Appl Microbiol Biotechnol (2004) 63:383–389
DOI 10.1007/s00253-003-1337-0

ORIGINAL PAPER

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Characteristics of fungal phytases from *Aspergillus fumigatus* and *Sartorya fumigata*

Received: 27 January 2003 / Revised: 4 April 2003 / Accepted: 11 April 2003 / Published online: 12 June 2003

Abstract *Aspergillus fumigatus* phytase has previously been identified as a phytase with a series of favourable properties that may be relevant in animal and human nutrition, both for maximising phytic acid degradation and for increasing mineral and amino acid availability. To study the natural variability in amino acid sequence and its impact on the catalytic properties of the enzyme, we cloned and overexpressed the phytase genes and proteins from six new purported *A. fumigatus* isolates. Five of these phytases displayed $\leq$2 amino acid substitutions and had virtually identical stability and catalytic properties when compared with the previously described *A. fumigatus* ATCC 13073 phytase. In contrast, the phytase from isolate ATCC 32239 (*Sartorya fumigata*, the anamorph of which was identified as *A. fumigatus*) was more divergent (only 86% amino acid sequence identity), had a higher specific activity with phytic acid, and displayed distinct differences in substrate specificity and pH-activity profile. Finally, comparative experiments confirmed the favourable stability and catalytic properties of *A. fumigatus* phytase.

Some of the data presented here, in particular the amino acid sequences of the phytases from different *A. fumigatus* and *S. fumigata* isolates, were first presented at the workshop on "The biochemistry of plant phytate and phytases", Copenhagen, Denmark, 25–28 October 1997

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Introduction

Vegetable feed ingredients typically contain a considerable number of components that cannot be digested efficiently by animals, and/or display antinutritional properties (Bedford 2000). One such compound is phytic acid (*myo*-inositol hexakisphosphate), the main storage form of phosphorus in plant materials. Depending on the plant species, phytic acid constitutes between 60% and 90% of all phosphorus present (Selle et al. 2000).

Monogastric animals such as pigs and poultry are unable to utilise phytic acid phosphorus, since they essentially lack enzymes for phytic acid hydrolysis (i.e. phytase) in the digestive tract. This can lead to phosphorus deprivation and, thus, to incomplete or irregular ossification and to demineralisation of existing bones. To maintain health and optimal growth rates in such animals, it is still common practice to supplement their feed with inorganic phosphate. Consequently, these diets contain far more phosphorus than the animal actually needs, with the excess being excreted in the manure. Especially in areas of intensive livestock production, disposal of the manure on the fields can result in phosphorus runoff and eutrophication of surface waters, thereby creating a major environmental problem.

With rock phosphate resources becoming increasingly precious, and with increasing environmental pressure, primarily in Europe and the United States, supplementation of animal feed with exogenous phytase becomes more and more attractive (for reviews, see Bedford 2000; Mullaney et al. 2000b; Lei and Stahl 2001). Exogenously added phytase improves the dietary availability of phytic acid phosphorus in monogastric animals, reduces or even alleviates the need for rock phosphate supplements, and thereby reduces environmental pollution by these animals' manure.

We have previously reported on the favourable characteristics of a fungal phytase from *Aspergillus fumigatus* (Pasamontes et al. 1997a; Wyss et al. 1998, 1999a, 1999b; Simões Nunes and Guggenbuhl 1998). It is more heat resistant and has more promising catalytic

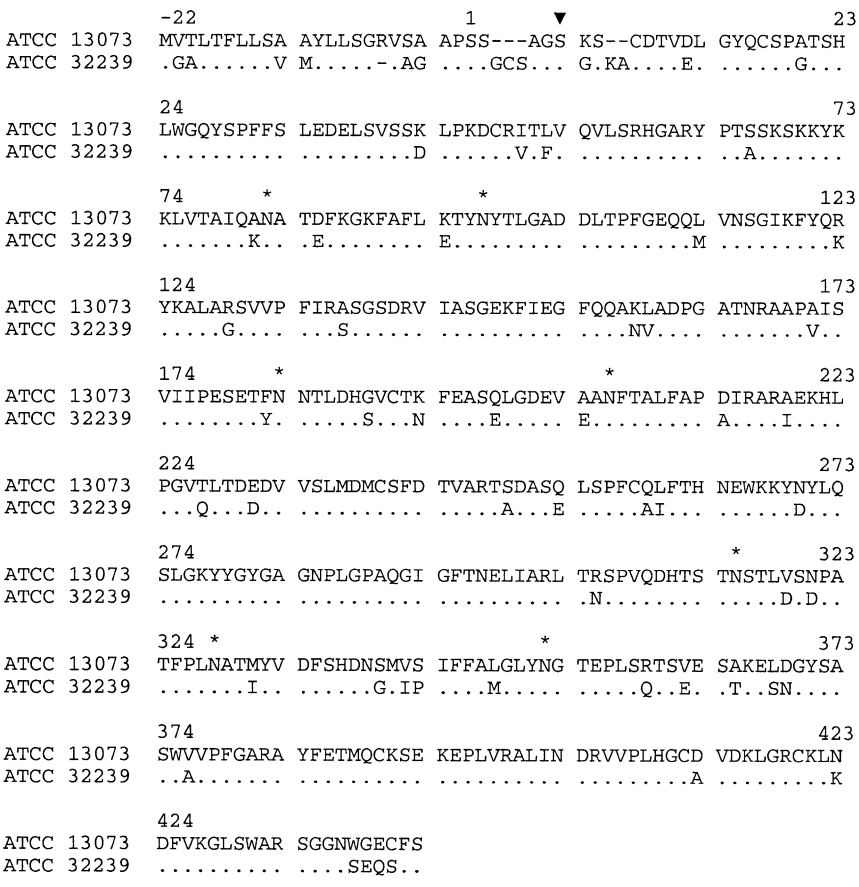

Fig. 1 Alignment of the amino acid sequences of *Aspergillus fumigatus* ATCC 13073 and *Sartorya fumigata* ATCC 32239 phytase. In the latter sequence, only the differing amino acids are shown, whereas identical amino acids relative to *A. fumigatus* ATCC 13073 phytase are represented by a *dot*. A *hyphen* stands for a gap in the sequence introduced for optimal alignment of the two sequences. Relative to *A. fumigatus* ATCC 13073 phytase, the other *A. fumigatus* phytases display the following amino acid substitutions: ATCC 26906, SRRC 45 and SRRC 46: P107A and E220K; ATCC 32722: E355G; ATCC 58128: P396S; SRRC 2397: Q27E and P396S. The *arrowhead* indicates the first amino acid of *A. fumigatus* ATCC 13073 phytase determined by N-terminal sequencing of the native protein (Wyss et al. 1999a). *Asterisks* Potential sites of *N*-linked glycosylation; all these sites, except Asn-183, are located on the surface of the molecule

properties when compared with the benchmark phytase from *Aspergillus niger*, which has been studied extensively in the past (for reviews, see Wodzinski and Ullah 1996; Mullaney et al. 2000b). Since some of our results and conclusions have been called into question by two recent publications (Mullaney et al. 2000a; Ullah et al. 2000), the purpose of the present article is to shed light on the apparent inconsistencies around *A. fumigatus* phytase and to unravel the diversity in amino acid sequences and catalytic properties of phytases from different *A. fumigatus* isolates.

Materials and methods

Isolation of phytase genes from different A. fumigatus isolates

Purified DNA was obtained from six (purported) *A. fumigatus* isolates: ATCC 26906, from Japanese soil; ATCC 26934, from human aspergillotic disease, Montana; ATCC 32239, from Aspen roots, Wyoming (*Sartorya fumigata*, the anamorph of which was erroneously listed as *A. fumigatus* in the print version of the ATCC catalogue); ATCC 32722, used for production of protein from starchy substrates; ATCC 34625, from submerged balsa wood, Maryland; and ATCC 58128, from grape marc compost, Hungary. The gene encoding wild-type *A. fumigatus* phytase used as reference in this paper and described in our previous publications (Pasamontes et al. 1997a; Wyss et al. 1998, 1999a, 1999b; Simões Nunes and Guggenbuhl, 1998) was obtained from strain ATCC 13073. Phytase genes were isolated by PCR as described previously (Mitchell et al. 1997; Pasamontes et al. 1997a, 1997b).

Production, purification and biochemical characterisation of phytases

Phytases were produced by recombinant DNA technology using *Saccharomyces cerevisiae*, *Hansenula polymorpha* or *A. niger* as production hosts. Unless stated otherwise, the data shown are from *A. fumigatus* ATCC 13073 phytase (accession no. tre_fun:o00092) expressed in *A. niger*, and from the other *A. fumigatus* (accession nos. Gsp_Aab20507, Gsp_Aab20508 and Gsp_Aab20509) and *Sartorya fumigata* phytases (Gsp_Aab20510) expressed in *Saccharomyces cerevisiae*. Phytase purification as well as determination of pH-activity profiles and substrate specificities were performed as described previously (Pasamontes et al. 1997a; Wyss et al. 1998, 1999a). One unit of phytase (or acid phosphatase, glucose-6-phosphatase, etc.) activity catalyses the liberation of 1 μmol inorganic phosphate per minute at 37°C.

Storage stability study

Samples of *A. fumigatus* phytase were sterile-filtered and stored at either room temperature or 4°C. Samples were withdrawn for activity measurements at 0, 1, 2, 4, 8 and 12 weeks. Phytase activity was determined by incubating 400 μl of appropriately diluted sample with 800 μl 7.5 mM phytic acid (dodecasodium salt from rice; Sigma, St. Louis, Mo.) for 30 min in 250 mM sodium acetate buffer, pH 5.5, containing 0.003% Tween-20. The reaction was stopped by adding 800 μl molybdate/vanadate reagent (5 mM ammonium monovanadate, 20 mM ammonium heptamolybdate in 1.7 M nitric acid and 0.06% ammonia). The absorbance was measured at 415 nm and the activity determined using a phosphate standard curve.

Differential scanning calorimetry

For differential scanning calorimetry (DSC), protein samples were concentrated to approximately 50 mg/ml and extensively dialysed against 10 mM sodium acetate, pH 5.0. DSC was performed on a Mettler DSC 821e heat flux calorimeter equipped with the STARe control and evaluation software (Version 4.00). Tightly sealed 40 µl aluminium crucibles were used for the protein samples, while the reference cell was empty. The instrument was calibrated using indium as standard. The proteins were subjected to three consecutive temperature cycles. The sample was cooled down from room temperature to 5°C, the starting temperature of the experiment. After equilibration for 5 min at this temperature, a constant heating rate of 10°C min^{-1} was applied up to 90°C. The temperature was held constant for 1 min at 90°C and subsequently decreased to 5°C with a constant cooling rate of 10°C min^{-1}. This cycle was repeated twice.

Other methods

Protein concentrations were calculated from the optical density at 280 nm by using theoretical absorption values calculated from the known protein sequences using DNA* software (DNASTAR, Madison, Wis.). One absorbance unit at 280 nm corresponds to 0.94 mg/ml *A. fumigatus* phytase and 1.03 mg/ml *S. fumigata* phytase. All amino acid numberings used in the present paper correspond to the numbering of mature *A. niger* phytase (Wyss et al. 1999a). Unless stated otherwise, all data are given as means±SD of at least three measurements; in many cases, the standard deviations were smaller than the size of the data symbols.

Results

Natural variation of phytase amino acid sequences from different *A. fumigatus* isolates

Phytase genes were obtained from six different isolates of purported *A. fumigatus* origin. Two of the six new isolates (ATCC 26934 and ATCC 34625) yielded identical phytase amino acid sequences when compared with the original wild-type *A. fumigatus* ATCC 13073 phytase. The phytases from three isolates (ATCC 26906, 32722, and 58128) displayed one or two amino acid substitutions (Fig. 1), none of which directly affecting the active site. The phytase from the sixth isolate (ATCC 32239) differed in 13 positions in the signal sequence and 51 positions in the mature part of the protein from the original wild-type *A. fumigatus* phytase (Fig. 1); several of these substitutions affect variable residues of the active site cavity. In agreement with the considerable sequence differences, this latter isolate is correctly classified as *S. fumigata* (see above).

Catalytic properties of the different *A. fumigatus* phytases

In agreement with the fact that none of the amino acid substitutions in the *A. fumigatus* phytases from isolates ATCC 26906, 32722 and 58128 affects the active site, these phytases displayed virtually identical catalytic properties in terms of pH-activity profiles (Fig. 2), substrate specificity (not shown) and specific activity [27.8±0.8, 26.6±0.8 and 24.6±2.6 U (mg protein)$^{-1}$ at pH 5.0, respectively; n=4 each] when compared with *A. fumigatus* ATCC 13073 phytase [26.5±5.2 U (mg protein)$^{-1}$; n=22]. In contrast, *S. fumigata* ATCC 32239 phytase displayed a higher specific activity with phytic acid as substrate [42.6±2.8 U (mg protein)$^{-1}$; n=4], lower specific activities with other phosphate compounds (Fig. 3), and a distinctly different pH-activity profile, with loss of enzymatic activity above pH 7.0 (Fig. 2).

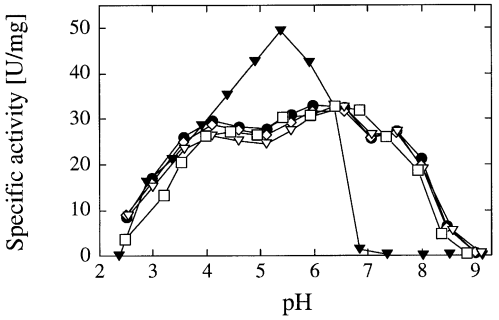

Fig. 2 pH-Activity profiles of different *A. fumigatus* and *S. fumigata* phytases. The pH-activity profiles were determined as described in Materials and methods. ❑ *A. fumigatus* ATCC 13073 phytase, ● *A. fumigatus* ATCC 26906 phytase, ◇ *A. fumigatus* ATCC 32722 phytase, ▽ *A. fumigatus* ATCC 58128 phytase, ▼ *S. fumigata* ATCC 32239 phytase

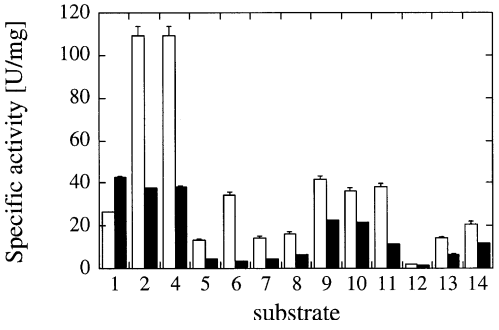

Fig. 3 Specific activities of *A. fumigatus* ATCC 13073 (*open bars*) and *S. fumigata* ATCC 32239 phytase (*black bars*) with different phosphate compounds. The substrate specificity profiles were determined as described in Materials and methods. Substrates: *1* Phytic acid; *2* *p*-nitrophenyl phosphate; *4* fructose-1,6-bisphosphate; *5* fructose-6-phosphate; *6* glucose-6-phosphate; *7* ribose-5-phosphate; *8* α-glycerophosphate; *9* β-glycerophosphate; *10* 3-phosphoglycerate; *11* phosphoenolpyruvate; *12* AMP; *13* ADP; *14* ATP

Storage stability of *A. fumigatus* phytase

In two recent articles (Mullaney et al. 2000a; Ullah et al. 2000), it has been stated that *A. fumigatus* phytase displays a rapid loss of activity at both 0 and 4°C. Investigation of the storage stability of *A. fumigatus* ATCC 13073 phytase (Fig. 4) shows that this is clearly not the case. Even at room temperature, purified *A. fumigatus* phytase retained >96% of the initial activity after 12 weeks.

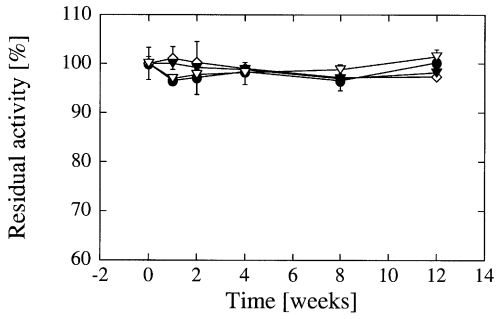

Fig. 4 Storage stability of *A. fumigatus* phytase. The storage stability study was performed as described in Materials and methods with the following samples: ● purified *A. fumigatus* phytase expressed in *Hansenula polymorpha*, storage at room temperature; ▼ same, but storage at 4°C; ◇ culture supernatant of a recombinant *Aspergillus niger* strain expressing *A. fumigatus* phytase, 4°C; ▽ culture supernatant of a recombinant *H. polymorpha* strain expressing *A. fumigatus* phytase, 4°C

Thermostability properties of *A. fumigatus* and *A. niger* phytase as investigated by DSC

When phytase samples were analysed in three consecutive rounds of heating and cooling to probe the reversibility of thermal unfolding, transitional enthalpy in the second and third heating periods was detected only for *A. fumigatus* phytase (Fig. 5A), but not for *A. niger* NRRL 3135 phytase (Fig. 5B), *A. terreus* phytases or *Emericella nidulans* phytase (not shown). These results show that *A. fumigatus* phytase is the sole enzyme that, under the conditions of the DSC experiment, is able to refold properly into a native-like conformation. The T_m values of *A. fumigatus* phytase in the three consecutive heating intervals were identical.

Several studies have shown that the extent of glycosylation of a protein may have an impact on its thermostability and on its refolding capacity (e.g. Owers Narhi et al. 1991; Kern et al. 1992). Interestingly, the extent of glycosylation of fungal phytases strongly depended on the expression system used (Wyss et al. 1999a). While glycosylation was moderate when *A. fumigatus* phytase was expressed in *A. niger*, it was excessive and variable when the same protein was produced in *S. cerevisiae* or *H. polymorpha*. The different extents of glycosylation had only a small effect on the T_m value of *A. fumigatus* phytase; however, they influenced protein refolding upon heat denaturation to some extent (62, 50 and 19% residual transitional enthalpy observed in the second as compared with the first round of consecutive heating intervals for *A. fumigatus* phytase expressed in *H. polymorpha*, *S. cerevisiae* and *A. niger*, respectively).

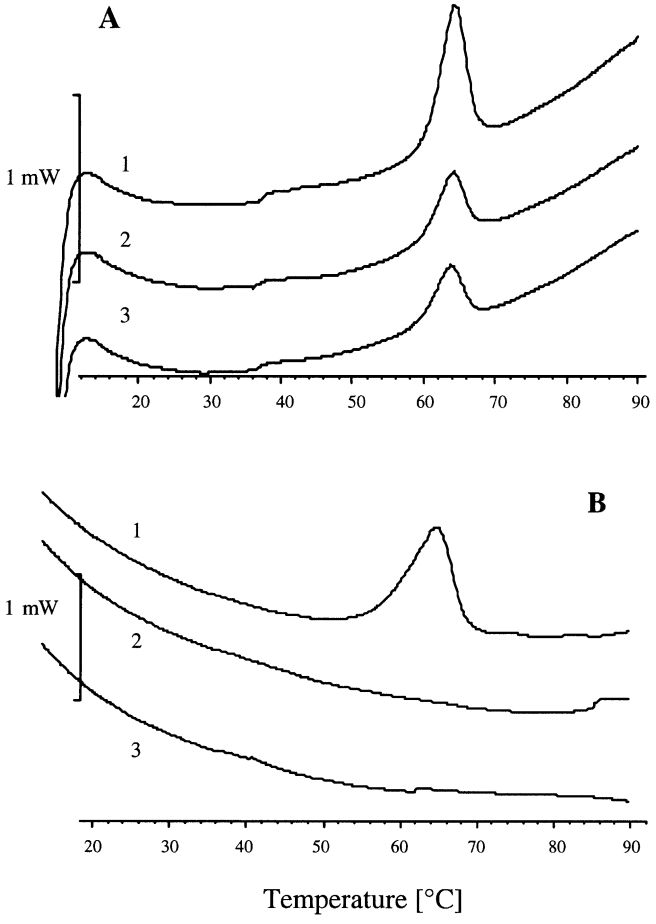

Fig. 5 Differential scanning calorimetry (DSC) of **A** *A. fumigatus* ATCC 13073 phytase (expressed in *H. polymorpha*) and **B** *A. niger* NRRL 3135 phytase. The scans of three consecutive heating intervals are shown. *1*, *2*, *3* First, second, and third heating intervals, respectively

Discussion

Natural variability and stability properties of *A. fumigatus* phytase

So far, the phytase amino acid sequences from 11 *A. fumigatus* isolates have been described (Pasamontes et al. 1997a; Mullaney et al. 2000a; this paper; and accession no. tr_other:q8wzj5). Compared with the "benchmark" *A. fumigatus* ATCC 13073 phytase described in our previous publications (Pasamontes et al. 1997a; Wyss et al. 1998, 1999a, 1999b; Simões Nunes and Guggenbuhl, 1998; Tomschy et al. 2000), four new *A. fumigatus* isolates (ATCC 26934, ATCC 34625, SRRC 322, and CCTCC AF93024) yielded identical phytase amino acid sequences. The phytases from two isolates (ATCC 32722 and ATCC 58128) had one amino acid substitution, and those of four isolates (ATCC 26906, SRRC 45, SRRC 46 and SRRC 2397) displayed two amino acid substitutions. Among the latter four phytase amino acid sequences,

three (from isolates SRRC 45, SRRC 46 and ATCC 26906) are identical.

From a catalytic standpoint, Mullaney et al. (2000a) proposed two "classes" of *A. fumigatus* phytases: group I (from isolates SRRC 45 and SRRC 2397) with a pH optimum of 4.0 and a temperature optimum at 70°C, and group II (from isolates SRRC 46 and SRRC 322) with a pH optimum of 5.0 and a temperature optimum at 58°C. These considerable apparent differences in catalytic properties are surprising, especially because the phytases from isolates SRRC 45 and SRRC 46 have identical amino acid sequences.

Although our collection of *A. fumigatus* phytases included examples with identical amino acid sequences to three of the four *A. fumigatus* phytases studied by Mullaney et al. (2000a), we failed to see differences in catalytic properties: specific activities with phytic acid as substrate (see Results), substrate specificities (not shown) and pH-activity profiles (Fig. 2) were virtually indistinguishable among the four *A. fumigatus* phytases studied biochemically (from isolates ATCC 13073, ATCC 26906, ATCC 32722 and ATCC 58128).

In terms of thermal stability, *A. fumigatus* ATCC 13073 phytase proved to unfold at a temperature similar to that of other fungal phytases, both in our previous studies and in the present investigation. However, after thermal unfolding (at up to 100°C), *A. fumigatus* phytase displayed the unique property of refolding to a considerable extent (Fig. 5) or, under certain conditions, almost completely (Pasamontes et al. 1997a; Wyss et al. 1998) into a fully active, native-like conformation. Storage stability of *A. fumigatus* phytase at 4°C was very good, particularly when the protein was purified (Fig. 4). Only when expressed in *A. niger* NW205 and stored as concentrated culture supernatants at 4°C, several fungal phytases (*A. fumigatus*, *E. nidulans*, *A. terreus* 9A1 and *Myceliophthora thermophila* phytase) had a tendency to undergo proteolytic degradation by proteases present in the culture supernatants (Wyss et al. 1999a).

Conflicting findings have been made by Drs. Ullah, Mullaney, and co-workers. They reported that *A. fumigatus* phytase displays inferior storage stability compared to *A. niger* phytase and, at 70 or 80°C, undergoes irreversible rather than reversible inactivation (Mullaney et al. 2000a; Ullah et al. 2000).

How to explain these apparent inconsistencies? The *A. fumigatus* SRRC 2397 phytase of Mullaney et al. (2000a) contained an amino acid substitution (Q27E) at an active site residue previously shown to have an impact on the specific activity of the enzyme (Tomschy et al. 2000). However, this phytase displayed properties identical to those of *A. fumigatus* SRRC 45 phytase (Mullaney et al. 2000a), which, in turn, has an identical amino acid sequence to "our" *A. fumigatus* ATCC 26906 phytase. Therefore, the Q27E mutation seems to be neutral.

Differences in posttranslational modifications between the various preparations used are also unlikely to explain the discrepancies observed: (1) we failed to see major differences in catalytic or stability properties between *A. fumigatus* phytases expressed in either *A. niger*, *S. cerevisiae*, or *H. polymorpha*, despite largely different extents of glycosylation (Wyss et al. 1999a, 1999b; and data not shown). (2) Although both investigations were carried out on *A. fumigatus* phytase expressed in *Pichia pastoris*, with most likely no difference in posttranslational modifications, Ullah et al. (2000) and Rodriguez et al. (2000) arrived at different conclusions. Whereas the latter authors virtually confirmed our previous and present findings, Ullah et al. (2000) obtained evidence for a much poorer performance of *A. fumigatus* phytase. (3) Finally, numerous studies in the literature have shown that fungal proteins expressed recombinantly in other fungi in most instances display identical or very similar properties when compared with the native enzyme isolated from the corresponding wild-type strain (e.g. Minning et al. 1998; Shibuya et al. 1998).

A more plausible reason for the discrepant findings may be the presence of minor protease impurities in the preparations of Drs. Ullah, Mullaney, and co-workers: (1) fungi and yeasts used as expression systems for (recombinant) proteins typically produce variable amounts of extracellular proteases (Gouka et al. 1997; van den Hombergh et al. 1997; Cregg et al. 2000). In the experiments of Ullah et al. (2000) and Mullaney et al. (2000a), *A. niger* NRRL 3135 phytase overexpressed in *A. niger* (Natuphos; BASF, Germany) was used as reference. Proteolytic degradation of phytase in this preparation is minimised, since this is an example of homologous expression, and since industrial production strains for recombinant proteins are normally selected and/or engineered to display low protease activity (e.g. by knocking out the relevant protease genes). Whereas the methylotrophic yeast *H. polymorpha* used in our company for high-level expression of *A. fumigatus* phytase (Mayer et al. 1999) is characterised by very low protease activity, it is questionable whether the wild-type *A. fumigatus* strains used by Mullaney et al. (2000a) or *P. pastoris* strain X33 from Invitrogen as used by Rodriguez et al. (2000) and Ullah et al. (2000) are similarly suited and/or optimised for recombinant protein production (see, e.g. Ferrarese et al. 1998). Therefore, contaminating proteases may still have been present in the preparations of Mullaney et al. (2000a) and Ullah et al. (2000), causing degradation and thus inactivation of *A. fumigatus* phytase both during storage at 0 or 4°C and upon unfolding of the protein at elevated temperatures. The latter may explain why these authors did not observe refolding of *A. fumigatus* phytase after thermal unfolding. (2) The fact that, on a unit basis, wild-type *A. fumigatus* phytase tended to be more potent than *A. niger* phytase in animal experiments (see below) is further proof that, in vivo, proteolytic susceptibility is not more pronounced for the former than for the latter. In addition, in feed pelleting experiments, recovery of enzymatic activity was considerably higher for *A. fumigatus* than for *A. niger* phytase (Wyss et al. 1998; and data not shown). Together with our in vitro results on highly purified phytases (Wyss et al. 1998, 1999a; this paper), the currently available data indicate that from a

stability point of view, *A. fumigatus* phytase is the wild-type fungal phytase of choice. Even if stability problems were encountered, as for heterologous expression in *A. niger*, we have described an efficient means [i.e. site-directed mutagenesis of the major site(s) of protease attack] of alleviating proteolytic susceptibility (Wyss et al. 1999a). In conclusion, these different lines of evidence argue against the idea that there are two classes of *A. fumigatus* phytase with distinct differences in catalytic and stability properties as suggested by Mullaney et al. (2000a).

The phytase of choice in terms of catalytic properties

Based solely on considerations of k_{cat} and/or k_{cat}/K_m values, Ullah et al. (2000) concluded that *A. fumigatus* phytase is inferior to *A. niger* phytase, and "that the benchmark phytase produced by *Aspergillus ficuum* (*niger*) is still the enzyme of choice from a catalytic standpoint". Even if specific activity, k_{cat} and/or k_{cat}/K_m values were the only catalytic properties to be considered, this statement would not be valid: specific activities of 140–200, $\geq$800 and 400–1,200 U (mg protein)$^{-1}$ have been reported for *A. terreus* phytases, *Escherichia coli* phytase and different basidiomycete phytases, respectively, at pH values of 5.0–5.5, in comparison to only approximately 100 U (mg protein)$^{-1}$ for *A. niger* NRRL 3135 phytase (Greiner et al. 1993; Wyss et al. 1999b; Lassen et al. 2001).

If other catalytic parameters were not important, one would, under practical conditions and at the same unit inclusion level, expect an identical performance of different phytases. This is, however, not the case. In in vitro experiments on phosphate liberation from animal feed samples, *A. fumigatus* phytase was significantly more potent than *A. niger* phytase (Wyss et al. 1999b). Similarly, in pig feeding experiments, *A. fumigatus* phytase caused a larger increase in plasma phosphate concentration and a larger decrease in fecal phosphorus excretion than *A. niger* phytase (Simões Nunes and Guggenbuhl, 1998; and data not shown; however, the differences were not statistically significant in these experiments). Finally, in whole-wheat flour during bread baking, *A. fumigatus* phytase (from strain SRRC 322) was more powerful than *A. niger* or *E. coli* phytase in degrading phytic acid and in increasing iron availability (Porres et al. 2001). Therefore, the broader substrate specificity (Fig. 3) and the more favourable pH-activity profile of *A. fumigatus* phytase (Fig. 2) seem to allow more complete degradation of phytic acid. In summary, at the same unit inclusion level, *A. fumigatus* phytase is the most powerful wild-type ascomycete phytase known to date.

Admittedly, the specific activity of *A. fumigatus* phytase is rather low, which limits its commercial feasibility. However, given its other favourable catalytic properties and its superior thermotolerance, *A. fumigatus* phytase seems to be an ideal starting point for enzyme engineering. In fact, an increase in specific activity of up to 7-fold has recently been achieved by replacement of the active site residue Gln-27 with several other amino acids (Tomschy et al. 2000).

Acknowledgements Dr. E.J. Mullaney is gratefully acknowledged for invaluable comments on the manuscript, and Mrs. Catherine Griggio and Michaela Schritz for technical assistance.

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