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Gene cloning, expression and characterization of novel phytase from *Obesumbacterium proteus*

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Abstract

The gene *phyA* encoding phytase was isolated from *Obesumbacterium proteus* genomic library and sequenced. The cleavage site of the PhyA signal peptide was predicted and experimentally proved. The PhyA protein shows maximum identity of 53% and 47% to phosphoanhydride phosphorylase from *Yersinia pestis* and phytase AppA from *Escherichia coli*, respectively. Based on protein sequence similarity of PhyA and its homologs, the phytases form a novel subclass of the histidine acid phosphatase family. To characterize properties of the PhyA protein, we expressed the *phyA* gene in *E. coli*. The specific activity of the purified recombinant PhyA was 310 U mg^{-1} of protein. Recombinant PhyA showed activity at pH values from 1.5 through 6.5 with the optimum at 4.9. The temperature optimum was 40–45 °C at pH 4.9. The K_m value for sodium phytate was 0.34 mM with a V_{max} of 435 U mg^{-1}.

Keywords: *Obesumbacterium proteus*; Phytase; Histidine acid phosphatase family; AppA-like protein; Phylogeny

1. Introduction

Phytate (*myo*-inositol hexakisphosphate) is the main storage form of phosphorus in seeds [1]. Monogastric animals are incapable of digesting phytate phosphorus due to lack or low level of the phytase activity in the intestine [2,3]. Phytates are regarded as decreasing feed quality because they bind proteins and mineral [4]. Further, undigested phytates cause significant environmental pollution [5].

Phytases (EC 3.1.3.8) belong to the family of histidine acid phosphatases, a subclass of phosphatases [6] capable of hydrolyzing phytates and releasing phosphate groups. They are successfully used as a feed additive. The characteristics phytase that important in practical applications are high specific activity, pH optima corresponding to various departments of the animal digestive tract, resistance to stomach proteases, drying and high temperature. This creates a need for phytases with an optimal combination of various properties.

A number of microorganisms, mainly of fungal and bacterial origin, are capable of phytase synthesis [7–15]. Recently, artificial phytases with superior thermostability were designed using semi-rational sequence comparison methods based upon several mesophilic homologues phytases from fungi (with sequences identity of about 50–70%) [16]. Phytase AppA from *Escherichia coli* is known to have the greatest specific activity [17] but it is inferior in terms of thermostability. In order to construct a more stable enzyme based on the *E. coli* phytase, it is necessary to have a set of homologs to

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E. coli phytase sequence. However, this protein has only a few homologs with low sequence identity not exceeding of 30% to other known phytases. We hypothesized that species from the Enterobacteriaceae family related to *E. coli* would have similar phytases. Indeed, a member of this family *Obesumbacterium proteus* was recently found to possess periplasmic phytase activity [18]. This activity is expected to be determined by a phytase similar to AppA.

In this paper, the phytase gene from *O. proteus* was cloned, sequenced, the enzyme recombinantly produced, characterized and compared with phytase from *E. coli*. Our aim was to expand the range of sequences close to the *E. coli* phytase in order to study this enzyme group and to develop high production of phytases.

2. Materials and methods

2.1. Strains, plasmids and chemicals

The following strains were obtained from the Russian Collection of Industrial Microorganisms (VKPM) *O. proteus* B-4567, B-6897, B-6898. *E. coli* strains and plasmids: XL-1 Blue (Strategne, La Jolla, CA), BL21 (DE3), pET22b(+) (Novagen, Madison, WI) and pUC19 [19]. All strains were grown at 37 °C in Luria–Bertani (LB) medium [20]. All chemicals were of the analytical grade, commercially available.

2.2. Cloning of the phytase gene

The *O. proteus* B-6898 genomic DNA was partially digested with *Mph*143I to obtain fragments of size 3–6 kb, and purified using DNA extraction Kit #K0513 (Fermentas AB, Vilnius, Lithuania). The DNA fragments were cloned into the *Bam*HI site of pUC19 and transformed into *E. coli* XL-1 Blue. Transformants were tested for the phytase activity on LB plates by pouring over the Test Upper Layer cooled to 37 °C and containing (w/v) 1% agarose, 1% phytate, and 0.5% $CaCl_2$. Positive clones caused clear zones around the colonies after 1–6 h of incubating with the upper layer. The clones were directly looped from beneath the upper layer in order to avoid the procedure of clone picking. The colonies harboring plasmids with DNA fragments encoding active phytase were named pPhyAn, where the last symbol n was the serial number. The size of insert was subcloned from the 4.2 kb insert by partial digestion with *Mph*143I to produce the final insert size of about 2.0 kb. The final pUC19 plasmid that harbored the subcloned DNA insert encoding the phytase activity, was named pPhyAmini. The insert DNA was sequenced by Syntol Co. (Moscow, Russia). The GenBank Accession No. is AY378096.

2.3. Expression of O. proteus phyA and E. coli appA

The templates for PCR were the total genomic DNA of *O. proteus* B-6898 and *E. coli* XL-1 Blue. PCR amplification of DNA containing the *phyA* gene including the signal peptide was performed using primers PphyaECF (forward, 5′-CCCATATGACAATTTCTCTGTTTACACA-3′) and PphyaECR (reverse, 5′-CCGAATTCTATTGGCACTCCACCAGTTCGT-3′) (the *Nde*I and *Eco*R1 restriction sites, respectively, are underlined). Amplified DNA was digested by *Nde*I and *Eco*RI and ligated into the respective sites of pET22b+ vector DNA. The obtained pETPhyA plasmid was used to transform *E. coli* BL21 (DE3). To express the *E. coli appA* gene, the same procedure was performed with different primers: PappaECF (forward, 5′-GCATATGAAAGCGATCTTAATCCCAT-3′) and PappaECR (reverse, 5′-GGGAATTCATTACAAACTGCACGCCG-3′) (the *Nde*I and *Eco*RI restriction sites, respectively, are underlined).

To obtain high level expression of recombinant phytases, cells were incubated for 20 h in 500 ml flasks containing 100 ml of LB plus the following components (w/v): 0.2% glucose, 1.5% lactose or 1 mM IPTG and ampicillin (100 μg ml^{-1}). Recombinant protein produced by *appA* in *E. coli* was named AppA-coli. Recombinant protein produced by *phyA* in *E. coli* was named PhyA-coli.

2.4. Purification of the phytases

After expression of phytase, cells were harvested, resuspended in 50 mM Tris–HCl buffer, pH 7.0 and sonicated at 4 °C. Phytases were purified in two steps (Table 1). At the first step, the majority of extraneous proteins were precipitated at low pH (AppA-coli and PhyA-coli are resistant to low pH of 2.0) by adding to the crude extract sample an equal volume of 0.5 M glycine–HCl buffer (pH 1.8). Solution was incubated 30 min at 37 °C and centrifugured at 14000*g*. At the second step, the enzymes were purified by FPLC gel-filtration Superdex 75-HR column.

2.5. Determining of the N-terminal amino acid sequence of the protein

The phytase samples to be analyzed were prepared by SDS–PAGE followed by semi-dry electroblotting of the separated material onto Immobilon-P membrane. The obtained Immobilon filter strip was used as a carrier in the automated Edman protein sequencing on an Applied Biosystems Procise cLC instrument. The N-terminal sequence was determined using the protein purified by a single-stage FPLC on a Superdex-75HR column.

Table 1
Purification scheme

Subject	Step	Total protein (mg)	Total U	U mg^{-1} of protein	Purification fold
AppA-coli	Crude extract	23.4	400	17.1	1
	Precipitation at pH 2.0	2.1	356	169.5	10.0
	FPLC	0.23	326	1420	83
PhyA-coli	Crude extract	26.0	250	9.6	1
	Precipitation at pH 2.0	3.2	236	73.8	7.7
	FPLC	0.7	217	310	32.3

2.6. Activity and properties of the phytase

The effect of pH on phytase activity was determined using the following buffer solutions: glycine–HCl, pH 1.5–3.5; Na acetate–acetic acid, pH 3.5–6.0; Tris–acetic acid, pH 6.0–7.0; and Tris–HCl, pH 7.0–8.0. Phytase activity was measured by accumulation of free phosphate in the reaction mixture detected according to Fiske–Subbarow [21]. The activity unit (U) causes releasing of 1 μmol of phosphate per min.

Phytase and phosphatase pH optima were determined using the above buffers with the concentration of 0.1 M at 37 °C. The temperature optima were determined at the pH optimum (4.9) ranging the temperature values from 20 to 90 °C. The temperature stability was estimated by incubation of the enzyme sample in 50 mM buffers for 30 min at 50, 60, 70, or 80 °C, followed by the 30 min incubation at 5 °C before measuring. The concentration of protein was determined by the Bradford method [22], the Lowry method [23] and by measuring the UV absorbance at 280 nm. The Bradford and Lowry assay was not adopted because of very low estimation of the AppA [17] and PhyA concentration. UV absorbance method was adopted as the most suitable routine method.

2.7. Sequence and phylogenetic analysis

The signal peptide was predicted using SignalP [http://www.cbs.dtu.dk/services/SignalP/] [24]. The molecular mass of the mature peptide was determined using PeptideMass [http://cn.expasy.org/tools/peptide-mass.html]. Homology searches in GenBank were done using the BLAST server [http://www.ncbi.nlm.nih.gov/BLAST/] [25]. Multiple alignment of protein sequences was done using the CLUSTALW program [http://www.ebi.ac.uk/clustalW/] [26]. The phylogenetic trees of proteins were constructed using various programs (SeqBoot, Protdist, Neighbor) in PHYLIP (Phylogeny Inference Package) version 3.6b [http://evolution.gs.washington.edu/phylip/] [27] with genetic distances computed by Kimura's two-parameter model [28]. The trees were visualized and drawn using the TreeView software version 1.6.6 [http://taxonomy.zoology.gla.za] [29].

3. Results

3.1. Isolation of the gene encoding phytase from the genomic library

Phytase activity of three *O. proteus* strains (VKPM B-4567, B-6897, B-6898) was measured at the pH optimum (4.9). *O. proteus* strain VKPM B-6898 (further referred to as *O. proteus*) has the highest phytase activity of 0.11 U mg^{-1} of total protein and was chosen to create a genomic library. The phytase activity was detected in 13 of 10,000 clones from the *O. proteus* genomic library. To compare the phytase activities, cells from stationary cultures of all 13 clones were subjected to sonication. The obtained values were about 3 U mg^{-1} of total protein with no significant variations for each of the clones. The clone harboring plasmid pPhyA8 with the shortest insert of 4.2 kb was selected and its insert was reduced to 2.6 kb to form the pPhyAmini. The latter insert was sequenced and the sequence deposited in GenBank under Accession No. AY378096. An ORF of 1.3 kb within this insert with homology to the phytase gene *appA* from *E. coli* was named *phyA*.

To detect the presence of the *phyA* gene in the remaining 12 active clones, PCR analysis of the internal portion of *phyA* (with primers 5′-GACGCAAACCATGCGCGACGTA-3′ and 5-CATCTGGCATGCCCTGCGCATA-3′) was carried out. This analysis demonstrated that all 13 clones contain the same gene *phyA* in the *O. proteus* phytase genomic library.

The orientation of the *phyA* gene in the plasmid pPhyA8 is in the direction opposite to pUC19 genes and thus cannot start at any of the plasmid promoters. However, the phytase activity of the strain that carries the phyA-containing plasmid considerably exceeds that of the *E. coli* and *O. proteus* wild-type cells. Therefore, we suppose that the *phyA* gene is expressed from a multi-copied vector, and the transcription initializes from the gene's own promoter which is contained within the inserted fragment. Indeed, this fragment contains several promoter-like sequences identified using the SignalX software [30]. However, the specificity of the existing algorithms is insufficient to reliably predict bacterial promoters [31], and thus the identified candidate sites are only tentative (data not shown).

3.2. Sequence and phylogenetic analysis

Several PhyA homologs were found in GenBank using BLAST and aligned (Fig. 1). Using this alignment we constructed a phylogenetic tree (Fig. 2). All these proteins were of bacterial origin. The closest homologs to PhyA were phosphoanhydride phosphorylase from *Yersinia pestis* (55% identity) and AppA from *E. coli*

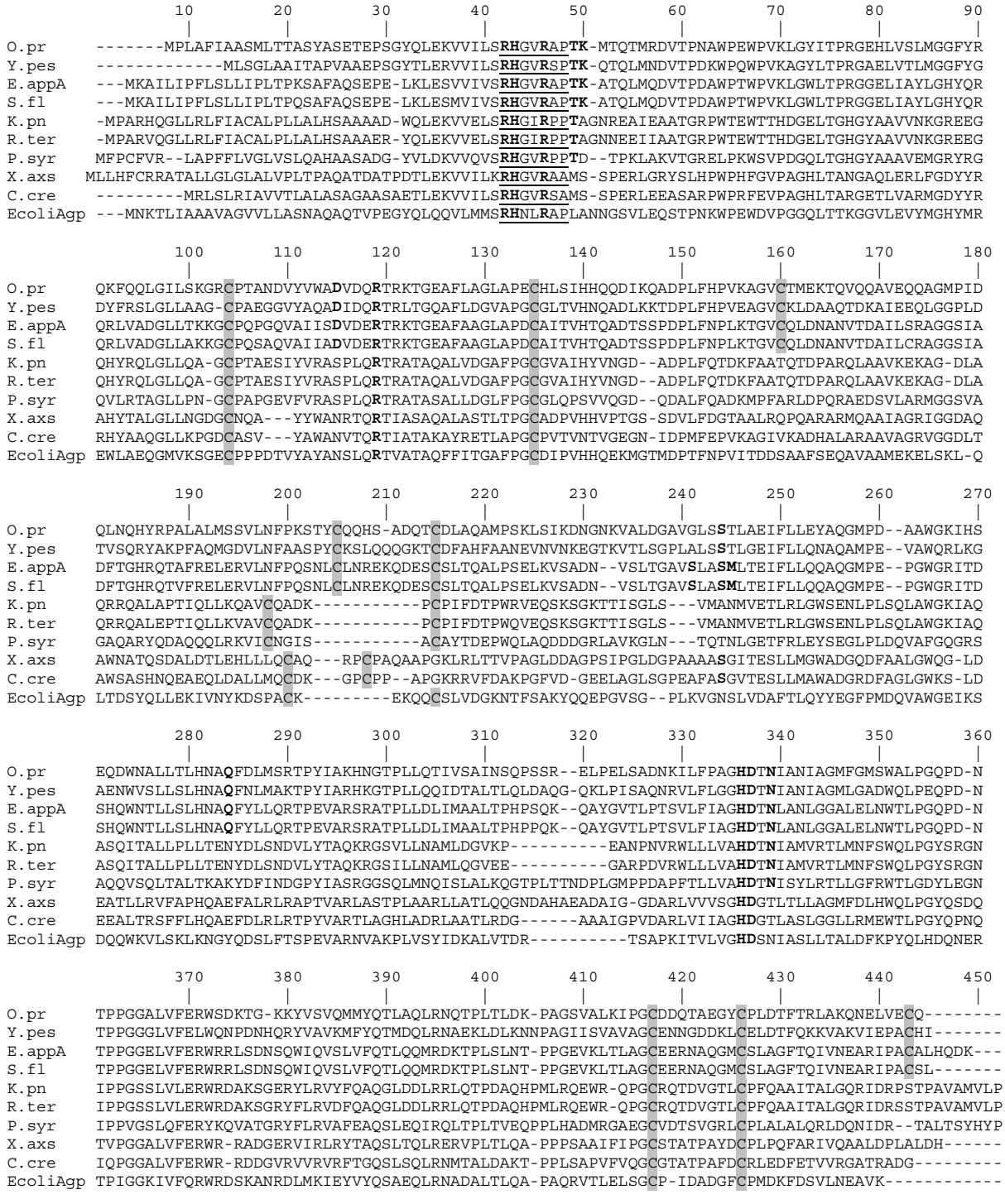

Fig. 1. Multiple alignment of homologs of the *O. proteus* phytase PhyA. Conserved histidine acid phosphatase family motif is underlined, residues of active site of AppA from *E. coli* and corresponding residues in homologs are marked as bold, cysteine residues are shown on a gray background. The abbreviations, source and GenBank Accession Nos. of proteins are: O.pr, *O. proteus*, AY378096; Y.pes, *Y. pestis* KIM, AE013783; E.appA, *E. coli* CFT073, AE016758; S.fl, *Shigella flexneri* 2a str. 301, AE015127; EcoliAgp, *E. coli* O157 H7 EDL933, AE005294; C.cre, *Caulobacter crescentus* CB15, AE005727; X.axs, *Xanthomonas axonopodis* pv. *citri* str. 306, AE011713; K.pn, *Klebsiella pneumoniae*, AY091638; R.ter, *Raoultella terrigena*, AJ575300; P.syr, *Pseudomonas syringae*, AY156083.

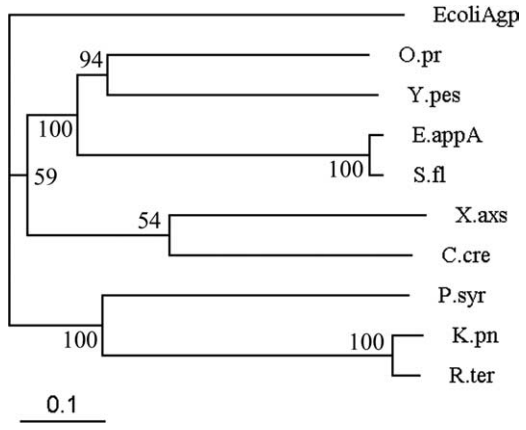

Fig. 2. Phylogenic tree of homologs of the *O. proteus* phytase PhyA. Bootstrap values (%) from analysis of 1000 bootstrap replicates are given at the respective nodes. The bar represents 1 substitution per 10 amino acids. For the abbreviations, see the Fig. 1 legend.

(48% identity). Other homologs have only about 30% identity to both *O. proteus* and *E. coli* phytases.

Positions of 12 out of 14 amino acid residues responsible for the enzyme–substrate binding in AppA [32] were identical to the corresponding residues in PhyA (Fig. 1). Positions of all 4 disulfide bonds were identical (Fig. 1).

3.3. *Overexpression of* phyA *and* appA *in* E. coli

Cloning of *phyA* into the pET22b+ vector and expression in *E. coli* BL21 (DE3) enhanced the enzyme production by two orders of magnitude compared with the wild type *O. proteus* VKPM-6898. Transcription of the *phyA* gene in this system depends on the lactose (or IPTG) induction. A completely analogous scheme using the pET22b+ vector was applied to overexpress the *appA* gene. The phytase activity in *E. coli* BL21 (DE3) crude extracts was 9.6 and 17.1 U mg^{-1} of total protein for PhyA-coli and AppA-coli, respectively (Table 1).

3.4. *Properties of the purified enzymes*

The activities of the purified enzymes were 310 U mg^{-1} and 1420 U mg^{-1} of total protein for PhyA-coli and AppA-coli, respectively (Table 1). Compared to other known phytases of bacterial and fungal origin, the specific activity of the *O. proteus* phytase was very high.

Samples of purified PhyA-coli displayed activity at acidic pH values (1.5–6.6) (Fig. 3(a)). The pH-optimum was observed at pH 4.9, and a local pH-optimum was also detected at pH 3.4. The maximum activity was observed at 40–50 °C (Fig. 3(b)). The stability of the enzyme at a short high temperature action is shown in Fig. 3(c). The enzyme is highly resistant to 30-min heating at 50 °C, but loses its activity very rapidly under further increase of the temperature. As a control, characteristics of AppA-coli were measured and plotted on the corresponding graphs (Fig. 3(a)–(c)).

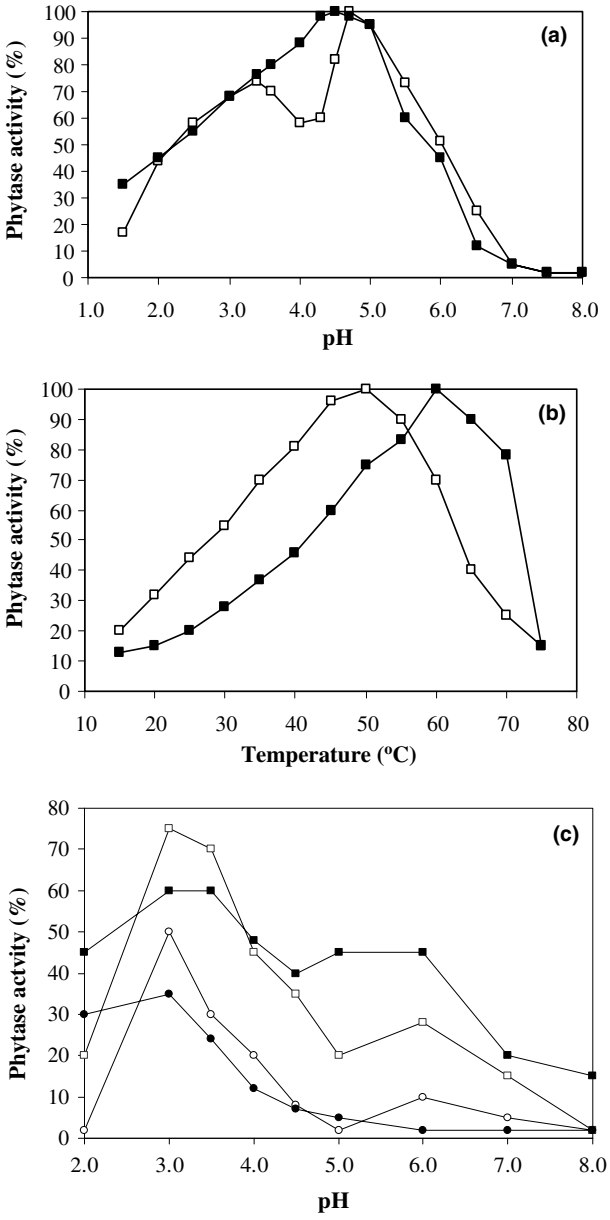

Fig. 3. Effect of pH (a) and temperature (b) on phytase activities of PhyA-coli (□) AppA-coli (■). Effect of 30 min treatment at 60 °C (PhyA-coli (□) AppA-coli (■)) and 70 °C (PhyA-coli (○) AppA-coli (●)) at different pH values (c) on the residual phytase activities, assayed thereafter (compared to untreated phytase corresponding to the 100% value).

PhyA-coli was very stable for long term incubation in 0.2 M buffer solutions at pH 1.5–8.0, 37 °C, and even after 24 h of incubation no significant changes in the activity could be detected. It is likely that disulfide bonds strongly contribute to the stability of the phytase molecules, which is clearly seen at low pH values. Most *E. coli* soluble proteins are denatured and precipitated in this range of pH values (Fig. 4).

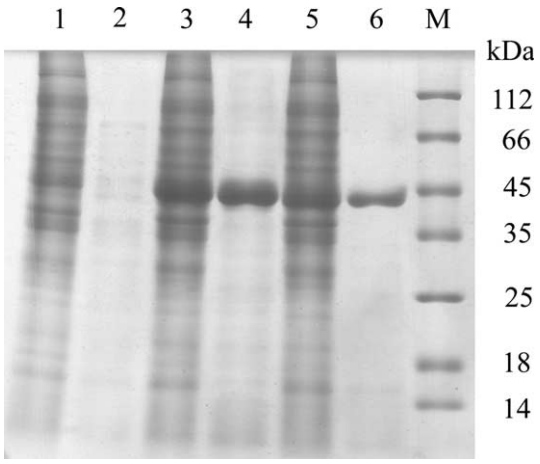

Fig. 4. SDS–PAGE analysis of phytases expression in *E. coli*. The analysis of proteins was carried out using 7.5% polyacrylamide gel and stained by Coomassie brilliant blue R: control (no phytase expression), PhyA-coli and AppA-coli crude extracts (lanes 1, 3 and 5, respectively). Effect of low pH (2.0) on crude extract of a previous line (lanes 2, 4 and 6, respectively) M: molecular weight standards.

The predicted mass of the peptide (45.2 kDa) was agreed with the SDS–PAGE value (about 45 kDa) (Fig. 4).

3.5. Identification of N-termini position of PhyA

The most likely position of the cleavage site at the N-terminus, Ser33–Ala34, was determined using the SignalP program and multiple sequence alignment. The sequence of the N-end of the mature PhyA protein purified from the *E. coli* crude extract, SETEPSGYQLEKVVI, confirmed this prediction. Unlike the mature enzymes, the leader peptides of PhyA homologs from *Y. pestis* and *E. coli* show no sequences similarity, nor even conservation of the peptide length. This data on the localization of the N-terminus of the mature PhyA protein is a necessary step in development of the enzyme expression in heterologous systems, including yeast, to reach high level expression of industrial proteins.

3.6. Substrate specificity of PhyA-coli

PhyA-coli was shown to cleave some phosphorus-containing organic substances other than phytate (Table 2). The kinetic parameters for the hydrolysis of phytate has a K_m of 0.34 mM and a V_{max} of 435 U mg^{-1} as determined from a Lineweaver–Burke plot. However, similarly to the *E. coli* phytase AppA [33], PhyA-coli degrades phytate with much higher rate than any other substrate under study, and thus it is a true phytase.

Table 2
Substrate specificity of PhyA-coli

Substrate	U mg^{-1} of protein[a]	pH optimum[a]
Phytate	310	4.9
Glucose-1-phosphate	0.8	3.5
Glucose-6-phosphate	3.1	3.5
Ribose-5-phosphate	8.4	3.0
Fructose-1-phosphate	2.2	3.5

[a] Values represent the arithmetic average of two measurements.

4. Discussion

The PhyA sequence from *O. proteus* showed high identity with the *E. coli* phytase. The high identity of *E. coli* AppA and *O. proteus* PhyA sequences, position of the active center and the cysteine residues allows us to suggest that the proteins may have similar three-dimensional structure and a similar mechanism of the enzyme action. The same similarity have been observed for both these phytases and their homolog with unknown function from *Y. pestis*. All these proteins form a particular branch in the phylogenetic tree. So it is possible that the protein from *Y. pestis* is also a phytase. Further, several bacterial species are known to have phytases with similar properties but unknown sequences. These bacteria are *Enterobacter cloaceae* [18], *Citrobacter freundii* [18] and *Citrobacter braakii* [11], from the Enterobacteriaceae family. It is likely that their phytases also have similarity to the *E. coli* phytase AppA.

Note that homologous proteins aligned and depicted in the tree (Fig. 2) are produced from Gram-negative bacteria most of which (7 of 10) belong to the Enterobacteriaceae family. These proteins were found using BLAST and the identity between the members of the group is about 30–50% throughout the entire protein length. Other known phytases of the fungal origin demonstrated no significant similarity to the bacterial phytases except the conserved motif RHGXRXP of the histidine acid phosphatase EC 3.1.3.8 family [6]. So, based on the protein sequence similarity, the studied proteins form a separate subclass of this protein family. Similarly, a separate subclass of fungal phytases was established earlier [34].

Unlike other phytases whose substrate specificity has been studied [33], the *E. coli* and *O. proteus* phytases show specificity to phytate that is 10- to 100-fold higher than specificity to other phosphorylated compounds. On the other hand, known phytases of Enterobacteriaceae are frequent in the animal intestine compartments (with neutral or weak alkaline pH of 7–8) [2] where the phytase activity toward phytate must be non-existent. Thus, the role of phytases in bacterial metabolism remains unclear. Nevertheless, the phytase activity at low pH values is exactly the feature required in agricultural manufacturing to use them as feed additives for farm animals with the stomach pH values of 2–6 [2].

We have obtained 100-fold increase in the PhyA synthesis during overexpression in *E. coli* cells. The recombinant enzyme PhyA-coli is not inferior to the

wild type enzyme in such industrial characteristics as temperature resistance, pH optimum, as well as activity at low pH values of 1.5–6.5 [18], (Fig. 3). AppA-coli, the closest homolog of PhyA-coli, has somewhat higher specific activity. Although the *O. proteus* phytase is inferior to *E. coli* phytase in specific activity, it also can be used as feed additives as an alternative enzyme.

This study demonstrated that the *phyA* gene could be used for developing strains-producers of the enzyme with higher expression level. The high identity between PhyA and AppA may be helpful in investigation of the structure, evolution, and mechanisms of the enzyme action. It can also be used to compute the modifications of the enzyme in order to enhance its industrial potential using rational or semi-rational sequence comparison methods.

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References

[1] Reddy, N.R., Sathe, S.K. and Salunkhe, D.K. (1982) Phytates in legumes and cereals. Adv. Food Res. 28, 1–92.
[2] Bitar, K. and Reinhold, J.G. (1972) Phytase and alkaline phosphatase activities in intestinal mucosae of rat, chicken, calf, and man. Biochim. Biophys. Acta 268, 442–452.
[3] Common, F.H. (1989) Biological availability of phosphorus for pigs. Nature 143, 370–380.
[4] Harland, B.F. and Morris, E.R. (1995) Phytate: a good or bad food component? Nutr. Res. 15, 73–754.
[5] Sweeten, J.M. (1992) Livestock and poultry waste management: a national overview. In: National Livestock, Poultry and Aquaculture Waste Management (Blake, J.D. and Magette, W., Eds.), pp. 4–15. American Agricultural Engineering, St. Joseph, MN.
[6] van Etten, R.L. (1982) Human prostatic acid phosphatase: a histidine phosphatase. Ann. N. Y. Acad. Sci. 390, 27–51.
[7] Lei, X.G. and Stahl, C.H. (2001) Biotechnological development of effective phytases for mineral nutrition and environmental protection. Appl. Microbiol. Biotechnol. 57, 474–481.
[8] Greiner, R., Haller, E., Konietzny, U. and Jany, K.-D. (1997) Purification and characterization of a phytase from *Klebsiella terrigena*. Arch. Biochem. Biophys. 341, 201–206.
[9] Mullaney, E.J., Daly, C.B. and Ullah, A.J. (2000) Advances in phytase research. Adv. Appl. Microbiol. 47, 157–199.
[10] Kim, Y.O., Lee, J.K., Kim, H.K., Yu, J.H. and Oh, T.K. (1998) Cloning of the thermostable phytase gene (*phy*) from *Bacollus* sp. DS11 and its overexpression in *Escherichia coli*. FEMS Microbiol. Lett. 162, 185–191.
[11] Kim, H.W., Kim, Y.O., Lee, J.H., Kim, K.K. and Kim, Y.J. (2003) Isolation and characterization of a phytase with improved properties from *Citrobacter braakii*. Biotechnol. Lett. 25, 1231–1234.
[12] Lambrechts, U.K., Boze, H., Moulin, G. and Galzy, P. (1992) Utilization of phytate by some yeast. Biotechnol. Lett. 14, 64–66.
[13] Pandey, A., Szakacs, G., Soccol, C., Rodriges-Leon, J.A. and Soccol, V.T. (2001) Production, purification and properties of microbial phytases. Biores. Technol. 77, 203–214.
[14] Pasamontes, L., Haiker, M., Wiss, M., Tessier, M. and van Loon, A.P.G.M. (1997) Gene cloning, purification, and characterization of a heat-stable phytase from the *fungus Aspergillus fumigatus*. Appl. Environ. Microbiol. 63, 1696–1700.
[15] Yanke, L.J., Bae, H.D., Selinger, L.B. and Cheng, K.J. (1998) Phytase activity of anaerobic rumminal bacteria. Microbiology 144, 1565–1573.
[16] Lehmann, M, Loch, C., Middendorf, A., Studer, D., Lassen, S.F., Pasamontes, L., van Loon, A.P.G.M. and Wyss, M. (2002) The consensus concept for thermostability engineering of proteins: futher proof of concept. Protein Eng. 15, 403–411.
[17] Golovan, S., Wang, G., Zhang, J. and Forsberg, C.W. (2000) Characterization and overproduction of the *Escherichia coli appA* encoded bifunctional enzyme that exhibits both phytase and acid phosphatase activities. Can. J. Microbiol. 46, 59–71.
[18] Zinin, N.V., Samsonov, V.V., Samsonov, V.V., Borshevskaya, L.N., Kanikovskaya, A.A., Gudima, M.U. and Syneokii, S.P. (2003) Phytase activity of several bacteria groups. Biotechnologia (Russia) 2, 3–10.
[19] Yanich-Perron, C., Vieira, Y. and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33, 103–119.
[20] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[21] Fiske, C.M. and Subbarow, Y.T. (1925) The colometric determination of phosphorus. J. Biol. Chem. 66, 375–400.
[22] Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein binding. Anal. Biochem. 72, 248–254.
[23] Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.
[24] Nielsen, H., Eengelbrecht, J., Brunak, S. and von Heijne, G. (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. 10, 1–6.
[25] Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. J. Mol. Biol. 215, 403–410.
[26] Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.
[27] Felsestein, J. (1989) PHYLIP: phylogeny inference package (version 3.6). Cladistics 5, 164–166.
[28] Kimura, M. (1980) A simple method for estimating evolutionary rates of base substitution through comparative studies of nucleotide sequence. J. Mol. Biol. 16, 111–120.
[29] Page, R.D. (1996) TreeView: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12, 357–358.
[30] Mironov, A.A., Vinokurova, N.P. and Gelfand, M.S. (2000) Software for analysis of bacterial genomes. Mol. Biol. 34, 222–231.
[31] Hertz, G.Z. and Stormo, G.D. (1996) *Escherichia coli* promoter sequences: analysis and prediction. Methods Enzymol. 273, 30–42.
[32] Lim, D., Golovan, S., Forsberg, C.W. and Jia, Z. (2000) Crystal structure of *Escherichia coli* phytase and its complex with phytate. Nat. Struct. Biol. 7, 108–113.

[33] Wiss, M., Brugger, R., Kronenberger, A., Remy, R., Fimbel, R., Oesterhelt, G., Lehmann, M. and van Loon, A.P.G.M. (1999) Biochemical characterization of fungal phytases (myo-inositol hexakisphosphate phoshohydrolazes): catalytic resistance. Appl. Environ. Microbiol. 65, 367–373.

[34] Mitchell, D.B., Vogel, K., Weimann, B.J., Pasamontes, L. and van Loon, A.P.G.M. (1997) The phytase subfamily of histidine acid phosphatases: isolation of genes for two novel phytases from the fungi *Aspergillus terreus* and *Myceliophthora thermophila*. Microbiology 143, 245–252.