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Purification and Characterization of a Soybean Root Nodule Phosphatase Expressed in *Pichia pastoris*

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Abstract

Soybean root nodules possess a developmentally regulated acid phosphatase (ACP) that exhibits the highest specificity for purine 5′-nucleoside monophosphates. The enzyme is a glycosylated dimer of 28- and 31-kDa subunits, which appear to be products of the same gene but differ in posttranslational modifications. In order to perform directed mutagenesis and more extensive biochemical characterization, a means of producing recombinant ACP was needed. Several attempts were made to express ACP in *Escherichia coli,* but all conditions employed resulted in protein that was found entirely in inclusion bodies, and resolubilization experiments were unsuccessful. Therefore, the methyltrophic yeast *Pichia pastoris* was chosen as a eukaryotic expression host. The coding sequence of ACP was cloned into the pPIC9 vector to create a fusion with the yeast α mating factor secretion signal. The ACP:pPIC9 construct was integrated into *P. pastoris* strain GS115. Expression of ACP was under the control of an alcohol oxidase methanol-inducible promoter. Methanol induction resulted in secretion of ACP to a level of 10 mg/L. The recombinant ACP was purified 550-fold to homogeneity by phenyl-Sepharose, hydroxyapatite, and MonoS chromatography. The purified enzyme had K_m values of 0.08 and 0.12 for 5′-AMP and 5′-GMP. These values were similar to those obtained for the native ACP heterodimer purified from soybean (0.08 and 0.15 mM for 5′-AMP and 5′-GMP). The specific activity of the recombinant enzyme for all substrates tested was 1.6- to 1.8-fold higher than the values for the purified soybean heterodimer.

Abbreviations: ACP, acid phosphatase; BMMY, buffered complex methanol medium; BMGY; buffered complex glycerol medium; MD, minimal dextrose; MM, minimal methanol; AOX, alcohol oxidase; HTP, hydroxyapatite.

Soybeans and other tropical legumes export fixed nitrogen as ureides, allantoin and allantoic acid (1,2). Nitrogen fixation takes place in specialized root nodules that harbor symbiotic bacteria which catalyze the reduction of atmospheric nitrogen. The first set of reactions in ureide biosynthesis serve to assimilate fixed nitrogen into the hypoxanthine base of IMP. The 10 reactions of IMP biosynthesis occur in the mitochondria and plastids of bacteria-infected cells (3). This portion of the ureide biosynthetic pathway is quite well characterized and appears to be present in all organisms (4). In the intermediate reactions of ureide biosynthesis, IMP is oxidized to xanthine, probably through the action of IMP dehydrogenase (5), followed by removal of the phosphate group by a 5′-nucleotidase or phosphatase and removal of the ribose moiety by a 5′-nucleosidase (6). The intermediate reactions presumably occur in the infected cell cytoplasm. These reactions have been particularly difficult to study because 5′-nucleotides can serve as substrates for many nonspecific phosphatases present in a crude extract. In the final stage of ureide biosynthesis, xanthine is exported to the uninfected cells where it is converted to allantoin by the sequential action of xanthine dehydrogenase and uricase (73).

We recently reported the purification and characterization of a phosphatase from soybean root nodules (ACP) which exhibits the highest specificity for purine 5′-nucleotides and is developmentally regulated in a manner consistent with a role in the intermediate reactions of ureide biosynthesis (9). The enzyme did not require metal ions for activity, but activity was stimulated by $MgCl_2$ and inhibited by $CaCl_2$. ACP was purified from soybean root nodules as a dimeric glycoprotein that existed as all three dimeric combinations of 28- and 31-kDa subunits. The difference in mass of the subunits was most likely due to differential glycosylation, as both subunits bound to concanavalin A-Sepharose (9).

Database searches with the translated ACP cDNA (Accession No. AJ223074) revealed homology to another plant acid phosphatase, vegetative storage proteins, and bacterial class B acid phosphatases. Multiple sequence alignments and secondary structure modeling revealed several conserved residues and a probable active site for the phosphatases. In order to perform directed mutagenesis and to obtain sufficient material for biochemical characterization, a heterologous expression host was needed. After several failed attempts to produce soluble protein in *Escherichia coli,* the methyltrophic yeast *Pichia pastoris* was chosen as a host. This organism has been used successfully to produce moderate to large amounts of heterologous protein. Vector preparation, transformation, and screening in shaker flasks can be performed in about 3 weeks, and the production is easily scaled-up by fermentation (10,11,12)

MATERIALS AND METHODS

Expression of Nodule ACP in E. coli

Several attempts were made to express nodule ACP in *E. coli.* First, ACP was amplified by PCR, T/A cloned, sequenced, and inserted into the *Nco*I/*Eco*RI site of pET28a (Novagen). The primers used for PCR contained the same complementary sequence as those listed for pPIC9 construction, and the forward primer contained a 5′-overhang to generate a *Bsp*HI site. The nodule ACP insert contained internal *Nco*I sites, so *Bsp*HI was used to generate an *Nco*I-compatible cohesive end. The ACP:pET28a construct was transformed into *E. coli* strain BL21. Cultures were grown to OD_{600} = 0.6 and induced at 28 or 37°C with 0, 0.4, or 1 mM IPTG. Second, the same insert used for pET28a was cloned into the *NcoI*/*Eco*RI site of pET27b to generate a fusion with the PelB periplasmic targeting signal. Finally, a 6-Histidine-tagged ACP was constructed by PCR with a 5′-overhang on the forward primer to generate an *Nde*I site. Inclusion bodies were dissolved in 6 M urea or 6 M guanidinium HCl in Tris-buffered saline (TBS) + 20 mM imidazole and applied to an immobilized nickel column. The column was washed with TBS and eluted with TBS + 50 mM EDTA or TBS + 400 mM imidazole according to the method of Holzinger et al. (13).

Construction of P. pastoris GS115 Secreting ACP

The nodule ACP insert was amplified by PCR, cloned into the TIA cloning vector (Invitrogen), and sequenced. The primers used for PCR were Fwd, 5′-CTCGAGA-AAAGAGAGGCTGAAGCT*ATTCCGGAGGTAT-CATGCCA*-3′; Rev, 5′-*GAGCCCTAGAGACCTCAAC-TAATG*-3′ (italicized sequences denote nucleotides complementary to the ACP cDNA). The forward primer used for PCR contained a 24-nt 5′-overhang to restore the *Xho*I site and the C-terminal eight residues of the α mating factor signal sequence of pPIC9. The reverse primer contained a stop codon directly after the coding sequence of ACP. The insert was excised from the TIA vector, cloned into the *Xho*I/*Eco*RI site of pPIC9, and transformed into *E. coli* TOP10F′ cells (Invitrogen) . Approximately 20 μg of purified ACP:pPIC9 was linearized with *Sal*I. The digest was extracted with phenol and then chloroform, ethanol precipitated, and resuspended in 10 μL TE. Seven and one-half microliters of plasmid was mixed with 80 μL electrocompetent *P. pastoris* GS115 cells prepared as described in the Pichia Expression Kit Manual, Version E (Invitrogen). Plasmid and cells were transferred to a 0.2-cm electroporation cuvette. Electroporation was carried out in a Bio-Rad Gene Pulser with charging voltage of 1500 V, capacitance of 85 μF, and resistance of 200 Ω. Immediately after electroporation, 1 mL of ice-cold 1 M sorbitol was added to the cuvette, and 200-μL aliquots were spread onto minimal dextrose (MD) plates lacking histidine. The plates were incubated for 2 days at 30°C for selection of His^+ transformants. Fifty His^+ transformants were screened for methanol utilization (Mut phenotype) by patching onto plates containing minimal methanol medium (MM) or MD medium. The plates were grown for 2 days at 30°C.

Screening of Transformants for Secretion of Nodule A CP

Five His^+/Mut^+ transformants were screened for the production of secreted nodule ACP. Colonies were grown for approximately 16 h in 50 mL buffered complex glycerol medium (BMGY) to an OD_{600} of 2-4. Aliquots of the cells were pelleted by centrifugation and resuspended in 75 mL of buffered complex methanol medium (BMMY) at a final OD_{600} = 1. Cultures were grown at 30°C in 250-mL baffled flasks with shaking at 300 rpm. Methanol was added to 0.5% (v/v) every 24 h. Aliquots were removed at various times and the medium was assayed for phosphatase activity against *p*-nitrophenyl phosphate (PNPP) or by Western blotting as described previously (9). One colony was found with a particularly high level of activity and protein and was used for all further experiments.

Scale-Up of Expression

One colony of GS115 expressing nodule ACP was transferred to 25 mL of BMGY and grown to OD_{600} = 5. This culture was added to 250 mL of BMGY and grown until the OD_{600} reached approximately 4. The cells were pelleted by centrifugation, resuspended in 1 L BMMY (OD_{600} = 1), and distributed to four 1-L baffled flasks. Flasks were shaken at 300 rpm at 30°C. Methanol was added to 1% (v/v) every 24 h. After 80 h of induction, cells were pelleted by centrifugation at 3000*g* for 5 min at 30°C.

Purification of Nodule ACP from the Medium

Dry ammonium sulfate was added to 500 mL medium to 30% saturation and stirred on ice for 30 min. The sample was then centrifuged at 10,000*g* for 20 min. The supernatant was applied with gravity flow to a 2.5 × 6 cm (30 mL) column of phenyl-Sepharose equilibrated in 1.8 M ammonium sulfate, 0.1 M Tris-HCl, pH 7.0. The column was washed with 5 column volumes of equilibration buffer and then 5 column volumes of 0.9 M ammonium sulfate, 0.1 M Tris-HCl, pH 7.0; and eluted with 5 column volumes of 0.225 M ammonium sulfate, 0.1 M Tris-HCl, pH 7.0. The eluted sample was concentrated over a YM-10 membrane (Amicon) in a stirred pressure cell and dialyzed exhaustively against 10 mM KP_i, pH 7.0. The dialyzed sample was then applied to 2.5 × 5 cm (25 mL) column of hydroxyapatite (BioGel HTP, Bio-Rad) and eluted with a linear gradient from 10 to 700 mM KP_i, pH 7.0, over 20 column volumes with gravity flow. Eight-milliliter fractions were collected and assayed for activity against PNPP. Fractions 17–25 contained the majority of ACP activity and protein. Pooled HTP fractions were concentrated and dialyzed into 30 mM NaAc, pH 5.0. The dialyzed sample was loaded onto a MonoS 5/5 column (Pharmacia; 1 column volume = 1 mL). The column was washed with 10 column volumes of 30 mM NaAC, pH 5.0, and eluted with a linear gradient from 0 to 0.8 M NaCl in 30 mM NaAc, pH 5.0, over 50 column volumes at a flow rate of 1 mL/min. Two-milliliter fractions were collected after injection. ACP eluted in fractions 19 and 20. Sample loading, gradient control, and fraction collection were performed on a BioCAD Workstation (PerSeptive Biosystems)

Activity, Protein Assays, and N-Terminal Sequencing

Activity assays against PNPP and other phosphorylated substrates, protein determinations, and N-terminal sequencing were performed as described previously for ACP purified from soybean root nodules (9).

SDS-PAGE of Crude P. pastoris GS115 Medium

Protein levels of the *P. pastoris* medium were too low to analyze directly by SDS-PAGE and Coomassie staining, so a method to concentrate the medium was employed. Three milliliters of medium was loaded onto an EconoPac 10-DG desalting column (Bio-Rad) equilibrated in water. The sample was eluted with 4 mL water. The desalted medium was then precipitated by the chloroform/methanol/water method (14). One to 2 mL of the desalted medium, 4 vol methanol, 1 vol chloroform, and 3 vol water were added to a conical bottom centrifuge tube and vortexed vigorously for 1 min. The tube was then centrifuged for 2 min at the highest setting in a clinical centrifuge. The upper phase was removed carefully, to avoid disturbing the protein at the interphase, and discarded. Four volumes of methanol was added to the lower phase, mixed, and centrifuged. The pelleted protein was resuspended in 50 μL SDS sample buffer.

RESULTS

Expression in E. coli

Several strategies were employed in an attempt to produce correctly folded soybean root nodule ACP in *E. coli.* All conditions tried resulted in the production of protein which was found solely in inclusion bodies (Fig. 1). Targeting to the periplasm also failed to produce soluble protein, although approximately 50% of the overexpressed protein was cleaved by the signal peptidase (not shown). Resolubilization, using His-tagged ACP immobilized on a nickel column, was also unsuccessful.

Expression in P. pastoris GS115

The root nodule insert encoding mature ACP was cloned into the pPIC9 plasmid to create a fusion with the α mating factor signal sequence (Fig. 2). *P. pastoris* strain GS115 was transformed with the linearized ACP:pPIC9 construct (Fig. 2) to create a His^+/Mut^+ strain which expressed ACP under the control of the AOX1 promoter and secreted the recombinant protein to the medium. Figure 3A shows SDS-PAGE analysis of the crude medium after induction. The *P. pastoris* expressed protein cross-reacted with ACP antibodies and was found to be slightly smaller than the small subunit of the native glycosylated heterodimer purified

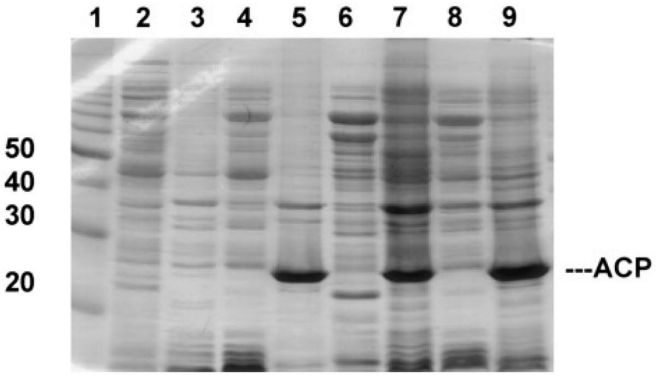

Figure 1. Coomassie-stained SDS-PAGE analysis of expression of nodule ACP in *E. coli* strain BL21 under various induction conditions. Lane 1, 10-kDa ladder; lanes 2, 4, 6, and 8, soluble fraction; lanes 3, 5, 7, and 9, insoluble fraction (inclusion bodies). Lanes 2 and 3, cells were grown for 3 h without IPTG. Lanes 4 and 5, cells were induced with for 3 h at 37°C with 1 mM IPTG. Lanes 5 and 6, cells were induced overnight at 28°C with 0.4 mM IPTG. Lanes 7 and 8, cells were grown overnight at 37°C without induction.

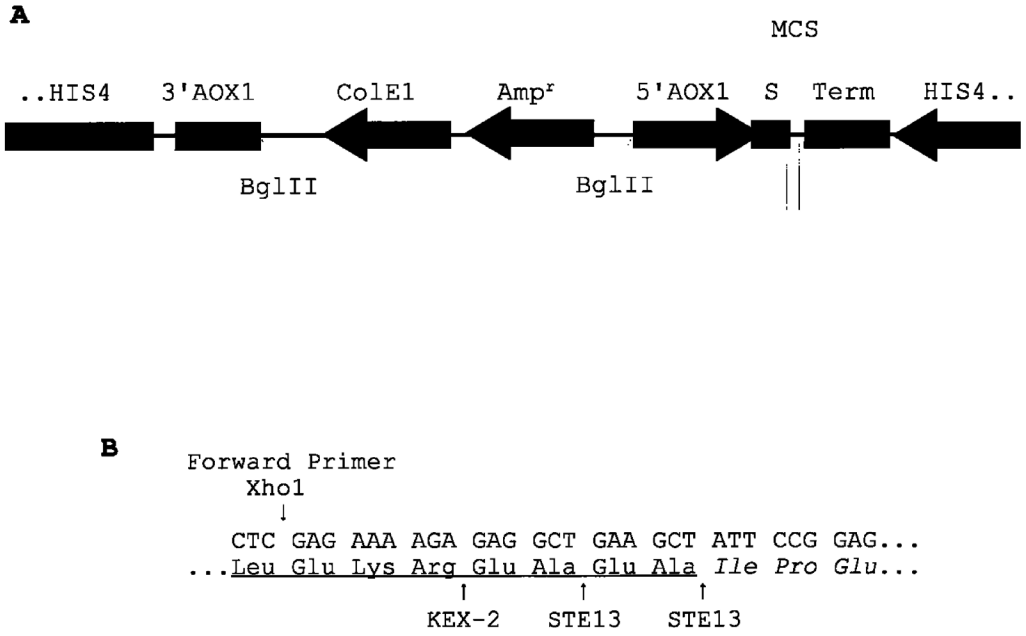

Figure 2. (A) Diagram of the ACP:pPIC9 construct linearized with *Sal*I. The ACP insert was cloned into the *Xho*I/*Eco*RI site of the multiple cloning site (MCS) to create a fusion with the α mating factor signal sequence (S). Expression of ACP was under control of the methanol- inducible alcohol oxidase promoter (AOX1). Ampicillin resistance (Amp^r) was used for selection in *E. coli*. The HIS4 gene product was used for selection in *P. pastoris* GS115. (B) Diagram of signal sequence processing. A 24-nt 5′-overhang was added to the forward PCR primer to restore the *Xho*I site and C-terminal portion of the α mating factor signal sequence. The C-terminal portion of the α mating factor signal is underlined, and the first residues of mature ACP are shown in italics. The signal peptide is cleaved by the KEX-2 protease. The Glu-Ala dipeptides are cleaved by the STE13 dipeptidyl aminopeptidase.

from soybean root nodules (Fig. 3B). The N-terminal sequence of the nodule ACP expressed in *P. pastoris* indicated that the protein was efficiently processed by the KEX-2 signal peptidase, but the cleavage of the Glu-Ala repeats by the STE13 protease was less efficient.

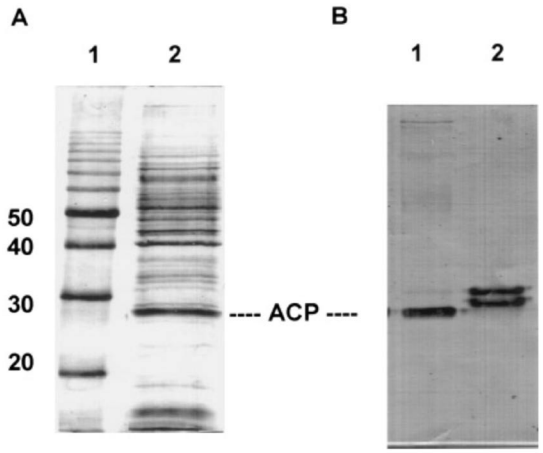

Figure 3. (A) Coomassie-stained SDS-PAGE of total medium protein from a *P.* pastoris GS115 His$^+$/Mut$^+$ transformant expressing nodule ACP. Lane 1, 10-kDa ladder; lane 2, *P. pastoris* medium concentrated by chloroform/methanol/water precipitation. (B)Western blot of total medium protein. Lane 1, medium from *P. pastoris;* lane 2, ACP heterodimer purified from soybean root nodules.

Approximately 60% of the protein was not processed by the STE13 protease. About 30% of the protein was cleaved once by the STE13 protease, and only 10% of the protein had both Glu-Ala sequences removed.

Purification of Nodule ACP

Nodule ACP was purified from the medium more than 550-fold by sequential chromatography on phenyl-Sepharose, HTP, and MonoS columns (Table 1). Phenyl-Sepharose chromatography served primarily to concentrate a large volume of dilute medium. The bulk of the purification came from HTP chromatography. The majority of the nontarget proteins secreted by *P. pastoris* did not bind to HTP or eluted very early in the gradient. MonoS chromatography served to remove two faint bands seen by SDS-PAGE in the HTP fractions and to further

Table 1. Purification of Recombinant Nodule ACP

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (-fold)	Recovery (%)
Crude	2573	296	0.12	1	100
P-Seph	201	135	0.67	6	46
HTP	1.5	80	53.3	444	27
MonoS	0.6	40	66.7	556	14

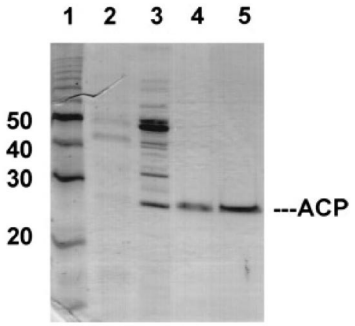

Figure 4. Coomassie-stained SDS-PAGE of ACP purification from *P. pastoris* medium. Lane 1, 10-kDa ladder; lane 2, crude medium (not concentrated); lane 3, eluted protein from phenyl-Sepharose column; lane 4, pooled fractions from HTP column; lane 5, pooled fractions from MonoS column.

concentrate the sample. The final specific activity of ACP purified from the medium of *P. pastoris* was 66.7 μmol min^{-1} mg^{-1} against 5 mM PNPP. This value is slightly higher than the heterodimer purified from soybean root nodules (9). The molecular mass of the purified protein was estimated to be approximately 26,000 by SDS-PAGE (Fig. 4). The predicted mass of ACP containing the Glu-Ala repeats at the N-terminus is 25,353. No shift in mobility was observed after digestion of purified ACP with endoglycosidase F, and the protein did not bind to concanavalin A-Sepharose. These data indicate that ACP was not N-glycosylyated in *P. pastoris.* The nature of the glycosylation of ACP in soybeans in unknown.

Kinetic Characterization of Recombinant A CP

Kinetic parameters were determined for the recombinant enzyme using purine nucleoside monophosphates as substrates (Table 2). The K_ms ranged from 0.08 mM for 5′-AMP and 5′-IMP to 0.18 mM for 5′-XMP. The K_ms for 5′-AMP and 5′-GMP of 0.08 and 0.12 mM compare to 0.08 and 0.15 mM reported for the native heterodimer purified from root nodules (9). V_{max} values ranged from 247 to 461 U/mg with the various substrates (Table 2). The V_{max} values for the recombinant enzyme against all substrates tested were 1.6 to 1.8 times higher than for the heterodimer from nodules. Like the soybean heterodimer the recombinant enzyme was stimulated by $MgCl_2$ and inhibited by $CaCl_2$.

DISCUSSION

Since ACP failed to fold correctly in *E. coli*, it was assumed that glycosylation and/or ER localized molecular chaperones might be required for folding. Therefore, *P. pastoris* was chosen as a eukaryotic host that would allow ER targeting and possible glycosylation. The secretion level of ACP was modest at 10 mg/L in comparison to several proteins which are secreted at levels approaching 1 g/L or greater (10,15,16). However, the level of expression of ACP is adequate for biochemical and mutagenesis studies and could be scaled-up by fermentation. The major difficulties in dealing with the modest level of expression were that the medium was so dilute that it needed to be concentrated for standard SDS-PAGE with Coomassie staining, and the high salt level in BMMY medium limited the types of chromatography which could be used as a first step in a purification scheme. The problem of SDS-PAGE analysis was solved by chloroform/methanol/water precipitation of the desalted medium. This enabled the total protein from 1-2 mL of medium to be loaded in a single well of a mini-gel. Several strategies have been employed for concentration and desalting of media from *P. pastoris* for protein purification. These typically include large-scale filtration to remove particulate matter, followed by concentration over molecular weight cut-off membranes. Buffer exchange must be accomplished by diafiltration or dialysis (1 1,12,17). Other researchers have chosen columns in which binding of their protein of interest is not hindered by the ionic strength of the medium (18,19). The method employed for ACP was to bring the medium to 30% saturation with ammonium sulfate, centrifuge to remove most of the particulate matter, and bind the dilute medium to a small column of phenyl-Sepharose. Five hundred milliliters of medium was loaded onto a 30-mL column. Since proteins bind to phenyl-sepharose in high salt, the medium did not require desalting prior to chromatography. Presumably hydrophobic interaction chromatography could be used as a first purification step for most proteins secreted in *P. pastoris,* and if substantial fold-purification is not required from the column, proteins can be eluted in a low-ionic-strength buffer for the next purification step.

The nodule ACP purified from the medium of *P. pastoris* was very similar to the heterodimer purified from soybean nodules in all properties tested, except that the specific activity of the *P. pastoris*-expressed enzyme was 1.6- to 1.8-fold higher. This may be due to the lack of

Table 2. Kinetic Properties of Recombinant Nodule ACP

Substrate	K_m (mM)	V_{max} (units/mg)[a]	V_{max}/K_m
5'-AMP	0.08	247	3088
5'-GMP	0.12	450	3750
5'-XMP	0.18	461	2561
5'-IMP	0.08	311	3888

Note. Kinetic constants were determined in triplicate using 0.25 μg purified enzyme per assay.

[a] One unit = 1 μmol phosphate released per minute at 25°C in 50 mM Mes-NaOH, pH 6.0, plus 1 mM $MgCl_2$.

carbohydrate moieties added to the enzyme. However, different purification procedures were employed for the soybean enzyme (9) and the recombinant enzyme. Thus, it is possible that the enzyme prepared from soybean was partially inactivated.

An additional advantage of *P. pastoris* as an expression host is that it does not appear to secrete any endogenous phosphatase (in BMMY, which contains 100 mM KP_i) which is active against the broad-range substrate PNPP at the pH and buffer conditions used for ACP assays. A negative control of His^+/Mut^+ GS115 transformed with empty pPIC9 exhibited no phosphatase activity in the medium of induced or uninduced cultures. This makes *P. pastoris* a particularly suitable host for the expression of phosphatases, in contrast to *E. coli*, which produces several specific and nonspecific phosphatases under the conditions normally employed for expression (20,21).

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