ISSN 1330-9862
(FTB-2761)

original scientific paper

A Comparative Analysis of Recombinant Expression and Solubility Screening of Two Phytases in *E. coli*

*Ushasree Mrudula Vasudevan, Sumayya Husaiba Beevi Salim and Ashok Pandey**

Biotechnology Division, National Institute for Interdisciplinary Science and Technology (NIIST), CSIR, 695 019 Trivandrum, Kerala, India

Received: November 20, 2010
Accepted: January 25, 2011

Summary

Microbial phytases, especially from fungal and bacterial sources, have received much attention as food additives in human nutrition and as feed supplements for monogastric animals. An effective expression screening method for recombinant production of this enzyme on a small scale is industrially desirable. An effort has been made in this work to clone and express phytase genes from *Aspergillus* sp. and *Escherichia coli* with the selected host, vector and inducer combination. Albeit the formation of insoluble inclusion bodies by fungal phytase, recombinant *E. coli appA* was effectively expressed in a cost-effective manner in the periplasm of BL21plysS using an inducer concentration of 0.01 mM in 4 h of growth. Enzyme was purified in three consecutive steps and functional studies were carried out.

Key words: A. niger phytase, inducer concentration, recombinant *appA*

Introduction

Phytases produced biotechnologically have emerged as indispensable entities in feed and food industry for increased bioavailability of essential minerals. In plant-derived foods, phytate acts as an antinutritive factor by causing mineral deficiency due to efficient chelation of metal ions and by forming complexes with proteins. Unlike ruminants, monogastric animals such as pigs and poultry lack phytase-producing microorganisms in their gut. The addition of microbial phytase to the feedstuff for monogastric animals can substantially improve phosphorus utilization, reduce excretion of phosphorus in the faeces and counteract the antinutritional properties of phytate. In addition, this phytase plays an important role in human nutrition in the hydrolysis of phytate (*myo*-inositol hexakisphosphate) during food processing as well as in the gastrointestinal tract (*1*). Several experiments and field trials have proved that 500 to 1000 units of phytase can replace approx. 1 g of inorganic phosphorus supplementation and decrease the total phosphorus excretion by 30–50 % (*2*).

The desirable characteristics of phytase relevant in practical applications are cost effectiveness, high specific activity, low optimal pH that corresponds to animal digestive tract environment, resistance to stomach proteases, and feed pelleting temperature (*3*). So far this so-called ideal phytase has never been encountered in nature or developed by any advanced strategies. Almost all microbes such as *Bacillus* sp. (*4*), *Klebsiella aerogenes* (*5*), *Pseudomonas* sp. (*6*), *Escherichia coli* (*7*), ruminal bacteria (*8*), *Aspergillus niger* (*9*) and *A. fumigatus* (*10*) have been reported to produce phytases. Phytases from fungal sources constitute a major component of feed industry, amongst them phytase (EC 3.1.3.8) from *A. niger* encoded by *phyA* gene, with favourable enzymatic characteristics, is the one which is best characterized. Besides this fungal phytase, phytase from *E. coli* has been shown to be more effective in diets for young chickens and pigs than other available phytases (*11*). *E. coli* phytase encoded by *appA* gene is a periplasmic phosphoanhydride phosphohydrolase, which cleaves phytate in a broad pH range (2.5–5.0). Hence, these two microbial phytases have received much

*Corresponding author; Phone: ++91 471 251 5279; Fax: ++91 471 249 1712; E-mail: pandey@niist.res.in, ashokpandey56@yahoo.co.in

attention due to their relatively high acid tolerance and specific activity that surpasses the activities of other phytases (*12*,*13*).

Expression of phytase gene in normally growing *E. coli* is extremely low (*7*) and this phytase activity increases when the bacterial culture enters the stationary phase of growth stimulated by anaerobiosis and exhaustion of inorganic phosphate. For these reasons, total yield of *E. coli* phytase is too low for practical applications necessitating its overexpression in suitable hosts. Effort has already been made to overproduce the enzyme in *E. coli* for subsequent characterization (*12*). A mutant derivative of *appA* gene has been effectively expressed in extracellular medium using Kil protein fusion signal (*14*). Likewise, despite the fact that *A. niger* phytase has been expressed successfully from a number of yeast systems, efforts to produce it in *E. coli* resulted in insoluble inclusion bodies (*15*).

There is enormous importance in finding economically competitive expression system for low-cost production of phytases through recombinant techniques. *E. coli* has been used as homologous as well as heterologous expression system for effective production of industrially relevant recombinant enzymes, including phytases. Here we made an effort to express recombinant *appA* from *E. coli* XL1-Blue and *phyA* from *A. niger* NII 08121 in BL21 pLysS cell using optimized inducer conditions from T7 polymerase-regulated promoter of pET expression system. Using this system, it has been demonstrated that periplasmic localization of recombinant protein could be achieved in reduced concentration of IPTG, making feasible a cost-effective commercial production of native or improved protein for practical application. The aim of the present work is the search for an optimized phytase from microbial sources and tailoring well-characterized and efficient catalyst using protein engineering.

Materials and Methods

Bacterial strains and plasmids

E. coli XL1-Blue was used as a source of *appA* gene, and genomic DNA of *A. niger* NII 08121 was used as a template for PCR amplification of *phyA* gene. *E. coli* DH5α was used for routine molecular cloning and *E. coli* BL21 pLysS as a host for expression of recombinant *appA* and *phyA*. Vectors pET-20b(+) and pET-28a(+) (Novagen, Madison, WI, USA) were used for cloning and expression of *appA* and *phyA* genes, respectively.

E. coli strain harbouring pET-20b(+)-*appA* was grown in Luria-Bertani (LB) medium supplemented with chloramphenicol (25 μg/mL), ampicillin (50 μg/mL) and 0.5 % glucose, while pET-28a(+)-*phyA* construct was grown in chloramphenicol (25 μg/mL) and kanamycin (30 μg/mL).

DNA manipulation and cloning of phytases

All DNA manipulations were carried out as described by Sambrook *et al.* (*16*). Gene encoding *appA* including signal sequence was PCR-amplified from genomic DNA of *E. coli* XL1-Blue using primers 5′-GCCGGCATATGAAAGCGATCTTAATCCCAT-3′ (AP FP) and 5′-GTCAAGCTTTTACAAACTGCACGCCGG-3′ (AP RP). These primers introduced restriction sites *Nde*I and *Hind*III at 5′ and 3′ end (underlined), respectively. *PhyA* gene was amplified using primers 5′-ATTGAATTCCTGGCAGTCCCCGCCTCGAGA-3′ (PhyFP) and 5′-ATCAAGCTTAGCGGAACACTCCGC-3′(PhyRP) having restriction sites *Eco*RI and *Hind*III at 5′ and 3′ end (underlined), respectively. The resulting amplicons were excised from the gel and isolated using QIAquick gel extraction kit (Qiagen, Hilden, Germany). Gel-purified DNA fragments were restriction digested, and ligated to the corresponding position of pET vector. The plasmid was transformed into the cloning host *E. coli* DH5α and sequenced using an ABI Prism 3.7.3.0 XL automated DNA sequencer (Scigenome Pvt. Ltd, Cochin, India). Plasmid encoding recombinant *appA* bearing native periplasmic signal and *phyA* encoding mature peptide under the control of a T7 inducible promoter was then transformed into BL21 pLysS for expression studies.

Recombinant phytase production and screening for inducer concentration

The expression of protein was performed as follows. Freshly transformed *E. coli* BL21 pLysS harbouring pET-20b(+)-*appA* construct was grown at 37 °C over night in 5-mL LB medium supplemented with glucose and appropriate antibiotics. The culture was pelleted and resuspended in fresh medium and cells were diluted 10 times (500 μL in 50 mL) for expression. Then it was grown in an incubator shaker at 37 °C until the absorbance at 600 nm ($A_{600\ nm}$) of 0.8 was reached. Protein production was induced by the addition of isopropylthio-β-D-galactoside (IPTG) at final concentrations of 0.01, 0.05, 0.1 and 0.4 mM. Following induction with IPTG, the cells were grown at 37 °C for 4 h. BL21 pLysS cells harbouring empty vector and uninduced cells were also kept as control. For SDS-PAGE analysis of total cell protein, 1 mL of culture was removed, normalized to $A_{600\ nm}$ of one and rotated at 4000×*g* for 5 min, the supernatant was removed, and the pellet was resuspended in 100 μL of 1× SDS-PAGE sample buffer, treated at 95 °C for 5 min, and vortexed at 10 000×*g* for 1 min.

Expression analysis of fungal phytase cloned in pET-28a(+) was carried out from BL21 pLysS. When cells reached an $A_{600\ nm}$ of 0.6, IPTG concentration of 0.1 mM was used for induction. The cells were shaken for additional 3 h at 37 °C and total cell protein, soluble and insoluble fractions were collected. Total cell protein, soluble cytoplasmic fraction and insoluble pellet fractions were mixed with SDS sample buffer and electrophoresed to determine the solubility of expressed protein.

Assay of acid phosphatase and phytase

Acid phosphatase activity was determined by incubation of recombinant phytase in 200 μL of assay mixture containing 20 mM *p*-nitrophenylphosphate and 100 mM sodium acetate at pH=4.5 and 37 °C for 30 min. The reaction was stopped by the addition of 800 μL of 1 M NaOH and the absorbance was measured at 405 nm to determine the formation of *p*-nitrophenol (*17*). One unit of acid phosphatase activity was defined as the amount

of enzyme catalyzing the formation of 1 μmol of *p*-nitrophenol per minute.

Phytase activity was analyzed by measuring the concentration of inorganic phosphate in a 200-μL incubation mixture consisting of 1 mM sodium phytate, 100 mM sodium acetate, pH=4.5, along with the recombinant enzyme (*18*). Following incubation of the assay mixture at 37 °C for 30 min, the reaction was stopped by adding 800 μL of freshly prepared acetone/2.5 M H_2SO_4/10 mM ammonium molybdate (2:1:1) and the absorbance was measured at 355 nm. One phytase unit was defined as the amount of activity that releases 1 μmol of inorganic phosphate from sodium phytate per minute at 37 °C.

Screening of recombinant phytase activity in periplasm and cytoplasm of the expression host

In order to analyze the expression of recombinant *appA*, periplasmic and cytoplasmic fractions were prepared as described below. The culture (50 mL) was harvested by centrifugation at 4000×*g* for 10 min and resuspended in 10-mL solution containing 20 % sucrose, 1 mM EDTA and 30 mM Tris-HCl (pH=8.0). After shaking at room temperature, cell suspension was centrifuged at 8000×*g* and 4 °C for 10 min to remove the supernatant and the resulting pellet was resuspended in 10 mL of ice-cold 5 mM $MgSO_4$, stirred for 10 min in ice water bath and centrifuged as above for the collection of osmotic shock fluid as supernatant. To prepare the cytoplasmic fraction, the pellet was resuspended in 5 mL of 20 mM Tris buffer (pH=8) and frozen briefly at –80 °C to disrupt the inner membrane and to allow resident T7 lysozyme to efficiently lyse the peptidoglycan layer. The lysed cells were sonicated on ice for 20 s and centrifuged at 12 000×*g* and 4 °C for 15 min. The supernatant was collected and enzyme activity was analyzed in periplasmic and cytoplasmic fractions. Enzyme activity of recombinant fungal phytase was also analyzed in the collected soluble fraction.

Purification of recombinant appA protein from periplasm

In order to purify the recombinant *appA*, expression was carried out in 200 mL of LB medium containing appropriate antibiotic and 0.5 % glucose according to the optimized conditions given above for BL21 pLysS cells. Periplasmic fraction was prepared and fractionation by salting out was carried out by ammonium sulphate. Powdered ammonium sulphate was added slowly to the fraction with constant stirring at 4 °C. The precipitated protein was collected by centrifugation at 15 000×*g* and for 30 min 4 °C. The second step of purification involved negative acid precipitation as follows: pellets from each fraction were dissolved in 0.5 M glycine-HCl buffer, pH=1.8, and kept at 37 °C for 30 min for precipitation of undesirable proteins due to their instability under acidic conditions. After that, samples were centrifuged at 13 000×*g* for 10 min, and the supernatants were collected. The fraction was then dialysed and diluted in 10 mM glycine--HCl buffer, pH=4.7, and loaded onto CM Sepharose™ cation exchange column. The enzyme was eluted in a linear gradient of sodium chloride (0–1 M), the most active eluted fractions were pooled, dialysed overnight at 4 °C against the standard buffer and used for further studies. Purified fraction was analyzed on 12 % SDS-PAGE and stained with silver nitrate.

Effect of temperature and pH on appA activity and stability

Optimum pH for the activity of purified enzyme was determined in different buffers ranging between 1.5 and 8.5. Enzyme activity profile of the purified enzyme depending on temperature was determined in the temperature range of 40–90 °C. The thermal stability of *appA* enzyme was determined by incubating the purified enzyme at 60, 70 and 80 °C for 20, 30, 45 and 60 min and the remaining activity was determined.

Stability of phytase at physiological temperature of monogastric animals

An experiment was performed to analyze the efficiency of *E. coli* phytase at physiological temperature of monogastric animals. Purified enzyme was incubated at temperature ranging from 39 to 44 °C for 1 h and the remaining enzyme activity was determined under optimum conditions.

Results and Discussion

Cloning of recombinant phytase

Two genes encoding phytase were amplified by PCR, one from genomic DNA of *E. coli* and the other from *A. niger* NII 08121. The 5′ oligonucleotides were designed so as to place the *appA* gene along with its periplasmic localization signal downstream of T7 promoter of pET--20b(+), whereas *phyA* gene was isolated excluding the signal peptide and intron, and inserted in the frame with N-terminal 6X histidine tag of pET-28a(+) and downstream of T7lac promoter. Both constructs were sequenced to confirm a proper orientation of the insert.

Optimization of inducer concentration for recombinant protein expression

Since the expression in pET-20b(+) was driven by T7 inducible promoter, even a low level expression of T7 RNA polymerase in the absence of IPTG may result in toxic basal expression of the target protein, as previously described (*19*). This basal level of expression may affect the stability of recombinant construct, so in the present study Bl21 pLysS, producing T7 lysozyme, a natural inhibitor of T7 RNA polymerase, was used (*20*). In an effort to optimize the production of recombinant *appA* in periplasm, native signal sequence was used as a means to target the enzyme to a preferred location. Glucose (0.5 %) was also added to expression medium for further regulation of enzyme production. Screening the effect of various concentrations of IPTG, examined on SDS-PAGE, showed the best yield of this 45-kDa monomer using the inducer concentration of 0.01 mM (Fig. 1).

It has been reported that IPTG concentration of 1 mM resulted in the formation of inclusion bodies (*15*) by unglycosylated *phyA*, hence, we made an effort to express *phyA* under reduced inducer concentration. However, this attempt did not give soluble expression in *E. coli* cytosol, which was evident from the presence of 56-

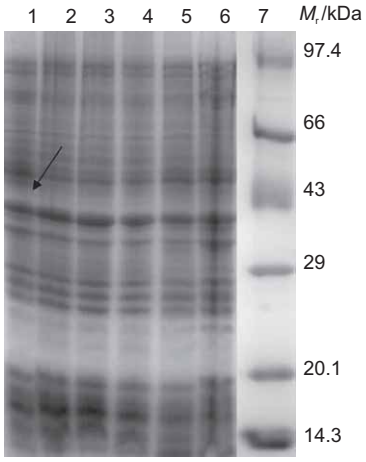

Fig. 1. SDS-PAGE analysis of total cell lysates showing the effect of inducer concentration on the expression of *appA*. Lanes 1 to 4: samples induced with 10, 50, 100 and 400 μM IPTG respectively, lane 5: uninduced control, lane 6: BL21 pLysS harbouring pET-20b(+) vector, lane 7: molecular mass standards in kDa

-kDa band in the pellet fraction and its subsequent absence from the soluble fraction on the SDS-PAGE (Fig. 2). Moreover, further solubilization of inclusion bodies was not promising for large-scale applications.

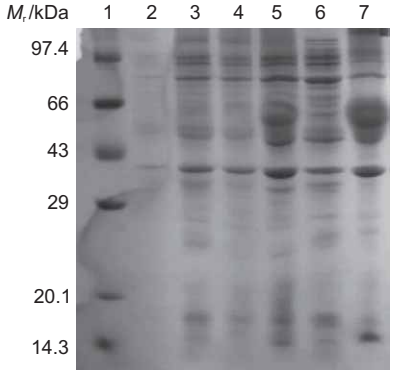

Fig. 2. Expressed fractions of *phyA* resolved by SDS-PAGE. Lane 1: molecular mass standards in kDa (GENEI), lane 2: control BL21 pLysS cells with vector, lane 3: uninduced total cell protein, lane 4: total cell protein of induced sample (0.1 mM), lane 5: soluble fraction of induced sample, lane 6: pellet fraction of induced sample

Screening of phytase activity in periplasm and cytoplasm

Acid phosphatase activity in periplasmic and cytoplasmic fractions induced with 0.01 mM IPTG was found to be 614.9 and 119.1 U/mL, respectively, after 4 h of post induction growth (Fig. 3). Enzyme activity observed in the uninduced culture and the host is indicative of basal level expression and endogenous phytase activity, respectively. These facts allowed to conclude that the highest protein yield can be attributed to lower inducer concentration under the experimental conditions determined here. As it was shown in a previous experiment, 1 mM IPTG was used to express the *E. coli appA* after 20 h of

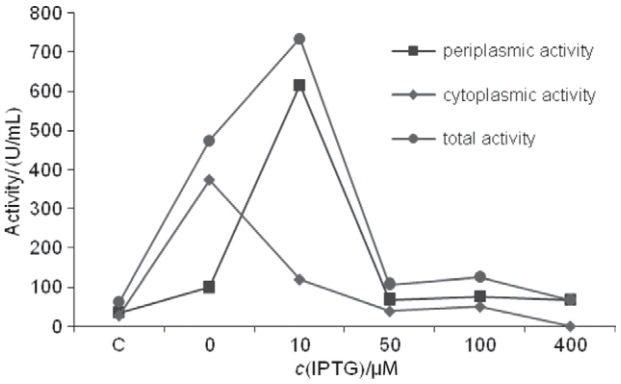

Fig. 3. Periplasmic and cytoplasmic activity of recombinant *appA*

growth (*21*). Our results seem to be promising since comparable yield was achieved using 100 times lower IPTG concentration and 5 times lower postinduction growth time compared to the previous report. These results are relevant since costs and time-consuming operations are common bottlenecks at industrial scale.

Inactive recombinant *phyA* expressed in *E. coli* cytosol clearly confirms the previous findings that glycosylation and proper disulphide bonds are mandatory for functional activity of fungal phytase (*22,23*). In addition, an N-terminal tag added to facilitate purification might have prompted misfolding or aggregation of protein, resulting in inclusion bodies lacking functional activity.

Purification of recombinant appA

Periplasmic space of *E. coli* contains lower number of proteins and this favours the purification of the recombinant protein expressed in periplasm. Periplasmic phytase activity was observed in 60–80 % saturation by ammonium sulphate fractionation. The procedure was followed by acid precipitation of contaminating protein in simple steps. The last step involves cation exchange chromatography, which allows obtaining homogenous purified protein. Purified recombinant *appA* was observed as 45-kDa band on 12 % SDS-PAGE gel (Fig. 4). The summary of the purification methodology is shown in Table 1.

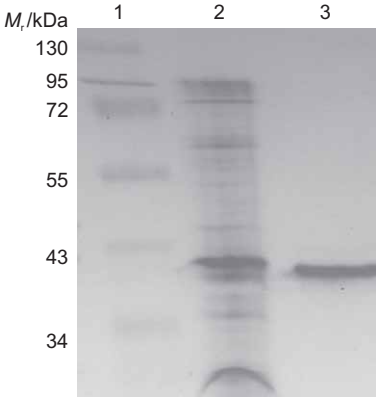

Fig. 4. Purification of *appA* by cation exchange chromatography. Lane 1: prestained molecular mass standards in kDa (Fermentas), lane 2: crude periplasm, lane 3: purified *appA* after ion exchange chromatography

Table 1. Purification of *appA* gene

Purification step	Activity U/mL	Total activity U	γ(protein) mg/mL	*m*(total protein) mg	Specific activity U/mg	Yield %
Crude ammonium sulphate	9.5	1900	0.30	60.00	31.6	100.0
Fraction 60–80 %	22.0	176	0.10	0.80	220.0	9.2
Ion exchange chromatography	9.1	162	0.01	0.18	900.0	8.5

Effect of pH and temperature

As shown in Fig. 5, purified recombinant *appA* enzyme preferentially acts under acidic pH conditions having optimum activity in the range of pH=3.0–4.5. This result is in agreement with a previous report (*12*). In spite of similar structural features among the microbial phytases, *E. coli appA* is well-suited for industrial application owing to its activity in acidic pH range, and this property unravels a marked difference betwen electrostatic environment at the active centre of this phytase and other microbial counterparts, as previously reported (*24*).

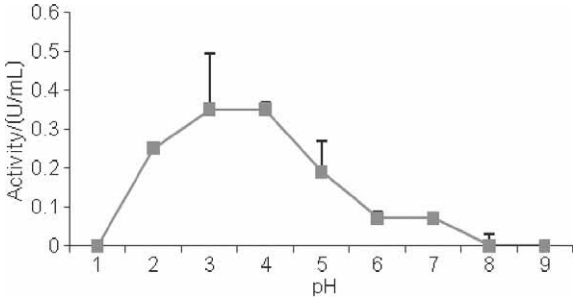

Fig. 5. Effect of pH on the activity of purified *appA*

The effect of temperature on *appA* activity shows that the optimum temperature for the enzyme is 60 °C (Fig. 6). Two *E. coli* phytases have already been characterized previously and both have an optimum temperature of 60 °C (*12*). Temperature stability analysis shows that the enzyme is moderately thermostable. It retained 50 % of its initial activity after 1 h of incubation at 60 °C, and 30 % activity after incubation at 70 °C for 20 min. At 80 °C beyond 30 min of incubation, the enzyme was fully inactivated (Fig. 7). Thermostability analysis was comparable to previous studies in which *E. coli appA* retained

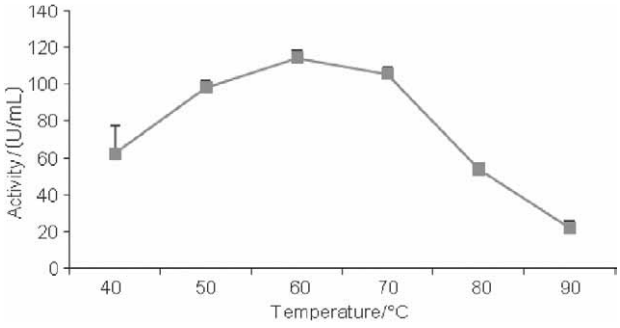

Fig. 6. Effect of temperature on the activity of purified *appA*

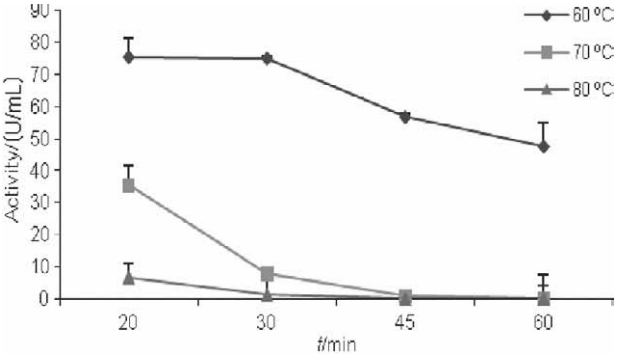

Fig. 7. Effect of temperature on the stability of purified *appA*

nearly half of its activity at 60 °C in 30 min of incubation and it diminished drastically in prolonged incubation at higher temperature (*21*).

Stability of phytase at physiological temperature of monogastric animals

To be used as an effective monogastric feed additive, phytase ought to be stable at acidic pH and physiological temperature, which prevail in the gut of swine and poultry. Temperature of monogastric gut generally ranges from 39 to 44 °C (for pig it is (39±2) °C, turkey about 39 to 40.5 °C and chicken between 39.8 and 43.6 °C). The analysis of stability of purified enzyme under these temperatures showed that more than 50 % of the enzyme activity was retained in this temperature range after 1 h of incubation, as compared to the control (Fig. 8).

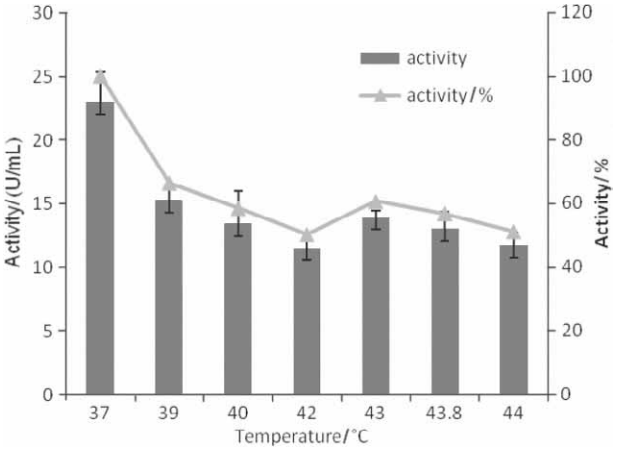

Fig. 8. Stability of *E. coli* phytase at physiological gut temperature of monogastric animals

Conclusions

Two microbial phytases, one from *Aspergillus niger* and other from *Escherichia coli*, were expressed under regulated expression conditions. Phytase from *E. coli* XL1--Blue was successfully targeted to the periplasm using its resident localization signal and by optimized combination of expression host and inducer concentration. This recombinant phytase showed a specific activity of 31.6 U/mg in crude periplasm under minimized inducer concentration of 10 μM during relatively lower postinduction incubation period. The enzyme was purified in three consecutive steps and functional characteristics were studied. It was not possible to solubilize the fungal phytase in *E. coli* system using reduced IPTG concentration and highly regulated T7lac promoter and BL21 pLysS host, as it formed insoluble inclusion bodies. In view of the fact that an unglycosylated form of fungal phytase produced in functional form in *E. coli* might permit effective high throughput screening in random and rational methods to improve the physicochemical properties of the enzyme, efforts to solubilize the protein using assistance of folding modulators are currently in progress.

Acknowledgements

The authors gratefully acknowledge the Department of Biotechnology, New Delhi, India, for financial support.

References

1. N.R. Nayini, P. Markakis, Effect of fermentation on the inositol phosphates of bread, *J. Food Sci. 48* (1983) 262–263.
2. P.A. Kemme, J.S. Radcliffe, A.W. Jongbloed, Z. Mroz, The effects of sow parity on digestibility of proximate components and minerals during lactation as influenced by diet and microbial phytase supplementation, *J. Anim. Sci. 75* (1997) 2147–2153.
3. X.G. Lei, C.H. Stahl, Biotechnological development of effective phytases for mineral nutrition and environmental protection, *Appl. Microbiol. Biotechnol. 57* (2001) 474–481.
4. M. Shimizu, Purification and characterization of phytase from *Bacillus subtilis* (natto) N-77, *Biosci. Biotechnol. Biochem. 56* (1992) 1266–1269.
5. M.P. Greaves, G. Anderson, D.M. Webley, The hydrolysis of inositol phosphates by *Aerobacter aerogenes*, *Biochim. Biophys. Acta, 132* (1967) 412–418.
6. G.C.J. Irving, D.J. Cosgrove, Inositol phosphate phosphatases of microbiological origin. Some properties of a partially purified bacterial (*Pseudomonas* sp.) phytase, *Aust. J. Biol. Sci. 24* (1971) 547–558.
7. R. Greiner, U. Konietzny, K.D. Jany, Purification and characterization of two phytases from *Escherichia coli*, *Arch. Biochem. Biophys*. 303 (1993) 107–113.
8. L.J. Yanke, H.D. Bae, L.B. Selinger, K.J. Cheng, Phytase activity of anaerobic ruminal bacteria, *Microbiology, 144* (1998) 1565–1573.
9. A. Ullah, K. Sethumadhavan, Differences in the active site environment of *Aspergillus ficuum* phytases, *Biochem. Biophys. Res. Commun. 243* (1998) 458–462.
10. L. Pasamontes, M. Haiker, M. Wyss, M. Tessier, A.P.G.M. Van Loon, Gene cloning, purification, and characterization of a heat-stable phytase from the fungus *Aspergillus fumigatus, Appl. Environ. Microbiol. 63* (1997) 1696–1700.
11. N.R. Augspurger, D.M. Webel, X.G. Lei, D.H. Baker, Efficacy of an *E. coli* phytase expressed in yeast for releasing phytate-bound phosphorus in young chicks and pigs, *J. Anim. Sci. 81* (2003) 474–483.
12. S. Golovan, G. Wang, J. Zhang, C.W. Forsberg, Characterization and overproduction of the *Escherichia coli appA* encoded bifunctional enzyme that exhibits both phytase and acid phosphatase activities, *Can. J. Microbiol. 46* (2000) 59–71.
13. M. Wyss, R. Brugger, A. Kronenberger, R. Rémy, R. Fimbel, G. Oesterhelt *et al.*, Biochemical characterization of fungal phytases (*myo*-inositol hexakisphosphate phosphohydrolases): Catalytic properties, *Appl. Environ. Microbiol. 65* (1999) 367–373.
14. G. Miksch, S. Kleist, K. Friehs, E. Flaschel, Overexpression of the phytase from *Escherichia coli* and its extracellular production in bioreactors, *Appl. Microbiol. Biotechnol. 59* (2002) 685–694.
15. B.Q. Phillippy, E.J. Mullaney, Expression of an *Aspergillus niger* phytase (*phyA*) in *Escherichia coli, J. Agric. Food Chem. 45* (1997) 3337–3342.
16. J. Sambrook, E.F. Fritsch, T. Maniatis: *Molecular Cloning: A Laboratory Manual, Vol. 3*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, NY, USA (2001).
17. A.H.J. Ullah, B.J. Cummins, Purification, N-terminal amino acid sequence and characterization of pH 2.5 optimum acid phosphatase (E.C. 3.1.3.2) from *Aspergillus ficuum, Prep. Biochem. 17* (1987) 397–422.
18. J.K. Heinonen, R.J. Lahti, A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphatase, *Anal. Biochem. 113* (1981) 313–317.
19. F.W. Studier, A.H. Rosenberg, J.J. Dunn, J.W. Dubendorff, Use of T7 RNA polymerase to direct expression of cloned genes, *Methods Enzymol. 185* (1990) 60–89.
20. F.W. Studier, B.A. Moffatt, Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes, *J. Mol. Biol. 189* (1986) 113–130.
21. N.V. Zinin, A.V. Serkina, M.S. Gelfand, A.B. Shevelev, S.P. Sineoky, Gene cloning, expression and characterization of novel phytase from *Obesumbacterium proteus*, *FEMS Microbiol. Lett. 236* (2004) 283–290.
22. Y. Han, D.B. Wilson, X.G. Lei, Expression of an *Aspergillus niger* phytase gene (*phyA*) in *Saccharomyces cerevisiae*, *Appl. Environ. Microbiol. 65* (1999) 1915–1918.
23. E.J. Mullanev, H. Locovare, K. Sethumadhavan, S. Boone, X.G. Lei, A.H. Ullah, Site-directed mutagenesis of disulfide bridges in *Aspergillus niger* NRRL 3135 phytase (*phyA*), their expression in *Pichia pastoris* and catalytic characterization, *Appl. Microbiol. Biotechnol. 87* (2010) 1367–1372.
24. A.H.J. Ullah, K. Sethumadhavan, E.J. Mullaney, Salt effect on the pH profile and kinetic parameters of microbial phytases, *J. Agric. Food Chem. 56* (2008) 3398–3402.