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Full Length Research Paper

Purification and identification of a phytase from fruity bodies of the winter mushroom, *Flammulina velutipes*

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A phytase, with a molecular mass of 14.8 kDa as determined by gel filtration and sodium dodecyl sulfate-polyacrylamide gel electrophoresis was isolated from fresh fruiting bodies of the mushroom *Flammulina velutipes*. The chromatographic procedure used for isolation of the phytase included ion exchange chromatography on DEAE-cellulose, CM-cellulose, Q-Sepharose, affinity chromatography on Affi-gel blue gel and fast protein liquid chromatography-gel filtration on Superdex 75. The enzyme was adsorbed on DEAE-cellulose, unadsorbed on CM-cellulose and Affi-gel blue gel, and adsorbed on Q-Sepharose. It presented an N-terminal sequence different from those of reported fungal phytases. It exhibited maximal activity at about 45°C and it did not manifest drastic fluctuations in activity over the pH of 3 to 9. The phytase presented activity towards a variety of phosphorylated compounds with the following ranking of activity: ATP > fructose-6-phosphate ≈ glucose-6-phosphate > AMP > ADP> β-glycerophosphate > sodium phytate. It did not present antifungal activity.

Key words: Phytase, *Flammulina velutipes*, Mushroom, Isolation.

INTRODUCTION

Phytic acid, the main form of phosphate and inositol stored in plants, make up 3 to 5% of the dry weight of cereal grains and beans (Reddy et al., 1982). It is complexed with calcium, magnesium, iron, zinc and proteins. Pigs, poultry and humans lack the enzymes to hydrolyze phytates (Lei and Porres, 2003) and pass them into the environment through fecal excretion, resulting in eutrophication in water in the vicinity (Kim et al., 2006). To circumvent the harmful effects of phytate, phytase has been added to animal feed (Choi et al., 2001; Lei and Stahl, 2001).

Phytases are phosphohydrolases that act on myoinositol hexakisphosphate to release myoinositol and phosphorus in plant foods such as oil seeds, cereal grains and legumes. Phytases can enhance phosphate bioavailability in foods of plant origin to humans (Martinez, 1996; Brask-Pedersen et al., 2011). Supplementation of animal feeds with phytase facilitates phytate digestion in phytase-deficient poultry (Pirgozliev et al., 2011) and pigs (Vats et al., 2009) and enables them to acquire phosphorus for skeletal growth. The need for supplementing pig and poultry feeds with inorganic phosphate is thus obviated. Fecal excretion of phosphate is reduced and environmental pollution is avoided.

Phytases of animal (Yang et al., 1991), plant (Greiner, 2002), yeast (Watanabe et al., 2009), fungal (Casey and Walsh, 2003; Zhang et al., 2010), mushroom (Lassen et al., 2001) and bacterial (Huang et al., 2008) origins have been reported. Mushroom phytases from only a few species including *Agrocybe pediades, Ceriporia* sp., *Periophora lycii*, *Trametes pubescens* (Lassen et al., 2001) and *Agaricus bisporus* (Collopy and Royse, 2004)

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Abbreviations: DEAE-cellulose, Diethylaminoethyl cellulose; **CM-cellulose,** carboxymethyl cellulose; **Q-Sepharose,** quaternary amine sepharose; **FPLC,** fast protein liquid chromatography; **SDS-PAGE,** sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **HPLC,** high performance liquid chromatography.

have been purified and characterized in spite of the numerous species available. The intent of the present study was to isolate a phytase from the edible mushroom *Flammulina velutipes* and compare its characteristics with those of mushroom phytases and other phytases reported earlier.

A variety of proteins including lectins (Beuth et al., 1995), ribosome inactivating proteins designated as flammulin, velutin (Wang and Ng, 2000) flammin and velin (Ng and Wang, 2004) have been reported from *F. velutipes*. A polysaccharide has also been isolated (Smiderle et al., 2006). The present report of a phytase would add to the literature on the protein constituents of this economically important mushroom.

MATERIALS AND METHODS

Isolation of phytase

Fresh fruiting bodies of winter mushroom, *F. velutipes* (2 kg) were extracted with 4 L water in a Waring blender. After centrifugation at 12000 rpm for 25 min, the supernatant obtained was ultrafiltered until the volume reached 100 ml. NH_4HCO_3 buffer (1M, pH 9.5) was added until the molarity of NH_4HCO_3 was 10 mM. Ion exchange chromatography on a diethylaminoethyl cellulose (DEAE-cellulose) (Sigma, America) column (2.5 x 30 cm) was performed in 10 mM NH_4HCO_3 buffer (pH 9.5). Following removal of fractions D1 and D2, the column was eluted with 1M NaCl in the 10 mM NH_4HCO_3 buffer (pH 9.5) to remove adsorbed materials. Fraction D2 containing phytase activity was dialyzed and then subjected to ion exchange chromatography on a 2.5 x 20 cm column of carboxymethyl cellulose (CM-cellulose) (Sigma, America) in 10 mM NH_4OAc buffer (pH 4.5). The unadsorbed fraction CM1 containing phytase activity was collected before desorption of inactive adsorbed materials (fraction CM2) with 10 mM NH_4OAc buffer (pH 4.5) containing 1 M NaCl. Fraction CM1 was then loaded on a 2.5 x 20 cm column of Affi-gel blue gel (Bio-Rad, America) in 10 mM Tris-HCl buffer (pH 7.5). Unadsorbed materials with phytase activity (fraction B1) were eluted with the same buffer, while adsorbed proteins without phytase activity (fraction B2) were eluted with 1M NaCl added to the 10 mM Tris-HCl buffer. Fraction B1 was subsequently chromatographed on a 1.0 x 30 cm column of quaternary amine cellulose (Q-Sepharose) (GE Healthcare, America) in 10 mM NH_4HCO_3 (pH 9.5). The unadsorded fraction Q1 presented no phytase activity, while adsorbed proteins were eluted into fractions Q2 and Q3. Fraction Q2 with phytase activity was further purified by gel filtration on a Superdex 75 HR 10/30 column (GE Healthcare, America) in 0.2 M NH_4HCO_3 buffer (pH 8.5). The second peak (SU2) represented purified phytase.

Molecular mass determination by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and fast protein liquid chromatography gel filtration (FPLC-gel filtration)

SDS-PAGE was carried out according to Laemmli and Favre (1973) using a 12% resolving gel and a 5% stacking gel. At the end of electrophoresis, the gel was stained with Commassie brilliant blue (Bio-Rad). FPLC-gel filtration was carried out using a Superdex 75 column (GE Healthcare, America) which had been calibrated with molecular mass standards (GE Healthcare, America) including blue dextran (to determine void volume), phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and lactalbumin (14.4 kDa).

Analysis of N-terminal amino acid sequence

Amino acid sequence analysis was carried out using an HP G1000A Edman degradation unit and an HP 1000 high performance liquid chromatography (HPLC) system.

Assay of phytase activity

Phytase activity was measured by incubating 0.1 ml of enzyme solution with 0.9 ml of 2 mM sodium phytate in 0.1 M Tris-HCl buffer (pH 7.0). The enzyme reaction was assayed at 37°C for 15 min and stopped by addition of 0.75 ml 5% trichloroacetic acid. The released phosphate was measured at 700 nm after adding 1.5 ml of color reagent, which was freshly prepared by mixing four volumes of 2.5% ammonium molybdate solution in 5.5% sulfuric acid with one volume of 2.5% ferrous sulfate solution. One unit of phytase activity was defined as the amount of enzyme needed to release 1 µmol of phosphate per minute under the assay conditions. Protein was determined according to Bradford using a protein assay kit (Bio-Rad Lab, Richmind, CA) with bovine serum albumin as the standard (Choi et al., 2001).

Assay of substrate specificity

In order to determine the substrate specificity of the purified phytase, several phosphorylated substrates instead of sodium phytate, all at 5 mM concentrations, were separately added to the assay buffer. They included AMP, ADP, ATP, fructose-6-phosphate, glucose-6-phosphate and β-glycerophosphate. The buffer used was 50 mM NaOAc (pH 5.0). The release of Pi was determined as mentioned earlier.

Effects of pH and temperature on phytase activity

Phytase activity was tested over the pH range (pH 3 to 9) and temperature range (20 to 100°C). Solutions of phytate in different buffers at different pH value including 50 mM NaOAc (pH 3 to 5), 50 mM Mes (pH 5 to 7) and 50 mM Hepes (pH 7 to 9) were used to determine the optimal pH value. To determine the optimal temperature, the reaction mixture was incubated at 20, 30, 37, 45, 50, 60, 70, 80 and 100°C in 50 mM NaOAc (pH 5) for 30 min, respectively.

Assay for antifungal activity

The assay for antifungal activity was carried out since some mushroom proteins demonstrated this activity (Lam and Ng, 2001) and also because some enzymes such as ribonucleases (Wang and Ng, 2000), deoxyribonucleases (Wang and Ng, 2001), chitinases (Ho and Ng, 2007), glucanases (Ng and Ye, 2003) and peroxidases (Ye and Ng, 2002) may have antifungal activity. The assay for antifungal activity toward *Fusarium oxysporum*, *Rhizoctonia cerealis*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* was carried out in 100 nm × 15 mm Petri dishes containing 10 ml of potato dextrose agar (PDA). After the mycelial growth, sterile blank paper disks (0.625 cm in diameter) were placed at a distance of 0.5 cm away from the rim of the mycelial colony. An aliquot (10 µl) of the phytase was added to a disk. The dishes were incubated at 23°C for 72 h to analyze possible inhibition halos. The mushroom ribosome-inactivating protein hypsin was used as a positive control (Wang and Ng, 2000).

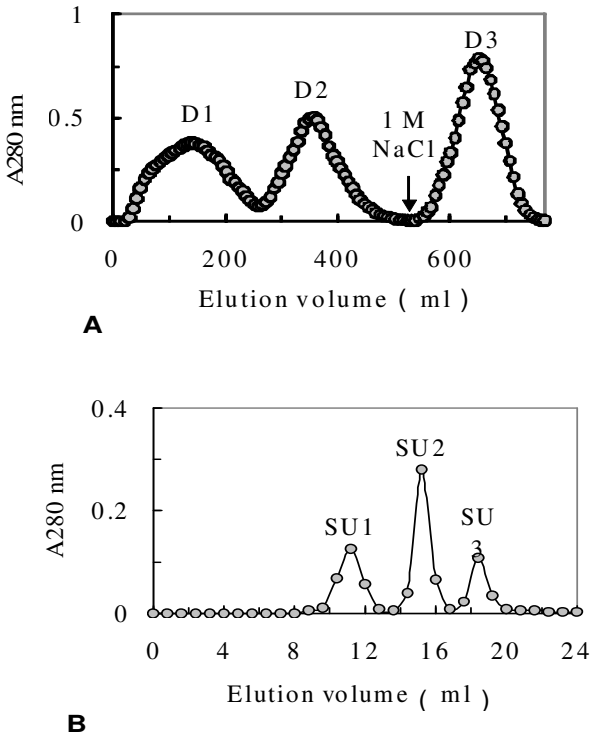

Figure 1. A: Ion exchange chromatography on a DEAE-cellulose column (2.5 x 30 cm). Sample: extract of *F. velutipes* fruiting bodies. Buffer: 10 mM NH_4HCO_3 buffer (pH 9.5). Phytase activity was enriched in fraction D2. B: Gel filtration by fast protein liquid chromatography on a Superdex 75 HR 10/30 column. Sample: fraction of fruiting body extract adsorbed on DEAE-cellulose (D2) and subsequently unadsorbed on CM-cellulose (CM1) and on Affi-gel blue gel (B1) and then adsorbed on Q-Sepharose (Q2). Buffer: 0.2 M NH_4HCO_3 buffer (pH 8.5). Phytase activity was enriched in fraction SU2.

Table 1. Yields and phytase activity of various chromatography fractions derived from 2 kg fresh fruiting bodies (enzyme assay condition: 37°C/15 min, 0.1 M NaOAc buffer, pH 5.0.).

Fraction	Proteins (total mg)	Activity (total units)	Protein (U/mg)	Recovery of activity (%)	Purification factor
Extract	3349.8	368.5	0.11	100	1
DEAE-cellulose (D2)	460.8	271.9	0.29	73.8	5.4
CM- cellulose (CM1)	246.9	197.5	0.8	53.6	7.3
Blue-gel affi-gel (B1)	135.1	135.1	1.0	36.7	9.1
Q-sepharose (Q2)	54.5	87.2	1.6	23.7	14.5
Superdex 75 (SU2)	20.6	70.0	3.4	19.0	30.9

RESULTS

Isolation of phytase

The extract of fruiting bodies was separated on DEAE-cellulose into two peaks, D1 and D2, and a large peak, D3 (Figure 1A). Phytase activity was enriched in peak D2 (Table 1). Peak D2 was fractioned on CM-cellulose into an unadsorbed peak CM1 with phytase activity. Phytase activity again appeared in the unadsorbed peak B1 upon affinity chromatography of peak CM1 on Aff-gel blue gel (Table 1). Upon ion exchange chromatography of peak

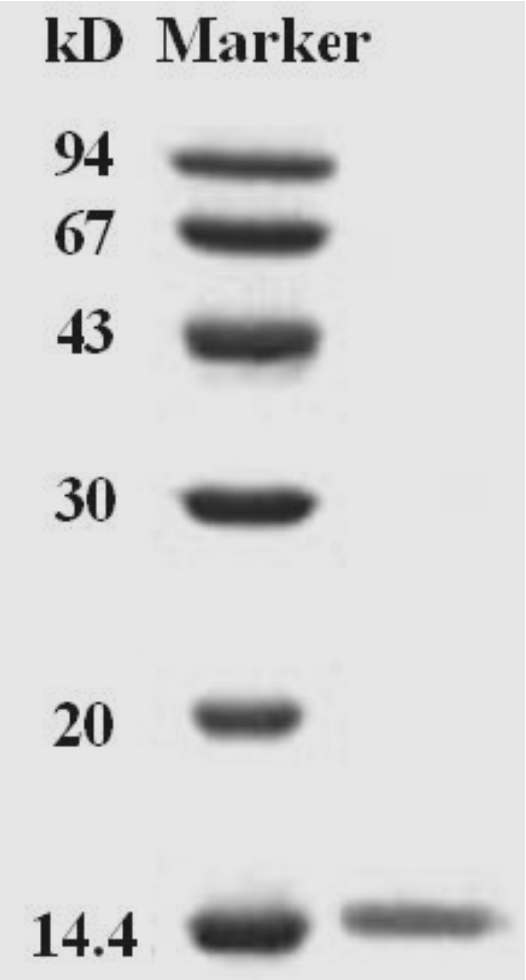

Figure 2. SDS-PAGE results. Right lane: Molecular mass markers from GE Healthcare. From top downward: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14.4 kDa). Left lane: purified phytase (9 µg).

B1 on Q-Sepharose, phytase activity was located in the second adsorbed peak Q2 (Table 1). Peak Q2 was separated into three peaks on Superdex 75 (Figure 1B). Phytase activity was enriched in the second and highest peak, SU2 (Figure 1B and Table 1). There was a 30-fold increase in specific activity.

Characterization of isolated phytase

SU2 appeared as a single band with a molecular mass of 14.8 kDa in SDS-PAGE (Figure 2). Its molecular mass as estimated by gel filtration on Superdex 75 was also 14.8 kDa (Figure 1B). The enzyme displayed a pH optimum at 5.0. However, the fluctuation of enzyme activity with ambient temperature was relatively moderate and remained within 80 and 90% of the activity at the optimal pH (Figure 3). Its optimal temperature was 45°C. The enzyme activity rose steadily as the temperature rose from 20 to 45°C. When the temperature was increased further, the enzyme activity began to fall. There was approximately 40% residual activity at 100°C (Figure 4).

Addition of 10 mM $CaCl_2$ had no effect on the activity of the phytase. The N-terminal sequence of the phytase was DFQVDTGNN, different from some of phytases reported in the literature (Table 2), but similar to others in some degree (Table 2). *F. velutipes* phytase manifested broad substrate specificity on a range of phosphorylated compounds. Its activity on ATP, AMP, fructose-6-phosphate and glucose-6-phosphate was higher than that on ADP and β-glycerophosphate (Table 3). It lacked antifungal activity when tested up to 100 µg/disk (data not shown).

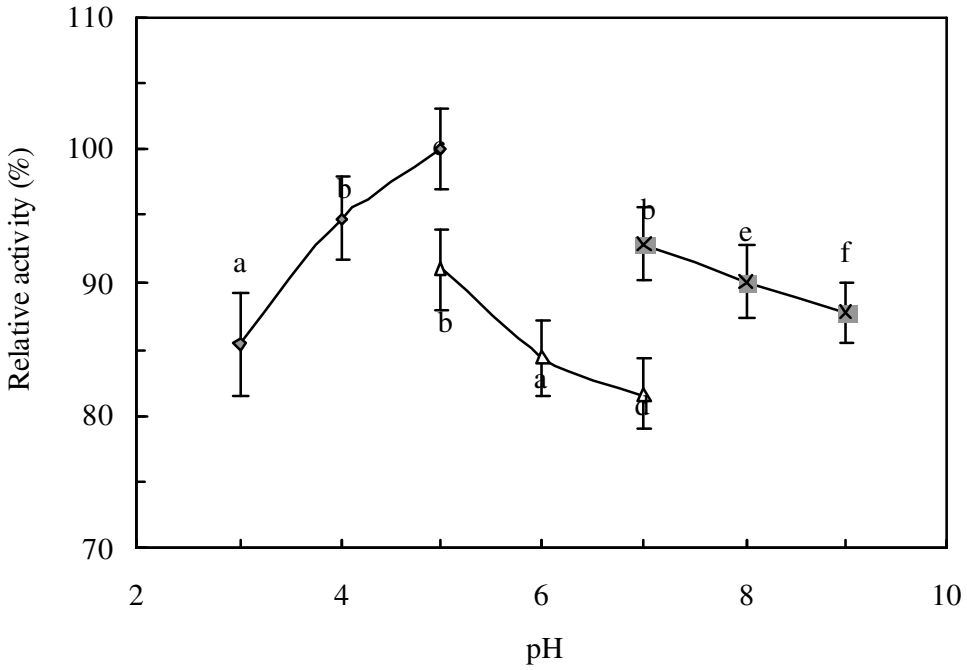

Figure 3. pH dependence of *F. velutipes* phytase activity. —◆—: NaOAc, —△—: Mes —▩—: Hepes. NaOAc buffer (0.1 M) at pH 3, 4 and 5, Mes buffer (0.1 M) at pH 5 and 6, and Hepes buffer (0.1 M) at pH 7, 8 and 9 were used. Maximal phytase activity at pH optimum was set at 100%. Results are presented as mean ±SD (n = 3). Different letters (a, b, c and d) next to the data points indicate statistically significant difference ($p < 0.05$) when the data are analyzed by analysis of variance followed by Duncan's multiple range tests.

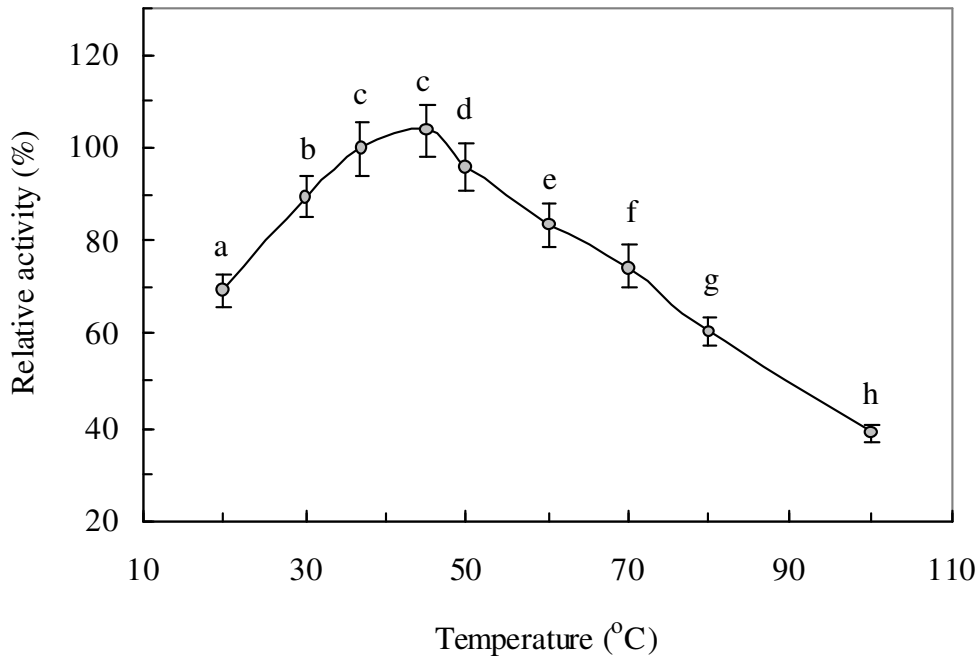

Figure 4. Temperature dependence of *F. velutipes* phytase activity enzyme assay was carried out in 0.1 M NaOAc buffer (pH 5) at 37°C. Maximal phytase activity at optimal temperature was set at 1005. Results are presented as mean ±SD (n = 3). Different letters (a, b, c and d) next to the data points indicate statistically significant difference ($p < 0.05$) when the data are analyzed by analysis of variance followed by Duncan's multiple range tests.

Table 2. Comparison of N-terminal sequences of phytases from *F. velutipes* and other fungal species.

Phytase	N-terminal sequence
Flammulina velutipes	DFQVDTGNN
Penicillium sp. Q7 (ABM92788.1)	DFTHDNGMI
Schwanniomyces capriottii (ABN04184.1)	DYYSNSAGNN
Wickerhamomyces anomalus (CBI71332.1)	DTAVKAGAA
Aspergillus fumigatus (AAU93517.1)	DFSHDNGMI
Aspergillus terreus (AAB52507.1)	NRVVDSATN
Aspergillus niger NW205 phyB[a]	FSYGAAIPQS
Aspergillus niger van Teighem[b]	FYYGAALPQS
Trametes pubescens PhyA[c]	SAXLDVTRDV

References: [a](Ehrlich et al., 1993; Kostrewa et al., 1997); [b](Vats and Banerjee, 2005); [c](Tomschy et al., 2002).

Table 3. Substrate specificity of *F. velutipes* phytase.

Substrate (5 mM)	Relative activity (%)
Sodium phytate	100.0 ± 4.9
β-Glycerophosphate	112.0 ± 6.8
ADP	129.1 ± 5.7
AMP	181.4 ± 5.0
G-6-P	206.3 ± 4.4
F-6-P	208.0 ± 7.3
ATP	263.7 ± 6.6

The phytase activity towards sodium phytate (5.0 mM) was regarded as 100%. Phytase activity was assayed with increasing concentration of Pi using the standard phytase assay. Results are presented as mean ±SD (n = 3).

DISCUSSION

Phytase has been purified from only a small number of mushrooms including *Agrocybe pediades, Ceriporia* sp., *Periophora lycii* and *Trametes pubescens* (Lassen et al., 2001). Ion exchange chromatographic media such as Q-Sepharose and Source 30 Q were used in that study as compared to DEAE-cellulose, CM-cellulose and Q-Sepharose employed in the present investigation. The phytases isolated in both studies were adsorbed on Q-Sepharose. The gel filtration chromatographic media Sephadex G-25 was used in the former study following the hydrophobic interaction chromatography step on Phenyl Toyopearl to remove salt, while the gel filtration chromatographic media Superdex 75 was used in the present study as the final step. Affinity chromatography on Affi-gel blue gel was also used as an intermediate step in this investigation. A number of absorbance peaks were obtained in each step of the purification scheme. The bulk of phytase activity was confined to only one of the absorbance peaks in each step, indicating the efficacy of the scheme used.

When *F. velutipes* phytase isolated in the present investigation was compared to the previously reported mushroom phytases, it was found that its molecular mass (14.8 kDa) was appreciably smaller than the value of 45 kDa cited for others (Lassen et al., 2001). Another characteristic of *F. velutipes* phytase is that it expressed maximal activity in the pH range of 7 to 9, in sharp contrast to the optimum pH at 4 to 5 for the other mushroom phytases (Lassen et al., 2001). The temperature optimum for *F. velutipes* phytase occurred at 45°C.

The phytase from *Aspergillus niger* ATCC 9142 exerted high activity on sodium phytate and calcium phytate. Activity on ATP, ADP, AMP, glucose-1-phosphate and *p*-nitrophenyl phosphate was lower by comparison (Casey and Walsh, 2003). A phytase from *A. niger* exhibited the highest activity toward phytate, lesser activity toward ATP and ADP, but no activity on AMP and cAMP (Dvorakova et al., 1997). *Aspergillus fumigatus* phytase and *A. niger* acid phosphatase displayed activity towards a number of substrates, while phytases from *A. niger*, *Aspergillus terreus* CBS, *A. terreus* 9A1, and *Escherichia coli* were rather specific for phytic acid. *A. niger* PhyB demonstrated broader substrate specificity than *A. niger* phyA (

Mullaney et al., 2000).

Phytases from yeast and *Aspergillus* species were much larger in molecular mass (around 80 kDa), had a higher optimal temperature (60 to 80°C), and displayed optimal activity in the pH range 4.5 to 6.0 when compared with *F. velutipes* phytase (Casey and Walsh, 2003).

F. velutipes phytase exhibited a molecular mass which was much smaller (46 kDa) than that of the phytase from *Bacillus* sp. KHU-10 (Choi et al., 2001). However, the two phytases are similar in optimum pH (7 to 9 for *F. velutipes* phytase and 6.5 to 8.5 for *Bacillus* phytase), optimum temperature (45°C for *F. velutipes* phytase and 40°C for *Bacillus* phytase) and chromatographic behaviour on DEAE-ion exchanger (both phytases were adsorbed).

Lupine seed phytases (Greiner, 2002) had a larger molecular mass (57 to 64 kDa) and a lower optimal temperature (35°C) than *F. velutipes* phytase. These seed phytases exhibited a pH optimum at pH 5.0. Rat intestinal mucosa phytase (Yang et al., 1991) displayed an even higher pH optimum at pH 7.5, and was made up of two large (70 and 90 kDa) subunits.

In summary, *F. velutipes* phytase showed many distinctive features including a unique N-terminal amino acid sequence, a small molecular mass, a low optimal temperature, and little variations in activity in the pH range of 3.0 to 9.0.

ACKNOWLEDGEMENT

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