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Molecular cloning, expression and evaluation of phosphohydrolases for phytate-degrading activity

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SUMMARY

Four acid phosphatase (phosphomonoesterase E.C.3.1.3.2) genes were cloned by polymerase chain reaction (PCR). These were pho3, pho5 and pho11 from *Saccharomyces cerevisiae* and the gene for a phosphate-respressible acid phosphatase from *Aspergillus niger*. The individual genes were subcloned into an *A. oryzae* expression vector downstream from a starch-inducible α-amylase promoter and the resulting expression constructs were transformed into a mutant strain of *A. oryzae*, AO7. Southern hybridization analysis confirmed that the acid phosphatase genes had been integrated into the host genome with estimates of integrated copy numbers ranging from 2 to 20 for individual transformants. Northern hybridization analysis of total RNA from individual transformants revealed the presence of a single transcript of the expected size of 1.8 kb. Production of recombinant protein was induced by the addition of 30 g L^{-1} of soluble starch in the fermentation media. Active acid phosphatases, not present in control cultures, were detected in the supernatant fractions of transformant cultures by acid phosphatase activity staining of non-denaturing polyacrylamide gels. The ability of the recombinant acid phosphatases to hydrolyze phytate was assessed by referenced phytase (myo-inositol hexakisphosphate phosphohydrolase E.C. 3.1.3.8) activity assay procedures. A two- to six-fold increase in phytase activity was measured in transformants compared to control, untransformed *A. oryzae*. Sufficient quantities of *A. niger* and pho5 recombinant acid phosphatases were generated from large-scale fermentations to assess the efficacy of these enzymes as phytate-degrading enzymes when included in poultry diets. Data indicated an increase in available phosphorus of 1 g kg^{-1} obtained with yeast acid phosphatase and *A. niger* acid phosphatase representing 40% utilization of unavailable dietary P compared to 48% utilization for commercial phytase.

INTRODUCTION

Phytate phosphorus is unavailable to monogastric animals due to the lack of suitable phosphohydrolyzing enzymes in their gut [4,20]. As a result, the majority of this form of phosphorus is excreted in the manure, causing environmental problems in areas of intensive livestock production [5]. In addition, phytate is an anti-nutritional factor, chelating essential minerals [10].

Phytases catalyze the hydrolysis of phytate to penta-, tetra-, tri-, di-, mono-phosphoinositol, myoinositol and inorganic phosphate [21,32]. A broad range of microorganisms produce phytases including bacteria such as *Bacillus subtilis* [28], yeasts such as *Saccharomyces cerevisiae* [2] and *Aspergillus* species including *A. niger* [27], *A. terreus* [39] and *A. ficuum* [9]. Phytase from *A. ficuum*, the greatest known producer of this extracellular enzyme, has been isolated, purified and well characterized [32,33,35]. Cloning and expression of this enzyme to increase production yields has been achieved [37]. Addition of this phytase to feed substantially improves phosphorus utilization and reduces excretion of phosphate in the manure of pigs and poultry [15,30]. Successful results have also been reported with dietary supplementation of a phytase produced by a mutant strain of *A. niger* var. *awamori* [6]. This phytase has been cloned and expressed in the already-overproducing mutant strain to improve production to cost-effective levels [23]. Supplementation of broiler diets with transgenic seeds containing *A. ficuum* phytase resulted in an improved growth rate, comparable with diets supplemented with fungal phytase or phosphorus [22].

Acid phosphatase enzymes (E.C.3.1.3.2) (APase) are a broad family of phosphohydrolase enzymes of which phytases are members. APase co-isolated with phytase from *A. ficuum* and *A. niger* var. *awamori* can hydrolyze phytic acid at pH 2.5 (pH 2.5 APase) [8,41]. Zyla [40] reported that phytase can split sodium phytate molecules in a selective manner, while APase can probably attack the inositol phosphate degradation intermediates independently and, as a result, accelerate the dephosphorylation process. Alignment of fungal pH 2.5 APase active site sequence with published APase and phytase sequences revealed significant homology over a stretch of seven amino acids, RHGXRYP, (Arg-His-Gly-variable amino acid-Arg-Tyr-Pro), between pH 2.5 APase, pho3 and pho5 from *S. cerevisiae* and *A. ficuum* phytase [23,34]. This region is conserved within a number of APase enzymes, indicating functional significance. Ullah and Dischinger [34] reported that Arg (first amno acid of the sequence of seven) is involved

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in the geometric alignment of the negatively-charged phosphate group in the active centre of the pH 2.5 APase. The high sequence homology of the active site regions of phytase, pH 2.5 APase with phytate-hydrolyzing activity and known APases suggests that other APases exist which possess phytate-degrading activity. The objectives of this research were to identify potential, alternative enzyme sources with phytate-degrading activity, to isolate the genes for these enzymes, to express them in a suitable host and to assess their efficacy relative to commercial phytase preparations in a relevant target species.

MATERIALS AND METHODS

Materials

Genomic DNA from *A. niger* 762 was kindly provided by G.S. May (Baylor College of Medicine, Houston, TX, USA). Restriction and modifying enzymes and Prime-a-Gene kit were purchased from Promega Biotech (Madison, WI, USA) and Boehringer Mannheim Biochemicals (Lewes, Sussex, UK). The TA cloning™ system was purchased from Invitrogen Corporation (San Diego, CA, USA). α-^{32}P dCTP (3000 Ci mmol^{-1}), Hybond-N and Hybond-ECL membranes were obtained from Amersham International PLC (Little Chalfont, Bucks, UK). All molecular biology grade reagents were purchased from Sigma Chemical Company (Poole, Dorset, UK). Alkaline phosphatase-conjugated goat anti-rabbit Ig was obtained from Sigma Immuno Chemicals (Poole, Dorset, UK). Casamino acids, yeast extract, peptone and soluble starch were obtained from Difco (Detroit, MI, USA).

Genomic DNA isolation

S. cerevisiae 2601 was grown to stationary phase in YPD broth (20 g L^{-1} glucose, 20 g L^{-1} peptone, 10 g L^{-1} yeast extract). Rapid, total DNA preparations were carried out according to the method of Hoffmann and Winston [14].

Cloning and construction of expression vectors for various phosphatase enzymes

Using the published nucleotide sequences of the coding regions of the APase structural genes, pho3, pho5 and pho11 from *S. cerevisiae* S288C [1,13], specific 5′ and 3′ oligonucleotide primers were constructed to isolate these genes from *S. cerevisiae* 2601 by polymerase chain reaction (PCR). The 5′ oligonucleotide primers used to amplify APases were so designed as to place them in frame, when subcloned into the pAG vector, immediately downstream from the *A. oryzae* α-amylase 11 gene (AMY11) sequences, thus placing them under control of this highly efficient starch-inducible promoter. The nucleotide sequence of the primers are shown in upper case letters and the added restriction enzyme sites are shown in lower case letters. (Enzymes which cut the added sequences follow in brackets).

PHO3 5′: 5′-AAT ccc ggg ACA ATT CCC CTC GGA GAG TTA GCC-3′ (*Sma*I); PHO3 3′: 5′-TAT gga tcc AAT TTA TTG TTT TAA TAG GGT ATC GTT-3′ (*Bam*HI); PHO5 5′: 5′-AAT ccc ggg ACC ATT CCC TTA GGC AAA CTA GCC-3′ (*Sma*I); PHO5 3′: 5′-TAG gga tcc AAA ACT ATT GTC TCA ATA GAC TGG CGT T-3′ (*Bam*HI); PHO11 5′: 5′-AAT ccc ggg ACC ATA CCC CTC GGA AAG TTA TCT-3′ (*Sma*I); PHO11 3′: 5′-ATg gat ccA ATT TAC TGT TTT AAT AAA GTG TCG TCG TTG TA-3′ (*Bam*HI). Additional 5′ and 3′ oligonucleotide primers were designed for APase gene isolation from *A. niger* 762 based upon the published sequence for this gene [16]. These primers include *A. niger* APase 5′: 5′-ACG GCC GCC gat atc CGT CCG GTG GTC-3′ (*Eco*RV) and *A. niger* APase 3′: 5′-AGC Tga att cAC TGa gat ctC TCA ATG-3′ (*Eco*RI and *Bgl*II respectively).

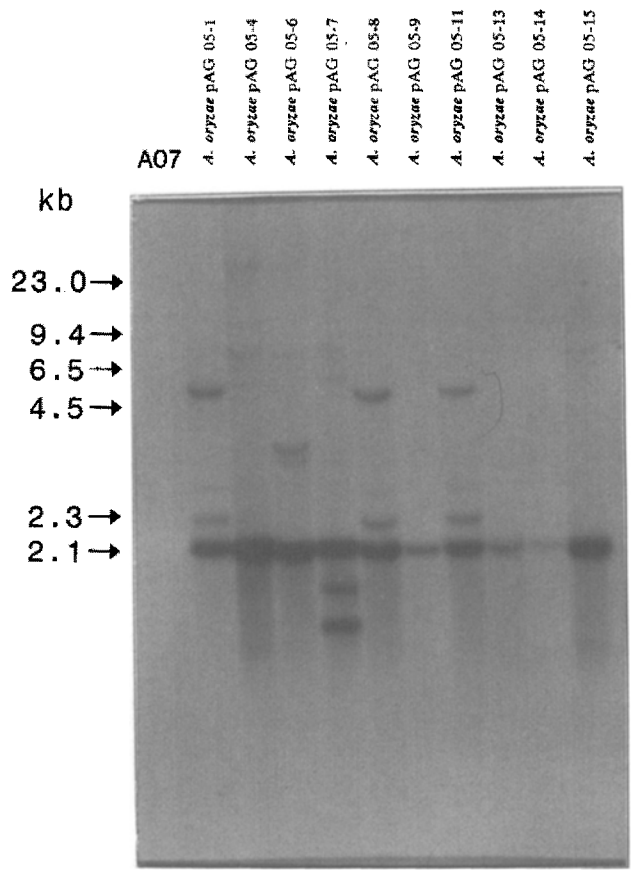

Fig. 1. Southern blot hybridization analysis of transformed *Aspergillus oryzae* strains. Genomic DNA from individual transformants and control AO7 were hybridized with the corresponding radiolabelled APase probe. A radiolabelled fragment is indicated at 2.1 kb, size estimated from standard molecular weight size markers.

For PCR, 1 μg of genomic DNA from *S. cerevisiae* 2601 and *A. niger* 762 were used as template in a total reaction mixture of 100 μl containing 50 mM KCl, 10 mM Tris HCl pH 8.3, 0.01 g L^{-1} gelatin, 1 μg of each primer, 200 μM of each of the four dNTPs and 5 mM $MgCl_2$. Following initial denaturation at 95 °C for 5 min, 2 units of Taq DNA polymerase were added and 30 cycles of PCR were carried out under the following conditions: denaturation at 94 °C for 1 min, primer annealing at 68 °C for 2 min, and primer extension at 72 °C for 3 min. PCR products were directly subcloned into a PCR™ II vector (Invitrogen, San Diego, CA, USA). The expression vector used, pAG [38] contains the promoter and secretory signal sequence of the *A. oryzae* AMYII [31],

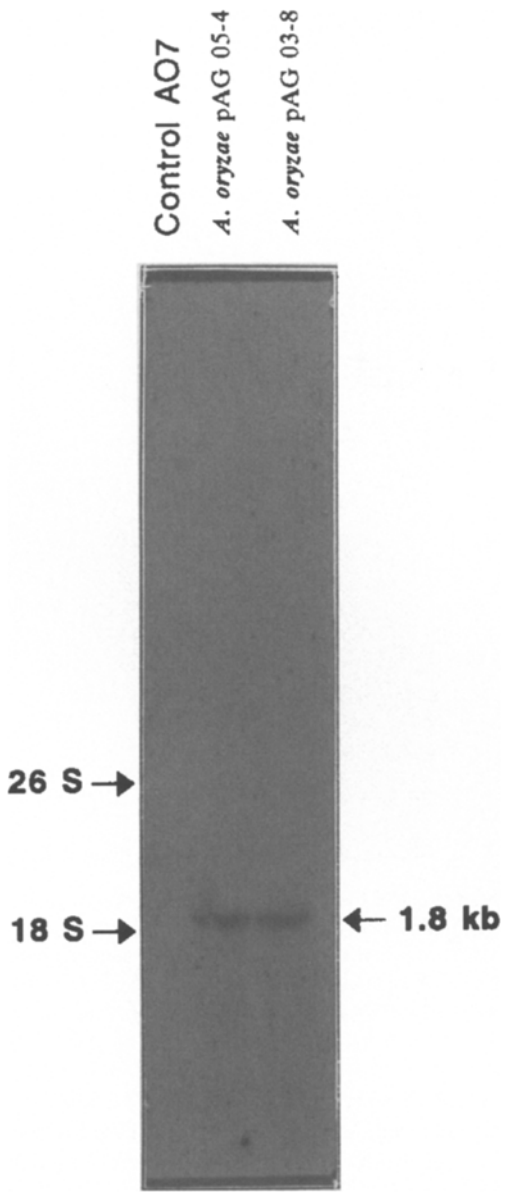

Fig. 2. Northern hybridization analysis of total RNA (10 μg) from control untransformed AO7 and transformants *A. oryzae* pAG05-4 and *A. oryzae* pAG03-8 using radiolabelled APase cDNA, as described in Materials and Methods.

a 160-bp 3′ untranslated region of the glucoamylase gene from *A. niger* to provide a transcription terminator, the *Neurospora crassa pyr* 4 selectable marker which complements a *pyr* G auxotrophic mutant of *A. oryzae* (AO7) and an ampicillin resistance gene. The amylase signal sequence also contains a codon for alanine (from the start of the α-amylase mature protein) located immediately downstream of the signal cleavage site (Ala-Leu-Ala) recognizable by an endogenous α-amylase peptidase. The pho3, pho5 and pho11 genes were excised from the PCR™ II vector using *Sma*I and *Bam*HI and ligated by standard procedures into the pAG vector which was digested with *Nsi*I, blunt-ended with T_4 DNA polymerase and digested with *Bam*HI. The *A. niger* APase gene was excised using *Eco*RV and *Bgl*II and ligated with prepared pAG vector as above. The resulting plasmids pAG03, pAG05, pAG011 and pAGN were transformed into the *pyr* G mutant strain of *A. oryzae*, AO7, as previously described [38] and transformants were selected by growth in the absence of uridine.

Southern hybridization analysis

Southern hybridization analysis was performed to confirm the integration of the respective APase genes into the *A. oryzae* host genome and to determine the number of copies of each gene which had been integrated. DNA was isolated from several tranformants as previously described [25] with one modification: isopropanol was used to precipitate DNA as opposed to ethanol. Genomic DNA (1 μg) isolated from individual transformants was digested with *Eco*RI and *Bam*HI and subjected to Southern hybridization analysis as previously described [38] using probes derived from the PCR products of the respective acid phosphatase genes under investigation. Correlation with different genomic equivalents of the expression plasmid was performed by visual comparison between the integrated radiolabelled fragment and the radiolabelled fragments corresponding to one, two and five copies of the gene.

Northern hybridization analysis

To determine if APase genes were correctly transcribed in *A. oryzae* under the regulatory control elements of the expression plasmid, northern hybridization analysis was performed. RNA was isolated from 100 to 200 mg of lyophilized mycelium by the procedure of Chomezynski and Sacchi [3]. RNA transfer, prehybridization and hybridization with the appropriate ^{32}P-radiolabelled APase probe was performed using previously-described methods [38].

Expression and analysis of recombinant APases

Transformants and control untransformed *A. oryzae* were cultured in limiting phosphate media [7] containing 30 g L^{-1} starch inoculated with 1×10^6 spores ml^{-1}. Limiting phosphate medium was utilized to avoid background inorganic phosphate interference in phytase activity assays which are based upon the measurement of enzymatically-liberated phosphate from sodium phytate. Cultures were incubated in shake flasks at 30 °C for 72 h at 100 r.p.m. Polyacrylamide gel electrophoresis (PAGE) of culture filtrates under non-denaturing conditions was performed on 6% polyacrylamide gels using 15 mM sodium acetate buffer, pH 5, in the gel and 50 mM glycine-HCl buffer, pH 3.1, as the running buffer. Under these conditions, the enzyme migrated towards the cathode. The gels were stained for APase activity using α-naphthyl phosphate as substrate and Fast Garnet GBC salt as developing reagent [17,19]. SDS/polyacrylamide gel electrophoresis was performed in 10% gels and separated proteins were transferred to Hybond-ECL membrane using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hemel Hempstead, Herts, UK). The filter was probed using rabbit anti *A. niger* APase polyclonal antibody which had been purified over protein-A Sepharose, and diluted 1:5000 in 4% dried milk, 50 mM Tris pH 7.5, 150 mM NaCl (TBS). This step was followed by incubation with alkaline phosphatase conjugated goat anti-rabbit

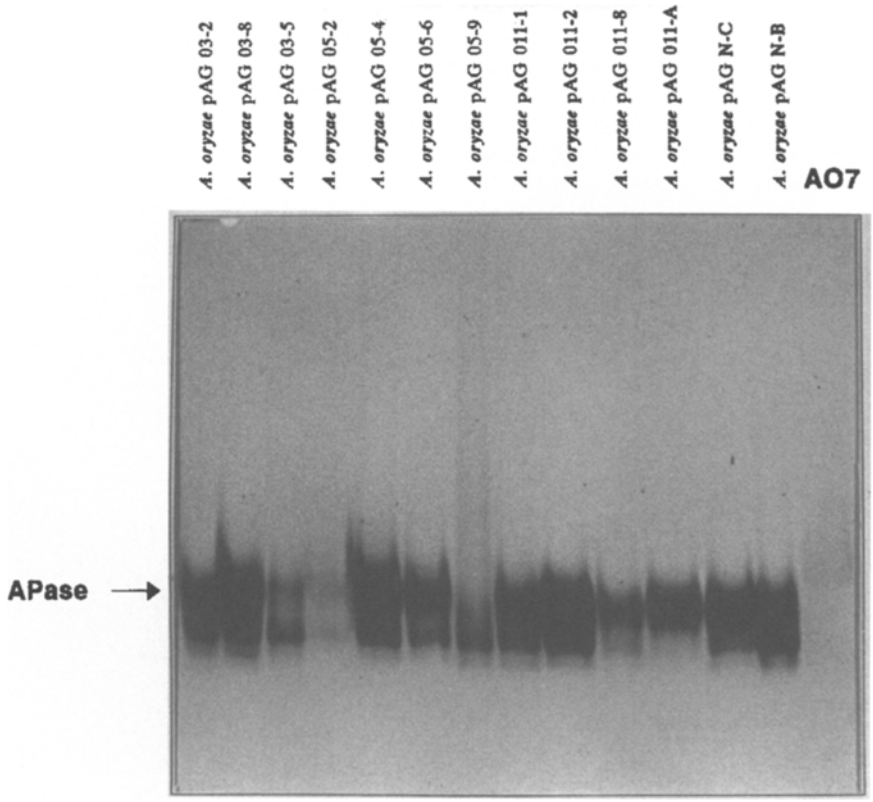

Fig. 3. Acid phosphatase activity stained non-denaturing polyacrylamide electrophoresis gel. Samples of growth medium (containing 10 μg of total protein) from control AO7 and selection of transformants were electrophoresed in 6% polyacrylamide gel under non-denaturing conditions and stained for APase activity, as described in Materials and Methods.

Ig diluted 1:30 000 in TBS. Phytase activity assays were performed on samples of culture filtrate to assess the ability of recombinant APase to utilize phytate as a substrate. Assays were performed using a standard phytase assay procedure [12]. One unit of phytase activity is defined as that amount of enzyme which liberates 1 μmol of orthophosphate from sodium phytate in 1 min at 37 °C and pH 5.5.

RESULTS

The four APase enzymes, pho3, pho5 and pho11 from *S. cerevisiae* and APase from *A. niger* were isolated by PCR. The PCR products and construction junctions were authenticated by nucleotide sequencing (data not shown). Integration of the expression plasmid into the *A. oryzae* genome was detected in all transformants analyzed. Figure 1 represents results obtained for a selection of pho5 transformants (Southern hybridization data for pho3 and pho11, and *A. niger* APase transformants not shown). A radiolabelled band (approximately 2.1 kb) corresponding to the α-amylase pho5 hybrid DNA sequence was detected in all transformants but was absent from control AO7 genomic DNA. Correlation with different genomic equivalents of the expression plasmid indicated that the number of plasmid copies integrated into the genome ranged from 2 (transformant *A. oryzae* pAG05-14) to 20 copies (transformant *A. oryzae* pAG05-4) per cell. The higher mobility radiolabelled bands detected in transformants *A. oryzae* pAG05-7, *A. oryzae* pAG05-9, *A. oryzae* pAG05-13, *A. oryzae* pAG05-14 and *A. oryzae* 05-15 are probably due to intramolecular rearrangements of the expression unit in the integration process. Northern blot analysis (Fig. 2) demonstrated correctly sized mRNA transcripts (1.8 kb) for the transformants analyzed. Varying levels of increased APase activity were observed in all transformants by activity-staining after non-denaturing PAGE (Fig. 3). Filtrates from untransformed *A. oryzae* cultures displayed very low levels of APase activity, demonstrating that functional recombinant APase enzyme is being produced and secreted by *A. oryzae* transformed with the expression unit. The highest producing transformants (*A. oryzae* pAG05-4) displayed a six-fold increase in phytase activity over untransformed *A. oryzae* (Table 1). Phytase activity asays on freeze-dried cells demonstrated that 20–40% of total activity was trapped within the cells. Further evidence to show that the expressed recombinant protein was indeed APase was confirmed by western blot analysis using a specific polyclonal antibody directed against a synthetic peptide fragment of APase protein (Fig. 4). The deglycosylated form of *A. niger* APase is reported to have a molecular weight of approximately 46 kDa. An immunoreactive component of approximately 50 kDa was detected in culture filtrate from *A. oryzae* transformed with *A. niger* APase (Fig. 4). The observed immunologically reactive protein in *S. cerevisiae* pho5 transformant culture filtrates was calculated to be approximately 60 kDa. APase prepared from yeast migrates during SDS poly-

TABLE 1

Phytase activities of different transformants and related copy numbers integrated

Transformant[a]	Gene inserted	Phytase activity[b] (μmol min^{-1} ml^{-1})	Estimated copy number
A. oryzae AO7	–	0.14	–
A. oryzae pAG05-2	pho5	0.12	4
A. oryzae pAG05-6	pho5	0.75	15
A. oryzae pAG05-4	pho5	0.84	20
A. oryzae pAG03-4	pho3	0.08	2
A. oryzae pAG03-5	pho3	0.32	5
A. oryzae pAG03-14	pho3	0.68	15
A. oryzae pAG03-8	pho3	0.67	20
A. oryzae pAG011-8	pho11	0.34	5
A. oryzae pAG011-1	pho11	0.42	10
A. oryzae pAG011-2	pho11	0.622	15
A. oryzae pAG011-C	pho11	0.32	20
A. oryzae pAGN-C	*A. niger* acid phosphatase	0.55	15
A. oryzae pAGN-B	*A. niger* acid phosphatase	0.75	20

[a] Transformant and untransformed control, AO7, cultures were harvested after growth for 72 h at 30 °C.

[b] Phytase activity assays were performed on supernatant fractions corrected to contain equivalent protein concentrations (150 mg L^{-1}) on three different supernatant fractions of each transformant and the mean values obtained.

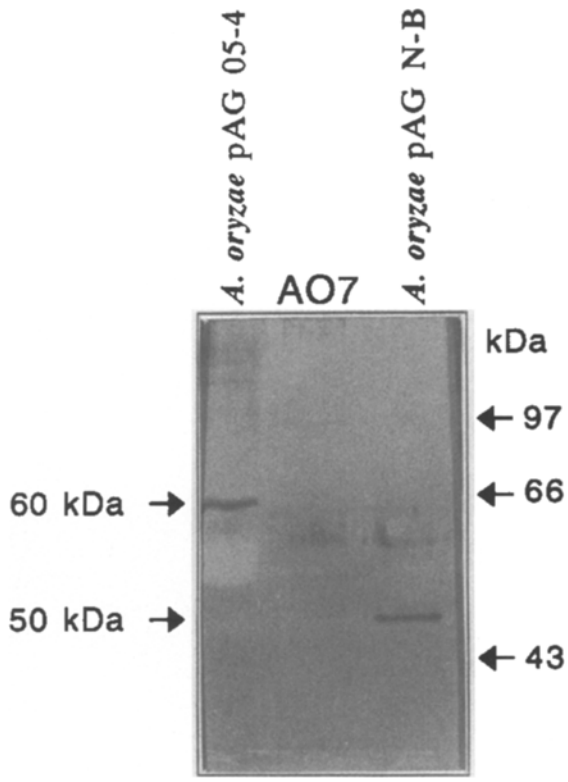

Fig. 4. Western immunoblot analysis of recombinant APases. Lane 1 contains a sample of growth medium (5 μg protein equivalent) from *A. oryzae* AO7 transformed with pAGN (containing *A. niger* APase) and lane 2 contains a sample of growth medium (5 μg protein) from *A. oryzae* AO7 transformed with pAG05 (containing yeast APase). *A. oryzae* AO7 denotes a sample of growth medium from control untransformed *A. oryzae* AO7. The filter was probed with an APase-specific antibody as described in the text. Protein bands were visualized using alkaline phosphatase conjugated secondary antibody.

acrylamide gel electrophoresis as a highly disperse component with molecular mass in excess of approximately 100 kDa [26]. Staining of SDS-polyacrylamide gels with Coomassie Blue proved to be insufficiently sensitive to detect the expressed APase enzymes in the respective culture filtrates (data not shown).

DISCUSSION

The *Aspergillus* expression system utilized takes advantage of the transcriptional and translational start sites of a gene endogenously expressed to high levels, and thus is suitable for high level production of APase. Levels of production vary between transformants isolated due to factors including copy number [24] and site of chromosomal integration [11] which is a random event. In general the estimated number of integrated gene copies correlated linearly with measured phytase activity and observed APase activities. However, transformant *A. oryzae* pAG011C was an exception as, while having high integration copy number, it did not show any appreciable increase in measured phytase activity over control *A. oryzae* AO7. This indicates that the site of integration into the host genome may have been in a tightly regulated region which exerted an inhibitory effect on the induction of transcription of the gene. Although the highest producing transformant showed a six-fold increase in phytase activity, manipulation of the expression vector may be expected to improve yields. Production of *A. ficuum* phytase in *A. niger* under the control of the *A. niger* amyloglucosidase (AG) promoter and AG signal sequence was improved when the AG signal sequence was substituted with the phytase leader sequence [36]. Although the yeast APase signal sequence is important for efficient translocation and core glycosylation of yeast APase, it is not absolutely necessary for entry of the protein into the yeast secretory pathway [29]. Replacement of the α-amylase signal sequence with the enzymes' native signal sequences in the pAG vector may improve production yields of functional recombinant yeast APase.

Native yeast APase displays size heterogeneity and has an apparent molecular weight in the range of 170–360 kDa. It has been suggested that the enzyme is a homogenous protein and that the heterogeneity resides in the carbohydrate portion [2]. The size of the recombinant yeast APase will depend on the degree of glycosylation of the protein in the *A. oryzae* system. Different forms of *A. niger* APase exist which vary in the degree of glycosylation and in the number of subunits in the multimeric forms. Activity staining of recombinant APases identifies one major band with at least two additional bands

of activity. The western analysis, however, identifies single protein bands for both recombinant yeast APase (60 kDa) and *A. niger* APase (50 kDa). Yeast APase is reported to be an octamer under physiological conditions [19] and the existence of multiple activity stained bands may be as a result of multimeric forms with variable degrees of activity. It was reported that APase which dissociated into dimers maintained not more than 10% of the original activity. Mrsa et al. [18] observed that protein glycosylation is necessary for enzyme secretion. We report 20–40% activity trapped within the *Aspergillus* cells which suggests that sufficient glycosylation may not have occurred to promote formation of the correct tertiary or quaternary structure which was suggested to be a primary requirement for efficient transport and secretion.

The degradation of phytic acid proceeds in a stepwise manner, producing five classes of intermediate products that cannot be absorbed [15]. It is only released *o*-phosphate ions from this dephosphorylation reaction that can pass through the gastrointestinal wall and become available for the animal. Zyla [40] reports that the predominant inositol form resulting from phytase activity on phytate was tetraphosphate and in conjunction with APase a synergistic effect was observed giving rise to an even distribution of lower inositol phosphates. The observed increase in phytase activity in transformants may be resulting from an additive effect between the expressed recombinant APase and the host *A. oryzae* phytase enzyme. This suggests that dual supplementation with phytase and APase may impart a synergistic effect in vivo leading to increased phytate–phosphorus utilization.

Feeding trials to compare the efficacy of the recombinant yeast APase, the recombinant *A. niger* APase and a commercial phytase preparation in improving phosphorus availability in corn-soyabean meal-based broiler diets have been conducted and the preliminary results are promising. In these experiments, dietary supplementation of the basal diet with each enzyme at a level of 1200 phytase activity units per kilogram of diet was compared to supplementation of the diet with graded levels of inorganic phosphorus at 0.06%, 0.12% and 0.18% respectively. Results showed that all three enzymes were effective in elevating plasma phosphorus to a level equivalent to that obtained with the highest level of supplemental dietary phosphorus. In addition, supplementation of the basal diet with yeast APase, *A. niger* APase and commercial phytase increased the available P in the diet by 0.10, 0.10 and 0.12% respectively. As the basal diet was calculated to contain 0.25% unavailable P, the corresponding increases in P utilization which can be attributed to the inclusion of the recombinant APases were 40% compared with a 48% increase for the commercial phytase preparation (A. Cantor, Univ. Kentucky, personal communication).

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