperature in the range 30°C-70°C.

Conclusion: Described gene and expression system have prospects of utilization in development of pilot industrial production of phytase in the country.

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Introduction

In plants, the predominant storage form of phosphorus is phytic acid salts (myo-inositol hexakisphosphate), which account for 50-80% of the total phosphorus content in cereals and legumes (Harland & Morris, 1995). Monogastric animals including hogs, poultry, and fish cannot digest phytates due to low concentration of enzyme (phytase) in their digestive tract (Selle, Ravindran, Caldwell, & Bryden, 2000). During the past two decades, industrially produced phytases have been added to farm animal diets to enhance the utilization of phosphorus (Luo et al., 2007). In general, phytase is added to feed before it is pelleted at elevated temperatures (60-80°C), thus the enzyme needs to survive elevated temperatures. Besides retaining activity after incubation at high temperatures, the thermotolerant phytase should have optimal activity at physiological temperatures (37°C).

Phytases are phosphatases cleaving ester bonds in the inositol phosphates (Konietzny & Greiner, 2002). Phytases were found in more than 200 species of microorganisms and plants, but vertebrates to day produce endogenous phytase (Liu, Rafiq, Tzeng, & Rob, 1998). Enzymes from actinomycetes and fungi (e.g. *Peniophora lycii* 6-phytase or *Aspergillus niger* 3-phytase) were the first commercialized phytases for feed additives (Vohoura & Satyanarayana, 2003). Phytases are classified into six classes according to structure of the active center: histidine acid phosphatases (HAP), cysteine phytases (CPhy), purple acid phosphatases (PAP), and beta-propeller phytases (BPP) (Mullaney & Ullah, 2003). Depending on the optimal pH, phytases are divided into acidic and alkaline phytases (Oh, Choi, Park, Kim, & Oh, 2004). Acidic phytases demonstrate the highest activity at pH 2,5-5,5. Acidic phytases include bacterial

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and fungal representatives. Their aminoterminal acid sequences have a conserved motif containing a histidine residue, which is involved in a nucleophilic attack (Wyss et al., 1999). Bacterial acid phytases demonstrate greater specific activity compared to fungal counterparts. Among all studied phytases, the highest specific activity was shown for *E. coli* phytaseAppA and *Citrobacter freundii* phytaseAppA. Disadvantage of natural bacterial phytases is their low thermal stability. Thermal stability of bacterial enzymes can be significantly improved by the enzyme engineering. Engineered mutant of the *E. coli* phytaseAppA with the name “Nov9x” has extremely attractive combination of high activity in stomach and impressive thermal tolerance. We aimed to produce the recombinant thermostable phytase Nov9x in *E. coli* and study its activity at elevated temperatures.

Materials and methods

Genetic engineering

Gene for Nov9x was synthesized de novo using construction PCR. Sequence of the Nov9x gene was designed in silico to be codon-optimized for expression in *E. coli*. Constructed DNA fragment contains 1236 bp of the phytase-specific sequence (without signal peptide for periplasmic export), and hexahistidine tag and linkers for cloning into expression vector. The synthetic DNA fragment was cloned into pGEM-T and subjected to bidirectional sequencing to verify the constructed sequence. The Nov9x gene was transferred into pET32 expression plasmid (Novagen) by cloning into NcoI-XhoI restriction sites. *E. coli* strains BL21(DE3) was transformed with the resulting plasmid pET32/Nov9x.

Small-scaled experiment to determine phytase accumulation in induced culture

Expression strain was inoculated into 100 ml of LB medium with ampicillin (100 µg/ml). When culture reached OD = 0.6, the IPTG was added to 0.2 mM. At time points of 1 hour, 2 hours, 4 hours, 6 hours, and 14 hours. Samples (10 ml) of culture were taken, and bacteria were pelleted by centrifugation. Bacterial pellet was resuspended in 1 ml of HN buffer (20 mM HEPES pH 7.5; 150 mM NaCl), and bacteria were disrupted by sonication (for pulses by 30 seconds at maximum power). Probes (15 µl) of sonicate were investigated in SDS-PAGE.

Production of recombinant phytase

The BL21(DE3)/pET32/Nov9x was inoculated into night culture (5 ml of LB medium with ampicillin, 100 µg/ml). On the next day, 100 µl of night culture were transferred into 200 ml of LB medium with ampicillin (100 µg/ml). Culture was incubated at 37°C, 150 rpm, for 14 hours. Bacteria were pelleted by centrifugation, resuspended in water, and re-pelleted. The pellet was suspended in 10 ml of lysozyme lysis buffer (20% sucrose; 20 mM HEPES pH 7.5; 5 mM EDTA; 0.1% Triton X100). To the suspension, lysozyme was added to 1 mg/ml, DNase I - 10 µg/ml, RNase - 100 µg/ml, and PMSF - 0.2 mM. Suspension was incubated for 1 hour at room temperature. Suspension was sonicated (10 pulses for 20 seconds, with 3-minute pauses between pulses). Bacterial debris was removed by centrifugation (10000 rpm, 20 minutes). The supernatant was subject to metal affinity chromatography (IMAC).

Purification of recombinant phytase by IMAC

Clarified supernatant was loaded onto column His GraviTrap (GE Healthcare) charged with Ni²⁺ ions. 3 ml of wash buffer (50 mM HEPES pH 7.0; 300 mM NaCl; 20 mM imidazole) was passed through the column. Proteins were eluted with buffers with increasing concentration of imidazole (100-300 mM). Elution buffers contained 50 mM HEPES pH 7.0, 300 mM NaCl, and imidazole (100 mM, 200 mM, or 300 mM). Eluted proteins were investigated in SDS-PAGE and tested for the enzymatic activity.

Tests for enzymatic activity of phytase

Two tests were employed to measure the enzymatic activity of phytase. One method employs the natural substrate for phytase—phytate. Sample (50 µl) of phytase was added to 616 µl of 0.25M NaAc pH 5.5.

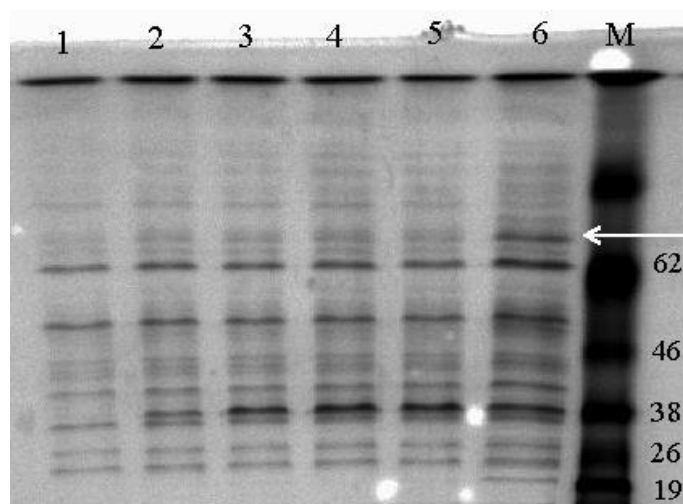
The solution was preincubated at selected temperature for 5 minutes, and then 333 ul of 10 mM water solution of sodium phytate (Sigma, P8810) was added. The mixture was incubated at selected temperature for 15 minutes. Then, the amount of released phosphate was measured using a colorimetric reaction with vanadium-molybdenum reagent. For the preparation of the vanadiummolybdenum reagent, 0.6 g of ammonium metavanadate (NH_4VO_3) was dissolved in 440 ml of water; then, 560 ml of 25% nitric acid and 25 g of ammonium molybdate ($(\text{NH}_4)_2\text{MoO}_4$) were added. The probe containing free phosphate was mixed with 1.5 ml of the vanadium-molybdenum reagent. The resulting mixture was incubated at 37°C for 1 hour. Any precipitates were removed by centrifugation, and the absorbance of the supernatant was measured at 415 nm.

The second test utilizes the ability of phytase to hydrolyze a non-natural colorigenic substrate pnitrophenyl phosphate (PNPP). This method is quick and less tedious than the test with the natural substrate. Sample (50 ul) of phytase was added to 850 ul of 0.25M NaAc pH 4.5. The solution was preincubated at 37°C for 5 minutes, and then 100 ul of water solution of PNPP (4 mM) was added. The reaction was incubated at 37°C for 15 minutes. Then, 100 ul of 10M NaOH was added. The resulting yellow color was quantified using absorbtion measurement at 410 nm. In all tests, imidazole elution buffer was used as a dummy probe to produce spectrophotometer blanks.

Results

Figure 1 presents SDS-PAGE (10%) of proteins accumulating in culture of the BL21(DE3)/pET32/Nov9x in the presence of chemical inducer IPTG. The expected product of recombinant expression, fusion protein Trx-Nov9x, has a molecular mass of 63.4 kDa.

Figure 1: SDS-PAGE of proteins accumulating in induced culture. Lanes: 1) before addition of IPTG; 2) 1 hour after addition of IPTG; 3) 2 hours; 4) 4 hours; 5) 6 hours; 6) 14 hours. Lane M - protein marker. Arrow points at band of the recombinant expression product.



Source: Authors

Figure 2 presents sequence of the synthesized Nov9x gene. DNA fragment was synthesized using the two-rounds PCR. Primers for the first round PCR (designated as P1-P32 in Figure 2) alternate in order Sense-Antisense-Sense-Antisense. A mixture of primers P1-P32 was PCR-amplified (all primers in equal concentration, concentrations of individual primers sum up to 100 pM/ul) using the high precision Phusionthermophilic polymerase. Reaction mixture of the first round amplification was used as template for the second round PCR with primers PNco + PXho (Figure 2).

Figure 2: Nov9x gene. Positions of primers used for the de novo gene synthesis are shown. Restriction sites Nco I and Xho I are denoted.

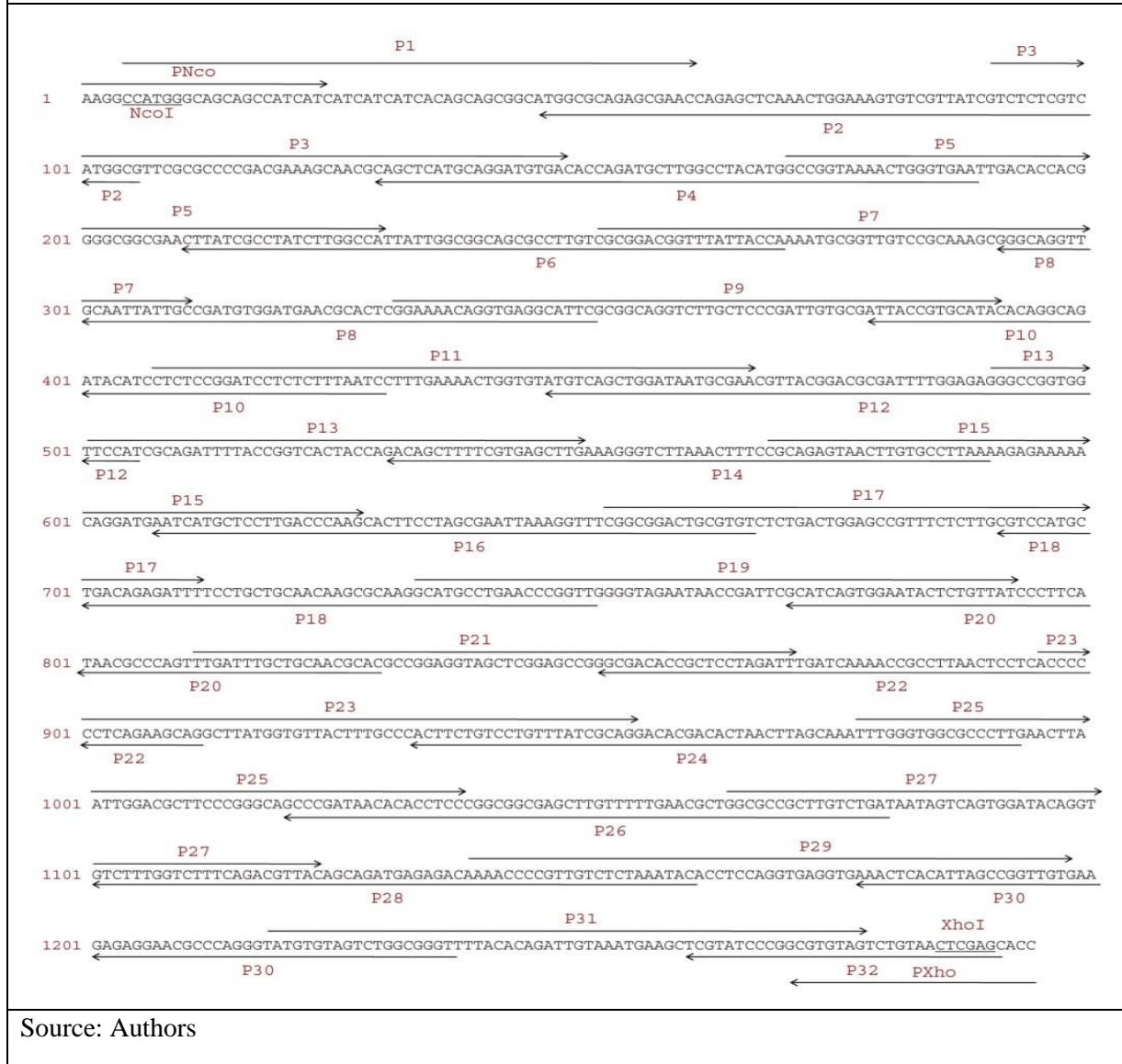
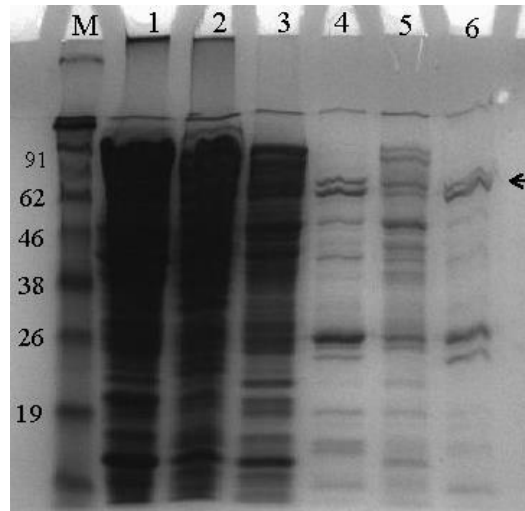


Figure 3 presents results of purification of the recombinant protein using IMAC. SDS-PAGE (15%) shows that protein with the expected molecular mass (63.4 kDa) elutes in a range of imidazole concentrations of 100-300 mM. Some contaminating proteins with lower Mw are also visible suggesting that single round IMAC is not sufficient to obtain pure recombinant phytase.

Figure 4 presents results of testing for phosphatase activity in the same samples for which the protein content is presented in Figure 3. This test was conducted with the PNPP substrate.

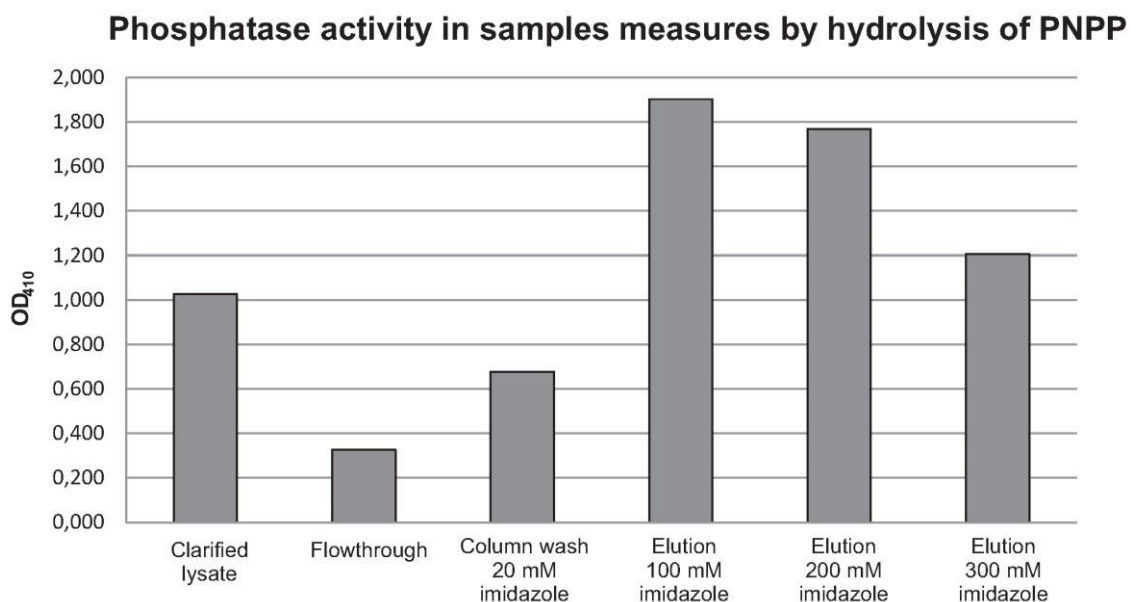
Combined samples of protein eluted into buffers with 100-300 mM imidazole was used to confirm thermostability of the obtained recombinant phytase. This test was performed with sodium phytate as substrate. Combined sample was mixed with sodium acetate buffer pH 5.5 and sodium phytate. The reaction mixture was incubated at various temperatures in the range of 30-80°C. It appeared that activity of the Nov9x almost linearly depends on the temperature in the temperature range of 30-70°C, and at 70°C enzymatic activity was two times higher than that at 30°C (Figure 5).

Figure 3: Purification of the recombinant phytase using the immobilized metal affinity chromatography, SDS-PAGE analysis of fractions. Lanes: M - protein marker; 1) clarified lysate; 2) flowthrough; 3) column wash (20 mM imidazole); 4) elution (100 mM imidazole); 5) elution (200 mM imidazole); 6) elution (300 mM imidazole). Arrow points at band of the recombinant expression product.



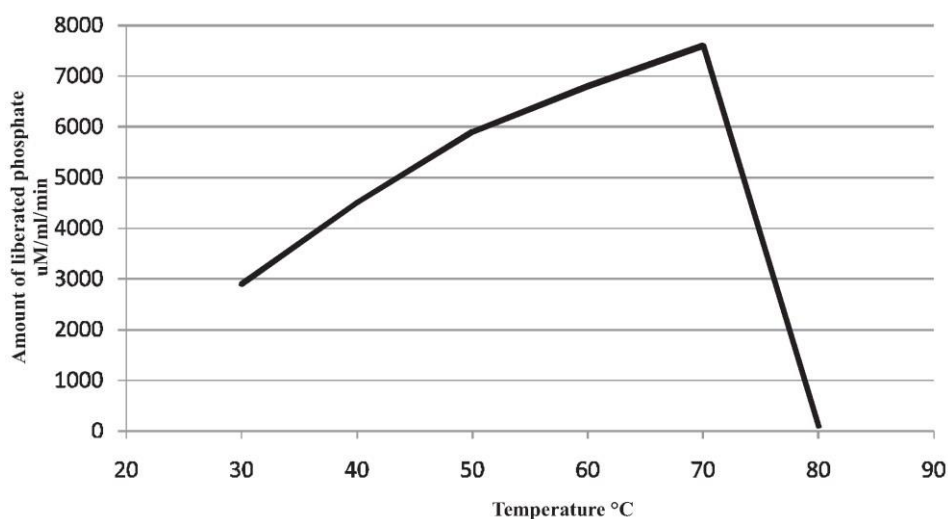
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Figure 4: Testing of phosphohydrolase activity of phytase with the p-nitrophenyl phosphate (PNPP). Phytase has activity for removing of the phosphate group from PNPP. Samples collected during purification of the recombinant phytase were tested. Accumulation of the coloured product of the reaction (p-nitrophenol) is measured by absorbance of light at 410 nm.



Source: Authors

Figure 5: Activity of the recombinant phytase Nov9x on sodium phytate. Reaction of enzymatic hydrolysis of phytate performed at various temperatures in the range 30°C-80°C.



Source: Authors

Conclusion

Industrially produced phytases are in high demand in the modern animal farming where they are used as feed additives. Phytases are added to animal diets to improve utilization of phosphorus from plant-derived components. Because of technological considerations, feed phytases have to withstand elevated temperatures (60-80°C), which are used during preparation of fodder. Significant success in development of engineered thermotolerant phytases was achieved by the method of directed evolution, which comprises repeating rounds of an in vitro mutagenesis and selection of best enzymes by high-throughput screening (Garret et al., 2004). Phytase Nov9x is a product of an exhaustive mutagenesis of the highly active *E. coli* phytase AppA. The Nov9x differs from the parental enzyme AppA by eight amino acid substitutions. The Nov9x is a generic name of the enzyme available on the market under brands Quantum Phytase (Syngenta Animal Nutrition, USA) and Quantum Blue Phytase (AB Vista, Germany). Nov9x withstands incubation for 1 hour at 62°C without a loss of activity and maintains 27% of initial activity after 10 minutes at 85°C.

We synthesized gene encoding the phytase Nov9x, expressed the gene in *E. coli*, and used enzymatic reactions to confirm specific biochemical properties of the product. Gene and expression system described has a prospective utilization in the development of pilot industrial production of phytase in the country.

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