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In Vitro Efficacies of Phosphorolytic Enzymes Synthesized in Mycelial Cells of *Aspergillus niger* AbZ4 Grown by a Liquid Surface Fermentation

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Activities of phytase, a pH 6.0 optimum nonspecific phosphomonoesterase and phosphodiesterase assayed toward bis(*p*-nitrophenyl)phosphate (phosphodiesterase I) and against *p*-nitrophenylphosphorylcholine (phosphodiesterase II), were partially purified from mycelial extracts of *Aspergillus niger* AbZ4 cultivated on a molasses medium by a liquid surface fermentation method. After elimination of phosphate from the medium, 7.3- and 3.5-fold enhancements in specific activities of phytase and phosphodiesterase II were observed. Efficacies of mycelial protein fractions in dephosphorylating a wheat-based broiler feed were determined in vitro according to a procedure that simulated digestion in the intestinal tract of poultry. The addition of 0.052 mg of protein from fractions, each of which was high in either pH 6.0 optimum phosphomonoesterase, phosphodiesterase I, phosphodiesterase II, or phytase per gram of a feed sample resulted in the enhancement of phosphorus release by 10, 11, 27, and 88%, respectively. In the presence of an excess of commercial phytase, the addition of the mycelial fraction high in phytase increased the dephosphorylation rate by 56%. The fraction high in phosphodiesterase II enhanced feed dephosphorylation by 8% in the presence of an excess of commercial phytase and commercial acid phosphatase.

KEYWORDS: ***Aspergillus niger*; phosphomonoesterase; phosphodiesterase; feed dephosphorylation; phytates**

INTRODUCTION

The original idea of Nelson and co-workers (*1*) to supplement poultry diets high in phytate phosphorus with microbial phytase has attracted much interest in the recent decade due to successful commercialization of the enzyme. Experimental evidence has accumulated, however, to show that the efficacy of phytase in poultry feeds is limited. This phenomenon is caused by phytase interactions with supplemental inorganic phosphorus and partial rather than complete dephosphorylation of feed phytates (*2*) and is reflected in no more than 60% phosphorus retention in broilers fed phytase-supplemented feeds. Bearing in mind that in plants phytate is occluded with other tissue components and its solubility varies in different parts of the intestinal tract of birds and among feed ingredients (*3*), it is not surprising that phytase is not a perfect biocatalyst. In an attempt to address that problem, an enzymic cocktail strategy has been undertaken (*2*, *4*, *5*). The strategy, based on a simultaneous application of phytate-degrading enzymes (phytase and acid phosphatase), pectinase, and citric acid, resulted in substantial increases in performance, bone mineralization, and phosphorus retention in growing broilers over values observed when phytase was fed as the sole supplemental enzyme. The most common source of phytase is *Aspergillus niger (ficuum)*, which synthesizes three extracellular acid phosphatases: phytase A with two pH optima at 2.5 and 5.0, acid phosphatase with pH optimum of 2.5, and an acid phosphatase with a pH optimum of 6.0 (*6*, *7*). The pH 2.5 optimum acid phosphatase has an active side sequence similar to that of phytase A and has been designated phytase B. Both enzymes belong to the histidine acid phosphatase family, whereas the pH 6.0 optimum acid phosphatase is a metallophosphoesterase.

Recently, there have been a few reports of an *A. niger* AbZ4 mycelium with exceptional dephosphorylating ability when incorporated into feeds fed to domestic poultry (*8*, *9*). Supplementation of low-phosphorus wheat-based diets with the mycelium enhanced performance, bone mineralization, and phosphorus and calcium retention of growing broilers to a much higher extent than a commercial phytase preparation. Furthermore, the efficacy of the mycelium was similar to or even higher than the combined effectiveness of phytase, acid phosphatase, and pectinase. In addition, preliminary results suggest that mycelial cells or cell fragments may modulate the immune system of birds and exert health benefits beyond inherent basic nutrition. The mycelium might partly or fully colonize the gut and exert a competitive exclusion effect against the environmental microflora. The main disadvantage of incorporating the biocatalyst into poultry feeds is a high effective dosage (4%).

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There was a need, therefore, to study intracellular phosphatases to optimize their biosynthesis during fermentation process. Effects of phytase supplementation on the dephosphorylation of different feeds have been studied throughly both in vitro and in vivo, and the role of acid phosphatase activity in the process has also been elucidated (*10*). However, there are very few literature data on intracellular phosphomonoesterases and phosphodiesterases synthesized by *Aspergillus* sp., and nothing is known on the possible participation of phosphodiesterases in feed dephosphorylation. The objective of this work was to identify both phosphomono- and phosphodiesterase activities present in the mycelium and to find the activities that contribute mostly to the dephosphorylating efficacy of the biocatalyst. This knowledge is necessary for optimizing conditions of the mycelium biosynthesis, so that the effective dosage of the mycelium in poultry diets could be reduced considerably. The in vitro effectiveness of phosphomono- and phosphodiesterase activities of the mycelium in feed dephosphorylation was assessed using a simple technique that simulates digestion in the intestinal tract of broilers.

MATERIALS AND METHODS

Chemicals. Dodecasodium phytate, *p*-nitrophenylphosphate, *p*-nitrophenylphosphorylcholine, bis(*p*-nitrophenyl) phosphate sodium salt, Polybuffer Exchanger 94, and Polybuffer 74 were purchased from Sigma Chemical Co. Sephadex G-100 was purchased from Pharmacia (Uppsala, Sweden). All other chemicals used were of analytical grade. Feed ingredients were obtained from commercial suppliers. Sugar beet molasses was obtained from Backer's Yeast Factory (Kraków-Bieżanów, Poland), whereas commercial unhopped wort was from the Krakòw Brewery (Poland).

Enzymes. Microbial phytase (EC 3.1.3.8) used in this study, a commercial preparation (Finase P) with a phytase activity of 5900 units g^{-1} (declared by the producer), and acid phosphatase (EC 3.1.3.2; Finase AP) with a declared activity of 225000 units g^{-1} were donated by Röhm Enzyme Finland Oy (Finland). Both preparations were characterized by the producer as being derived from a genetically modified *Trichoderma reesei* strain and have side β-glucanase, cellulase, and xylanase activities. Pepsin (declared activity of 225000 units mg^{-1}) and pancreatin (activity = 8× U.S. Pharmakopeia) were purchased from Sigma Chemical Co.

Enzyme Assays. The activities of acid phosphatase and phytase were determined as described previously (*9*). One unit of acid phosphatase activity (ACPU) was defined as the amount of enzyme required to liberate 1 μM of *p*-nitrophenol per minute from 5.5 mM disodium *p*-nitrophenylphosphate at 40 °C and pH 6.0. Finase AP was found to have acid phosphatase activity equal to 13150 ACPU g^{-1}. One unit of phytase activity (FTU) was equal to 1 μM min^{-1} of inorganic phosphorus liberated from 5.5 mM sodium phytate at 40 °C and pH 5.0. Under these conditions the phytase activity in the Finase P preparation was 5150 FTU g^{-1}. The activity of phosphodiesterase I was determined using 5.5 mM disodium bis(*p*-nitrophenyl)phosphate (bis-pNPP) in 0.1 M acetate buffer (pH 4.5) as the substrate. To 3.9 mL of the substrate was added 0.1 mL of the enzyme, and the reaction mixture incubated at 40 °C for 15 min. The reaction was stopped by the addition of 0.5 mL of 0.4 M NaOH. The amount of nitrophenol released was determined spectrophotometrically at 405 nm. One unit of phosphodiesterase I activity (NU) was defined as 1 μM of *p*-nitrophenol liberated per minute under the above conditions. The activity of phosphodiesterase II was determined with 5.5 mM *p*-nitrophenylphosphorylcholine (pNPP-choline) in 0.1 M acetate buffer (pH 4.5) as a substrate. Incubation conditions were the same as described for phosphodiesterase I. One unit of phosphodiesterase II (PLCU) was defined as 1 μM of *p*-nitrophenol liberated per minute under the assay conditions.

Fermentation Medium and Culture Conditions. *A. niger* AbZ4 (a strain from the culture collection of the Department of Food Biotechnology, University of Agriculture, Kraków, Poland) was maintained on a nutrient medium containing commercial unhopped wort diluted to the density of 1033.0 g L^{-1} (8 °Blg), agar (20 g L^{-1}), and NaCl (3 g L^{-1}). To prepare a seed culture, conidia from eight agar slants were scrapped into 200 mL of sterilized distilled water. The optical density of the spore suspension was measured at 750 nm and adjusted with water to the value of 1.78 ± 0.02. Each experimental tray containing 1 L of the fermentation medium was inoculated with 50 mL of the inoculum. This was equivalent to $\sim 3 \times 10^9$ spores per fermentation tray. The relationship between optical density of the spore suspension and the number of spores was determined using a spectrophotometer and a cell counter. Fermentation medium was prepared as follows: Molasses was diluted with distilled water to the density of 1055.4 g L^{-1} (15 °Blg), and the pH of the solution was adjusted to 6.5 with 2 M H_2SO_4. The medium was enriched with $K_4Fe(CN)_6 \times 3H_2O$ (1.2 g L^{-1}), $ZnSO_4$ (0.01 g L^{-1}), and $NaH_2PO_4 \times 2H_2O$ (0.1 g L^{-1}), boiled for 1 h, cooled, and filtered. To prepare a phosphate-limiting medium, the sodium phosphate was eliminated from salts that were added at this stage. The pH of the clear solution was adjusted again, 1 L of the medium was poured into each fermentation tray, and the trays were sterilized at 121 °C for 1 h. After 8 days of cultivation in a liquid state fermentation system at 30 °C, fungal mycelium was washed free from the medium, dried by air at 35 °C for 2 h, milled on a laboratory grinder, and stored at 4 °C.

Preparation of Crude Extract. Weighed amounts of mycelium were wetted with 37.5 mM imidazole buffer, pH 6.0, and homogenized with sand in a mortar blender for 30 min. The slurry was filled with the buffer to the concentration of 10% (w/v) and left at room temperature overnight. Then, the extract was centrifuged at 10000*g* for 20 min. The supernatant was used as a source of intracellular enzymes.

Partial Purification of Crude Extract. A 50 mL portion of the supernatant was ultrafiltered against several volumes of Polybuffer 74 (diluted 3.3 times with water and adjusted to pH 4.0 or 5.0) on an Amicon ultrafiltration unit (membrane cutoff = 30 kDa) to a final volume of 10 mL, concentrated to 3 mL, and passed through a 0.45 μm syringe filter. The sample was applied to a chromatofocusing column (0.9 × 55 cm) filled with Polybuffer Exchanger 94 gel and equilibrated with 0.025 M imidazole buffer, pH 7.4, for 24 h. Proteins were eluted from the column with the Polybuffer 74 diluted 1:8 and adjusted to pH 4.0 or 5.0. The flow rate was 18 mL h^{-1}, and 3 mL fractions were collected. The active enzyme fractions containing phosphorolytic enzymes were pooled and concentrated to 5 mL. The Polybuffer 74 was removed from selected pooled fractions by gel filtration on Sephadex G-100. The column (2.6 × 35 cm) was equilibrated with 0.005 M acetate buffer, pH 4.5, and eluted at a flow rate of 18 mL h^{-1}. Fractions (3 mL) were collected, pooled according to enzyme activity, and concentrated to 5 mL. As the Polybuffer 74 absorbed at 280 nm and interfered with the Lowry reagent (*11*), the Bradford protein assay (*12*) was applied to confirm the location of fractions to which Polybuffer 74 was not eluted.

Optical Spectra. Selected pooled and concentrated (to 5 mL volume) fractions were equilibrated with 0.1 M acetate buffer, pH 4.5, and scanned from 400 to 700 nm against a buffer blank using the Genesys 2 spectrophotometer with the measurement density of 1 point/3.0 nm.

Assays. Total phosphorus in feed was determined colorimetrically by using the molybdovanadate method (*13*) in duplicate samples that were digested by a wet-ash procedure. The procedure was validated by including standard reference material 1572 (citrus leaves) from the National Institute of Standards and Technology. Protein was determined either spectrophotometrically at 280 nm, according to the method of Lowry et al. (*11*), or by using the Bradford procedure (*12*) with bovine serum albumin as a standard.

Experimental Diet Composition. The detailed composition of a wheat-based broiler feed used in the experiments is given in **Table 1**. The total phosphorus content of the feed was 4.58 mg g^{-1}.

In Vitro Digestions and Measurements. The in vitro procedure of Żyła et al. (*14*), which was designed for simulating the intestinal tract of the turkey and subsequently modified for broilers (*9*), was used. In this model, pepsin and pancreatin digestion periods are preceded by a preincubation at pH 5.80 to simulate digestion in the crop of broilers. Triplicate samples (1 ± 0.001 g) of a diet, ground through a 1 mm screen, were weighed into 5 mL plastic syringes without Luer-lock

Table 1. Composition and Nutrient Content of the Wheat-Based Diet

ingredient	
wheat (g kg^{-1})	550.55
soybean meal (g kg^{-1})	371.00
vegetable oil (g kg^{-1})	51.00
limestone (g kg^{-1})	12.00
lucerne meal (g kg^{-1})	10.00
salt (g kg^{-1})	3.00
DL-methionine (g kg^{-1})	1.20
vitamins and minerals[a]	1.25
composition	
metabolizable energy (kcal/kg)	2972.00
crude protein (g kg^{-1})	225.20
P nonphytate	1.70
P total (g kg^{-1})	4.10
P total analyzed (g kg^{-1})	4.58
Ca (g kg^{-1})	6.90
Ca analyzed (g kg^{-1})	7.32
lysine (g kg^{-1})	11.90
methionine (g kg^{-1})	4.60

[a] Supplied per kilogram of feed: retinyl acetate, 12000 IU; cholecalciferol, 3000 IU; DL-α- tocopheryl acetate, 20 mg; menadione sodium bisulfite, 3 mg; thiamin mononitrate, 2 mg; riboflavin, 6 mg; pyridoxine, 2 mg; cyanocobalamin, 15 μg; nicotinic acid, 20 mg; calcium pantothenate, 12 mg; folic acid, 1 mg; biotin, 50 μg; choline-HCl, 200 mg; Mn, 65 mg; Zn, 50 mg; Fe, 20 mg; Cu, 6 mg; I, 0.5 mg; Se, 0.1 mg; Co, 0.2 mg; ethoxyquin, 125 mg; virginiamycin, 15 mg; diclasuril, 1 mg.

tips. The feed samples were hydrated with double-distilled water and HCl solution so that a pH value of 5.80 was obtained. When enzyme solution was applied, water was partly (or completely) substituted for by the investigated enzyme fractions. In each experiment, the fractions were added at 0.052 mg of protein/g of feed. The contents of each tube were vortexed, and the tubes were sealed with Parafilm and incubated in a water bath at 40 °C for 30 min. Then 0.5 mL of 1.5 M HCl and 3000 units of pepsin were added to each tube, mixed well, vortexed, sealed with Parafilm, and reincubated for 45 min at the same temperature. (Hydrochloric acid should be handled with caution, with dispensation in a fume hood and proper usage of personal protective equipment.) At the end of this period, 0.455 mL of 1 M $NaHCO_3$ containing 5.6 mg mL^{-1} pancreatin was added dropwise with constant stirring into each tube. The slurry was transferred quantitatively to segments of dialysis tubing by means of the syringe piston. Segments were placed in 250 mL Erlenmeyer flasks containing 100 mL of 0.1 M NaCl in a 0.05 M succinate buffer (pH 6.10) and incubated in a shaking water bath at 41.1 °C (temperature of dialysate was 40 °C). Samples of the dialysate were withdrawn after 240 min for determining inorganic phosphate as described previously (*14*). In the current study, the procedure was used to determine the effects of selected protein fractions purified from *A. niger* AbZ4 mycelium on the dephosphorylation of experimental feed. The effects were measured by determining the contribution of each fraction, either alone or in the presence of excess phytase [1500 FTU (kg of feed)$^{-1}$], or excess phytase and acid phosphatase [3156 ACPU (kg of feed)$^{-1}$], to the amounts of inorganic phosphate released from a feed sample.

Statistical Analysis. Data collected from the in vitro procedure were subjected to one-way analysis of variance using Statgraphics Plus for Windows 4.0 statistical package (*15*). Mean differences were determined using Fisher's least significant difference test. Statistical significance was accepted at $P < 0.05$.

RESULTS

Phosphorolytic Enzymes in *A. niger* AbZ4 Mycelium. Proteins synthesized in the mycelium of *A. niger* AbZ4 grown for 8 days on a molasses medium were partially purified and fractionated by chromatofocusing. Proteins high in different phosphorolytic activities were found in fractions ranging from 62 to 143 (**Figure 1**). The proteins were partially combined according to their prevailing activity, grouped into new fractions numbered 1a, 1b, 2, 3, 4 and 5, and concentrated to 5 mL.

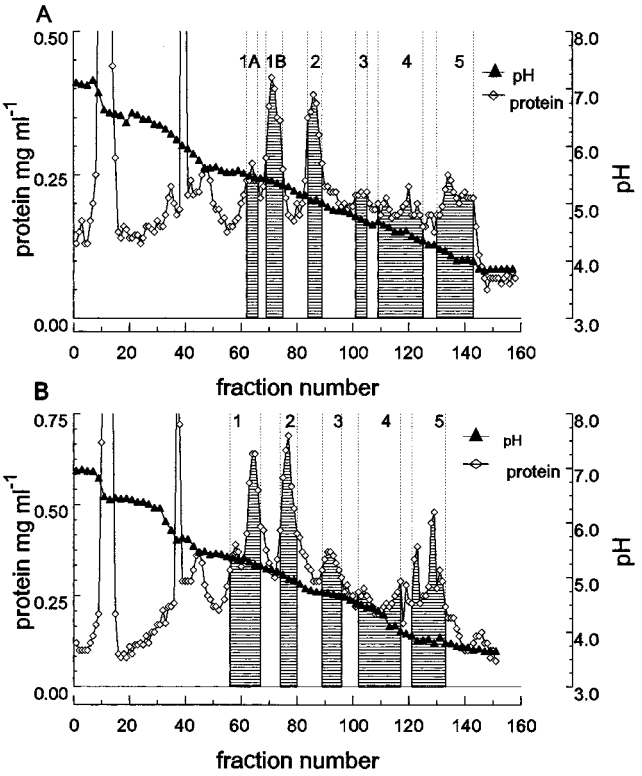

Figure 1. Chromatography of mycelial extract of *A. niger* AbZ4 grown on a standard medium (A) and on a phosphorus-deficient medium (B) by chromatofocusing on Polybuffer Exchanger 94. Conditions: column, Pharmacia K 9/60; bed height, 53.5 cm; sample, 110 mg of protein; pH gradient, 7.0–4.0; flow rate, 18 mL h^{-1}. The shaded areas indicate fractions with high activity of phosphatases: 1 and 5, pH 6.0 optimum phosphomonoesterase; 2, phosphodiesterases active against *p*-nitrophenylphosphorylcholine and bis(*p*-nitrophenyl)phosphate; 3 and 4, phytase.

Table 2. Characteristics of Protein Fractions Resulting from Chromatofocusing of a Mycelial Extract from *A. niger* AbZ4 Grown on a Phosphorus-Adequate Medium

			specific activity[b] (units/mg of protein)			
peak[a]	elution pH	protein (mg)	ACPU (6.0)	NU × 10^3	PCLU × 10^3	FTU
1A	5.5	1.40	2.255	0.314	0.096	0.0305
1B	5.4	1.15	3.773	0.622	0.253	0.0770
2	5.1	1.75	0.405	6.651	2.769	0.0505
3	4.8	1.70	0.086	1.371	0.676	6.1683
4	4.7	1.95	0.031	0.700	0.138	3.9504
5	4.2	3.05	4.213	0.248	1.054	0.2236
Σ		11.00	10.763	9.906	4.986	10.500

[a] For peak designation refer to **Figure 1A**. [b] One unit of acid phosphatase activity (ACPU) was defined as the amount of enzyme required to liberate 1 μM of *p*-nitrophenol per minute from 5.5 mM disodium *p*-nitrophenylphosphate at 40 °C, pH 6.0. One unit of phytase activity (FTU) was equal to 1 μM min^{-1} of inorganic phosphorus liberated from 5.5 mM sodium phytate at 40 °C, pH 5.0. One unit of phosphodiesterase I activity (NU) was defined as equal to 1 μM of *p*-nitrophenol liberated per minute from 5.5 mM disodium bis(*p*-nitrophenyl)phosphate at 40 °C, pH 4.5. One unit of phosphodiesterase II (PLCU) was defined as 1 μM min^{-1} of *p*-nitrophenol liberated from 5.5 mM *p*-nitrophenylphosphorylcholine at 40 °C, pH 4.5.

Specific activities of phosphodiesterase I, phosphodiesterase II, pH 6.0 optimum acid phosphatase, and phytase found in the fractions are summarized in **Table 2**. At the pH values 5.5 to 5.4 two fractions with similar enzyme activity profiles were eluted. The fractions were high in pH 6.0 optimum acid

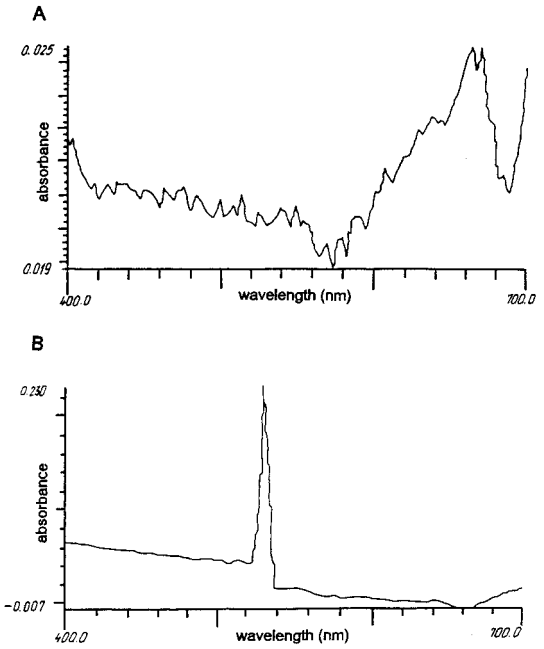

Figure 2. Visible absorption spectrum of the phosphomonoesterase 2 fraction (A) and fraction 3 high in phosphodiesterases (B). The protein concentration was held at 0.23 mg mL^{-1} in fraction 2 and at 0.35 mg mL^{-1} in fraction 3. Fraction numbering refers to **Figure 1A**.

phosphatase and accounted for 56% of this activity recovered during purification. Most of the remaining pH 6.0 optimum acid phosphatase activity was localized in fraction 5. The activity in fractions 1a and 1b was ~10 times higher at pH 6.0 than at pH 4.5 (data not shown). Sixty-seven percent of phosphodiesterase I activity and 46% of phosphodiesterase II activity were found in fraction 2 that was eluted at pH 5.1. A substantial portion (21%) of the phosphodiesterase II activity was eluted at pH 4.2 into fraction 5. Fractions 3 and 4, eluted at pH values of 4.8 and 4.7, respectively, accounted for 96% of total phytase activity recovered during purification. Fractions 1a−5 equilibrated with 0.1 M acetate buffer, pH 4.5, were scanned on a spectrophotometer from 400 to 700 nm against a buffer blank. Only in fractions 1b and 2 were signals of different strengths observed (**Figure 2A**,**B**, respectively). Maximum absorption in fraction 1b was found at 660 nm, whereas fraction 2 absorbed strongly at 530 nm.

Effects of Phosphorus Limitation in the Medium. Fractionation of proteins from mycelial extracts of *A. niger* AbZ4 grown on the medium deprived of inorganic phosphate resulted in a chromatogram similar to that described above (**Figure 1B**). Phosphorolytic activities were found in proteins eluted into fractions ranging from 56 to 137. The fractions were partially combined according to their prevailing activity and concentrated to 5 mL as new fractions numbered 1−5. Specific activities of phosphodiesterase I, phosphodiesterase II, pH 6.0 optimum acid phosphatases, and phytase found in the fractions are given in **Table 3**. The specific activity of phosphomonoesterase assayed at pH 6.0 was at a level similar to that observed when inorganic phosphate was added to the medium. To the contrary, the specific activity of phosphodiesterase II was 3-fold higher, and the specific activity of phytase was increased as much as 7-fold. Furthermore, >90% of the whole phytase activity recovered after purification was eluted at pH 4.8 into the single fraction 3. To achieve better separation of phosphodiesterase I from phosphodiesterase II, the original pH gradient of elution of 7 to 4 was narrowed to 7 to 5. As a result, proteins high in

Table 3. Characteristics of Protein Fractions Resulting from Chromatofocusing of Mycelial Extract from *A. niger* AbZ4 Grown on a Phosphorus-Deficient Medium

			specific activity[b] (units/mg of protein)			
peak[a]	elution pH	protein (mg)	ACPU (6.0)	NU × 10^3	PCLU × 10^3	FTU
1	5.4	3.25	2.298	0.121	0.000	0.0425
2	5.1	3.30	1.872	4.652	14.574	0.3300
3	4.8	2.15	0.228	1.261	2.436	70.9516
4	4.6	3.85	0.034	0.000	0.155	4.9241
5	4.0	2.25	5.365	0.050	0.500	0.7613
Σ		14.80	9.797	6.084	17.665	77.010

[a] For peak designation refer to **Figure 1B**. [b] For definitions of enzyme activity units see **Table 2**.

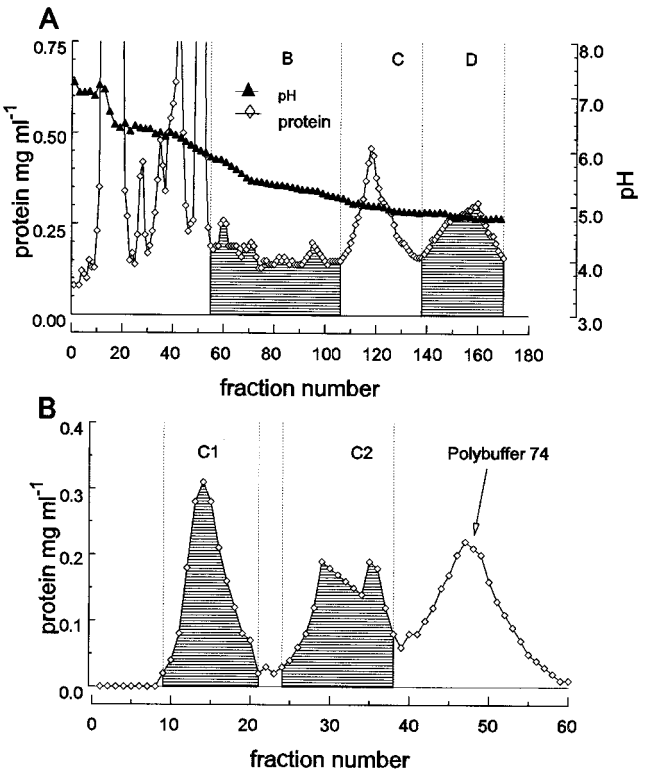

Figure 3. Chromatography of mycelial extract of *A. niger* AbZ4 grown on a phosphorus-deficient medium by chromatofocusing on Polybuffer Exchanger 94 (A) and subsequent gel filtration of fraction C on Sephadex G-100 (B). Chromatofocusing conditions: column, Pharmacia K 9/60; bed height, 53.5 cm; sample, 280 mg of protein; pH gradient, 7.0–5.0; flow rate, 18 mL h^{-1}. Gel filtration conditions: column, K 26/40; bed height, 34 cm; flow rate, 18 mL h^{-1}. Fraction designation: B, high in pH 6.0 optimum phosphomonoesterase; C, phosphodiesterase active against *p*-nitrophenylphosphorylcholine and bis(*p*-nitrophenyl)phosphate; C1, phosphodiesterase active against *p*-nitrophenylphosphorylcholine; C2, phosphodiesterase active against bis(*p*-nitrophenyl)phosphate; D, phytase.

phosphorolytic activities were found in fractions ranging from 55 to 170 (**Figure 3A**). They were combined and grouped into new fractions B, C, and D and concentrated to 5 mL. Subsequent gel filtration of fraction C on Sephadex G-100 yielded two peaks of catalytically active proteins designated C1 and C2 (**Figure 3B**). Specific activities of phosphorolytic enzymes in fractions B, C1, C2, and D are presented in **Table 4**. Fraction B had high specific activity of pH 6.0 optimum phosphomonoesterase, was devoid of phytase activity and displayed negligible activity

Table 4. Characteristics of Protein Fractions Resulting from Chromatofocusing (Narrow pH Gradient) and Gel Filtration of Mycelial Extract from *A. niger* AbZ4 Grown on a Phosphorus-Deficient Medium

			specific activity[b] (units/mg of protein)			
peak[a]	elution pH	protein (mg)	ACPU (6.0)	NU × 10^3	PCLU × 10^3	FTU
B	5.8	0.204	732.05	0.904	3.408	0.00
C1	5.1	0.696	57.86	18.39	325.35	0.01806
C2	5.1	1.008	43.20	964.30	40.33	0.03194
D	4.8	2.05	0.414	1.007	1.857	74.4297

[a] For designation of peaks refer to **Figure 3A**,**B**. [b] For definitions of enzyme activity units see **Table 2**.

of phosphodiesterases. The activity of phosphodiesterase I was found mainly in fraction C2, whereas more than 88% of phosphodiesterase II was located in the fraction C1. Fraction D was abundant in phytase activity and had negligible activities of other phosphorolytic enzymes.

Effects of phosphomono- and phosphodiesterases − high proteins on the dephosphorylation of a broiler feed. Equal amounts of protein (0.052 mg) from fractions B, C1, C2, and D were added to 1 g feed samples and digested by the in vitro procedure that simulated conditions of the intestinal tract of broilers. The same amounts of proteins were added to feed samples in the presence of an excess of the commercial phytase [1500 FTU (kg of feed)$^{-1}$] or in the presence of the excess of phytase and acid phosphatase [3156 ACPU (kg of feed)$^{-1}$]. Amounts of phosphorus liberated under such conditions from a wheat-based feed are given in the **Table 5**. The process of feed dephosphorylation was significantly enhanced by fractions high in either phytase or phosphodiesterase II. As compared to the control, as much as 88% increase in the amounts of freed phosphorus was observed when the phytase-high fraction was added to the diet. The fraction that was high in phosphodiesterase II improved feed dephosphorylation by almost 27%. Significant increases in feed dephosphorylation were caused by each of the fractions studied in the presence of an excess of commercial phytase. As compared to the dephosphorylation yield produced by commercial phytase alone, fractions B, C1, C2, and D added on top of commercial phytase enhanced dephosphorylation by 43, 49, 48, and 56%, respectively. In the presence of an excess of both phytase and acid phosphatase, however, the addition of fraction D into feed sample was ineffective. Under such conditions, only fraction C1 produced a significant increase in feed dephosphorylation, which amounted to 8% over the control and was equivalent to 89% of total phosphorus content in feed. Similar amounts of phosphorus were liberated from feed supplemented with optimal dosage (4%) of fungal mycelium.

DISCUSSION

Phosphomono- and phosphodiesterase activities accumulated in the mycelium of *A. niger* AbZ4 grown on a molasses medium in a liquid surface fermentation system were separated by chromatofocusing into several protein fractions. Two fractions (isoenzymes probably), which were eluted at pH 5.5 and 5.4, high in pH 6.0 optimum acid phosphatase, had similar activity profiles against all tested substrates. The main fraction that absorbed visible light at 660 nm was most probably a metallophosphoesterase similar to the well-characterized extracellular enzyme secreted into cultures of *A. niger (ficuum)* (*16*, *17*). After multiple copies of the *aphA* gene that encodes a pH 6.0 optimum acid phosphatase in *A. niger (ficuum)* (*18*) had been cloned into the promoter region of *Aspergillus oryzae* phytase, the host phytase activity increased by >5-fold (*19*). This phenomenon was caused most probably by the synergistic action of phytase and pH 6.0 optimum acid phosphatase on the phytic acid molecule. In the study presented here the protein fraction high in the pH 6.0 optimum acid phosphatase improved feed dephosphorylation when an excess of commercial phytase was present. This suggests that the phosphomonoesterase active around pH 6.0 may hydrolyze partially dephosphorylated substrate provided by the action of phytase but cannot hydrolyze phytic acid molecules present in the feed matrix.

Scarce information is available about *A. niger* phosphodiesterases. Two fractions, a nonspecific phosphodiesterase that hydrolyzed bis(*p*-nitrophenyl)phosphate, designated phosphodiesterase I, and a phosphodiesterase II, which did not attack bis(*p*-nitrophenyl)phosphate but was active against glycerophosphocholine, have been found in commercial extracts of *A. niger* (*20*). An enzyme that hydrolyzed glycerophosphocholine, very active toward *p*-nitrophenylphosphorylcholine but free from an activity against bis(*p*-nitrophenyl)phosphate, has also been found in mouse brain (*21*). In our studies, a protein fraction eluted at pH 5.1 was active against both bis(*p*-nitrophenyl)phosphate and *p*-nitrophenylphosphorylcholine. The strong light absorption at 530 nm by the phosphodiesterase fraction indicates a possible presence of metals in the cocatalytic or active site of an enzyme similar to mold nuclease P1 (*22*) or Zn^{2+} requiring glycerophosphocholine cholinephosphodiesterase (*23*). The activity toward bis(*p*-nitrophenyl)phosphate was separated from that against *p*-nitrophenylphosphorylcholine by narrowing the chromatofocusing gradient and subjecting the phosphodiesterases fraction to gel filtration. The activity against *p*-nitrophenylphosphorylcholine (phosphodiesterase II) proved to be more effective in enhancing feed dephosphorylation in vitro than the activity toward bis(*p*-nitrophenyl)phosphate (phosphodiesterase I). Phosphodiesterase II enhanced dephosphorylation of a wheat−soybean feed when assayed as the sole supplemental activity, in the presence of commercial phytase, as well as in the presence of an excess of commercial phytase and commercial acid phosphatase. To the contrary, the activity toward bis(*p*-nitrophenyl)phosphate required an excess of commercial phytase to affect dephosphorylation yield. A phosphodiesterase partially purified from *A. nidulans*, which hydrolyzed bis(*p*-nitrophenyl)-phosphate, present at high levels in a nitrogen-starved mycelium possessed both phosphomono- and phosphodiesterase catalytic functions (*24*). In the study presented here, the protein fraction high in the activity of phosphodiesterase I does not seem to belong to that category as it contributed only 0.04−0.05% of total phosphomonoesterase activity assayed at pH 2.5 and 6.0, respectively (data not shown). It should be emphasized, however, that the fraction eluted at pH 4.2 had considerable activity against both *p*-nitrophenylphosphate and *p*-nitrophenylphosphorylcholine. *p*-Nitrophenylphosphorylcholine is commonly used as a chromogenic artificial substrate to determine the activity of glycerophosphocholine cholinephosphodiesterase (EC 3.1.4.38) or phospholipase C (EC 3.1.4.3) activity (*25*−*27*). Critical evaluation of that substrate revealed, however, that it can also be hydrolyzed by a phosphomonoesterase activity, at least at pH 7.2 (*28*). More detailed studies with lipid substrates are needed, therefore, for correct characterization of intracellular *A. niger* phosphodiesterase II, which contributed substantially to feed dephosphorylation.

The mycelium proved to be an abundant source of phytase, an activity that was purified >200-fold from crude extract in two simple steps. The protein fraction high in phytase was

Table 5. Dialyzable Phosphorus Released in Vitro from 1 g of the Wheat-Based Diet by 0.052 mg of Protein from Fractions That Differed in Phosphomono- and Phosphodiesterase Activities[a]

		in the presence of the excess of		
peak[b]	fraction main activity	no enzyme	phytase	phytase and acid phosphatase
B	pH 6.0 ACP	$2.015^{ab} \pm 0.032^{3}$	$3.497^{b} \pm 0.110$	$3.801^{a} \pm 0.053$
C1	phosphodiesterase II	$2.365^{c} \pm 0.103$	$3.636^{bc} \pm 0.076$	$4.085^{b} \pm 0.061$
C2	phosphodiesterase I	$2.034^{ab} \pm 0.045$	$3.592^{bc} \pm 0.051$	$3.831^{ab} \pm 0.061$
D	phytase	$3.449^{d} \pm 0.052$	$3.792^{c} \pm 0.052$	$3.813^{a} \pm 0.037$
none (control)		$1.839^{a} \pm 0.049$	$2.432^{a} \pm 0.033$	$3.774^{a} \pm 0.032$
	4% mycelium	4.055 ± 0.036		

[a] The in vitro procedure of Żyła et al. (*14*, *32*) was applied. [b] For proper designation of peaks refer to **Figure 3A**,**B**. [c] Means within columns with different superscript letters differ significantly ($P < 0.05$).

exceptionally active toward phytates of wheat−soybean feed and also enhanced dephosphorylation in the presence of an excess of commercial phytase. This finding suggests that the mycelial phytase was more resistant to proteolytic digestion than the commercial enzyme or that the phytase fraction was not homogeneous and included multiple forms or side activities that stimulated the process of feed dephosphorylation. Fujita and co-workers (*29*) found that *Aspergillus oryzae* secreted a single phytase when grown in submerged culture, but two different forms of the enzyme were accumulated in the koji culture. It is also known that phytase sources vary in terms of sensitivity to digestive proteolytic activity. *A. niger* phytase is more resistant to proteolytic digestion than the enzymes from plant sources such as wheat (*30*), and it appears that phytase A is more resistant to trypsin digestion but less resistant to pepsin than phytase B. In our study, the addition of mycelial phytase to the feed that had already been supplemented with an excess of commercial phytase and commercial acid phosphatase did not influence amounts of phosphorus released from feed. Fractions high in phytase were eluted at pH 4.8, which is quite close to the electrophoretically determined isoelectric point (pH 4.6) of intracellular acid phosphatase from *A. niger* (*31*). It is possible therefore that acid phosphatase has not been completely separated from phytase activity. As compared to the commercial enzyme, which was a genetically engineered pure phytase A, the efficacy of intracellular *A. niger* AbZ4 phytase was impressive when determined by a reliable in vitro technique (*14*, *32*, *33*). The fraction high in phytase released 75%, whereas the commercial phytase released only 53% of total feed phosphorus content. Further 6% improvement in the yield of dephosphorylation resulted from the addition of the fraction high in phosphodiesterase II activity. Konieczny-Janda and Richter (*34*) found that the activity of lysophospholipase was crucial for reducing viscosity of glucose syrups produced by enzymatic conversion of wheat starch. It seems very probable that similar phenomena may take place in the intestine of birds fed diets based on wheat and that phosphodiesterase II improves dephosphorylation by degrading wheat lipids. Lipid degradation makes phytate (the substrate) more accessible to the action of endogenous wheat phytase, microbial phytase A, microbial phytase B, or to a combination of phosphorolytic enzymes. The research presented here provides evidence for the necessity to incorporate phosphodiesterase II into a cocktail of enzymes intended for optimal dephosphorylation of feeds based on wheat. In the past couple of years there has been an intensive search for new sources of phytase with improved functional properties and high efficacy. The findings reported here point to the necessity for enhancing phytase and phosphodiesterase II levels in the *A. niger* AbZ4 mycelium so that a new alternative source of a nongenetically modified, highly effective biocatalyst is available.

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