Molecular Characterization of a Novel Yeast Cell-wall Acid Phosphatase Cloned from *Kluyveromyces marxianus*

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Received August 9, 1999; Accepted September 9, 1999

A novel *Kluyveromyces marxianus* gene that encodes an acid phosphatase, Pho610, was cloned in *Saccharomyces cerevisiae*. The deduced amino acid sequence was distinct from *S. cerevisiae* phosphatases but similar to some fungal enzymes. A peculiar feature of the sequence is that it has hydrophobic stretches both at the N- and C-termini, which is a characteristic of the precursors of glycosylphosphatidylinositol(GPI)-anchored proteins. When the gene was expressed in *S. cerevisiae*, the active enzyme was recovered in the periplasmic fraction by glucanase digestion. The Pho610 polypeptide was highly glycosylated and a significant portion was covalently linked to the cell-wall glucan. The enzyme was identified when the C-terminal region was truncated to remove the GPI signal. Therefore, Pho610 is a novel cell-wall protein having an enzyme activity.

**Key words:** GPI-anchor protein; cell wall; acid phosphatase; *Saccharomyces cerevisiae*; *Kluyveromyces marxianus*

Acid phosphatases (EC 3.1.3.2) with a broad substrate specificity are common in secretory proteins of a wide variety of yeasts and fungi.1 They generally function at the cell surface of these microorganisms to release inorganic phosphate from various phosphorylated organic compounds for their efficient uptake by the cell. These phosphatases are transported to the cell surface via a general protein secretion pathway.2

The repressible acid phosphatase encoded by the *PHO5* gene of *Saccharomyces cerevisiae* has an N-terminal signal sequence of 17 amino acids to translocate it into the endoplasmic reticulum and is transported via the Golgi complex to the periplasmic space.3 Digestion of the cell-wall glucans with glucanases or its mechanical disruption is necessary to release soluble Pho5 phosphatase efficiently.4 This is mainly because the Pho5 polypeptide is present in multimeric forms and the large size prevents most of the enzyme from being released into the medium through the diffusion barrier of the cell wall, as it is the case for invertase and α-galactosidase.5

The structural mannoproteins of the cell wall were shown to constitute the outer surface layer by electron microscopic observation that the electron-sparse glucan layer was resistant to glucanase digestion unless the electron-dense protein layer was removed by proteinase digestion.6 Biosynthesis of the wall mannoprotein has been extensively studied with α-agglutinin.7 The precursor polypeptide of α-agglutinin translocated in the endoplasmic reticulum was shown to be anchored to the membrane via the GPI-anchor and then transported to the cell surface where it became bound to cell-wall glucan.8 Most known wall proteins and candidates were included in the recent compilation of possible GPI-proteins9,10 and so are suggested to follow the same pathway. When they arrived at the cell wall the mature polypeptides were covalently linked to the β-1,6-glucan via residues of GPI.11-15 A chimeric gua α-galactosidase with the C-terminal 30 amino acids of α-agglutinin was incorporated in the yeast cell wall.16

During the course of our genomic search for secretory proteins of the yeast *Kluyveromyces marxianus* (formerly *K. fragilis*), we cloned a structural gene that encodes a novel protein with acid phosphatase activity. The activity was detected by cleavage of phosphate from α-naphthylphosphate under acidic conditions upon transformation of a phosphatase-negative *S. cerevisiae* mutant.17 Our experiments indicated this protein was a covalently-linked constituent of the cell wall. This may be the first example of a naturally occurring cell-wall protein which has enzyme activity. In this paper, we describe the cloning and novel characteristics of the gene product.

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**Abbreviations:** GPI, glycosylphosphatidylinositol; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis
Materials and Methods

Strains, plasmids and media. Kluyveromyces marxianus var. marxianus (formerly K. fragilis) Y-610 was made from our laboratory stock and identical to ATCC12424 (American Type Culture Collection). Saccharomyces cerevisiae NA87-11A (MATa pho3-1 pho5-1 leu2-3,112 trpl his3) and SH1089 (MATa ura3-52::HIS4-lacZ/leu2-3,112 trpl pho3-1 pho5-1) were generous gifts from Yasuji Oshima and Satoshi Harashima, respectively, and used as the host for cloning of the phostase gene. Escherichia coli DH5α (F−, ϕ80lacZΔM15, supE44 ΔlacU169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1) was used in plasmid propagation. pHN114 is a derivative of pUC19 carrying the S. cerevisiae EcoRI fragment containing TRP1 and ARS1 (Harushi Nakajima, personal communication). A genomic library of K. marxianus was constructed by inserting DNA digested partially with Sau3A1 into the BamHI site of pHN114. pRS series plasmids and pKT10 were also used as vectors. Yeast was usually grown at 30°C in YEPD (1% Bacto yeast extract, Difco), 2% Bacto peptone (Difco), 2% glucose, or SD (0.67% Bacto yeast nitrogen base without amino acids (Difco), 2% glucose, and appropriate supplements) medium. Burkholder low phosphate medium was prepared as described and used in phostase assays. Solid media were made with 2% agar.

Molecular genetic techniques. Standard methods were use unless otherwise stated. Enzymes were purchased from either Boehringer Mannheim or Takara Shuzo Co. The DNA sequence was analyzed by the dyeoxy chain termination method using a DNA sequencer model 373A (Applied Biosystems Inc.). As the promoter of PHO610 did not work efficiently in S. cerevisiae, the coding region was subcloned in pKT10 under the control of the GAPDH promoter when necessary.

Preparation of the periplasmic fraction. Cells were grown to mid-logarithmic phase in 1 ml of YEPD medium, and incubated by centrifugation, washed once, and incubated in 100 μl of 10 mM Tris-HCl (pH 8.0) containing 0.9 M sorbitol, 0.1 M EDTA, 10 mM diethiothreitol, and 100 μg/ml of Zymolyase 10T (Seikagaku Kogyo) at 30°C for 30 min. The released periplasmic fraction was separated from the spheroplasts by centrifugation.

Laminarinase and endo-N-acetylg glucosaminidase H (endo H) treatments of the cell wall fraction. A 100 ml culture of transformants was grown in SD medium at 30°C to an OD600 of 1.0, washed in distilled water, and then suspended in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF). Cells were broken with glass beads in lysis buffer containing 2% SDS and protease inhibitor mixture as described. The cell wall was collected by centrifugation at 12,000 × g for 5 min at 4°C, extracted twice with lyss buffer containing 2% SDS by heating at 95°C for 5 min, and washed 5 times with lamarinase buffer (100 mM sodium acetate, pH 5.5, 1 mM EDTA, 1 mM PMSF, protease inhibitor mixture). The cell wall (150 mg) was incubated with 0.25 unit of lamarinase (Sigma) in 180 μl of laminarinase buffer at 37°C for 16 h. The soluble fraction was separated by centrifugation at 12,000 × g for 5 min. To the half of the supernatant (90 μl), SDS and 2-mercaptoethanol were added to a final concentration of 0.1% and 0.5%, respectively. After this was boiled for 5 min, 5 μl of recombinant endo H (Boehringer-Mannheim) was added and the mixture was incubated at 37°C for 16 h. These samples were analyzed by SDS-PAGE and immunoblotting.

Phosphatase assays. Activity staining of colonies was done by overlaying molten soft agar (0.05% α-naphthylphos- phate, 0.25% Fast blue salt B, and 0.8% Bactoagar (Difco) in 0.1 mM sodium acetate, pH 5.2) and plates were incubated at 30°C. Enzyme activity was assayed using p-nitrophenylphosphate as the substrate.

Immunological methods. Anti-Pho610 antiserum was prepared by immunizing rabbits with His-SA ‘Pho610’ (amino acid 40–424) fusion protein produced in E. coli. The ‘PHO610’ fragment was prepared by PCR and inserted in the BamHI site of the His-tag expression vector pRESET A (Invitrogen).

Results and Discussion

Cloning of a K. marxianus acid phosphatase gene in S. cerevisiae

The S. cerevisiae phosphatase-negative mutant NA87-11A was transformed with a K. marxianus genomic library and a phosphatase-positive transformant was selected by activity staining of the colonies. It was noticed that the phosphatase reaction was restricted in the colony of the S. cerevisiae transformant although K. marxianus Y-610 formed a large halo around the colony. As described below, the cloned phosphatase was distinct from the ones that are similar to Pho5 in their sequences and general in most ascomycete species, which form halos around their colonies. The plasmid (pIKD50) DNA was recovered in E. coli. The insert fragment was about 2.8 kb. The phosphatase-coding region could be trimmed down to about 1.7 kb by exonuclease digestion. We tentatively named this gene PHO610 as it originated from K. marxianus Y-610.
Fig. 1. The Nucleotide Sequence and the Derived Amino Acid Sequence of Pho860 Acid Phosphatase.

Hydropathic regions at the N- and C-termini are underlined. Asparagine residues of 14 possible N-glycosylation sites are highlighted. The sequence was registered in the DDBJ database under the accession number E02615. By reexamination of the sequence, we found two mistakes; the 333rd nucleotide was C instead of T, and the number of A between nucleotide 1619–1625 was 6 instead of 7.
Characteristics of Pho610 phosphatase

The Pho610 phosphatase activity was recovered in the periplasmic fraction of S. cerevisiae NA87-11A, which was released after Zymolyase digestion of the yeast cell wall. The periplasmic preparation was used for preliminary characterization of the enzyme as that from the host yeast had no detectable phosphatase activity. The optimal pH of Pho610 phosphatase was 5.2 using p-nitrophenylphosphate as the substrate. Substrate specificity was examined for several organic phosphate compounds; 5 mM each of glucose-1-phosphate, glucose-6-phosphate, glucose-1,6-diphosphate, fructose-1-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, galactose-1-phosphate, galactose-6-phosphate, mannose-1-phosphate, and guanosine-5'-phosphate. The periplasmic preparation of NA87-11A (PHO610) liberated inorganic phosphate from all these substrates at similar rates. It also liberated phosphate from casein. So we concluded that Pho610 is a phosphomonoesterase with low substrate specificity.

Nucleotide sequence and characterization of PHO610

The nucleotide sequence of 1769 bp was analyzed. The open reading frame of PHO610 was suggested to be 1449 bp, which can encode a 52.6-kDa protein of 483 amino acids (Fig. 1). The predicted amino acid

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Fig. 2. Comparison of the Amino Acid Sequences of Pho610 and Fungal Phosphatases.

(A) K. marxianus Pho610 phosphatase precursor (B) ORF2 of K. lactis LAC12 mRNA,26 (C) A. fumigatus acid phosphatase precursor,26 and (D) P. chrysogenum phosphate-repressible acid phosphatase precursor.26 The amino acids identical with that of Pho610 are highlighted.
sequence did not have significant similarity to \textit{S. cerevisiae} acid phosphatases. A significant identity with the fungal phosphatases of \textit{Aspergillus fumigatus} \textsuperscript{54} (45.8\%) and \textit{Penicillium chrysogenum} \textsuperscript{55} (46.0\%) was found by a BLAST search (Fig. 2). Pho610 had the highest identity with an ORF (1266 bp) of \textit{Kluyveromyces lactis} (76.1\%), which has been discovered in the 3' region of the lactose permease (\textit{LAC12}) mRNA but suggested to be latent and not translated in this organism \textsuperscript{56} (Fig. 2).

The hydrophobic plot (Fig. 3) as described by Kyte and Doolittle \textsuperscript{27} indicated that Pho610 protein has two hydrophobic stretches at the N- and C-terminals that span 21 and 22 amino acids, respectively (Fig. 1, underlined). The N-terminal hydrophobic region should represent the signal sequence for translocation across the endoplasmic reticulum membrane, which is common for secretory enzymes. The C-terminal hydrophobic region of Pho610 seemed unusual for secretory acid phosphatases. The analysis of the function of this region will be described in the latter part of this paper.

\textbf{Characterization of Pho610 protein}

Trials to produce the intact Pho610 phosphatase in the periplasm of \textit{E. coli} were unsuccessful, but a truncated protein (amino acid 40–424) after removal of the terminal hydrophobic regions (amino acids 1–39 and 425–483) and addition of a His\textsubscript{6}-tag was efficiently produced as inclusion bodies. The His\textsubscript{6}-'Pho610' was purified by affinity chromatography after solubilization in 8 M urea, and used for antiserum preparation. After dialysis, the His\textsubscript{6}-'Pho610' protein showed very weak but clearly detectable phosphatase activity to form nitrophenol from p-nitrophenyl-phosphate (data not shown). This indicated that amino acid 40–424 of Pho610 is responsible for the enzyme activity.

The periplasmic localization of Pho610 protein coincided well with the prediction that the N-terminal hydrophobic region may function as the signal sequence for protein secretion. As for the C-terminal hydrophobic region, there are a group of proteins in the cell surface which have a C-terminal modification with glycolipid named glycosylphosphatidylinositol (GPI-anchor)\textsuperscript{7–10}. The C-terminal sequence is cleaved at the ω site where the GPI-anchor is attached to these proteins. The residues Asn, Ser, Gly, Ala, Asp and Cys were shown to be most effective as an ω site residue for GPI-anchorage\textsuperscript{28,29}. The two adjacent residues on the carboxyl side, ω + 1 and ω + 2, are also important. They should have rather short side chains with the second one being more important than the first. The C-terminal sequence of PHO610 has Gly at position 461, Ala at 462, and Ala at 463, which fulfill the requirement for the GPI-anchor attachment. In \textit{S. cerevisiae}, GPI-anchored mannoproteins are further processed to crosslink to β-1,6-glucan of the cell wall\textsuperscript{10–16}. Crosslinking to the cell wall might explain why the staining of Pho610 phosphatase activity was restricted in colonies in contrast to halos of other acid phosphatases as Pho5.

\textbf{Localization of Pho610 protein}

To address this issue, we examined the diffusibility of the products of deletion derivatives of PHO610 from the 3'-end by a halo assay (Fig. 4). Nucleotide sequencing showed that deletions removed 22 (Pho610Δ22), 55 (Pho610Δ55), or 167 (Pho610Δ167) amino acids from the C-terminus. There was no color reaction in the colony of Pho610Δ167, probably because the large deletion removed the essential region for its activity. Pho610Δ22 and Pho610Δ55 had large, clear halos around their colonies in contrast to the wild-type Pho610, which had a little halo around the stained colony. The halo of Pho610Δ22 was larger in diameter than that of Pho610Δ55. These results indicate that the C-terminal region is responsible for retention of Pho610 protein on the cell surface.

\textbf{Covalent linkage of Pho610 to glucan}

We prepared the cell wall fraction by glass-bead disruption of the transformant cells and the materials which were not covalently linked to glucan were removed by boiling in sodium dodecylsulfate. The presence of Pho610 polypeptide was detected by SDS-PAGE and immunoblotting with anti-'Pho610' antiserum. The signals was only detected after treatment of the cell wall with laminarinase (endo-β-1,3-glucanase) when the full length Pho610 was expressed from the GAPDH promoter. The protein was detected as a smear band by immunoblotting (Fig. 5, lane 5). Amino acid sequence of Pho610 suggested there are 14 possible N-glycosylation sites (Fig. 1). After endoglycosidase H treatment, the immunoreactive band became more sharp and dense at a lower molecular mass region (lane 6), which indicated the polypeptide had been highly N-glycosylated. No signal was detected when Pho610Δ22 was
expressed (lanes 7-9). This indicates the C-terminal hydrophobic region is essential for the covalent linkage of Pho610 protein to the cell wall.

As a conclusion, Pho610 acid phosphatase of *K. marxianus* origin is a novel cell wall constituent that has an enzyme activity. With the aid of genetic manipulation, enzymes could be localized on the cell surface and this technology has been successfully applied to molecular bleeding of the yeast. Our study discovered that cell wall proteins could have enzyme activity by nature.

**Acknowledgments**

We thank Drs. Yasuji Oshima (Kansai University), Satoshi Harashima (Osaka University), and Harushi Nakajima (University of Tokyo) for strains or plasmids, and Koichi Tachibana (SS Pharmaceutical) for antisera. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan and a grant for “Biodesign Research Program” from the Institute of Physical and Chemical Research (RIKEN).

**References**


