PHO13 phosphoglycolate phosphatase from Saccharomyces cerevisiae (baker’s yeast), a member of the p-nitrophenylphosphatase family of the haloalkanoic acid dehalogenase (HAD) superfamily

Regina Puts
PHO13 Phosphoglycolate Phosphatase from *Saccharomyces cerevisiae* (Baker’s Yeast), a Member of the *p*-Nitrophenylphosphatase Family of the Haloalkanoic Acid Dehalogenase (HAD) Superfamily

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ABSTRACT

The function of phosphoglycolate phosphatase (PGPase) in photosynthetic organisms such as green algae (*Chlamydomonas reinhardtii*) is to recycle the 2-phosphoglycolate that is formed as a by-product of the Calvin cycle. The role of PGPase in nonphotosynthetic organisms is less understood, but one potential role that has been suggested is to remove the 2-phosphoglycolate that is formed as a result of DNA repair. This may be the biological role of PHO13 PGPase in *Saccharomyces cerevisiae* as well.

PHO13 PGPase from *S. cerevisiae* is a member of the PGPase subfamily within the *p*-nitrophenylphosphatase (*p*-NPPase) family, which is within the Haloalkanoic Acid Dehalogenase (HAD) superfamily. *pho13* has been subcloned into pET19b to create PHO13(His•Tag). PHO13(HT) was overexpressed and found to be soluble. It purified well by Ni\(^{2+}\)-NTA affinity chromatography followed by size-exclusion chromatography. Its expression, solubility, and activity appeared comparable to the native PHO13, but PHO13(HT) was easier to purify due to the histidine tag. Both enzymes had comparable specific activities for both *p*-nitrophenylphosphate (*p*-NPP) and 2-phosphoglycolate (PG), pH optima around pH 8.0, optimal activity with \(\geq 7 \text{ mM } \text{Mg}^{2+}\), activity with Co\(^{2+}\) and Mn\(^{2+}\), negligible activity in the presence of Zn\(^{2+}\), and no activity for Ca\(^{2+}\).

PHO13(HT) is now ready for x-ray crystal structure determination with our collaborator, Joseph Wedekind, at the University of Rochester.
INTRODUCTION
The Haloalkanoic Acid Dehalogenase (HAD) enzyme superfamily was given its name because the first member discovered catalyzed halogen moiety transfer (Burroughs et al, 2006). The HAD superfamily is a large protein superfamily containing more than 3000 members, from organisms belonging to all three superkingdoms of life, with only a few of them characterized (Tirrell et al, 2006).

Families within the HAD superfamily are classified in 5 major groups based on the chemical mechanism that they utilize. Haloalkanoic acid dehalogenases hydrolyze carbon-halogen bonds. Phosphonoacetalddehyde hydrolases cleave phosphate off of the carbon directly. ATPases are active towards phosphate-oxygen bonds, where oxygen is attached to another phosphate group. Phosphomutases perform phosphate cleavage of the phosphate-oxygen bond followed by intramolecular phosphoryl group transfer. The majority of the enzymes of the HAD superfamily perform the phosphate monoesterase function, hydrolyzing the phosphate-oxygen bond, where the oxygen is attached to carbon (Lahiri et al, 2004).

Each member of the HAD superfamily is comprised of core and cap domains. The core domain forms a Rossmannoid fold (Fig. 1), which consists of repetitive $\beta/\alpha$ secondary structures and is the location of the active site (Burroughs et al, 2006).

![Figure 1. Core domain of the HAD superfamily enzyme (Burroughs et al, 2006).](image)
The core catalytic domain contains 4 conserved structural motifs (Fig. 2) (Burroughs et al, 2006).

Figure 2. Amino acid sequence alignment of four enzymes of the HAD superfamily. Amino acids were highlighted based on sequence identity and similarity. Similar amino acids are designated as follows: (1) G; (2) P; (3) I, L, V, A, M, F, W; (4) T, S, N, Q; (5) D, E; R, K; and (6) Y, H (Tirrell et al, 2006).

The first structural motif contributes two aspartate residues. The first aspartate acts as a nucleophile while the second is involved in acid/base catalysis (Lu et al, 2008).

The second conserved motif contains either serine or threonine residue, which forms hydrogen bonding with the phosphoryl group of the substrate, stabilizing the intermediate of the catalytic reaction (Fig. 3).
A conserved lysine or arginine residue of the third motif also stabilizes negatively charged intermediates through a salt bridge and as it coordinates the side chain of the aspartate residue of the first conserved motif.

The fourth domain contains either two aspartate or glutamate residues, which position the divalent metal ion cofactor. The known metal cofactors utilized by the HAD superfamily members are Mg\(^{2+}\), Ca\(^{2+}\), Zn\(^{2+}\), Co\(^{2+}\) and Mn\(^{2+}\). The metal cofactor is also coordinated by the side chain of the first aspartate residue and the backbone of the second aspartate residue of the first motif. The metal cofactor in turn shields the negative charge on the nucleophilic aspartate residue and stabilizes the phosphoryl group of the substrate (Fig. 3) (Burroughs et al, 2006).

While the core domain is conserved throughout the superfamily, the cap domain differs from one family to another, and it defines the substrate specificity of the enzyme. Karen Allen differentiates between three cap domains, which vary by their structure and by the location of insertion within the catalytic domain. They are cap 1, cap 2 and cap 0 (Tremblay et al, 2005). Cap 1 has an alpha helical structure and is inserted between
motifs one and two. It provides good solvent exclusion and the enzymes with the cap 1 utilize relatively small substrate molecules. Cap 2 has an alpha/beta fold, and it is positioned between motifs two and three. Cap 2 results in worse solvent exclusion and allows the enzyme to act on larger substrate molecules. Cap 0 is a beta hairpin region between the second and third motifs. The enzymes with the cap 0 are active on even larger substrate molecules compared to members with either cap 1 or cap 2 domains (Fig.1) (Burroughs et al, 2006).

In 1994 Koonin and Tatusov using a computational iterative approach first identified the $p$-nitrophenylphosphatase ($p$-NPPase) family within the HAD superfamily. At that time two known enzymes of the family were from *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Koonin and Tatusov, 1994). Most members of $p$-NPPase family are active towards the substrate analog $p$-nitrophenyl phosphate ($p$-NPP) and the family owes its name to that.

There are several subfamilies that are distinguished within the $p$-NPPase family. Enzymes are differentiated from one subfamily to another based on the sequence homology. Each member of each subfamily utilizes the same substrate. Figure 4 shows a sequence alignment of enzymes of the various subfamilies within the $p$-NPPase family. The multiple conserved amino acids are highlighted in red, which indicates a significant similarity between all of these enzymes, more so than between those in other families of the HAD superfamily.
**Figure 4.** Amino acid sequence alignment of members of the various subfamilies within the $\beta$-NPPase family of the HAD superfamily. Amino acids were highlighted based on sequence identity and similarity. Similar amino acids are designated as follows: (1) G; (2) P; (3) I, L, V, A, M, F, W; (4) T, S, N, Q; (5) D, E, R, K; and (6) Y, H (Tirrell et al., 2006). The subfamilies include the phosphoglycolate phosphatases (PGPases), the pyridoxal phosphatases (PLPases), the uridine 5'-monophosphatases (UMPases) and the sugar phosphatases of which AraL belongs.
Figure 5 shows a sequence alignment of four members of the phosphoglycolate phosphatase (PGPase) subfamily of the \( p \)-NPPase family. The enzymes within this subfamily have even more sequence homology between them as compared to the sequence alignment of the \( p \)-NPPase family members shown in the Figure 4.

**Figure 5.** Amino acid sequence alignment of the members of the PGPase subfamily of the \( p \)-NPPase family of the HAD superfamily. Amino acids were highlighted based on sequence identity and similarity. Similar amino acids are designated as follows: (1) G; (2) P; (3) I, L, V, A, M, F, W; (4) T, S, N, Q; (5) D, E; R, K; and (6) Y, H (Tirrell et al, 2006).

Biologically significant substrates have been identified for some members of the \( p \)-NPPase family. NagD from *Escherichia coli* utilizes uridine 5’-monophosphate as a substrate. We believe it is involved in regulatory cell wall and polysaccharide biosynthesis because *nagd* is within the NAG operon and N-acetylglucosamine is a precursor to those pathways (Neidhardt, 1995). It is the first enzyme of the \( p \)-NPPase family, whose x-ray crystal structure has been determined (Tremblay et al, 2005). YZGD
from *Paenibacillus thiaminolyticus* has a pyridoxal phosphate phosphatase (PLPase) activity and is a unique enzyme of the family containing a second catalytic domain belonging to the NUDIX hydrolase superfamily. (Tirrell et al, 2006). There is also a human PLPase enzyme that does not contain the NUDIX domain, and it is suggested to play a role in vitamin B\textsubscript{6} metabolism in erythrocytes (Fonda, 1992; Fonda and Zhang, 1995). AraL from *Bacillus subtilus* has sugar phosphatase activity and its gene resides within the L-arabinose operon (Sa-Nogueira et al, 1997).

The PGPase from *Chlamydomonas reinhardtii* was determined to have a photorespiratory function. It catalyzes hydrolysis of 2-phosphoglycolate formed as a byproduct of the Calvin cycle. Glycolate, the product of hydrolysis, gets further utilized in the cell’s metabolism involving amino acids synthesis and further incorporation into the Calvin cycle through formation of 3-phosphoglycerate (Mamedov et al, 2001). The PGPase from *Staphylococcus aureus* was determined to be a virulence factor of the pathogen in the *Caenorhabditis elegans* model system (Begun et al, 2004). There is also an enzyme with PGPase activity from *E. coli*, which is a member of the HAD superfamily but belongs to a different family within the superfamily based on sequence homology. The PGPase from *E. coli* is believed to be a housekeeping enzyme. DNA damage induced by oxidative stress is repaired by DNA repair enzymes, releasing 2-phosphoglycolate, which is further hydrolyzed by the *E. coli* PGPase (Pellicer et al, 2003).

Approximately 40 years ago the \textit{p}-NPPase activity of PHO13 enzyme from the baker’s yeast, *S. cerevisiae* was discovered (Gorman et al, 1968). Further, it was determined that the enzyme strongly depends on the presence of Mg\textsuperscript{2+} and is inhibited by
Ca$^{2+}$, Zn$^{2+}$ and Be$^{2+}$. It was noted that the enzyme has the highest activity at pH 8.0-8.2 and inorganic phosphate acts as a competitive inhibitor (Attias et al., 1972). Protein phosphatase activity of PHO13 was suggested but never proven (Galabova et al., 1998). In 2008, phosphoglycolate phosphatase (PGPase) activity of PHO13 was predicted through BLAST searches and bioinformatics sequence alignments and was proven experimentally (unpublished results. Strassner, O’Handley).

PHO2 from *Schizosaccharomyces pombe* was also discovered to have p-NPPase activity. Its pH optimum is also around 8.0 and the rate of the enzyme is accelerated in presence of Mg$^{2+}$ and inhibited by Zn$^{2+}$ (Yang et al., 1991). Due to its strong sequence homology to the PGPase from *S. cerevisiae* (Fig. 6) we predict that this enzyme will also have PGPase activity.

**Figure 6.** Amino acid sequence alignment of PHO13 from *S. cerevisiae* and PHO2 from *S. pombe*. Amino acids were highlighted based on sequence identity and similarity. Similar amino acids are designated as follows: (1) G; (2) P; (3) I, L, V, A, M, F, W; (4) T, S, N, Q; (5) D, E; R, K; and (6) Y, H (Tirrell et al., 2006).
The biological function of the yeast PGPase is presently not known, and it is of particular interest. Future plans of the O’Handley lab include phenotypic studies of a pho13 knockout in collaboration with the Phizicky lab from the University of Rochester. *S. cerevisiae* is an eukaryotic single-cell model organism and thus the information gained working with it can be further applied to more complex organisms possibly including human. For example *S. cerevisiae* is currently used to study cancer due to the fact that the genes controlling the cell cycle in the yeast are significantly similar to the ones in humans (Pray et al, 2008). Baker’s yeast is also used for potential drug screening (Auerbach et al, 2005).

The *pho13* gene encoding for the PHO13 PGPase has been previously cloned into a pET11b plasmid and expressed, showing good expression levels and solubility. The expressed PHO13 protein has been purified using streptomycin precipitation, ammonium sulfate fractionation and size exclusion chromatography. The multiple steps of purification retained protein impurities and significantly decreased the yield of PHO13 (unpublished results. Lof, O’Handley).

This current project was created to increase the yield and purity of the PHO13 PGPase enzyme through utilization of affinity chromatography. The project includes subcloning, protein purification and enzyme characterization. Future research includes x-ray structure determination.

The first step of this project was to subclone the *pho13* gene into a pET19b plasmid, which encodes a string of ten histidines to create a protein containing a His•Tag to aid in purification by affinity chromatography.
The pET19b plasmid expression system is controlled by a T7lac promoter. The T7lac promoter has a lac operator sequence located downstream from the promoter region. Lac repressor binding to this region prevents T7 RNA polymerase transcription (Fig. 7) and therefore reduces basal expression. A similar lac promoter regulates expression of the T7 RNA polymerase encoded by (DE3) strains of *E. coli* ensuring tight regulation of expression (Novagen. pET system manual, 2006).

**Figure 7.** Control of the T7lac promoter (Novagen. pET system manual, 2006).

Isopropyl β-D-1-thiogalactopyranosidase (IPTG) is used as an inducer of the lac promoter. Its structure is similar to allolactose, the natural inducer of the lac promoter in *E. coli*. IPTG binds to the lac repressor triggering T7 RNA polymerase transcription of DNA. The *E.coli* (DE3) cells containing T7 RNA polymerase encoded in the chromosomal DNA are used as an expression host (Novagen. Competent cells, 2004).
The expressed PHO13 protein had ten histidines attached to it and will be referred to as PHO13(HT) (Novagen. pET-19b vector, 1998). The imidazole ring (Fig. 8), the side chain of histidine has a strong affinity to divalent nickel cations (Ni^{2+}).

Figure 8. Structure of imidazole (Sundberg and Martin, 1973).

PHO13(HT) was purified by a His•Tag affinity column, which contains an agarose matrix and Ni^{2+} ions located on its surface. Nitrilotriacetic acid (Fig. 9) used as a chelating agent immobilizes the Ni^{2+} ions in the His•Tag binding resin (Novagen. Ni-NTA His•Bind Resins, 2001).

Figure 9. Nitrilotriacetic acid (DrugBank Version 2.5. Nitrilotriacetic acid, 2005).

The PHO13(HT) protein was retained by the column while other proteins passed through and was washed off the column later with an elute buffer containing imidazole to out compete PHO13(HT) (Novagen. Ni-NTA His•Bind Resins, 2001).
Size exclusion chromatography was used to remove the residual impurities from the PHO13(HT) sample. Size exclusion chromatography, which is also known as gel filtration chromatography, is a technique that separates macromolecules based on their molecular weight. The size exclusion resin contains a porous matrix. Larger molecules do not fit within the pores of the resin and therefore freely move around the matrix. Smaller molecules are retained in the pores and travel through the matrix more slowly. The technique is widely used for the separation of biomolecules and is very convenient to approximate the molecular weight of the selected compounds (Docstoc. Protein Purification Tutorial).

The pH optimum, the best divalent metal cofactor and optimum concentration were determined for both PHO13 and PHO13(HT). Characterization of enzyme kinetics was also attempted. Each of the parameters was evaluated by colorimetric enzyme assay, the Ames and Dubin assay, which uses ammonium molybdate for the colorimetric studies. The method is based on the reaction between ammonium molybdate and inorganic phosphate released by the enzyme. As a result phosphomolybdate is formed. Sulfuric acid acting as oxidizing agent and ascorbic acid acting as reducing agent further reduce the phosphomolybdate into a blue compound. An intensity of the resulting blue-colored solution is proportional to the concentration of inorganic phosphate in it. (Ames and Dubin, 1960).
As a final part of the project the enzyme kinetics of PHO13(HT) was attempted to be studied.

Many enzymes including those of the \(\rho\)-NPPase family of the HAD superfamily obey Michaelis-Menten kinetics, which can be described by the equation 1:

\[
V_0 = \frac{V_{\text{max}} \cdot [S]}{K_m + [S]} \quad \text{(eq. 1)}
\]

The Michaelis-Menten equation shows the relationship between the initial velocity \(V_0\), the maximum velocity \(V_{\text{max}}\), the initial substrate concentration \([S]\) and the Michaelis constant \(K_m\). The Michaelis constant \(K_m\) represents a substrate concentration when \(V_0 = \frac{1}{2} \cdot V_{\text{max}}\).

If the plot of the dependence of \(V_0\) on \([S]\) of the selected enzyme exhibits hyperbolic character then it designates that enzyme follows the Michaelis-Menten kinetics (Fig. 10) (Nelson and Cox, 2005).

![Figure. 10 Michaelis-Menten dependence of initial velocity on substrate concentration (The Chemistry Encyclopedia. Michaelis-Menten Kinetics, 2007).](image)

Another representation of the \(V_{\text{max}}\) and \(K_m\) values can be obtained from a double-reciprocal Lineweaver-Burk plot, which is described by equation 2:
\[
\frac{1}{V_0} = \frac{K_m}{V_{max} \cdot [S]} + \frac{1}{V_{max}} \quad \text{(eq. 2)}
\]

The Lineweaver-Burk plot describes the dependence of \( \frac{1}{V_0} \) value on \( \frac{1}{[S]} \) (Fig. 11).

For the enzymes following the Michaelis-Menten kinetics the plot gives a straight line with a slope of \( \frac{K_m}{V_{max}} \), an intercept of \( \frac{1}{V_{max}} \) on the \( \frac{1}{V_0} \) axis and an intercept of \( -\frac{1}{V_0} \) on the \( \frac{1}{[S]} \) axis. The Lineweaver-Burk plot allows visually easier determination of \( V_{max} \) and \( K_{max} \) than the simple plot of \( V_o \) versus \([S]\) (Fig. 10) (Nelson and Cox, 2005).

![Lineweaver-Burk plot diagram]

**Figure 11.** Lineweaver-Burk plot (The Medical Biochemistry Page. Enzyme Kinetics).

The general rate constant \( k_{cat} \) defines the limiting rate of the reaction at enzyme saturation. The \( k_{cat} \) is equal to the rate constant of the rate limiting step of an enzyme reaction. For enzymes following Michaelis-Menten kinetics, \( k_{cat} = \frac{V_{max}}{[E_t]} \), where \([E_t]\) is
the total enzyme concentration or the sum of free and substrate-bound enzyme. The Michaelis-Menten equation is modified to equation 3:

\[
V_0 = \frac{k_{\text{cat}}[E][S]}{K_m + [S]} \quad \text{(eq. 3)}
\]

The general rate constant \( k_{\text{cat}} \) is also known as the turnover number, which is defined as the amount of substrate converted to product within the selected unit of time within a single enzyme molecule saturated with substrate.

To evaluate catalytic efficiencies of enzymes, the \( \frac{k_{\text{cat}}}{K_m} \) ratio or specificity constant is taken into account. The ratio can also be interpreted as the rate constant for the conversion \( E+S \) to \( E+P \). Therefore the higher the specificity constant, the higher the efficiency of the selected enzyme (Nelson and Cox, 2005).

The kinetics parameters \( K_m, V_{\text{max}} \) and \( k_{\text{cat}} \) will be attempted to be determined for PHO13(HT) towards both substrate analog \( p \)-nitrophenyl phosphate and the biologically significant substrate 2-phosphoglycolate.
MATERIALS AND METHODS
**pho13 Subcloning into pET19b Plasmid**

**Plasmid Midipreps**

_Escherichia coli_ (E.coli) DH5α cells containing pETpho13 (pho13 cloned into pET11b, previously by Brandon Wahler and Megan Walling) were grown in overnight cultures. Freezer stocks of these plasmid containing cells were suspended in 50 ml of Luria-Bertani (LB) media with 100 µg/ml ampicillin. Cells were grown in a shaker incubator at 37°C for approximately 16 hours. The cells were harvested by centrifugation at 13,148 RCF (relative centrifugal force) for 2 minutes. Harvested cells were treated with a solution containing 50 mM glucose to increase the osmotic pressure and 10 mM EDTA to inactivate DNase (solution I). A solution containing 0.13 M NaOH to rupture the cells and 0.66% sodium dodecyl sulfate (SDS) to disrupt the phospholipid bilayer was added (solution II). A solution containing 1M potassium acetate was used to precipitate _E. coli_ chromosomal DNA leaving plasmid DNA in solution (solution III). The sample was further treated with 0.044 mg/ml DNase-free bovine pancreatic RNase A (Sigma) solution in TE buffer (10 mM Tris•HCl (pH 8.0), 1mM EDTA). Extractions with phenol:chloroform (1:1) and chloroform were used to denature and remove proteins from the sample. The plasmid DNA was precipitated with 66.7 % ethanol at -70°C and the centrifuged pellet was rinsed with ice-cold 70% ethanol. The precipitant was dried using a Speed•Vac centrifuge and dissolved in cold sterile water. The plasmid DNA was treated with 6.5 % polyethylene glycol (PEG) : 0.4 M NaCl solution overnight at 4°C in order to remove tRNAs from the sample. The solution was centrifuged at 13,148 RCF for 30 min and the pellets were rinsed with ice cold 70% ethanol. Pellets were resuspended in cold sterile water. The plasmid DNA concentrations were determined by measuring
absorbance at 260 nm and the plasmids were qualitatively analyzed by 0.7 % agarose gel electrophoresis.

**Plasmid Maxipreps**

*E. coli* DH5α cells containing pET19b plasmids were grown in 2 L of LB media at 37°C. After 26 hours the cells were centrifuged at 604 RCF for 15 minutes, pellets saved and resuspended in the ice-cold solution I. Fresh lysozyme solution was added to the sample for a final concentration of 0.9 mg/ml lysozyme. Next this mixture was treated with the solution II followed by the solution III. DNA was precipitated with 37.5 % isopropanol, centrifuged at 40,248 RCF for 15 minutes and pellets were resuspended in TE buffer (pH 8.0). LiCl solution was added to the sample for a final concentration of 2.5 M and it was centrifuged. Pellets were saved, rinsed with 70% ethanol, centrifuged and the pellets were resuspended in TE (pH 8.0) buffer containing 20 µg/ml DNase free pancreatic RNase A solution. This plasmid DNA solution was treated with 6.5 % PEG and 0.4 M NaCl overnight on ice. After approximately 24 hours incubation on ice samples were centrifuged and pellets were resuspended in TE buffer (pH 8.0). Residual proteins were removed from the DNA solution by chloroform-phenol and then chloroform extractions. The plasmid DNA was precipitated with room temperature 70 % ethanol for 10 minutes, centrifuged and rinsed twice with 70 % ethanol. Pellets were dried and resuspended in 500 µl of TE buffer (pH 8.0). Absorbance at 260 nm was measured to find the concentration of purified pET19b plasmids. Plasmids’ purity and quantity was further evaluated by 0.7 % agarose gel electrophoresis.
**Restriction Enzymes Double-Digest and Gene Purification**

NdeI and BamHI endonucleases (New England Biolabs) were used to excise the *pho13* gene from the pET*pho13* plasmid. To obtain complementary ends pET19b plasmid was treated with the same restriction enzymes. The efficiency of the digest was verified by also treating pET19b plasmid with each of the restriction enzymes separately. Samples were analyzed by 0.7% agarose gel electrophoresis.

BamHI and NdeI endonucleases were added to pET19b and pET19*pho13* just prior to incubation. NdeI endonuclease was also added to each every 30 minutes because of its shortened half life. The plasmids were digested with these restriction enzymes for a total of 2 1/2 hours. Both double digested pET19*pho13* and pET19b plasmids were run on the 0.7% agarose gel and stained with ethidium bromide. Bands containing the digested pET19b and the excised *pho13* gene were cut out the gel and purified using the Gene-clean kit following the kit’s protocol (MPBiomedicals). The DNA concentration was approximated by running the samples on agarose gel electrophoresis and using 100 ng of pET19b as a standard.
Ligation

Four different ratios (molecule : molecule) of \textit{pho13} : digested pET19b were prepared (Table 1) and T4 DNA ligase (Invitrogen) was added to each of the mixtures. Ligations were carried out at 15°C for 24 hours.

Table 1. Ligation of the \textit{pho13} gene in the pET19b plasmid.

<table>
<thead>
<tr>
<th>Ligation Mixture</th>
<th>Ratios</th>
<th>ng of pET19b</th>
<th>ng of \textit{pho13}</th>
<th># of Colonies</th>
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<tr>
<td>A</td>
<td>1:0</td>
<td>100</td>
<td>0</td>
<td>73</td>
</tr>
<tr>
<td>B</td>
<td>2:1</td>
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<td>34</td>
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<tr>
<td>C</td>
<td>1:1</td>
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</tr>
<tr>
<td>D</td>
<td>1:2</td>
<td>100</td>
<td>36</td>
<td>20</td>
</tr>
<tr>
<td>PUC19</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>many colonies</td>
</tr>
</tbody>
</table>

\textit{pho 13}: 940 basepairs; digested pET19b: 5705 basepairs.

Transformation

5 µl of each of the four ligation mixtures were added to separate 50 µl \textit{E. coli} DH5α (Novagen) competent cells. PUC19 was transformed into DH5α cells as a positive control. Cells were incubated on ice and heat shocked for 25 seconds in a 37°C water bath. Cells were placed back on ice immediately for at least 2 min. 1 ml of LB media was added to each of the samples and were incubated at 37°C for 1 hour with mixing every 5-
10 minutes. The samples and positive control were grown on LB plates containing 100 µg/µl ampicillin in order to select the cells containing the pET19pho13 plasmids, which have the ampicillin resistance gene. Plates were incubated at 37°C overnight for approximately 15 hours.

Colonies were streaked to single colonies and cell cultures were grown from the single colonies. Cells were harvested, plasmids were purified as described by Sambrook and Russell (2001), similar to the procedure for plasmid midipreps. The success of the subcloning was verified by running the purified plasmids out on a 0.7% agarose gel.

**Verification of Obtained Subclone**

To verify that isolated plasmids actually represent recombinant pET19pho13, two digestion tests were performed. For the first test, purified plasmids were digested with NdeI and BamHI endonucleases; this would excise the pho13 gene from the plasmid. The other verification test was an EcoRV (New England Biolabs) endonuclease digest. 1µl of each restriction enzyme was added to 8 µl plasmid solution. For both tests, reactions were carried out at 37°C for one hour. All of the enzymes were added just prior to the incubation except for NdeI, which was added prior to the incubation and 30 minutes passed into the incubation.

*E. coli* BLR (DE3) (Novagen) and *E. coli* Rosetta (DE3) (Novagen) cells were transformed with purified pET19pho13 plasmid to be used for expression. Freezer stocks were created from cells (DH5α, BLR(DE3) and Rosetta (DE3)) containing the pET19pho13 recombinant plasmid (5 mL cell culture grown to 1-2 OD; 0.4 ml of 80 % glycerol is added to 1.6 ml of culture). Cultures were started from these freezer stocks.
Purification of the PHO13(HT) protein

100 ml Overexpression of PHO13(HT) in *E. coli* BLR (DE3) and *E. coli* Rosetta (DE3)

An inoculum of pET19*pho13* in *E. coli* BLR (DE3) was started by adding a scraping of freezer stock to 7.5 ml of LB media containing 100 µg/µl ampicillin. The cells were grown at 37°C with shaking until an $A_{600}$ of 1.0 – 1.5 OD was reached. The inoculum was transferred to prewarmed 100 ml LB and continued to incubate at 37°C. Once the $A_{600}$ reached 0.8 OD the cells were induced with 250 µg/µl isopropyl β-D-1-thiogalactopyranoside (IPTG) (Research Organics). The cells were incubated for another 3 hours and harvested by centrifugation at 1,207 RCF for 15 minutes. The pellet was rinsed twice with 50 ml of buffered saline (50 mM Tris•HCl (pH 7.5), 85 mM NaCl, 67 mM KCl) and stored at -80°C.

The same procedure was followed for the 100 ml overexpression of PHO13(HT) protein in *E. coli* Rosetta (DE3) cells.

2 L Overexpression of PHO13(HT) Protein in *E. coli* Rosetta (DE3)

PHO13 protein containing a string of 10 histidines at the N-terminal end (PHO13(HT)) was overexpressed in two 1 L cultures of *E. coli* Rosetta (DE3). 7.5 ml inoculum containing 100 µg/µl ampicillin was grown to an $A_{600}$ of approximately 1.0 OD. When the inoculums reached ~1.0 OD, 5 ml was transferred into 100 ml LB containing 100 µg/µl ampicillin. Once the $A_{600}$ reached 0.6 OD, 2 ml of 50 mg/ml ampicillin and 40 ml of the 100 ml inoculum was added to each of two 1 liters of LB. Cultures were incubated at 37°C with shaking. When $A_{600}$ reached 0.3, both 1 L cultures
were moved to 15°C. Expression at 15°C was shown to increase solubility and expression of PHO13 (unpublished results. Lof, O’Handley). Once A_{600} reached 0.6 OD, each 1 L culture was induced with 250 µg/µl of IPTG and PHO13(HT) was expressed overnight at 15°C. Next day the incubated cultures were harvested by centrifuging at 1,207 RCF for 15 minutes. The pellets were rinsed twice with cold buffered saline and stored at -80°C.

**Cell Harvesting and Sonication**

The saved pellets were resuspended in two volumes of 20 mM Tris•HCl (pH 8.0). Cells were disrupted by sonication using a Branson sonifier. Sonication was carried out at an output of 6.5, 50% duty cycle in 15 second intervals for a total of 5 minutes. Samples were centrifuged at 13,148 RFC for 5 minutes (harvesting 100 ml culture overexpression) and at 13,416 RFC for 20 minutes (harvesting 2 L culture overexpression). Supernatant and pellet were separated. The pellets were resuspended in 1 ml (harvesting 100 ml culture overexpression) and in 6.5 ml (harvesting 2 L culture overexpression) of 20 mM Tris•HCl (pH 8.0) and both the supernatants and resuspended pellets were stored at –80°C.

The same procedure was followed for overexpression of PHO13(HT) protein in *E. coli* Rosetta (DE3) cells.
His•Tag Affinity Chromatography Purification

A 10 ml Ni-NTA affinity column (Novagen) was prepared by pouring the 20 ml 1:1 suspension of Ni-NTA resin in ethanol into a biorad Econo-column and rinsing the column with 200 ml binding buffer (5 mM imidazole, 20 mM Tris•HCl (pH 8.0), 0.5 M NaCl) to equilibrate the column. Once the column was equilibrated sample was loaded on the column 100 ml wash buffer (60 mM imidazole, 20 mM Tris•HCl (pH 8.0), 0.5 M NaCl) was used to elute all of the proteins except PHO13(HT) and 18 fractions containing 5 ml of sample were collected. Finally the column was rinsed with 50 ml of elute buffer (250 mM imidazole, 20 mM Tris•HCl (pH 8.0), 0.5 M NaCl) to elute the PHO13(HT) and 22 fractions containing 2 ml of the sample were collected. The protein concentration of each of the fractions was determined by Bradford assay (Bradford, 1976) and the fractions containing protein were examined by SDS-PAGE to analyze PHO13(HT) purity and yield.

Dialysis and Concentration

Fractions containing PHO13(HT) were pulled together and dialysed in Pierce Slide-A-Lyzer cassettes (10 KDMWCO). Dialysis was performed in buffer 1 (100 mM imidazole, 250 mM NaCl, 20 mM Tris•HCl (pH 8.0)) and buffer 2 (50 mM imidazole, 250 mM NaCl, 20 mM Tris•HCl (pH 8.0)) for 1 hour each and overnight in buffer 3 (250 mM NaCl, 50 mM Tris•HCl (pH 8.0), 1 mM EDTA, 0.1 mM DTT). The concentration of imidazole was gradually decreased from 100 mM to 50 mM and finally to no imidazole. This step by step process was necessary to prevent protein precipitation.
The dialysed PHO13(HT) was concentrated using Amicon Ultra centrifugal filter units (Millipore) and centrifuged at 2,147 RCF until the final volume was approximately 1.5 ml.

**Size-Exclusion Chromatography Purification**

The concentrated PHO13(HT) was loaded onto a S-100 sephacryl HR column (3 cm × 80 cm) and rinsed with 400 ml of 250 mM NaCl, 50 mM Tris•HCl (pH 8.0), 1mM EDTA, 0.1 mM DTT. The collected 2 ml fractions were analyzed for PHO13(HT) by an enzyme assay with \( p \)-nitrophenylphosphate (\( p \)-NPP) substrate analog. In wells of a microtiter plate were added 19 µl of 53 mM Tris•HCl, 5.3 mM MgCl\(_2\), 5.3 mM \( p \)-NPP and 1 µl of fraction for a total volume of 20 µl. \( A_{280}/A_{260} \) was measured for each fraction and those containing PHO13(HT) \( p \)-NPPase activity were examined by SDS-PAGE. Those fractions containing pure PHO13(HT) were pulled together and concentrated as described above.
Characterization of PHO13 and PHO13(HT)

Enzyme Assay to Determine Specific Activity

Specific activity was determined for both PHO13 and PHO13(HT). The standard reaction mixture contained 50 mM Tris•HCl (pH 7.5), 5 mM MgCl₂, 1 mM DTT and either 4 mM \( p \)-nitrophenylphosphate (\( p \)-NPP) or 4 mM phosphoglycolate (PG) in 50 µl. Reactions with varying enzyme concentrations were carried out for 30 min at 37°C and quenched with 250 µl of 2 mM EDTA solution. Diluent was used in place of the enzyme in the negative control. The Ames and Dubin colorimetric assay (Ames and Dubin, 1960) was used to measure the concentration of inorganic phosphate released by the enzyme.

Enzyme Assay to Determine the pH Optimum

The standard reaction mixture consisted of 50 µl of 5 mM MgCl₂, 1 mM DTT and 4 mM \( p \)-NPP. 50 mM Tris Maleate (pH) 5.5-6.5 or 50 mM Tris•HCl (pH 7.0-9.5) were added to determine the pH optimum of the enzymatic reaction. Reactions with varying enzyme concentrations were carried out for 30 minutes at 37°C and quenched with 250 µl of 2 mM EDTA. The Ames and Dubin colorimetric assay (Ames and Dubin, 1960) was used to measure the concentration of inorganic phosphate released by the enzyme.
Enzyme Assay to Determine the Best Divalent Metal Cofactor

The standard reaction mixture consisted of 50 µl of 50 mM Tris•HCl (pH 8.0), 1 mM DTT and 4 mM \( p - \text{NPP} \). MgCl\(_2\), MnCl\(_2\), ZnCl\(_2\), CaCl\(_2\) and CoCl\(_2\) were added to 1mM or 10 mM total divalent metal ion concentrations. Reactions with varying enzyme concentrations were carried out for 30 minutes at 37ºC and quenched with 250 µl of 2 mM EDTA. The Ames and Dubin colorimetric assay (Ames and Dubin, 1960) was used to measure the concentration of inorganic phosphate released by the enzyme.

Enzyme Assay to Determine the Optimum Metal Concentration

Enzyme assays were carried out for solutions with concentrations of MgCl\(_2\) ranging from 1mM to 50 mM for both PHO13 and PHO13(HT). The standard reaction mixture consisted of 50 µl of 50 mM Tris•HCl (pH 8.0), 1 mM DTT and 4 mM \( p - \text{NPP} \). Varying concentrations of MgCl\(_2\) were used for each of the reaction mixtures. The reactions were carried out for 30 minutes at 37ºC and quenched with 250 µl of 20 mM EDTA. Note that more EDTA was needed in these assays to chelate the larger concentrations of \( \text{Mg}^{2+} \). The Ames and Dubin colorimetric assay (Ames and Dubin, 1960) was used to measure the concentration of inorganic phosphate released by the enzyme.
**Kinetic Analysis**

Enzyme assays were carried out at pH 8.0 in presence of 7.5 mM MgCl$_2$. The standard reaction mixture consisted of 50 µl of 50 mM Tris•HCl (pH 8.0), 1 mM DTT. Each reaction contained 1 mM – 40 mM p-NPP. The enzymatic reactions were carried out for 30 minutes at 37°C, quenched with 250 µl of 20 mM EDTA. The Ames and Dubin colorimetric assay (Ames and Dubin, 1960) was used to measure the concentration of inorganic phosphate released by the enzyme.

**Bioinformatics**

BLAST searches were performed using standard protein-protein BLAST (blastp). Sequence alignment of the amino acid sequences were generated using CLUSTAL W software. Amino acids were highlighted based on sequence identity and similarity. Similar amino acids were designated as follows: (1) G; (2) P; (3) I, L, V, A, M, F, W; (4) T, S, N, Q; (5) D, E; R, K; and (6) Y, H (Tirrell et al, 2006).
RESULTS AND DISCUSSIONS
Plasmid Purification

The pETpho13 and pET19b plasmids were purified from E. coli DH5α cells. Once purified, the quantity of each was determined as follows. The concentration of each purified plasmid was calculated based on A₂₆₀ using equation 4:

\[
\text{DNA concentration (ng/µl)} = \frac{A_{260} \text{ (OD)} \cdot 50 \, \mu g/(ml\cdot OD)}{\text{dilution factor}} \quad \text{(eq. 4)}
\]

To find the total mass of each purified plasmid, the concentrations of pETpho13 and pET19b were multiplied by the total volume of each plasmid solution (Table 2).

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Dilution Factor</th>
<th>A₂₆₀ OD</th>
<th>Concentration (ng/µl)</th>
<th>Total Volume (µl)</th>
<th>Total Mass (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pETpho13</td>
<td>10</td>
<td>0.034</td>
<td>170</td>
<td>120</td>
<td>20</td>
</tr>
<tr>
<td>pET19b</td>
<td>10</td>
<td>0.072</td>
<td>360</td>
<td>500</td>
<td>180</td>
</tr>
</tbody>
</table>
Plasmid purity and quantity are displayed in Figure 12. pET11b was used as a positive control.

**Figure 12.** Analysis of purified pETpho13 and pET19b via agarose gel electrophoresis.  
A. pETpho13: Lane 1. λ HindIII molecular weight markers; Lane 2. pET11b, 100 ng; Lane 3. pETpho13, 100 ng.  
B. pET19b: Lane 1. λ HindIII molecular weight markers; Lane 2. pET11b, 100 ng; Lane 3. pET19b, 100 ng.

As can be seen in Figure 12 the intensity of the band for the control pET11b plasmid is approximately the same as the intensity of the band for the pETpho13 and pET19b plasmids indicating that the concentrations of both purified plasmids have been accurately determined.
Figure 13. Restriction map of the pET19b plasmid (Novagen, pET-19b vector, 1998). The restriction sites for NdeI and BamHI endonucleases used for subcloning are circled in red.
The pETpho13 and pET19b plasmids were both digested with NdeI and BamHI restriction enzymes. Single digests of the pET19b plasmid by either of the enzymes proved that both enzyme digestions were complete within 1 hour, where the digested linear pET19b plasmid travels more slowly through the agarose gel (Fig. 14). If the enzymes digest pET19b completely separately, they will also do so jointly on pETpho13 and pET19b, thus Figure 14 shows that the plasmids were completely digested.

**Figure 14.** Digestion of pET19b with NdeI and BamHI endonucleases. Lane 1. λ HindIII molecular weight markers; Lane 2. pET19b digested with NdeI; Lane 3. pET19b digested with BamHI; Lane 4. pET19b, 100 ng.
Digested Plasmid and Gene Purification

Figure 15 shows the digestions of the pETpho13 and the pET19b plasmids with the NdeI and BamHI endonucleases.

Figure 15. Gel purification of the pho13 gene and digested pET19b plasmid. Lane 1. pET19b digested with NdeI and BamHI; Lane 2. pho13 (lower band) cleaved from pETpho13 by NdeI and BamHI; Lane 3. 100 ng pETpho13 digested with BamHI and NdeI; Lane 4. λ HindIII molecular weight markers.

The pho13 gene was cleaved from pETpho13 (lower band in lane 2, Fig. 15) and ran as expected for DNA of 940 bp (as compared to the molecular weight markers). The band in lane 1 (Fig. 15) at ~ 5.7 kbp corresponds to the digested pET19b plasmid. The pho13 gene and digested pET19b plasmid were excised from the gel and purified using the Gene-clean kit.
Agarose gel electrophoresis was used to estimate concentrations of the purified \textit{pho13} gene and pET19b plasmid.

\textbf{Figure 16.} Quantification of the gel-purified \textit{pho13} gene and pET19b plasmid. Lane 1. \textit{λ} HindIII molecular weight markers; Lane 2. 1 µl of digested gel-purified pET19b; Lane 3. 2 µl of digested gel-purified pET19b; Lane 4. 1 µl of gel-purified \textit{pho13}; Lane 5. 2 µl of gel-purified \textit{pho13}; Lane 6. pET19b, 100 ng.

A band in the lane 6 (Fig. 16) represents 100 ng of the pET19b plasmid, and it is approximately twice as intense as the band in the lane 3, corresponding to 2 µl of digested gel-purified pET19b. Therefore the concentration of digested pET19b plasmid is approximately 25 ng/µl.

A band in lane 5 (Fig. 16) corresponding to 2 µl of the \textit{pho13} gene is about half of the intensity of the band in lane 2 representing the 25 ng/µl of the pET19b. Therefore, the concentration of the \textit{pho13} gene was approximately 10 ng/µl.
Ligation and Transformation

Molecular size of the pET19b plasmid and *pho13* gene are approximately 5.5 kbp and 1 kb respectively. Therefore, for 100 ng of digested pET19b, 18 ng of the *pho13* gene is needed to create a 1:1 ratio. The ratios of digested pET19b and *pho13* used for ligations are shown in the Table 3.

### Table 3. Ligation of *pho13* and digested pET19b.

<table>
<thead>
<tr>
<th>Ligation Mixture</th>
<th>Ratios</th>
<th>ng of pET19b</th>
<th>ng of <em>pho13</em></th>
<th># of Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1:0</td>
<td>100</td>
<td>0</td>
<td>73</td>
</tr>
<tr>
<td>B</td>
<td>2:1</td>
<td>100</td>
<td>9</td>
<td>34</td>
</tr>
<tr>
<td>C</td>
<td>1:1</td>
<td>100</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>1:2</td>
<td>100</td>
<td>36</td>
<td>20</td>
</tr>
<tr>
<td>PUC19</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>many colonies</td>
</tr>
</tbody>
</table>

Ligation mixture A containing the digested gel-purified pET19b plasmid was used as a negative ligation control. PUC19, used as a positive transformation control, yielded many colonies. Transformation with ligation mixtures A, B, C and D resulted in 73, 34, 0 and 20 colonies, respectively.

Transformation with ligation mixture A, the negative control containing no *pho13*, yielded the most colonies indicating that although the double digestions with NdeI and BamHI appeared to be 100% complete, at least one of these reactions did not go to
100% completion. Instead of noncomplementary sticky ends, some of the plasmids had been digested by only one restriction enzyme and thus these complementary sticky ends could reanneal easily.

Twenty four colonies were selected from the B and D, and plasmids were purified and analyzed on a 0.7% agarose gel.

**Figure 17.** Purified plasmids from transformed DH5α *E. coli* cells. Lanes 1, 14, 15, 28. 100 ng pET19b; Lanes 2-13 and 16-27. Religated pET19b. The lower bands are supercoiled DNA and the top bands are relaxed.

In the Figure 17, bands corresponding to the purified plasmids have the same molecular weight as the bands corresponding to the undigested pET19b plasmid used as a standard. Therefore it was concluded that each of the twenty four purified plasmids represented religated pET19b.
Another twenty four colonies were selected from plates B and D, and plasmids were purified and examined by 0.7 % agarose gel electrophoresis.

Figure 18. Purified plasmids from the DH5α E. coli transformed with ligation mixtures. Lanes 1, 14, 15, 28. pET19b, 100 ng; Lane 2-7, 10-13, 17-27. Religated pET19b; Lanes 8 and 9. Unknown; Lane 16. pET19pho13.

A band in lane 16 ran slower than the other bands thus indicating a higher molecular weight than that of pET19b in lane 15 (Fig. 18). pET19b is 5.7 kbp and pET19pho13 would be 5.8 kbp. Of the 48 colonies screened, one colony appeared to contain the recombinant pET19pho13. This colony was number 13 from plate B and is thus designated colony B13.
Subcloning Verification

The purified plasmid from colony B13, the pET19pho13 was digested with NdeI and BamHI restriction enzymes and as expected pho13 was cleaved from the plasmid yielding 2 bands (Fig. 19, lane 7). One is approximately 5700 bp corresponding to the size of digested pET19b (Fig. 19, lane 4 and 5) and the other is 940 bp, corresponding to the size of pho13 gene (Fig. 19, lane 8).

Figure 19. Verification of the subcloning of recombinant pET19pho13. Lanes 1, 6, 10. λ HindIII molecular weight markers; Lane 2. pET19b, 100 ng; Lane 3. Potential recombinant pET19pho13; Lanes 4 and 5. pET19b digested with NdeI and BamHI; Lane 7. Recombinant pET19pho13 digested with NdeI and BamHI; Lane 8. pho13; Lane 9. Recombinant pET19pho13 digested with EcoRV; Lane 11. pET19b digested with EcoRV.

The purified plasmid from colony B13, the potential recombinant pET19pho13 was also digested with EcoRV endonuclease (Fig.19). pET19b has two EcoRV restriction sites on the enzyme at 187 bp and 1675 bp, yielding fragments of 1488 b and 4229 bp when digested with EcoRV (Fig. 19, lane 11). Digestion of pET19pho13 plasmid with
EcoRV yielded two fragments of 2416 bp and 4229 bp as was expected based on the location of the EcoRV restriction sites (Fig. 19, lane 9)

A midirep of the subcloned pET19pho13 was purified form the E.coli DH5α cells and absorbance at 260 nm was measured to determine the concentration (Table 4) and the purified subclone appears pure as determined by agarose gel electrophoresis (Fig. 20).

<p>| Table 4. Amount of the purified recombinant pET19pho13 plasmid. |
|--------------------------------|-----------------|--------------|--------------|--------------|--------------|</p>
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Dilution Factor</th>
<th>A260 OD</th>
<th>Concentration ng/µl</th>
<th>Total Vol µl</th>
<th>Total Mass µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET19pho13</td>
<td>10</td>
<td>0.042</td>
<td>210</td>
<td>120</td>
<td>25</td>
</tr>
</tbody>
</table>

![Image](image.png)

**Figure 20.** Purified recombinant pET19pho13.
Lane 1. λ HindIII molecular weight markers; Lane 2. Recombinant pET19pho13; Lane 3. pET19b, 100 ng
Overexpression of PHO13(HT) in *E. coli* BLR (DE3) in 100 ml LB

PHO13(HT) protein was overexpressed in *E. coli* BLR (DE3). Cells were sonicated and generated fractions are shown in Figure 21.

![Figure 21](image)

**Figure 21.** Overexpression of PHO13(HT) in *E. coli* BLR (DE3) cells. Lane 1. Molecular weight markers; Lane 2. Prior to induction; Lane 3. 1 hour after induction; Lane 4. 2 hours after induction; Lane 5. 3 hours after induction; Lane 6. Crude extract; Lane 7. Freeze thaw extract; Lane 8. Sonicated supernatant; Lane 9. Sonicated pellet.

The PHO13(HT) protein expresses; however there are two major bands that can be seen on the gel. The upper major band is approximately 35 kDa, which corresponds to the size of the PHO13(HT) protein. The lower major band probably represents truncated PHO13(HT) protein. The gene sequence of *pho13* gene was examined and it was determined that some of the codons found within the sequence are rare in *E. coli*. This means that cells either have no tRNAs corresponding to these codons or a limited quality. Therefore we transformed pET19*pho13* into *E. coli* Rosetta (DE3) cells, which encodes for the synthesis of some tRNAs common in other organisms but not *E. coli*. 
Overexpression of PHO13(HT) in *E. coli* Rosetta (DE3) in 100 ml LB

*E. coli* Rosetta (DE3) were transformed with pET19pho13. The PHO13(HT) protein was expressed in the cells, which were sonicated and fractions can be found in Figure 22.

![Image of gel electrophoresis](image)

**Figure 22.** Overexpression of PHO13 (HT) in *E.coli* Rosetta (DE3). Lane 1. Molecular weight markers; Lane 2. Prior to induction; Lane 3. 1 hour after induction; Lane 4. 2 hours after induction; Lane 5. 3 hours after induction; Lane 6. Crude extract; Lane 7. Sonicated supernatant; Lane 8. Freeze thaw extract; Lane 9. Sonicated pellet.

The PHO13(HT) protein expresses in *E.coli* Rosetta (DE3), as one major product as seen by one major band with no evidence of truncated protein and a significant portion of PHO13(HT) is in the sonicated supernatant as soluble protein.
Overexpression of PHO13(HT) in *E. coli* Rosetta (DE3) in 2 L LB

The overexpression of PHO13(HT) in *E. coli* Rosetta (DE3) was scaled up to 2 L, expressing in two 1 L cultures (sample A + B). Fractions generated after overexpression and sonication are shown in Figure 23.

**Figure 23.** 2 L Overexpression of PHO13(HT) in *E. coli* Rosetta (DE3).
Lane 1. Molecular weight markers; Lanes 2-5. Sample A (Lane 2. Prior to induction; Lane 3. Crude extract; Lane 4. Sonicated supernatant; Lane 5. Sonicated pellet); Lanes 6-9. Sample B (Lane 6. Prior to induction; Lane 7. Crude extract; Lane 8. Sonicated supernatant; Lane 9. Sonicated pellet).

As a result of sonication a significant amount of PHO13(HT) was retained in the pellets as seen in Figure 23, thus the resuspended sonicated pellets were sonicated for an additional 5 minutes to increase the amount of soluble PHO13(HT) in the supernatant.
This second round of sonication allowed more PHO13(HT) to be released as soluble protein in the supernatant (Fig. 24).

**Figure 24.** Additional sonication of PHO13(HT). Lanes 1-4. Sample A (Lane 1. 1\textsuperscript{st} sonicated supernatant; Lane 2. 1\textsuperscript{st} sonicated pellet; Lane 3. 2\textsuperscript{nd} sonicated pellet; Lane 4. 2\textsuperscript{nd} sonicated supernatant); Lanes 5-8. Sample B (Lane 5. 1\textsuperscript{st} sonicated supernatant; Lane 6. 1\textsuperscript{st} sonicated pellet; Lane 7. 2\textsuperscript{nd} sonicated pellet; Lane 8. 2\textsuperscript{nd} sonicated supernatant); Lane 9. Molecular weight markers.
His•Tag Affinity Purification of PHO13(HT)

Sonicated supernatant containing PHO13(HT) (Lane 1, Fig. 24) was purified by His•Tag affinity chromatography using a “Wash” solution to elute proteins other than PHO13(HT) first, followed by an “Elute” solution to elute PHO13(HT) from the column by increasing the concentration of imidazole, which competes with the PHO13(HT) for the Ni$^{2+}$-NTA resin of the column.

Figure 25. His•Tag affinity chromatography of PHO13(HT)
Lane 1. Molecular weight markers; Lanes 2-3. Wash fractions 2 and 4; Lanes 4-6. Elute fractions 5-7; Lanes 7-10. Elute fractions 9-12.

Most other proteins and almost no PHO13(HT) were eluted in the “Wash” fractions (Fig. 25, Lanes 2-3). The PHO13(HT) protein eluted off the column with the “Elution buffer” (Fig. 25, Lanes 4-10).
Size-Exclusion Chromatography of PHO13(HT)

Size-exclusion chromatography was used to remove as many remaining impurities in the sample as possible leaving PHO13(HT) significantly purified (Fig. 26).

![Size-exclusion chromatography purification of PHO13(HT)](image)

**Figure 26.** Size-exclusion chromatography purification of PHO13(HT)
Lanes 1-3. Fractions 45-47; Lanes 4-10. Fractions 52-58

Purified PHO13(HT) protein (Fig. 27) was concentrated and stored at – 80°C for characterization and crystal growth.
Purification of PHO13(HT) vs PHO13

The main reason that we subcloned *pho13* into pET19b (His•Tag vector) was to increase both the purity and final yield of PHO13. As can be seen in Figure 27, A, PHO13(HT) has been purified significantly by His•Tag affinity chromatography followed by size-exclusion chromatography. In comparison to the original PHO13 protein purification (Fig. 27, B), where the streptomycin precipitation, ammonium sulfate fractionation and size-exclusion purification steps retained significant amount of protein impurities.

**Figure 27.** Purification of PHO13(HT) and PHO13
A. PHO13 (HT). Lane 1. Molecular weight markers; Lane 2. Crude extract; Lane 3. Sonicated supernatant; Lane 4. His•Tag affinity chromatography; Lane 5. Size-exclusion chromatography.
B. PHO13. Lane 1. Molecular weight markers; Lane 2. Sonicated supernatant; Lane 3. Streptomycin precipitation; Lane 4. Ammonium sulfate fractionation; Lane 5. Size-exclusion chromatography.
Characterization of the PHO13 and PHO13(HT) Proteins

The characterization assays were carried out once and they will be performed in triplicate in the future. Therefore no statistical treatment of the data was done.

Units of Enzyme Activity

PHO13(HT) and PHO13 are active on the biologically significant substrate 2-phosphoglycolate (PG) and the unnatural substrate analog p-nitrophenylphosphate (p-NPP). Initial enzyme assays indicate that both the native protein (PHO13) and the one modified with a strand of histidines (PHO13(HT)) have comparable specific activities on these substrates. One would expect nearly identical specific activity and thus these assays will be repeated. Unit of p-nitrophenylphosphatase specific activity is defined as 1 μmol of substrate cleaved per minute by 1 mg of enzyme. It can be concluded from Table 4 that PHO13 and PHO13(HT) have comparable activities towards p-NPP.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Vol ml</th>
<th>Activity units/ml</th>
<th>Total units</th>
<th>Conc mg/ml</th>
<th>Specific Activity units/mg</th>
<th>Total Protein mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHO13</td>
<td>0.75</td>
<td>1273</td>
<td>866</td>
<td>2.07</td>
<td>615</td>
<td>1.55</td>
</tr>
<tr>
<td>PHO13(HT)</td>
<td>1.40</td>
<td>1154</td>
<td>1782</td>
<td>1.79</td>
<td>645</td>
<td>2.51</td>
</tr>
</tbody>
</table>

Similarly enzyme assays were carried out for both PHO13 and PHO13(HT) with 2-phosphoglycolate (PG) and results are shown in Table 6. PHO13 and PHO13(HT) have comparable activities towards PG.
Table 6. Enzymatic activity of PHO13 and PHO13(HT) towards PG

<table>
<thead>
<tr>
<th>Sample</th>
<th>Vol ml</th>
<th>Activity units/ml</th>
<th>Total units</th>
<th>Conc. mg/ml</th>
<th>Specific Activity units/mg</th>
<th>Total Protein mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHO13</td>
<td>0.75</td>
<td>689</td>
<td>517</td>
<td>2.07</td>
<td>333</td>
<td>1.55</td>
</tr>
<tr>
<td>PHO13(HT)</td>
<td>1.40</td>
<td>490</td>
<td>686</td>
<td>1.79</td>
<td>274</td>
<td>2.51</td>
</tr>
</tbody>
</table>

**Determination of pH Optimum**

The pH optimum was determined for PHO13(HT) and PHO13. Magnesium chloride was used as the source of metal cofactor and p-nitrophenyl phosphate (substrate analog) was used as a substrate. Buffers of 50 mM Tris Maleate or 50 mM Tris•HCl with pH values ranging from 5.5 to 9.5 were used. As shown in Figure 28 PHO13(HT) and PHO13 both have maximum activity at pH 8.0. This is in agreement with what has been reported in the literature (Attias et al, 1972).

![Figure 28. Effect of pH on PHO13 and PHO13(HT) activities.](image)
This experiment will be repeated with the 2-phosphoglycolate substrate, which has never been reported before.

**Determination of the best metal cofactor**

PHO13(HT) and PHO13 absolutely require a divalent metal cofactor for activity. The best divalent metal cofactor was determined for PHO13(HT) and PHO13 with solutions containing Mg$^{2+}$, Ca$^{2+}$, Co$^{2+}$, Mn$^{2+}$ and Zn$^{2+}$ at 1 mM or 10 mM concentrations. As shown in Figure 29 the metal yielding the highest activity was Mg$^{2+}$ followed by Co$^{2+}$ and then Mn$^{2+}$. Zn$^{2+}$ supported minimal activity and Ca$^{2+}$ did not support PHO13(HT) or PHO13 p-NPase activity.
B

Figure 29. Effect of divalent metal ion cofactor on activities: A. PHO13; B. PHO13(HT).

Attias (1972) reported strong dependence of p-NPPase activity on \( \text{Mg}^{2+} \) with inhibition by \( \text{Ca}^{2+} \) and \( \text{Zn}^{2+} \). This experiment will be repeated with 2-phosphoglycolate which has not been reported to date.

**Optimum \( \text{Mg}^{2+} \) Concentration**

Since \( \text{MgCl}_2 \) was determined to be the divalent metal ion that supported the largest amount of PHO13(HT) and PHO13 activity, \( \text{Mg}^{2+} \) concentrations were varied from 0 mM to 50 mM.
Figure 30. Optimum concentration of Mg$^{2+}$ for PHO13 and PHO13(HT).

Figure 30 shows that $p$-NPPase activity increases with increasing MgCl$_2$ concentrations where it reaches a maximum at 7 mM MgCl$_2$ and plateaus up through 50 mM MgCl$_2$. This experiment will be repeated with 2-phosphoglycolate as the substrate which has not been reported.

**Kinetic analysis**

Preliminary kinetic studies have been carried out for PHO13(HT). Enzyme assays that assess the qualitative aspects of its activity show that PHO13(HT) and the untagged native PHO13 have comparable activities (comparable pH optima, comparable metal ion requirements, comparable specific activities), and thus we can now focus on the His-tagged PHO13(HT). Both $p$-NPP, with concentrations varying from 0.1 mM to 40 mM, and phosphoglycolate, with concentrations varying from 1 mM to 10 mM, were used for the kinetic studies. $V_{max}$ and $K_m$ values were determined from nonlinear regression plots (Fig.31 A, B and C), where [S] is a substrate concentration and V is the specific activity,
measured as µmol of substrate cleaved per min per mg of enzyme.

**Figure 31.** Non-linear regression plot of PHO13(HT) with **A.** 1-40 mM p-NPP; **B.** 0.1-10 mM p-NPP; **C.** 0.1-10 mM 2-PG.
From these initial experiments we can see that PHO13(HT) follows Michaelis-Menten kinetics. $k_{\text{cat}}$ was calculated assuming PHO13(HT) is a monomer, as was indicated by how it ran on a size exclusion column as compared to molecular weight standard proteins. The kinetic parameters $V_{\text{max}}$, $k_{\text{cat}}$, $K_m$ and $k_{\text{cat}}/K_m$ are shown in Table 7.

Table 7. Kinetic parameters for PHO13(HT)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$V_{\text{max}}$ ($\text{umol}$)</th>
<th>$k_{\text{cat}}$ ($\text{s}^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{\text{cat}}/K_m$ ($\text{M}^{-1}\text{s}^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$p$-NPP</td>
<td>191.1±16.9</td>
<td>114.6±8.8</td>
<td>0.235±0.094</td>
<td>487.7±8.8</td>
</tr>
<tr>
<td>2-PG</td>
<td>673.7±27.9</td>
<td>404.2±2.0</td>
<td>3.436±0.315</td>
<td>117.6±2.0</td>
</tr>
</tbody>
</table>

The Lineweaver-Burk plots of PHO13(HT) were also generated and are shown in Figure 32 (A and B).
Figure 32. Lineweaver-Burk plot of PHO13(HT) with A. 0.1-10 mM p-NPP; B. 0.1-10 mM 2-PG.

Therefore the $k_{cat}/K_m$ values of PHO13(HT) for p-NPP and 2-PG are $487.7 \cdot 10^3$ M$^{-1} \cdot $s$^{-1}$ and $117.6 \cdot 10^3$ M$^{-1} \cdot $s$^{-1}$ respectively.

Since only one set of experiments with one enzyme concentration was run for each of the substrates, in the future PHO13(HT) kinetic experiments will be carried out in triplicate with three enzyme concentrations for both p-NPP and 2-PG and statistical treatment of data will be performed.
CONCLUSIONS

The gene \textit{pho13} has been subcloned for overexpression into pET19b to create a protein with a His\textbullet{}Tag to aid in purification over a Ni\textsuperscript{2+} affinity column. PHO13(HT) has been expressed, determined to be soluble, purified and partially characterized. Purification included Ni\textsuperscript{2+} affinity chromatography followed by size-exclusion chromatography.

Enzyme assays to ascertain pH optimum (pH 8.0), divalent metal ion optimum (7 mM Mg\textsuperscript{2+}), and preliminary kinetics parameters have been initiated for the substrate analog \textit{p}\textsuperscript{-}nitrophenylphosphate and the biologically significant substrate 2-phosphoglycolate and will be continued for both of them in the future.

The purified enzyme is now ready to set up microtiter plates of various conditions to screen for crystal formation. The conditions we will initially screen are those that allow the growth of crystals of other \textit{p}\textsuperscript{-}NPPase family members and more specifically other PGPases. The conditions that are favorable for crystal formation will be optimized to yield bigger crystals, and these will be used to determine the structure of PHO13 PGPase. This structural work is in collaboration with Joseph Wedekind at the University of Rochester Medical Center.

Additional future work will include phenotypic studies of a \textit{S. cerevisiae} PHO13 knockout, in collaboration with Eric Phizicky at the University of Rochester Medical Center.
REFERENCES


