*ScienceAsia* 30 (2004): **383-390**

# Purification, Characterization, Gene Cloning and Sequence Analysis of a Phytase from *Klebsiella pneumoniae* subsp. *pneumoniae* XY-5

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Received *7 Jul 2004*
Accepted *13 Sep 2004*

**ABSTRACT:** A phytase produced by the soil bacterium *Klebsiella pneumoniae* subsp. *pneumoniae* strain XY-5 was isolated and purified. The enzyme was a single chain protein with a molecular mass of 41.7 kDa, as determined by SDS-PAGE. The isoelectric point (p*I*) of the native enzyme was found to be 8.7. It exhibited two pH optima (3.7 and 5.5) when assayed both at 37°C (297 units/ mg protein) and 55°C (318 units/ mg protein) and it was found to be stable up to 60°C for 4.0 hours. The enzyme was found to have broad substrate specificity. It was activated by EDTA, $Al^{3+}$ and $Co^{2+}$, but was strongly inhibited by $Hg^{2+}$. The putative gene encoding the phytase was cloned by PCR, and DNA sequencing revealed an ORF of 1269 nucleotides downstream from a potential ribosome-binding site. The deduced amino acid sequence of the mature protein comprised 394 aa with a calculated molecular mass of 43.4 kDa and a signal peptide consisting of 28 aa. The mature enzyme contained the conserved active site RHGXRXP and an HD motif that placed it in the histidine acid phosphatase (HAPs) family. Expression of the cloned gene in an *E. coli* yielded active phytase. Due to its relatively high specific activity, broad substrate specificity, good pH profile and temperature stability, the enzyme could be a good candidate for industrial applications.

**KEY WORDS**: Phytase; *Klebsiella*; Purification; Gene cloning

## INTRODUCTION

Phosphorus, like nitrogen, is essential for all forms of life. Phytate (myo-inositol 1,2,3,4,5,6-hexakisphosphate, IP6) is the principal storage form of phosphorus in plants, particularly in cereal grains and legumes, and it typically represents approximately 75%-80% of the total phosphorus found in nature.[1] The phytate form of phosphorus is not easily utilizable by monogastric animals, so it contributes to phosphorus pollution problems in areas of intensive livestock production.[2] On the other hand, an external source of phosphorus must be supplied to monogastric animals and humans in sufficient quantity to meet their daily mineral requirements.[3] This can be costly, and excess use of dietary phosphorus can also cause environmental problems.[4]

Facing the problem of phosphorus deficiency in animal feed and the problem of phosphorus pollution in areas of intensive livestock production, phytase seems destined to become increasingly important. Phytase, or *myo*-inositol-hexakisphosphate phosphohydrolase (EC 3.1.3.8), was first discovered by Suzuki *et al*, in 1907.[5] This enzyme can catalyse the hydrolysis of phytic acid to inositol and orthophosphoric acid. Commercialization requires not only a practical use and delivery of the enzyme but also the ability to produce the enzyme economically. So far, large-scale production of cheap phytase for animal feed has been solved through fermentation of genetically modified microorganisms[6], however, one key problem that has not been solved is instability of the phytase upon heating.

The pelletization process for production of animal feeds uses high transient temperatures of 60-90°C. [7,8] These temperatures can cause phytases to lose substantial activity irreversibly. Therefore, a usable phytase must have good thermostability and high activity at the body temperature of target animals usually

around 37°C.[8] These characteristics are not found with most of the industrial phytases currently available.

In the present study, we selected the bacterial strain *Klebsiella pneumoniae* subsp. *pneumoniae* XY-5 for phytase production. The phytase (PhyK-W) from this strain had high activity and thermostability and was purified and characterized. Its putative gene was isolated, sequenced and proven produce functional enzyme.

## Materials and Methods

### Chemicals

Phytic acid and its dodecasodium salt were purchased from Sigma Chemical Co., St. Louis, Mo. All other chemicals were analytical grade and commercially available.

### Screening of Bacterial Strains for Phytase Production

Bacterial isolates (from soil, composes, and waste water samples) were screened for phytase production on phytase screening medium as described by Kerovuo *et al* with modification by adjustment to pH 5.0.[9]

### Phytase Production

Strains that produced clear zones on the screening medium were tested for phytase production in phytase production medium (containing in 1 litre: sodium phytate, 10 g;

$(NH_4)_2SO_4$, 1 g; $MgSO_4.7H_2O$, 0.1 g; $CaCl_2.2H_2O$, 0.1 g; trace element solution, 1.0 ml; KCl, 0.7 g; glucose, 1 g; and D-mannose, 1 g; the pH was adjusted to 5.0, and sterilized by filtration). A single colony was streaked on a LB agar plate and incubated at 37°C for 24 h. One loop-full of bacteria was transferred into 100 ml of phytase production broth (PPB) in a 500-ml Erlenmeyer flask and the culture was incubated at 37°C with 200 rpm shaking for 4 days. One ml of the culture was centrifuged and both the supernatant and the cell pellet were separately used as enzyme sources for phytase activity assays.

### Phytase Activity Assays

Phytase activity assays were routinely performed as described by Engelen *et al.* [10] Briefly, place 1.0 ml enzyme solution and 2.0 ml substrate solution (5.1 mmol/L of sodium phytate) were mixed and incubate for 65 minutes at 55°C, unless or otherwise stated. Then, 1.0 ml of the mixture was taken and added to 0.5 ml color-stop mix. The color-stop mix was prepared by mixing 250 ml ammonium heptamolybdate (10% of ammonium molybdate in 0.25% ammonia solution) with 250 ml ammonium vanadate (2.35 g ammonium vanadate in one liter of 2 % (v/v) nitric acid solution), adding 165 ml nitric acid (65%) while mixing slowly, and diluting to 1 L with water. The mixture was centrifuged at 10,000 rpm in a microfuge to obtain a clear supernatant and the absorbance was measured at 415 nm. Distilled water was used as a blank. One unit of the phytase activity was defined as the amount of enzyme able to hydrolyse phytate to give 1 mmol of inorganic phosphate ($P_i$) per min under the assay conditions. Specific activity was expressed in units of enzyme activity per milligram protein. When cell pellets were used in the enzyme assay, the amount of cell pellet equal to that from one ml of culture broth was suspended in 1 ml buffer solution and used as the enzyme source. The assay was done as with the supernatant. Protein concentrations were determined by the Bradford method. [11]

### Purification of Phytase

Cells were cultured in 100 ml PPB in 500-ml Erlenmeyer flasks at 37°C with 200 rpm shaking. They were harvested from 800 ml pooled broth after 4 days culture by centrifugation at 5,000 g for 15 min at 4°C. The supernatant was discarded and the cell pellet was washed twice with 20 mM Tris-HCl buffer (pH 7.0) containing 1 mM $CaCl_2$ to remove any P*i* contamination. Then, the P*i*-free cell pellet was stored at -20°C until use. Cells were disrupted by ultrasonication (Vibra cell, Sonics & Materials Inc., U.S.A.). Proteins in the clear cell-free extract were fractionated by stepwise precipitation with ammonium sulphate powder at 0-40%, 40%-60%, 60%-80%, and 80%-100% saturation. The protein precipitate formed at each step was collected by centrifugation at 13,000 g (Sorvall RC 5C, SS-34) for 45 min at 4°C. The protein pellet from 20-80% ammonium sulphate saturation was dissolved in 20 mM Tris-HCl pH 8.5 buffer with 1 mM $CaCl_2$. The resultant solution was subjected to column chromatography with Sephadex G-25 to desal it with 20 mM Tris-HCl, pH 8.5, containing 1 mM $CaCl_2$ as the mobile phase. The column used was XK26/70 (Pharmacia) with a bed volume of 60 ml. Elution was done at a flow rate of 1.0 ml $min^{-1}$. Next, the desalted enzyme was subjected to ion-exchange chromatography using a DEAE-Sepharose Fast Flow (Pharmacia) chromatographic column [XK16/20 (Pharmacia) with the bed volume of 25 ml] pre-equilibrated with 20 mM Tris-HCl buffer, pH 8.5, containing 1 mM $CaCl_2$, and eluted with a linear gradient of 0-0.5 M NaCl. The protein sample collected from the DEAE-Sepharose Fast Flow column was concentrated by centrifugal ultrafiltration with a Centricon membrane (MW cut off 10,000) and applied to an SP-Sepharose Fast Flow column [XK16/20 (Pharmacia) with the bed

volume of 25 ml] pre-equilibrated with 20 mM sodium acetate-acetic acid buffer, pH 5.0, containing 1 mM $CaCl_2$. The enzyme was eluted with a linear gradient of 0-1.0 M NaCl (100 ml) at a flow rate of 1.0 ml $min^{-1}$.

### Molecular Weight and Isoelectric Point (p*I*) Determination

The relative molecular weight ($M_r$) of the native enzyme was determined using gel filtration chromatography with a Superdex 200 HR 10/30 (Pharmacia) column eluted with a buffer containing 50 mM sodium phosphate, pH 7.0, 0.15 M sodium chloride and 1 mM $CaCl_2$, at a flow rate of 0.25 ml $min^{-1}$. Protein molecular weight markers were: α-lactalbumin ($M_r$ 14,200), trypsin inhibitor ($M_r$ 20,100), trypsinogen ($M_r$ 24,000), carbonic anhydrase ($M_r$ 29,000), glyceraldehyde-3-phosphate dehydrogenase ($M_r$ 36,000), ovalbumin ($M_r$ 45,000) and bovine serum albumin ($M_r$ 66,000). The molecular weight of the denatured enzyme was estimated using SDS-PAGE with 10%, 12% and 14% separating gels and 4 % stacking gels. The p*I* of the enzyme was determined using a broad p*I* calibration kit (Pharmacia) on a 5% PAA gel containing Pharmalyte pH range 3-10.

### Determination of Amino Acid Sequences

The N-terminal sequence of the first 28 amino acids of PhyK-W was determined by the Biotechnology Resource Laboratory, Medical University of South Carolina. The internal amino acid sequences were determined at the University of Newcastle, Australia.

### Effects of Temperature and pH on Phytase Activity

Effect of temperature on phytase activity was determined at 35°C, 37°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C, and 70°C, while stability was tested at 60°C by incubation for 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, and 4.5 hours before the residual activity was assayed at 55°C, pH 5.5 using the routine method. The optimum pH for the activity was determined by mixing 50 ml of the purified enzyme with 950 ml 0.22 M NaOAc-HOAc buffer at various pH values, ranging from 3.2 to 7.0, for assays at 37°C and 55°C. The pH stability was examined at pH 2.0 – 11.0. The 0.1 M buffers used were as follows: A. Glycine-HCl (pH 2-3); B. NaOAc-HOAc (pH 4-7); C. Tris-HCl (pH 8); D. Glycine-NaOH (pH 9-11). The mixtures were kept at 25°C at those pH's for 15 h before residual activity was determined at 55°C and pH 5.5 by the routine assay.

### Substrate Specificity

The reaction velocity for hydrolysis of various phosphate-containing compounds was determined with 2 mM disodium pyrophosphate, D-glucose-6-phosphate, *p*-nitrophenyl phosphate, β-glycerophosphate, tripolyphosphate, DL-α-glycerophosphate or phytic acid as the substrates. The phosphate compounds were dissolved in 0.22 M NaOAc-HOAc buffer, pH 5.5, supplemented with 1 mM $CaCl_2$ to make 2 mM substrate solutions. The rate of P*i* released from these compounds by PhyK-W was measured and compared.

### Enzyme Inhibitors and Metal Ions

Effects of inhibitors and metal ions on the enzyme activity were examined using sodium phytate pH 5.5 as substrate in the presence of each inhibitor or metal ion. The inhibitors and metal ions tested at 5 mM were $NaN_3$, EDTA, $MgCl_2$, $HgCl_2$, $MnCl_2.H_2O$, $NiCl_2.6H_2O$, $CoCl_2$, $ZnCl_2$, $CdCl_2$, $BaCl_2$, $CuSO_4.5H_2O$, $FeSO_4.7H_2O$, and $AlCl_3$.

### Cloning of the Phytase Gene

Based on the N-terminal and internal amino acid sequences and the *Klebsiella pneumoniae* phytase gene information at GenBank (AF453254), four primers were designed to cover the whole phytase gene. The PCR products obtained using both *Taq* polymerase and *Pfu* polymerase were sequenced. PCRs were carried out in a Mastercycler gradient machine (Eppendorf-Netheler-Hinz, GmbH, Hamburg). For *Taq* polymerase PCR, cycling parameters were: 1) denaturation at 94°C for 3 min (1 cycle), 2) denaturation at 94°C for 45 sec, 3) annealing at 58-62°C for 45 sec, 4) extension at 72°C for 60 seconds, 5) repetition of steps 2-4 for 30 cycles, and 6) extension at 72°C for 5 min (1 cycle). For *Pfu* polymerase PCR, cycling parameters were: 1) denaturation at 94°C for 3 min, 2) denaturation at 94°C for 45 sec, 3) annealing at 51°C for 45 sec, 4) extension at 72°C for 4 min, 5) repetition of steps 2-4 for 30 cycles, and 6) extension at 72°C for 5 min.

The PCR product from the *Pfu* polymerase experiment was ligated with pBluescript®II SK (+) and transformed into *E. coli* DH5a. *E. coli* DH5a harboring pBluescript®II SK (+) plasmid with the inserted *PhyK-W* gene and that with the pBluescript®II SK (+) plasmid without insert were inoculated on phytase screening agar plates containing 20 µl ampicillin (50 mg $ml^{-1}$). They were incubated at 37°C for 48 h. A positive recombinant clone that produced the largest phytase hydrolysis zone was selected for further study.

## Results and Discussion

### Screening for Phytase Producers and Enzyme Production

The strain *Klebsiella pneumoniae* subsp. *pneumoniae* XY-5 produced an interesting phytase when grown in

minimal medium containing phytate as the sole source of phosphorus. Enzyme production paralleled the amount of phosphate released into the broth and reached a plateau around days 4-5. Thereafter, enzyme activity dropped drastically (Fig. 1). Thus, the concentration of phosphorus in the culture broth was a good indicator of when to harvest the enzyme.

### Enzyme Purification and Substrate Specificity

### Molecular Weight and Isoelectric Point

PhyK-W was purified 181 fold from the cell extract with 15.9 % recovery (Table 1) and specific activity was $5.3 \times 10^3$ nkat $mg^{-1}$ (318 U $mg^{-1}$) for sodium phytate hydrolysis. The purified enzyme gave a single protein band on SDS-PAGE (Fig. 2), indicating that it was probably a single chain protein or a homomultimeric protein. The molecular mass of PhyK-W in the native form as estimated by gel filtration was found to be 61 kDa while that estimated by SDS-PAGE was 41.7 kDa. The p*I* value determined by isoelectric focusing analysis was 8.75. This was higher than the previously reported highest value of 8.56 from *A. fumigatus*.

Phytases can be divided into two classes based on specificity: i.e., those with broad substrate specificity (e.g., *Aspergillus fumigatus*, *Emericella nidulans* and *Myceliophthora thermophila*) and those with rather high specificity for phytic acid (e.g., *A. niger*, *A. terreus* and *E. coli*).[11,13] Most belong to the first group, as did PhyK-W, because it exhibited relatively high activity toward a wide spectrum of substrates (Table 2). Even substrates such as ADP, ATP, *p*-nitrophenyl phosphate, phenyl phosphate, fructose 1,6-bisphosphate, glucose 6-phosphate, a- and b-glycerophosphate, and 3-phosphoglycerate that are not structurally similar to phytic acid are hydrolysed by this group. However, PhyK-W differed from other members of the group in having high activity with many more substrates and highest activity with phytic acid. The specific activity of PhyK-W was 318 U $mg^{-1}$. Other phytases with broad substrate specificity have rather low specific activity for phytic acid (23 to 43 U $mg^{-1}$), whereas phytases with

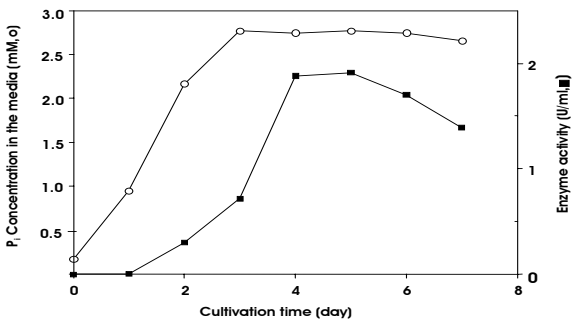


**Fig. 1.** Phytase expression (■) and phosphate liberation (o) profiles of *K. pneumoniae* subsp. *pneumoniae* XY-5 cultured in minimal medium containing 10 mM sodium phytate as the sole source of phosphorus. The enzyme source used was from lysis of the washed cell pellet.

**Table 1.** Purification of phytase from *K. pneumoniae* subsp. *pneumoniae* XY-5

| Purification step | Total protein (mg) | Total activity (unit) | Specific activity (unit/mg) | Percent Yield | Fold purification |
|---|---|---|---|---|---|
| 1. Crude cell extract | 237 | 417.1 | 1.76 | 100 | 1 |
| 2. $(NH_4)_2SO_4$ precipitate | 167 | 382.4 | 2.29 | 91.6 | 1.3 |
| 3. Sephadex G-25 | 130 | 345.8 | 2.66 | 82.9 | 1.5 |
| 4. DEAE-Sepharose | 74 | 213.9 | 2.89 | 51.3 | 1.6 |
| 5. SP-Sepharose | 0.22 | 70.0 | 318 | 16.7 | 181 |

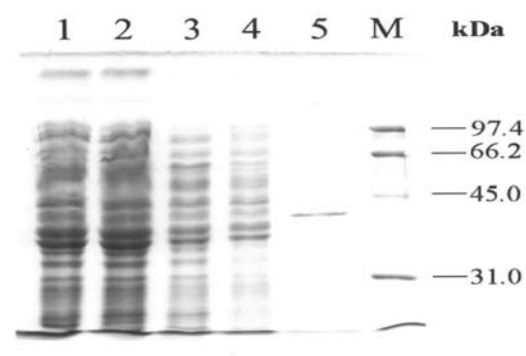


**Fig. 2.** SDS-PAGE analysis of PhyK-W purification products with proteins visualised by Coomassie blue staining. Lane 1 = cell extracts, Lane 2 = ammonium sulphate precipitate, Lane 3 = Sephadex G-25 eluate, Lane 4 = DEAE Sepharose FF eluate, Lane 5 = SP Sepharose FF eluate, Lane M = protein markers.

**Table 2.** Relative activity of PhyK-W towards different substrates.

| Substrate | Relative Activity (%) |
|---|---|
| Phytic acid | 100 |
| *p*-Nitrophenyl phosphate | 68 |
| Tripolyphosphate | 60.4 |
| Disodium pyrophosphate | 38 |
| β-Glycerophosphate | 28.3 |
| DL-α-Glycerophosphate | 12.7 |
| D-Glucose 6-phosphate | 11 |

narrow substrate specificity generally have high specific activities of 103 - 811 U $mg^{-1}$. [13]

Only a few phytases have been described as highly specific for phytic acid. These include *Bacillus* phytases [9] and alkaline phytase isolated from lily pollen.[12] Although very specific for phytic acid, *Bacillus* phytases have low specific activity and this is likely to hinder their use in industrial applications. The specific activities of fungal phytases for phytic acid vary almost 10-fold (e.g., from 23 to 198 U $mg^{-1}$ protein for *A. fumigatus* and *A. terreus*, respectively), while those of bacteria vary almost 100-fold (e.g., from 8.7 to 811 U $mg^{-1}$ for *B. subtilis* and *E. coli*, respectively). [9, 13, 14]

## Effects of pH and Temperature on Activity and Stability

PhyK-W was found to have two pH optima, i.e., it was most active at pH's 3.7 and 5.5 when assayed at both 37°C (297 units/ mg protein) and 55°C (318 units/ mg protein). This was different from most other bacterial phytases but similar to phytases from fungi such as *A. niger* NRRL 3135, *A. niger* 963,[15] *Myceliophthora thermophila,*[16] *Schwanniomyces occidentalis* and *Citrobacter freundii.* [17] Previously reported pH optima for phytases varied from 2.2 to 8.[18, 19] Most microbial phytases and especially those of fungal origin have pH optima between 4.5 and 5.6. In contrast to most fungal phytases, *A. fumigatus* phytase has activity over a broad pH range (i.e., at least 80% of maximum activity between pH 4.0 and 7.3). Some bacterial phytases and especially those from *Bacillus* have pH optima at 6.5 - 7.5.[9, 20] The pH optima of plant seed phytases range from 4.0 to 7.5, but most fall between 4.0 and 5.6. Two alkaline plant phytases having pH optima around 8.0 have been described from legume seeds [19] and lily pollen. [21]

Optimal temperatures of most phytases vary from 37 to 77°C. [21] PhyK-W falls into this range with two temperature optima at 37°C and 55°C. When left at pH 3.0 to 5.0 PhyK-W was stable for 15 hours while at pH 2.0, 20% of the activity was lost, and all was lost at pH higher than 6.0. At pH 5.0, PhyK-W was stable at 60°C for up to 4.0 hours and at 65°C for 10 minutes with only 50% loss in activity, while at 68°C, only 20% of the activity remained after 10 min (data not shown). This was considered to be relatively stable compared to most bacterial phytases.[22] However, a phytase from *A. fumigatus* was found to be more thermostable, with only 10% loss in activity was found when it was exposed at 100°C for 20 minutes. [23]

## Enzyme Inhibitors

PhyK-W was activated to 125% by 5 mM $Al^{3+}$, 120% by $Co^{2+}$ and 110% by EDTA as compared to 100% without inhibitors. $Ba^{2+}$, $Ni^{2+}$, $Cd^{2+}$ and $Fe^{2+}$ had little or no effect on its activity, while $Mg^{2+}$, $Cu^{2+}$, $Mn^{2+}$, $Zn^{2+}$, and $NaN_3$ moderately inhibited activity by about 15-20%. $Hg^{2+}$ completely inhibited the enzyme at this concentration (data not shown). By contrast, phytase from *Enterobacter* sp.4 was strongly inhibited by $Zn^{2+}$, $Ba^{2+}$, $Cu^{2+}$ and $Al^{3+}$.[17] Similarly, phytase from *B. subtilis* (natto) N-77 was greatly inhibited by various metal ions including $Zn^{2+}$, $Cd^{2+}$, $Ba^{2+}$, $Cu^{2+}$, $Fe^{2+}$, and $Al^{3+}$.[9] Also in contrast to PhyK-W, phytases from *Enterobacter* sp. 4, *B. subtilis* (natto) N-77 and two other *Bacillus* were greatly inhibited by EDTA. [9,20] Similar to PhyK-W, EDTA activated *A. fumigatus* phytase to 150%, although it had no major effect on other fungal phytases (*E. nidulans*, *A. niger* and *A. terreus*). The lack of EDTA inhibition is related to the lack of metal ions in the crystal structure of *A. niger* phytase. [24] This phytase, other fungal phytases and PhyK-W are clearly different from the $Ca^{2+}$-dependent phytases of *Bacillus*, *Typha latifolia* pollen and some other plants. [21] PhyK-W also resembles fungal phytases in showing considerable activity depression with $Cu^{2+}$, as reported for *E. nidulans* and *A. terreus* phytases. [14]

The activation of the enzyme activity by $Al^{3+}$ and $Co^{2+}$ was quite unexpected, however, this might be due to the interaction of the metals with the enzyme in such a way that the conformation of the enzyme became more active. The metal requirement for Ca with phytase from *Bacillus* has been reported. [20] On the other hand, the interaction of the metal ions, $Mg^{2+}$, $Cu^{2+}$, $Mn^{2+}$, and $Zn^{2+}$, with the enzyme occurred in a negative manner, such that the new conformation of the enzyme is less active. Thus, the positive effect of EDTA might be due to removal of the metal ion inhibitors, which were present as impurity in the assay system. It is also possible that the metal ions may react with the negative charges of the substrate resulting in changing the nature and concentration of the substrate. However, because positive, negative and neutral effects were seen with different positively charged metal ions, we believe that the observed effects were less likely to be from the interaction of the metal ions with the substrate.

## N-terminal and Internal Peptide Sequencing, and Gene Cloning

The N-terminal amino acid sequence and two internal sequences of PhyK-W were: ADWQLEKVVELSRHGIRPPTAGNREAIE, GNIPPGSSLVLER and FQAQGLDDLR, respectively. These exactly matched the phytase gene sequence of *Klebsiella pneumoniae* deposited at GenBank under the accession number AF453254. The two primers used to amplify the full gene sequence were TCT GAC CTG CTT CCT TGG and CGG ATT GTA AAC GGC AGC. PCR and gene cloning products using both *Taq* polymerase and *Pfu* polymerase were identical in size (Fig. 3). The

PCR product from *Pfu* polymerase was ligated with pBluescript®II SK (+) and transformed into *E. coli* DH5a.

## DNA Sequence and Deduced Amino Acid Sequence

A positive recombinant *E. coli* clone that produced the largest phytate hydrolysis zone in minimal medium was selected. Its recombinant plasmid was shown to contain an ORF of 1,269 bp beginning with an ATG (nt 309-311) start codon and ending with a TAG (nt 1575-1577) stop codon (Fig. 4). A putative ribosome-binding site TAAGGAAG (nt 297-303) was found at a position 5 bp upstream of the ATG start codon. The ORF was translated into a putative sequence of 422 amino acids that matched exactly the N-terminus (a.a. 29-56) and the two internal amino acid sequences (a.a. 330-342 and a.a. 357-366) previously determined from the purified enzyme. This confirmed that the ORF encoded the phytase. The first 28 amino acids were assumed to be a signal peptide because the N-terminus of the mature enzyme began with alanine at position 29. Thus, the mature enzyme contained 394 putative amino acid residues having a calculated molecular mass of 43.37 kDa. This was in good agreement with the value of 41.75 kDa obtained experimentally. The sequence also contained the conserved active site, RHGXRXP, and the HD motif (a.a. 41-47 and a.a. 69-70, respectively), which placed the enzyme in the histidine acid phosphatase (HAPs) family. The calculated p*I* was 9.85, in agreement with the value of 8.57 determined with the purified enzyme.

Comparison of the amino acid sequence of PhyK-W to those in the database suggested that PhyK-W might have originated from the same origin as PhyK-RS1, as they were 98% identical and 99% similar, while


**Fig. 3.** Agarose gel electrophoresis of the PCR products amplified from *K. pneumoniae* subsp. *pneumoniae* XY-5 genomic DNA using phytase gene specific primers. Lane M, l DNA/Hind III markers; Lanes 1 and 2, PCR products obtained using *Taq* and *Pfu* polymerase, respectively.

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CGGGCTGATCGCCGTCGATATCTCTTCGCGCAAGGCCGCCGGGGCCGCGC 50
  G  L  I  A  V  D  I  S  S  R  K  A  A  G  A  A
TCGGCACCATCGGCATCGCCAGCTACGCCGGCGCCGGCCTGGGCGAGTTT 100
L  G  T  I  G  I  A  S  Y  A  G  A  G  L  G  E  F
CTCACCGGGATCATTATTGATAAAACGGCTATCCTTGAAAACGGCAAAAC 150
 L  T  G  I  I  I  D  K  T  A  I  L  E  N  G  K  T
GCTGTATGATTTCAGCACATTGGCGCTGTTCTGGGTGGGTACCGGTCTGG 200
  L  Y  D  F  S  T  L  A  L  F  W  V  G  T  G  L
GTTCCGCGCTACTCTGTTTTACCACTGCCGCCATCGTCGCCCGGCGCCAT 250
G  S  A  L  L  C  F  T  T  A  A  I  V  A  R  R  H
GCCGTCGAACGGCAGACCTCGTTTTCCTCATAACCGATTAACGAATaagg 300
  A  V  E  R  Q  T  S  F  S  S  *
aagAAGATATGATGCCTGCAAGACATCAGGGGCTGTTACGCCTGTTTATC 350
          M  M  P  A  R  H  Q  G  L  L  R  L  F  I  a14
GCCTGCGCGCTGCCGCTGCTGGCGCTGCAATCTGCCGCCGCCGCGGACTG 400
  A  C  A  L  P  L  L  A  L  Q  S  A  A  A  A  D  W a31
GCAGCTGGAGAAAGTGGTCGAGCTCAGCCGCCACGGTATTCGTCCGCCGA 450
  Q  L  E  K  V  V  E  L  S  R  H  G  I  R  P  P  a47
CGGCCGGCAACCGGGAAGCCATCGAGGTCGCCACCGGACGACCGTGGACC 500
T  A  G  N  R  E  A  I  E  V  A  T  G  R  P  W  T  a64
GAGTGGACCACCCATGACGGGGAGCTCACCGGCCATGGCTATGCCGCCGT 550
  E  W  T  T  H  D  G  E  L  T  G  H  G  Y  A  A  V a81
GGTCAACAAAGGCGTGCGGAAGGCCAGCATTACCGCCAGCTCGGCCTGT 600
  V  N  K  G  R  A  E  G  Q  H  Y  R  Q  L  G  L  a97
TGCAGGCCGGATGCCCGACGGCGGAGTCGATATACGTGCGCGCCAGCCCG 650
L  Q  A  G  C  P  T  A  E  S  I  Y  V  R  A  S  P a114
CTGCAGCGGACGCGAGCGACCGCCCAGGCGCTGGTGGATGGCGCCTTCCC 700
  L  Q  R  T  R  A  T  A  Q  A  L  V  D  G  A  F  P a131
CGGCTGCGGCGTCGCTATCCATTATGTCAGCGGGGATGCCGATCCCCTGT 750
  G  C  G  V  A  I  H  Y  V  S  G  D  A  D  P  L  a147
TTCAGACCGACAAGTTCGCCGCCACGCAAACCGACCCCGCCCGCCAGCTG 800
F  Q  T  D  K  F  A  A  T  Q  T  D  P  A  R  Q  L a164
GCGGCGGTGAAAGAGAAGGCCGGGGATCTGGCGCAGCGTCGGCAGGCGCT 850
  A  A  V  K  E  K  A  G  D  L  A  Q  R  R  Q  A  L a181
GGCGCCGACCATCCAGCTATTGAAACAGGCGGTTTGTCAGGCCGATAAGC 900
  A  P  T  I  Q  L  L  K  Q  A  V  C  Q  A  D  K  a197
CCTGCCCGATCTTCGATACCCCGTGGCAGGTCGAGCAGAGCAAAAGTGGG 950
P  C  P  I  F  D  T  P  W  Q  V  E  Q  S  K  S  G a214
AAGACCACCATTAGCGGACTGAGCGTGATGGCCAATATGGTGGAGACGCT 1000
  K  T  T  I  S  G  L  S  V  M  A  N  M  V  E  T  L a231
GCGTCTCGGCTGGAGTGAAAACCTGCCTCTCAGCCAGCTGGCGTGGGGCA 1050
  R  L  G  W  S  E  N  L  P  L  S  Q  L  A  W  G  a247
AGATCACCCAGGCCAGGCAGATCACCGCCCTGCTGCCGCTGTTAACGGAA 1100
K  I  T  Q  A  R  Q  I  T  A  L  L  P  L  L  T  E a264
AACTACGATCTGAGTAACGATGTGTTGTATACCGCGCAAAAACGCGGGTC 1150
  N  Y  D  L  S  N  D  V  L  Y  T  A  Q  K  R  G  S a281
GGTGCTGCTCAACGCTATGCTCGACGGCGTCAAACCGGAGGCGAATCCGA 1200
  V  L  L  N  A  M  L  D  G  V  K  P  E  A  N  P  a297
ACGTACGCTGGCTGCTGCTAGTGGCCCATGACACCAATATCGCCATGGTG 1250
N  V  R  W  L  L  L  V  A  H  D  T  N  I  A  M  V a314
CGCACGCTGATGAACTTTAGCTGGCAGCTGCCGGGCTACAGCCGGGGAAA 1300
  R  T  L  M  N  F  S  W  Q  L  P  G  Y  S  R  G  N a331
TATCCCGCCGGGCAGTAGCCTGGTGCTGGAGCGCTGGCGCAACGCGAAGA 1350
  I  P  P  G  S  S  L  V  L  E  R  W  R  N  A  K  a347
GCGGAGAACGCTATCTGCGGGTCTATTTCCAGGCCCAGGGCCTCGACGAC 1400
S  G  E  R  Y  L  R  V  Y  F  Q  A  Q  G  L  D  D a364
CTGCGTCGTCTGCAGACGCCGGACGCGCAGCACCCGATGCTGCGTCAGGA 1450
  L  R  R  L  Q  T  P  D  A  Q  H  P  M  L  R  Q  E a381
GTGGCGTCAGCCGGGCTGCCGTCAGACCGACGTCGGTACGCTGTGTCCCT 1500
  W  R  Q  P  G  C  R  Q  T  D  V  G  T  L  C  P  a397
TCCAGGCGGCTATTACCGCCCTCGGTCAGCGTATCGACCGATCATCCGCC 1550
F  Q  A  A  I  T  A  L  G  Q  R  I  D  R  S  S  A a414
CCGGCGGTAGCCATGGTCCTGCCGTAGCGGCGCGGTGTTGTCCGGGCCC 1600
  P  A  V  A  M  V  L  P  *                        a422
GGGAAAACCTTTTTTTCCAGGCCCGGCACGACGTCCGTTATCCGTTGTCC 1650
GGCGCAAACGCCCCGGCGGCGACCTGCGCCGGGGTGACCACCCCGCTGTC 1700
CAGCACCCAGCCGCTTATCAGCCCAGCAGGCGTGACGTCGAACGC      1745
```

**Fig. 4.** Nucleotide sequence (nt 309-1577) and deduced amino acid sequence (a 1-422) of *K. pneumoniae* subsp. *pneumoniae* XY-5 phytase gene. The putative ribosome binding site (rbs) is indicated by lower case letters. The signal peptide cleavage site is indicated by a vertical arrow. The N-terminal amino acid sequence and the two internal peptide sequences determined from the purified enzyme are underlined. The conserved acid phosphatase active site residues are indicated by open boxes. A possible transcription terminator downstream of the phytase gene is indicated by horizontal arrows under the sequence. Upstream of the phytase ORF is the partial sequence of an uncharacterized protein similar to a putative hexose phosphate transport protein of *Bacteroides thetaiotaomicron* VPI-5482.

other sequences were up to 41% identical and 62% similar.[25] A phylogenetic tree of related phytase sequences constructed by PHYLIP v. 3.6 and viewed by Tree View v. 1.6.6 is shown in Fig. 5. [26,27]

Inspection of the DNA sequence (Fig. 4) upstream from the *PhyK-W* ORF revealed no sequence motifs characteristic of -10 and -35 prokaryotic transcription promoter sequences. This suggested that another ORF might precede the *PhyK-W* ORF and be co-transcribed with it in a common polycistronic mRNA. In support of this argument, an ORF encoding a 93-amino acid polypeptide (nt 1-280), ending in a TAA stop codon (nt 281-283) was found 12 bases upstream of the ribosome-binding site for the *PhyK-W* ORF. A BLAST [28] search against amino acid sequences in the NCBI GenBank database revealed that this putative 93-amino acid peptide had high similarity to a putative hexose phosphate transport protein of *Bacteroides thetaiotaomicron* VPI-5482 (accession no. NP_813639; 35% identity, 55% similarity) and a sugar phosphate permease of *Vibrio vulnificus* CMCP6 (accession no. NP_762189; 31% identity, 41% similarity). This suggested that the 93 amino-acid ORF preceding *PhyK-W* might encode a protein that functions to transport inositol phosphate hydrolysis products of the PhyK-W phytase catalyzed reaction into the cell. A detailed study of this putative ORF and its encoded product is yet to be carried out. A palindromic sequence which could form a stem-loop structure characteristic of a transcription terminator was found closely (15 nt) downstream of the TAG stop codon of the *PhyK-W* ORF (Fig. 4), suggesting that this might be the last gene in a polycistronic mRNA.

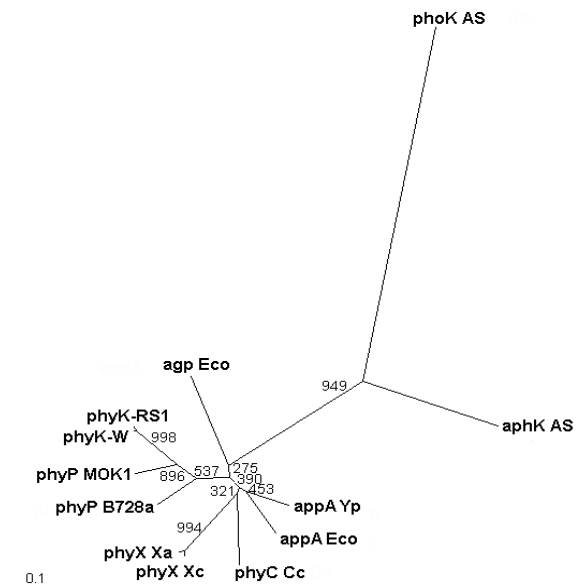


**Fig. 5.** Phylogenetic tree of deduced proteins of phosphatases and phytases constructed by PHYLIP version 3.6 (beta release) and TreeView version 1.6.6. phyK-RS1, *Klebsiella* sp. RS1 3-phytase; phyK-W, *Klebsiella pneumoniae* subsp. *pneumoniae* XY-5 phytase; phyP MOK1, *Pseudomonas syringae* MOK1 putative phytase; phyP B728a, *Pseudomonas syringae* B728A putative phytase; phyX Xa, *Xanthomonas axonopodis* putative phytase; phyX Xc, *Xanthomonas campestris* putative phytase; phyC Cc, *Caulobacter crescentus* putative phytase; appA Yp, *Yersinia pestis* KIM acid phosphatase; appA Eco, *Escherichia coli* phytase; agp Eco, *E. coli* glucose-1-phosphatase; aphK AS, *Klebsiella* sp. ASR1 acid phosphatase; phoK AS, *Klebsiella* sp. ASR1 alkaline phosphatase. Bootstrap values indicating reproducibility out of 1000 bootstraps are written by internal branches.

Up to now, more than ten phytase genes have been cloned and characterized. The *PhyK-W* gene sequence which contains 1422 bp of nucleotides showed 98 % identity with a phytase gene from *Klebsiella* sp. RS1 deposited in GenBank (AY091638). However, despite their high percentage of amino acids sequence similarity, these enzymes they show some differences in the enzyme properties. These include pH optima, temperature stability and substrate specificity. It should be pointed out that the *phyK-W* gene has high G+C content (63.86 %), which is very close to that of the *phyA* gene from *Aspergillus ficuum* NRRL3135 (68.3 %). Yao and Fan [6] concluded that high G+C content is one of the characteristics of highly expressed genes in filamentous fungi. The *PhyK-W* gene of *K. pneumoniae* subsp. *pneumoniae* XY-5 may have similar properties of expression to the *phyA* gene of *Aspergillus ficuum* NRRL3135.

In conclusion, bacterial *PhyK-W* has a number of good properties and shares several characteristics with fungal phytases. Cheap nitrogen and carbon sources can be used in relatively short cultivation times at 37C to obtain relatively high yields of the enzyme with a relatively high specific activity. The enzyme gives good activity at both 37°C and 55°C and has two pH optima at 3.5 and pH 5.5. Most importantly, it is stable at 60°C for up to 4 hours and is not inhibited by most normal phytase inhibitors. Thus, the bacterial enzyme has some advantages for industrial production.

## Acknowledgements

We wish to thank Prof. T. W. Flegel for careful reading of the manuscript and helpful suggestions.

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