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Secretion of Active Recombinant Phytase from Soybean Cell-Suspension Cultures[1]

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Phytase, an enzyme that degrades the phosphorus storage compound phytate, has the potential to enhance phosphorus availability in animal diets when engineered into soybean (*Glycine max*) seeds. The phytase gene from *Aspergillus niger* was inserted into soybean transformation plasmids under control of constitutive and seed-specific promoters, with and without a plant signal sequence. Suspension cultures were used to confirm phytase expression in soybean cells. Phytase mRNA was observed in cultures containing constitutively expressed constructs. Phytase activity was detected in the culture medium from transformants that received constructs containing the plant signal sequence, confirming expectations that the protein would follow the default secretory pathway. Secretion also facilitated characterization of the biochemical properties of recombinant phytase. Soybean-synthesized phytase had a lower molecular mass than did the fungal enzyme. However, deglycosylation of the recombinant and fungal phytase yielded polypeptides of identical molecular mass (49 kD). Temperature and pH optima of the recombinant phytase were indistinguishable from the commercially available fungal phytase. Thermal inactivation studies of the recombinant phytase suggested that the additional protein stability would be required to withstand the elevated temperatures involved in soybean processing.

Phytate (*myo*-inositol hexakisphosphate) is the major storage form of phosphorus in most mature plant seeds and pollen (Reddy et al., 1989). In cereals and legumes, phytate accumulates in seeds during maturation and accounts for 50 to 80% of total phosphorus. Soybean (*Glycine max* L. Merr.) meal is a major component of animal feed and contains adequate phosphorus levels to meet animal growth requirements if phosphorus from phytate could be made available. However, monogastric animals utilize phytate extremely poorly, which necessitates supplementation of animal rations with Pi to meet dietary requirements (Cromwell, 1992; Ravindran et al., 1995). Undigested phytate is excreted in manure, which is generally applied as fertilizer to pastures and croplands. The resulting elevation of soil phosphorus in areas of intensive poultry and swine production contributes to environmental phosphorus pollution (Sharpley et al., 1994). Phytate is also an antinutrient because it chelates important cations (including iron, magnesium, zinc, and calcium) and forms phytate-cation-protein complexes, thus lowering the bioavailability of minerals and amino acids in feed (Prattley and Stanley, 1983; Swick and Ivey, 1992).

Phosphorus availability has been improved by the supplementation of animal feed with phytase (EC 3.1.3.8) from the fungus *Aspergillus niger* (Nelson et al., 1971; Simons et al., 1990; Swick and Ivey, 1992; Cromwell et al., 1995; Denbow et al., 1995). Phytase sequentially dephosphorylates phytate to yield Pi and *myo*-inositol. Fungal phytase is an extracellular glycoprotein that exhibits enzyme activity over a broad temperature range, with an optimum at 58°C. The enzyme has two pH optima, one at approximately 2.5 and the other at 5.0 to 5.5 (Howson and Davis, 1983; Ullah and Gibson, 1987). The fungal phytase is commercially available as Natuphos (BASF, Mt. Olive, NJ), but enzyme supplementation adds to the cost of feed preparation.

The fungal phytase gene (*phy*A) has been cloned from *A. niger* (Mullaney et al., 1991; Piddington et al., 1993; van Hartingsveldt et al., 1993). An alternative approach for producing supplemental phytase is to synthesize the fungal enzyme in seeds of transgenic plants (Pen et al., 1993; Verwoerd et al., 1995). The feasibility of this method was illustrated by the production of transgenic tobacco (*Nicotiana tabacum* L.) seeds expressing fungal phytase, which efficiently substituted for the microbial enzyme in poultry-feeding studies (Pen et al., 1993). These studies also demonstrated the potential of using plant seeds as enzyme production and delivery systems. Unlike tobacco seeds, soybean meal is a major component of animal rations. Soybean meal has a high phytate content, with 61% of the total phosphorus present as phytate (Swick and Ivey, 1992). Making the phosphorus in soybean meal more available by introducing phytase genes may provide a less expensive alternative to phytase supplementation.

Transgene expression and targeting of transgene products to subcellular compartments in soybean have not been well studied because of the low efficiency of transformation and regeneration. Therefore, an examination of

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Abbreviations: CaMV, cauliflower mosaic virus; TEV, tobacco etch virus; TFMS, trifluoromethanesulfonic acid.

phytase gene expression is essential before committing resources to whole-plant studies. We report here the use of a soybean cell-suspension culture coupled with microprojectile bombardment for expression and enzyme localization studies. We predicted that the gene product obtained using a constitutive promoter to express fungal *phyA* fused to a plant signal sequence would follow the default secretory pathway and appear in the culture medium. Extracellular localization of phytase in cell-suspension cultures would provide an ideal system for assaying the biochemical properties of the recombinant enzyme. Phytase was characterized with respect to thermal stability, temperature, and pH optima.

MATERIALS AND METHODS

Growth and Maintenance of Soybean Cell-Suspension Cultures

Soybean (*Glycine max* L. Merr. cv Williams 82) cell-suspension cultures were obtained from Dr. J.M. Widholm (University of Illinois, Urbana). The cells were maintained in the dark at 28°C in Murashige-Skoog medium (Murashige and Skoog, 1962) supplemented with 0.4 mg/L 2,4-D and 30 g/L Suc in an orbital shaker-incubator at 120 rpm. The cells were subcultured every 5 d by diluting 10 mL of the old culture into 50 mL of fresh medium.

Construction of Phytase Expression Vectors

Phy*A* Gene

Four *phyA*-containing plasmids were constructed, two under the control of a constitutive (dual-enhanced CaMV 35S) promoter and two under the control of a seed-specific (soybean β-conglycinin α′-subunit) promoter, as shown in Figure 1. For each promoter, plasmids were constructed with and without a plant signal sequence. DNA amplification of the mature phytase sequence included the creation of flanking *Xba*I sites (underlined below) to facilitate cloning of the 1.3-kb fragment. Two different upstream oligonucleotides were used, the choice of which depended on whether a signal sequence would be inserted. The upstream oligonucleotide for control constructs without a signal sequence, which included an initiation codon (ATG is in bold in the primer sequence below), was 5′-GCGTCTAGA**ATG**CTGGCAGTCCCCGCCTC-3′ (primer 1 in Fig. 1A), and for constructs with a signal sequence the upstream oligonucleotide was 5′-GCGTCTAGACTGGCAGTCCCCGCCTCG-3′ (primer 2 in Fig. 1B). The downstream oligonucleotide (primer 3) was 5′-TGCTCTAGACTAAGCAAAACACTCCG-3′. The *phyA* gene (from Dr. E. Mullaney, U.S. Department of Agriculture, Southern Regional Research Center, New Orleans, LA) contains a 102-bp intron located within the fungal signal sequence. The fungal signal sequence and intron were eliminated by the amplification strategy. Amplification reactions were carried out at 94°C for 1 min, at 57°C for 1 min, and at 75°C for 165 s for 35 cycles with 25 ng of template plasmid DNA. *Pfu* DNA polymerase (Stratagene) was used for amplification.

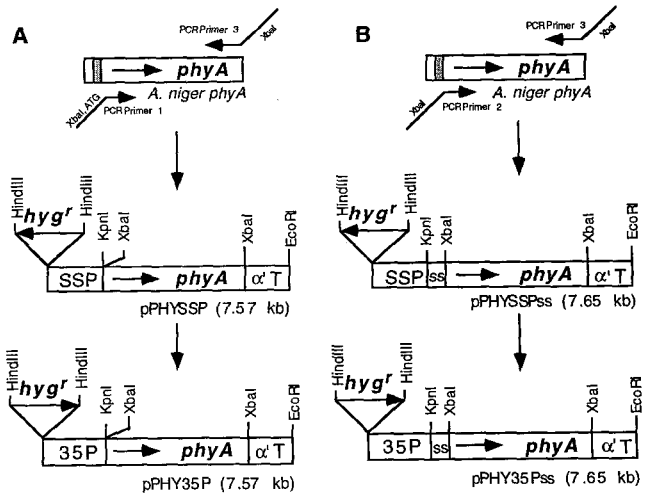

Figure 1. Amplification strategy and restriction maps are shown for *phyA* constructs lacking a signal sequence (A) or with a signal sequence (B). Oligonucleotide primers contained *Xba*I restriction sites to facilitate cloning. Expression of the mature *phyA*-coding sequence was controlled by the soybean β-conglycinin α′-subunit seed-specific promoter (SSP) or by the constitutive dual-enhanced CaMV 35S promoter (35P). Constructs were made with and without the patatin signal sequence (ss). Constructs containing the seed-specific promoter were created by insertion into the multiple cloning site of a soybean expression vector provided by Dr. R. Beachy (see "Materials and Methods"). An *Hin*dIII-*Kpn*I fragment containing the dual-enhanced CaMV 35S promoter was used to replace the seed-specific promoter to generate the constitutive constructs. The hygromycin resistance gene expression cassette was inserted as a 2.2-kb *Hin*dIII fragment (not to scale).

Plant Signal Sequence

The region of the patatin gene encoding the signal peptide (Iturriaga et al., 1989) was amplified from 100 ng of potato (*Solanum tuberosum* L.) genomic DNA using primers to create flanking restriction sites (underlined below), a 5′ *Kpn*I site, and a 3′ *Xba*I site. The upstream oligonucleotide was 5′-GCGGGTACCAATGGCAACTACTAAATCT-3′ and the downstream oligonucleotide was 5′-GGGTCTAGACGTAGCACATGTTGAACT-3′. The cloning of the signal sequence resulted in the addition of two amino acid codons (for Ser and Arg) at the fusion site with the *phyA* sequence. The *phyA* sequence alone or the signal and *phyA* sequences were inserted into the seed-specific expression cassette described below.

Promoter Regions

Two promoters, the constitutive dual-enhanced 35S CaMV promoter (Kay et al., 1987; Fang et al., 1989) with a TEV leader sequence (Carrington and Freed, 1990) and the seed-specific soybean β-conglycinin α′-subunit promoter (Chen et al., 1986), were used to direct *phyA* expression. The dual-enhanced 35S CaMV promoter was obtained from pRTL2, a vector provided by Dr. J. Mullet (Texas A&M University, College Station). A portion of the sequence from *Nco*I to *Sac*I in pRTL2 was deleted by endonuclease digestion and religation to eliminate the ATG

initiation codon at the *Nco*I site. The soybean seed-specific promoter was provided by Dr. R. Beachy (The Scripps Research Institute, La Jolla, CA) as an expression cassette. The soybean seed-specific cassette contains 0.9 kb of the promoter region and 0.45 kb of the terminator region from the soybean β-conglycinin gene flanking a multiple cloning site in the plasmid pUC19. Following insertion of the *phy*A constructs into the seed-specific expression cassette, the two constitutive expression plasmids were created by replacing the seed-specific promoters with a dual-enhanced CaMV 35S:TEV leader sequence as an *Hin*dIII-*Kpn*I fragment (Fig. 1).

Hygromycin Resistance Gene

An expression cassette containing the hygromycin phosphotransferase gene (*hyg*) was generated to utilize hygromycin resistance as a selectable marker. The plasmid pRTL2 (see above), containing the dual-enhanced CaMV 35S promoter and 35S terminator, was modified by endonuclease digestion and religation of the region from *Nco*I to *Kpn*I prior to insertion of *hyg*. The 1.0-kb *Bam*HI fragment used for insertion contains the *hyg*-coding region from vector pHYG^r (obtained from Dr. J. Finer, Ohio State University, Wooster). The plasmid with the hygromycin cassette was used as a vector control for transformations (pHYG2). The hygromycin cassette, including plant promoter and terminator, was excised from pHYG2 and inserted into the phytase constructs as an *Hin*dIII fragment, as shown in Figure 1. All constructs were sequenced to verify that there were no DNA amplification or cloning errors. Plasmids were purified by cesium chloride density-gradient ultracentrifugation prior to gene gun bombardment.

Particle Bombardment and Recovery of Transgenic Cell-Suspension Cultures

Soybean cells, collected 5 d after transfer, were placed on sterile, 3.0-cm Whatman no. 1 filters (equivalent to 300 μL of packed cell volume/filter) and bombarded once with tungsten particles (0.6–0.9 nm in diameter) that had been coated with plasmid DNA (0.4 mg of tungsten coated with 0.6 μg of supercoiled DNA/discharge). Construction of the particle inflow gun and soybean transformation methods were based on reports by Finer et al. (1992) and Finer and McMullen (1991). The filters and cells were transferred to Murashige-Skoog medium containing 30 g/L Suc, 0.4 mg/L 2,4-D, and 2 g/L Phytagel (Sigma) to recover. After 2 d cells were transferred to selection plates (in the same medium supplemented with 50 μg/mL hygromycin B) and maintained under selection for 4 weeks. Surviving cell foci were transferred three times to solid medium containing hygromycin, and cell-suspension cultures were subsequently reinitiated.

DNA and RNA Analyses

Genomic DNA was isolated from soybean cell cultures by a modification of the procedure by Dellaporta et al. (1983). The DNA was treated with RNase Plus (5 Prime→3 Prime, Boulder, CO) and extracted with phenol:chloroform before ethanol precipitation. DNA samples (10 μg each) were digested with *Eco*RI and separated by 0.8% agarose gel electrophoresis. DNA was transferred to nylon membranes (Schleicher & Schuell) by capillary blotting in 10× SSC. Total RNA was extracted from soybean cell-suspension cultures by the method of Silflow et al. (1979), modified by the addition of 1 mM aurintricarboxylic acid as an inhibitor of RNases in the extraction and resuspension buffers. Cells were extracted by phenol:chloroform to remove proteins and the RNA was separated from DNA by LiCl precipitation. RNA samples (15 μg) were separated by electrophoresis in a 1.2% agarose-formaldehyde gel. The RNA was transferred to a nylon membrane by capillary blotting in 20× SSC.

Hybridization probes were synthesized from the gel-purified *phy*A (1.3-kb *Xba*I fragment) and *hyg* (1.0-kb *Bam*HI fragment) sequences using a random-priming kit (Boehringer Mannheim) and [α-^{32}P]dATP. Hybridizations and washes were performed according to the membrane manufacturer's specifications (Schleicher & Schuell). Blots were exposed on Kodak X-Omat AR film at −80°C with intensifying screens.

Phytase Activity Assay

Phytase assays were performed as previously described (Ullah and Gibson, 1987). Phytase activity was expressed as picokatals (pmol Pi released s^{-1} μg^{-1} protein). Incubations were performed for 15 min at 63°C and pH 5.0 unless otherwise specified. Released Pi was detected by the method of Heinonen and Lahti (1981). Culture media samples were prepared by centrifugation of the medium at 4°C for 15 min at 8000*g* to remove cells. Soybean cells were collected 5 d after transfer and 100 mg (fresh weight) was homogenized in an ice-cold mortar with 2.0 mL of protein-extraction buffer containing 100 mM sodium acetate, pH 5.5, 20 mM $CaCl_2$, 1 mM DTT, and 1 mM PMSF. Homogenates were centrifuged at 8000*g* for 15 min at 4°C, and the supernatants were used for phytase activity assays.

Determination of Temperature and pH Optima

Assays were conducted according to the method of Ullah and Gibson (1987) for direct comparison to previously published results for fungal phytase. Activity was assayed over a temperature range of 20 to 75°C in 50 mM sodium acetate at three pH levels (4.5, 5.0, and 5.5) to determine optimal temperature. Assays to determine optimal pH were performed at 58, 63, and 66°C over a pH range of 2.0 to 7.5. Three different buffers were used in the pH assays: 50 mM Gly-HCl for pH 2.0 to 3.0, 50 mM sodium acetate for pH 3.5 to 5.5, and 50 mM Mes for pH 6.5 to 7.0. As a control in the pH optima studies commercial *A. niger* phytase (Sigma) was prepared in culture medium from untransformed soybean cells collected 5 d after transfer.

Enzyme Stability

The thermal stability of recombinant and fungal phytase was compared following incubation at different temperatures. Activity was also assayed after prolonged incubation

at 63°C. A commercial preparation of fungal phytase was dissolved in culture medium from untransformed cells collected 5 d after transfer. The fungal phytase activity was adjusted to match the activity in culture medium from transformed cells 5 d after transfer (5–10 nKat/mL). The medium was adjusted to pH 4.5 for fungal and recombinant phytase samples. Enzyme samples were preincubated for 10 min at temperatures ranging from 50 to 100°C, or for 0 to 60 min at 63°C, and allowed to cool to room temperature. Phytase activity was then assayed at 63°C as described above.

Immunodetection of Phytase

For immunoblot analysis cell-culture media were concentrated with a 30-kD exclusion limit concentrator (Centricon, Amicon, Beverly, MA). Cell extracts were prepared as described by Gibson and Ullah (1988). Chemical deglycosylation with TFMS was carried out by the method of Edge et al. (1981). Endoglycosidase F/*N*-glycosidase F digestion was performed according to the manufacturer's specifications (Boehringer Mannheim). Electrophoresis was performed in a 12% SDS-polyacrylamide gel. Electrophoretic transfer was carried out according to the manufacturer's specifications (Bio-Rad). Chemiluminescent detection protocols (Clontech Laboratories, Palo Alto, CA) were used for immunoblotting using *A. niger* phytase polyclonal antibody (provided by Dr. J. Ullah, Southern Regional Research Center) as the primary antibody and horseradish peroxidase-conjugated secondary antibody.

RESULTS

Generation of Soybean Cell-Suspension Cultures Transformed with *phyA* Constructs

Plasmids containing the *phyA* gene were introduced into soybean cell-suspension cultures to test for expression of the phytase constructs. The amplification strategy, *phy*A constructs, and partial restriction maps of four *phyA* expression cassettes are shown in Figure 1. After particle bombardment and selection for hygromycin resistance, 2 control cultures and 11 cultures containing *phy*A plasmids were analyzed. The growth rates of transformed cultures were indistinguishable from untransformed cells as measured by packed cell volume (data not shown).

To demonstrate the presence of the phytase and hygromycin resistance genes, DNA-hybridization analyses were performed on *Eco*RI-digested genomic DNA from the 2 control cultures and the 11 transformed cultures. Duplicate blots were used for hybridizations with the *phy*A and *hyg* probes (Fig. 2). The results indicated the presence of a variable number of copies of the constructs in soybean cells. No hybridization was observed to DNA from untransformed cells used as the negative control (Fig. 2, lanes 1). Cells transformed with a control plasmid (pHYG2) containing the hygromycin resistance gene alone showed hybridization to only the *hyg* probe as expected (Fig. 2, lanes 2). The fragments observed for DNA samples from transformed cells resulted from the presence of multiple *Eco*RI sites within the plasmids (located in the TEV leader sequence and the hygromycin resistance gene). From the identical results observed in Figure 2, lanes 3 and 4, we concluded that these two samples must have been derived from the same initial transformation event. The lack of fragments of the predicted sizes in Figure 2, lanes 6, 7, and 13, could have resulted from disruption of the phytase-coding region during integration. For samples 6 and 7, this agrees with the absence of an RNA of the predicted size and lack of phytase activity as described below.

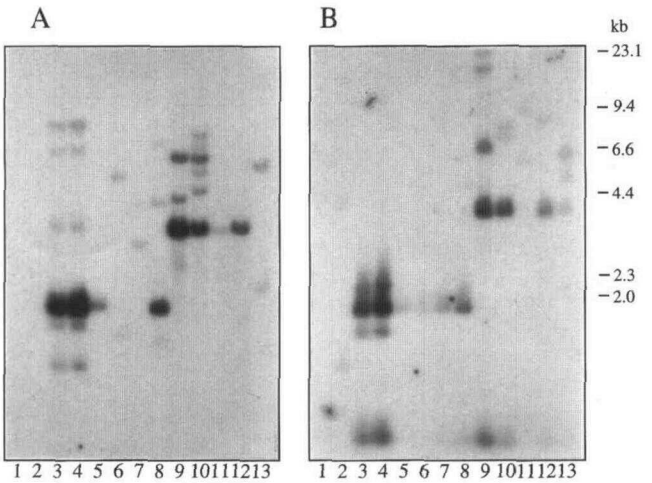

Figure 2. Analysis of genomic DNA from soybean cell-suspension cultures. Genomic DNA (10 μg/lane) was digested with *Eco*RI and transferred to nylon membranes after separation in duplicate 0.8% agarose gels. Genomic DNA was probed with the *phyA*-coding region (A) and the hygromycin resistance gene-coding region (B). Lane 1, Untransformed cv Williams 82 cells; lane 2, cells transformed by a plasmid containing the hygromycin resistance gene alone (pHYG2); lanes 3 and 4, cells transformed with the constitutive construct lacking the signal sequence (pPHY35P); lanes 5 to 8, cells transformed with constitutive construct containing the signal sequence (pPHY35Pss); lanes 9 to 11, cells transformed with the seed-specific construct lacking the signal sequence (pPHYSSP); and lanes 12 and 13, cells transformed with the seed-specific construct containing the signal sequence (pPHYSSPss). Size markers were *Hin*dIII-digested bacteriophage λDNA.

Phytase Gene Expression in Transgenic Cells

To examine expression of the transgenes, RNA was isolated from transformed and control cell-suspension cultures and analyzed for recombinant phytase transcripts by RNA-hybridization analysis (Fig. 3). No *phy*A transcripts were observed in RNA from the untransformed cells or from cells containing the control hygromycin resistance vector alone (Fig. 3, lanes 1 and 2, respectively). Cells from four of six cultures (representing five independent transformants) containing *phy*A constructs under control of the constitutive CaMV 35S promoter with or without a signal sequence (pPHY35Pss and pPHY35P, respectively) produced an abundant transcript of the approximate size expected for *phy*A expression (1.6 kb, Fig. 3, lanes 3–5 and 8). Two additional cultures transformed with the pPHY35Pss constructs showed smaller hybridizing bands (Fig. 3, lanes 6 and 7). These RNAs may represent aberrant transcripts

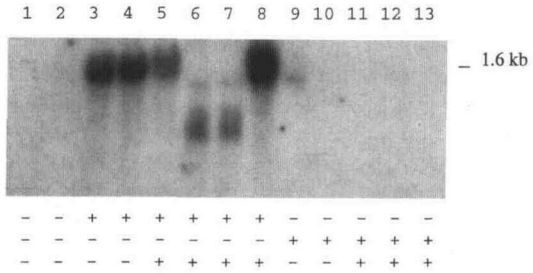

Figure 3. RNA analysis of *phy*A transcripts from transgenic soybean cells. The composition of each *phy*A construct is summarized below the autoradiograph. The dual-enhanced 35S CaMV promoter (35P) or soybean seed-specific promoter (SSP) and presence (+) or absence (−) of the signal sequence are indicated. Lane 1, Untransformed cv Williams 82 cells; lane 2, cells transformed by a control plasmid containing the hygromycin resistance gene alone (pHYG2); lanes 3 and 4, cells transformed with the constitutive construct lacking the signal sequence (pPHY35P); lanes 5 to 8, cells transformed with constitutive construct containing the signal sequence (pPHY35Pss); lanes 9 to 11, cells transformed with the seed-specific construct lacking the signal sequence (pPHYSSP); and lanes 12 and 13, cells transformed with the seed-specific construct containing the signal sequence (pPHYSSPss).

resulting from disruption of the transgene during integration. These two cultures also failed to produce active phytase, as described below.

As expected, four of the five cultures transformed with plasmids under control of the seed-specific promoter showed no expression of *phy*A (Fig. 3, lanes 10–13). However, one sample containing the pPHYSSP seed-specific construct (Fig. 3, lane 9) showed a low level of one of the small RNAs similar to those observed for RNA samples from cells containing the constitutive constructs. This may be the result of errors during integration. No enzyme activity was observed in those cells.

Phytase Activity Expressed by Transgenic Soybean Cell-Suspension Culture

Recombinant phytase was assayed in soybean cell-suspension cultures to demonstrate enzyme activity and site of enzyme accumulation. Phytase activity was assayed in culture media and cell extracts at 63°C and pH 5.0 (Fig. 4). High phytase activity was detected in the media from two cultures, but only background levels were observed in any of the cell extracts. The two active cell cultures contained constructs with the constitutive promoter and plant signal sequence (Fig. 4A). The highest level of phytase activity observed was approximately 920 pKat μg^{-1} total soluble protein. No phytase activity was detected in two additional cell cultures transformed with the constitutive plasmid pPHYG35ss, although the signal sequence was present in the construct. This agrees with the absence of a *phy*A transcript of the appropriate size in these cultures, as shown in Figure 3 (lanes 6 and 7).

Cells transformed with constructs lacking the signal sequence failed to secrete active phytase into the medium (Fig. 4B). We would predict accumulation of the phytase protein in the cytoplasm of these cells. The lack of appreciable intracellular phytase activity suggested that glycosylation of the recombinant phytase is necessary for enzyme activity and/or stability. It is also possible, although we consider it less likely, that the abundant *phyA* transcript lacking the signal sequence is not efficiently translated in soybean cells.

Temperature and pH Optima of Recombinant Phytase

The activity of recombinant phytase was assayed over a temperature range of 20 to 75°C for comparison with the enzyme characteristics reported for fungal phytase (Ullah and Gibson, 1987). The culture medium from cells with the highest level of phytase activity (PHY35Pss-4) was chosen for enzyme activity studies. As shown in Figure 5A, the recombinant phytase had temperature optima at 66, 63, and 58°C, determined at pH 4.5, 5.0, and 5.5, respectively. The culture medium from untransformed cells was assayed at pH 5.0 and did not demonstrate phytase activity. The pH optima of the recombinant phytase were determined at three temperatures (Fig. 5B). At 58°C two pH optima (pH 3.0 and 5.5) were observed, which agrees with previous reports of bimodal pH optima for the fungal enzyme (Ullah and Gibson, 1987). Above 63°C the lower peak disappeared, indicating that the activity at lower pH is temperature-dependent.

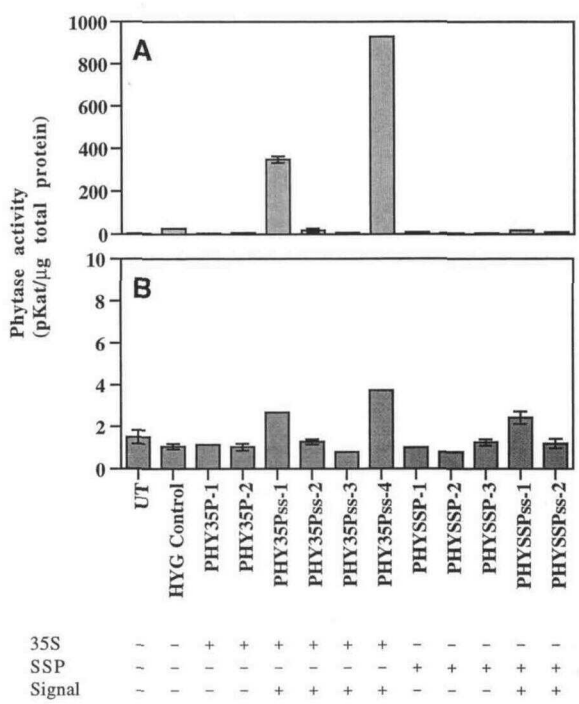

Figure 4. Phytase activity in cell culture media and cell extracts. Phytase assays of cell culture media (A) and cell extracts (B) were conducted at 63°C and pH 5.0. Cultures are presented in the same order as the DNA and RNA analysis results (Figs. 2 and 3). There is a 100-fold difference between the enzyme activity scale for A versus B. Components of the *phy*A expression cassettes are indicated: CaMV 35S promoter (35P), seed-specific promoter (SSP), and presence (+) or absence (−) of a signal sequence. Each point is the mean of three independent assays. SES were calculated for all points and error bars are shown where differences were observed. UT, Untransformed cv Williams 82 cells.

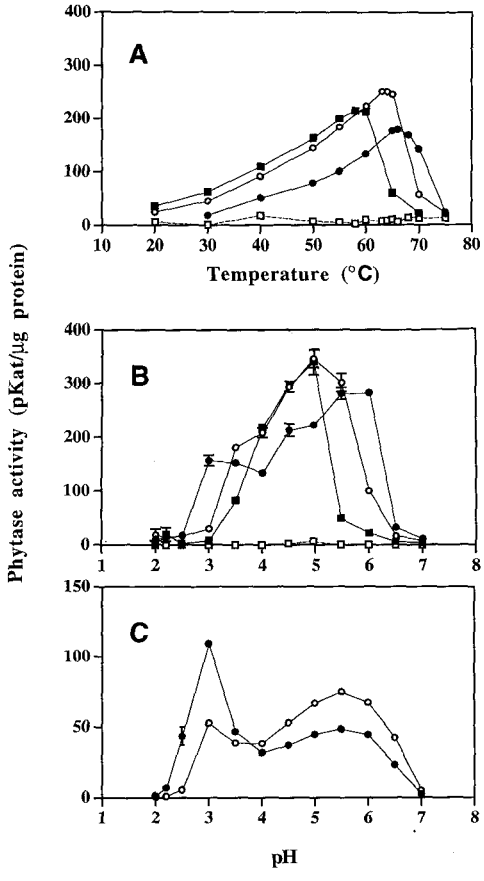

Figure 5. Temperature and pH optima for recombinant phytase. Temperature optima (A) were determined under three different pH conditions: pH 4.5 (●), pH 5.0 (○), and pH 5.5 (■). Phytase activity levels in the medium from untransformed cells assayed at pH 5.0 were used as a control (□). Phytase pH optima (B) were determined at three different temperatures: 58°C (●), 63°C (○), and 66°C (■). Phytase activity in the culture medium of control, untransformed culture was assayed at 63°C (□). Comparison of pH optima (C) for fungal (●) and recombinant (○) phytase was performed at 58°C. Commercial fungal phytase was prepared in medium from untransformed cells at 0.1 mg/mL. Activity for the recombinant enzyme was normalized to equivalent total values to the fungal enzyme for data presentation. All data points for fungal and recombinant enzyme represent means of three assays. SEs were calculated for all samples and error bars are shown where differences were observed.

The highest activity for the recombinant enzyme was observed at 63°C and pH 5.0, which were used for routine enzyme assays. Untransformed cells were included as a control to show that the phytase activity was due to the introduction of the *phy*A constructs. In a direct comparison between the recombinant phytase from the culture medium and a commercial *A. niger* phytase preparation (Sigma) diluted in culture medium from untransformed cells, identical pH optima were observed (Fig. 5C).

Comparison of Thermal Stability between Recombinant and Fungal Phytase

The temperature stability of the plant-synthesized, recombinant phytase was compared with the phytase enzyme from *A. niger* following preincubation at temperatures from 50 to 100°C. Little decrease in activity was detected for preincubations up to 60°C for either recombinant or fungal phytase (Fig. 6A). After preincubation at 63°C for 10 min, 90% of the activity remained for recombinant phytase, whereas only 75% remained for fungal phytase. Above 63°C activity declined rapidly for the fungal and recombinant enzyme. No Pi was detected in assays of the medium from untransformed cells, indicating that thermal breakdown of phytate was not responsible for the presence of Pi detected in the enzyme assays. Thermal stability of the recombinant phytase was also assayed by measuring the loss of activity during prolonged preincubation (up to 60 min), as illustrated in Figure 6B. Preincubation at 63°C for 60 min resulted in a 20% loss of activity for recombinant phytase and a 60% loss of activity for fungal phytase. The small enhancement in thermal stability observed for the recombinant phytase may be attributed to

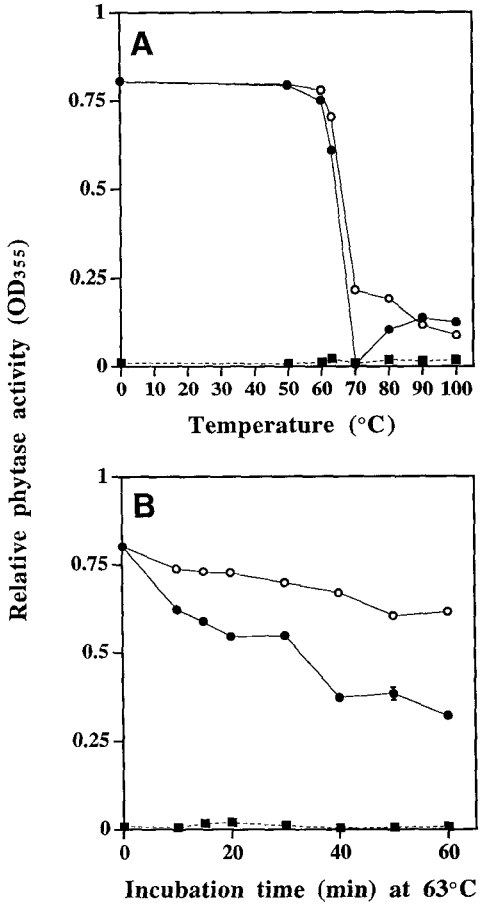

Figure 6. Thermal stability of recombinant and fungal phytase. Phytase activity was assayed for commercial (●) and recombinant (○) enzyme preparations following 10 min of preincubation at temperatures ranging from 50 to 100°C (A) or after preincubation at 63°C from 0 to 60 min (B). All samples were assayed at pH 5.0 and 63°C. For both experiments each point represents the mean of three separate samples. Culture medium from untransformed cells (■) was assayed to demonstrate that detected phosphorus release was due to the presence of phytase enzyme. SEs were calculated for each point and error bars are presented where differences were observed.

possible differences in plant protein processing (e.g. glycan composition) or to the addition of two amino acids at the translational fusion site.

The recombinant phytase is also very stable at 4°C. No loss of activity was observed in the culture medium (cells removed) after 2 months of storage (data not shown). During growth of two phytase-producing cultures, phytase activity increased in the medium up to 7 d following transfer. After 7 d, activity declined rapidly, presumably due to protease activity from senescent cells.

Immunodetection of Phytase

The molecular mass of the mature fungal phytase based on the predicted amino acid sequence is 49 kD. The reported molecular mass of 85 to 100 kD (Ullah and Gibson, 1987) reflects the extensive glycosylation of the enzyme. To determine the mass of the recombinant phytase synthesized in soybean cells and to verify that it was glycosylated, recombinant phytase secreted into culture medium was compared with commercial fungal phytase by immunoblot analysis using polyclonal antiserum to *A. niger* phytase (Fig. 7). Chemiluminescent detection of protein bands showed that the fungal phytase migrated with an apparent molecular mass of approximately 85 kD (Fig. 7, lanes 2 and 8), whereas the recombinant phytase from soybean cells appeared as a doublet at approximately 69 to 71 kD (Fig. 7, lane 7), similar to the size observed for recombinant phytase produced in tobacco (Pen et al., 1993).

To confirm that the proteins were glycosylated both fungal and recombinant phytases were digested with endoglycoside F/*N*-glycosidase F and chemically deglycosylated by treatment with TFMS (Edge et al., 1981). Enzymatic digestion resulted in a shift in size of the fungal phytase to the predicted molecular mass of 49 kD (Fig. 7, lane 3). A small size shift for the recombinant phytase was observed (Fig. 7, lane 6), indicating incomplete removal of glycans. The predicted molecular mass (49 kD) was observed for both the fungal and recombinant enzymes following TFMS treatment (Fig. 7, lanes 4 and 5). A control lane contained culture medium from cells transformed with the hygromycin vector alone (Fig. 7, lane 1). The doublet observed for the recombinant protein disappears upon complete deglycosylation, indicating that it was a consequence of the protein glycosylation patterns. The small size shift observed for the enzymatic digestion of the recombinant protein can be attributed to the presence of unique glycosidic linkages in plant glycoproteins that are not recognized by the glycosidases used (Tretter et al., 1991; Chrispeels and Faye, 1996).

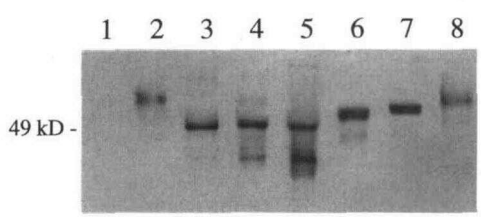

Figure 7. Immunoblot analysis of fungal and recombinant phytase. Protein samples from purified fungal phytase and culture medium containing recombinant phytase (untreated or treated with endoglycosidase F/*N*-glycosidase F or TFMS) were separated by electrophoresis in a 12% SDS-polyacrylamide gel. Lane 1, Medium from control *hyg*-transformed cells (no treatment); lane 2, untreated fungal phytase; lane 3, fungal phytase treated by endoglycosidase F/*N*-glycosidase F digestion; lane 4, TFMS-deglycosylated fungal phytase; lane 5, TFMS-deglycosylated recombinant phytase; lane 6, recombinant phytase treated by endoglycosidase/*N*-glycosidase F digestion; lane 7, untreated recombinant phytase; and lane 8, untreated fungal phytase. Polyclonal antibody raised against purified *A. niger* phytase was used for immunodetection. Purified phytase and polyclonal antibodies were obtained from Dr. J. Ullah. The chemiluminescent detection protocol using horseradish peroxidase-conjugated secondary antibody was used to visualize protein bands.

DISCUSSION

As an initial step in evaluating the feasibility of engineering soybean for improved phosphorus availability, we examined expression of a modified fungal phytase gene in a cell-suspension culture system. Two phytase plasmids under the control of a seed-specific promoter and two plasmids in which *phyA* was controlled by a constitutive dual-enhanced 35S CaMV promoter were introduced by particle bombardment. Although the seed-specific promoter would be unlikely to function in cell culture to produce active phytase, the plasmids were generated in parallel with the constitutive constructs for future introduction into regenerable soybean cultures for recovery of transgenic plants. Cells transformed with the constitutive *phy*A plasmids were capable of producing active recombinant phytase, indicating successful cloning and expression of the *phy*A gene construct.

The fact that phytase produced by *A. niger* is a glycoprotein necessitated a strategy to direct the recombinant phytase to the plant endomembrane system for glycosylation. The addition of a plant signal sequence-coding region to the *phy*A constructs resulted in correct sorting of the recombinant phytase through the default secretory pathway in soybean cells. Cells that contained the *phy*A constructs lacking the signal sequence synthesized mRNA, but no activity was detected in either cell extracts or culture media. High activity of recombinant phytase was found only in the media of transgenic cultures containing a signal sequence. This suggested that glycosylation is required for enzyme stability and/or activity or that the protein is not efficiently translated in the absence of a signal sequence. The apparent molecular mass of the secreted recombinant phytase was higher than predicted by amino acid composition, which provides evidence that the recombinant enzyme is glycosylated. Phytase activity in cell extracts was low, further suggesting that secretion was quite efficient following glycosylation in soybean cells.

Recombinant phytase from the culture medium was used to characterize enzyme activity. For comparison, a commercially available *A. niger* phytase enzyme was assayed in culture medium from the untransformed cells. The pH and temperature optima for the two enzymes were identical. At 58°C both enzymes showed two optima. It had previously

been suggested that the presence of two peaks was due to contamination with a second phytase enzyme (Howson and Davis, 1983). A second phytase gene (*phyB*) has been reported for *A. niger*, with a pH optimum of 2.5 (Ehrlich et al., 1993; Piddington et al., 1993). Since the recombinant phytase from soybean cultures resulted from the introduction of a single *phyA* gene, our results resolved any uncertainty about the presence of two pH optima for the *phyA* gene product. Two differences between the secreted recombinant phytase and the commercial fungal phyase can be noted from Figure 6. The pH profile for the comparison with the fungal phyase is presented on a different scale to reflect the lower specific activity of the crude commercial protein preparation. In addition, the relative distribution of activity between the two optima differs between the recombinant phytase and the commercial fungal enzyme. This may be a result of *phyB* gene product present in the commercial phytase.

Current strategies for phytase supplementation of animal feed rely on retention of phytase activity after consumption by the animal. For use in animal diets, soybeans are toasted to inactivate trypsin inhibitors and other antinutrient proteins and are further processed to produce soybean meal (Cowan, 1973; Mounts et al., 1987). In addition, manufactured feed is subjected to high temperatures during the pelleting process. For soybeans expressing recombinant phytase, this would result in enzyme inactivation unless enhanced stability of the phytase can be achieved. Recombinant phytase showed a relatively small increase in thermostability, but it did not appear sufficient to withstand processing temperatures.

Another strategy for increasing phosphorus availability would be to lower phytate content during seed maturation. The addition of specific targeting sequences should facilitate the localization of phytase to the protein bodies, which is the site of phytate accumulation. From our results confirming secretion of the recombinant phytase into the culture medium, we concluded that the current constructs are not suitable for obtaining co-localization of enzyme and substrate. This result also highlights the utility of tissue culture for testing constructs prior to plant transformation and regeneration studies. Our future goal is to alter the fungal phytase constructs to include subcellular targeting sequences. This approach to lowering phytate content should eliminate the need to maintain phytase activity during processing of soybean meal and soybean-based animal feed.

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