Active site residue 297 of *Aspergillus niger* phytase critically affects the catalytic properties

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Abstract The wild-type phytases from the *Aspergillus niger* strains NRRL 3135 and T213 display a three-fold difference in specific activity (103 versus 32 U/mg protein), despite only 12 amino acid differences that are distributed all over the sequence of the protein. Of the 12 divergent positions, three are located in or close to the substrate binding site. Site-directed mutagenesis of these residues in *A. niger* T213 phytase showed that the R297Q mutation (R in T213, Q in NRRL 3135) fully accounts for the differences in catalytic properties observed. Molecular modelling revealed that R297 may directly interact with a phosphate group of phytic acid. The fact that this presumed ionic interaction – causing stronger binding of substrates and products – correlates with a lower specific activity indicates that product (*myo*-inositol pentakisphosphate) release is the rate-limiting step of the reaction.

Key words: Phytase; Acid phosphatase; Site-directed mutagenesis; Three-dimensional structure; Active site; *Aspergillus niger*

1. Introduction

Phytic acid (*myo*-inositol hexakisphosphate) occurs abundantly in plant seeds where it serves as the main reservoir of phosphorus for the build-up of biomass during germination of the plant [1,2]. Since monogastric animals (e.g. pigs and poultry) have little or no phytase (*myo*-inositol hexakisphosphate phosphohydrolase, EC 3.1.3.8 or 3.1.3.26) activity in their digestive tract, phytic acid phosphorus is largely unavailable to these animals. It is, therefore, common practice to supplement pig or poultry feed either with inorganic phosphate or with a phytase of microbial (fungal) origin. Phytase supplementation is preferable to supplementation with inorganic phosphate since the latter increases the total phosphate burden and intensifies environmental problems (eutrophication), especially in areas with intensive livestock production. Low-phytic acid plants – with unchanged total phosphorus, but decreased phytic acid concentration in the seeds [3] – are a potential alternative for improving phosphorus availability in seed-based animal feed.

In the course of a program for developing an improved, heat-stable, recombinant fungal phytase [4–10], we identified an *Aspergillus niger* wild-type phytase (from strain T213) which is also a monomeric, glycosylated protein [9], but has a three-fold lower specific activity than the *A. niger* phytase (from strain NRRL 3135) that is currently in widespread use. In the present article, we compare the catalytic properties of these two *A. niger* wild-type phytases. In addition, inspection of the 3D structure [11] and site-directed mutagenesis allowed us (i) to identify the residue(s) that are responsible for the differences in biochemical properties, and (ii) to propose a hypothesis about the rate-limiting step of the reaction.

2. Materials and methods

2.1. Source of purified proteins

A. niger NRRL 3135 phytase was obtained commercially (Natuphos, BASF, Ludwigshafen, Germany). *A. niger* T213 (= *A. niger* CB) phytase was overexpressed initially in *A. niger* [9]. For the analysis of mutations, however, both *A. niger* T213 phytase and all site-directed mutants were expressed in *Saccharomyces cerevisiae*. Phytases are more extensively glycosylated when expressed in *S. cerevisiae* than in *A. niger*, but this additional glycosylation has no effect on the catalytic properties of the enzymes [9]. Protein purification was performed as described previously for phytases expressed in *A. niger* and *S. cerevisiae* [9].

2.2. Molecular biology

Site-directed mutagenesis was done on template pScer-Ro11, a *S. cerevisiae* expression plasmid carrying the *A. niger* T213 phytase gene [9]. Mutations resulting in the amino acid exchanges E89D, H292N, R297Q and combinations thereof were introduced using the 'quick exchange™ site-directed mutagenesis kit' from Stratagene (La Jolla, CA, USA) according to the manufacturer's instructions. The following sense and antisense primers were used: mutation E89D (sense primer 5′-GCGGTATCCGACCGACTCCAAGGGCAAGAAA-3′, antisense primer 5′-TTTCTTGCCCTTGGAGTCGGTCGGATACCGC-3′); mutation H292N (sense primer 5′-CATGACGAATGGATCAACTACGACTACCTCC-3′, antisense primer 5′-GGAGGTAGTCGTAGTTGATCCATTCGTCATG-3′); mutation R297Q (sense primer 5′-CTACGACTACCTCCAGTCCCTGAAAAAA-3′, antisense primer 5′-TTTTTTCAGGGACTGGAGGTAGTCGTAG-3′). The changed nucleotide causing the desired mutation is underlined. Plasmids harboring the respective mutation were verified by DNA sequence analysis using the ABI Prism Dye Terminator cycle sequencing kit and the ABI Prism 310 genetic analyzer following protocols provided by Applied Biosystems (Warrington, UK).

S. cerevisiae YMR4 [9] was used as transformation host. Individual transformants were grown initially for 1–2 days in minimal medium before being transferred to YPD medium for further growth.

2.3. Measurements of enzymatic activity

Phytase activity (or other phosphatase activities) was measured in an assay mixture containing 5 mM phytic acid (or other phosphate

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compounds), 200 mM sodium acetate, pH 5.0, as previously described [9,10]. pH activity profiles were determined at a phytic acid concentration of 5 mM, also as described before [10]. One unit of phytase (or acid phosphatase, glucose 6-phosphatase, etc.) activity catalyzes the liberation of 1 μmol of inorganic phosphate per minute at 37°C.

2.4. Other methods

Protein concentrations were calculated from the OD at 280 nm, using theoretical absorption values calculated from the known protein sequences with the DNA* software (DNASTAR, Madison, WI, USA). An absorption of 1 Å at 280 nm corresponds to 1.03 mg/ml of *A. niger* NRRL 3135 phytase and to 1.04 mg/ml of *A. niger* T213 phytase.

3. Results

3.1. Amino acid sequences of A. niger wild-type phytases, and location of the divergent residues in the 3D structure

The numbering of the amino acid residues begins with the initiating Met, thus includes the signal sequence, and is therefore shifted by 23 residues (+23) relative to the numbering used in our previous article on the crystal structure of *A. niger* NRRL 3135 phytase [11]. *A. niger* T213 phytase differs from *A. niger* NRRL 3135 phytase [12] at 12 amino acid residues: S14A (S in NRRL 3135, A in T213), S30T, E66D, D89E, A106V, V155I, K171E, V236A, N292H, Q297R, S345N, V438I [5]. In addition, *A. niger* T213 phytase differs at only two amino acid residues (Q297R, S466F) from the phytase of *A. niger* var. *awamori* (ATCC 38854) [13]. Residue 14 is located in the signal sequence that is cleaved off upon secretion of the protein and can therefore not account for the observed differences in catalytic properties. The remaining 11 divergent residues are distributed throughout the sequence and are exposed on the surface of the protein (Fig. 1). Only three divergent residues are within (No. 297) or close to the active site (No. 89 and 292) which is located at the interface between the larger α/β domain (lower half of Fig. 1) and the smaller α domain of the molecule (upper half). The impact of changes at the latter three residues on the catalytic properties was tested by site-directed mutagenesis (see below).

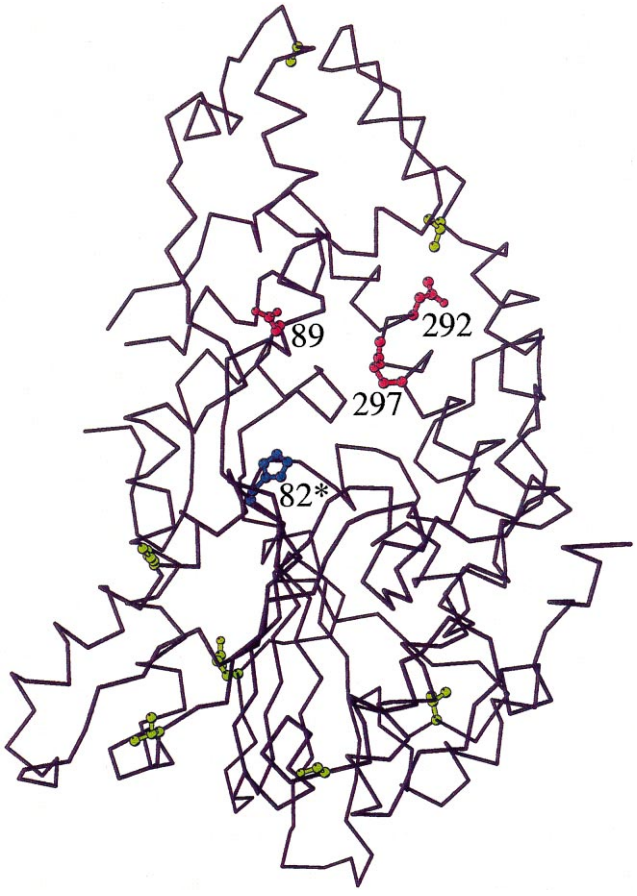

Fig. 1. Location of the residues in the 3D structure of *A. niger* NRRL 3135 phytase that differ from the respective residues in *A. niger* T213 phytase (represented in green and red). The three divergent residues located in or close to the active site (Asn-89, Asn-292 and Gln-297 in the *A. niger* NRRL 3135 phytase shown; Glu-89, His-292 and Arg-297 in *A. niger* T213 phytase, respectively) are numbered and shown in red. In addition, the catalytically active His-82 is marked with a star.

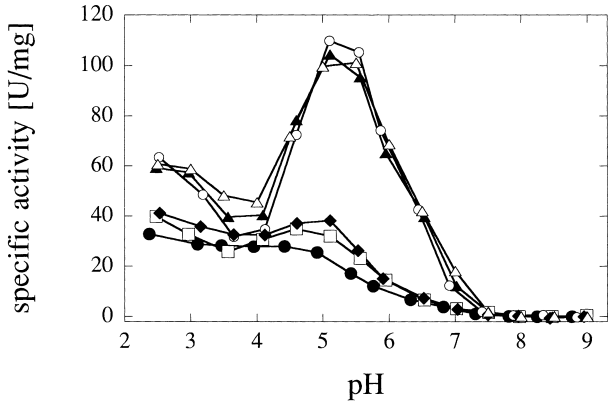

Fig. 2. pH activity profiles of *A. niger* NRRL 3135 phytase (△), *A. niger* T213 phytase (□), and the single mutants R297Q (▲), E89D (●) and H292N (♦) as well as the triple mutant E89D, H292N, R297Q (○) of *A. niger* T213 phytase.

3.2. Catalytic properties of A. niger wild-type phytases and site-directed mutants

The specific activity at pH 5.0 was three-fold lower for *A. niger* T213 phytase than for *A. niger* NRRL 3135 phytase (Table 1). Investigation of the three single mutants (E89D, H292N, and R297Q), two double mutants and the triple mutant revealed that a three-fold increase in specific activity from approx. 30 to 90 U/mg protein is invariably linked to the R297Q mutation.

Two pH optima, at 2.5 and 5.0–5.5, were observed for *A. niger* NRRL 3135 phytase (Fig. 2), as previously reported [2,10,14]. In *A. niger* T213 phytase, activity was depressed primarily between pH 4.5 and 7.0, and to a lesser extent between pH 2.5 and 3.0, with the result that the two pH

Table 1
Specific activities of *A. niger* wild-type phytases and mutant proteins at pH 5.0

Protein	U/mg (mean ± S.D.)	*n*
A. niger NRRL 3135 phytase	102.5 ± 19.9	8
A. niger T213 phytase	32.0 ± 4.7	15
A. niger T213 E89D	26.2 ± 2.6	6
A. niger T213 H292N	31.7 ± 4.7	6
A. niger T213 R297Q	91.5 ± 13.6	6
A. niger T213 E89D, R297Q	84.2 ± 4.1	5
A. niger T213 H292N, R297Q	96.8 ± 14.2	6
A. niger T213 E89D, H292N, R297Q	97.3 ± 18.1	6

n = number of measurements.

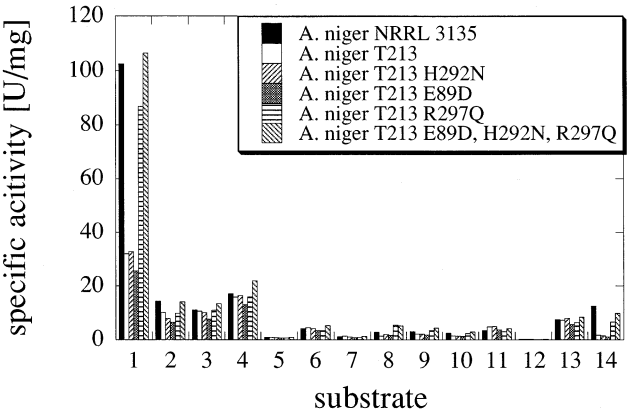

Fig. 3. Substrate specificities of *A. niger* NRRL 3135 phytase, *A. niger* T213 phytase, and the single mutants R297Q, E89D and H292N as well as the triple mutant E89D, H292N, R297Q of *A. niger* T213 phytase. (1) phytic acid; (2) *p*-nitrophenyl phosphate; (3) phenyl phosphate; (4) fructose 1,6-bisphosphate; (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.

optima could no longer be clearly distinguished. Again, the R297Q mutation is responsible for most, if not all, of the difference between the *A. niger* T213 and NRRL 3135 phytases over the whole pH range tested.

With regard to substrate specificity, the specific activities of the wild-type and mutant phytases were tested with 5 mM concentrations of 14 different phosphate compounds (phytic acid, 'classical' acid phosphatase substrates like phenyl phosphate or *p*-nitrophenyl phosphate, sugar phosphates, glycerophosphates, adenine nucleotide phosphates, etc.; Fig. 3). The specific activities with only two large and strongly negatively charged substrates – phytic acid and ATP – differed between *A. niger* T213 and *A. niger* NRRL 3135 phytase. Once more, the R297Q mutation alone fully accounted for the differences observed.

Finally, time course experiments with excess phytase and limiting substrate concentrations (phytic acid; *myo*-inositol-1-monophosphate; and *myo*-inositol-2-monophosphate) confirmed that *A. niger* T213 phytase – identical to *A. niger* NRRL 3135 and other fungal phytases [10] – is able to liberate five of the six phosphate groups of phytic acid, and that, most likely, *myo*-inositol-2-monophosphate is the end product of phytic acid degradation (data not shown).

4. Discussion

The identification of residues that critically determine the catalytic properties is of prime interest when attempting to optimize an enzyme for a particular application. Different approaches are available, for instance (i) random mutagenesis, followed by screening for the property of interest (e.g. high specific activity, activity at low pH); (ii) determination of the 3D structure of the enzyme – preferably complexed with its substrate – and structure-based identification of the residues involved in substrate binding and/or catalysis; or (iii) comparison of the sequences of highly homologous proteins with distinct differences in catalytic properties, and identification of the divergent residues responsible for those differences by site-directed mutagenesis.

We combined approaches (ii) and (iii). The 3D structure of *A. niger* NRRL 3135 phytase had previously been solved at 2.5 Å resolution [11]. In addition, the wild-type *A. niger* phytases from strains NRRL 3135 and T213, which differ at only 12 amino acid residues, displayed a three-fold difference in specific activity (103 versus 32 U/mg protein). Nine of the divergent residues are distant from the active site and unlikely to influence the catalytic properties of the enzyme. Of the remaining three residues, the R297Q mutation was found to (almost) fully account for the observed differences in catalytic properties.

Molecular modelling shows that the guanidino group of R297 of *A. niger* T213 phytase can be positioned in such a way as to allow ionic interaction with a phosphate group of the substrate and/or product, while the side chain of Q297 of *A. niger* NRRL 3135 phytase is too short to establish a direct interaction (data not shown). Consequently, R297 may cause stronger substrate and product binding and thereby reduce the rate of product release during catalysis. Since R297 is associated with a lower specific activity with phytic acid as substrate (but not with a variety of smaller and/or less negatively charged phosphate compounds), we hypothesize that product (*myo*-inositol pentakisphosphate) release is the rate-limiting step of the reaction. This hypothesis requires further experimental support, since (i) the neighboring side chains of Q50, Y51 and K300 require structural readjustments in order to allow R297 establishment of a salt bridge with phytic acid, and (ii) the binding mode of phytic acid in *A. niger* phytase is hypothetical rather than proven by X-ray crystallography [11]. Just very recently, the crystal structure of *Escherichia coli* phytase in complex with phytic acid has been solved [15]. Despite almost no homology at the level of amino acid sequence between *A. niger* and *E. coli* phytase, the two proteins display a very similar overall 3D structure. In particular, the hypothetical binding mode of phytate to *A. niger* phytase [11] closely resembles the actual binding of phytate to *E. coli* phytase [15], thereby supporting our hypothesis of a direct interaction between R297 and phytic acid.

Although different titratable groups may be responsible for the two pH optima of *A. niger* NRRL 3135 phytase at 2.5 and 5.0–5.5 (with phytic acid as substrate; Fig. 2), the fact that both peaks were affected by the R297Q mutation indicates that product release may be the rate-limiting step of the reaction at both pH 2.5–3.0 and 4.5–7.0. On the other hand, between pH 3.5 and 4.0 where almost no differences were seen, another step of the catalytic cycle may become rate-limiting. It remains a challenge for the future to find an explanation for this particular behavior.

With regard to the rate-limiting step of the reaction, the findings of the present experiments differ from previous reports on other high molecular weight acid phosphatases or alkaline phosphatases. For both human prostatic acid phosphatase and *E. coli* acid phosphatase (= phytase; AppA) tested with *p*-nitrophenyl phosphate as substrate, cleavage of the covalent phosphohistidine intermediate was suggested to be the rate-limiting step [16,17]. Site-directed mutagenesis of D304 of *E. coli* AppA to Ala yielded a variant where *formation* of the phosphoenzyme intermediate became rate-limiting [16]. Finally, for *E. coli* alkaline phosphatase, the rate-determining step is pH-dependent: at acidic pH, the hydrolysis of the covalent phosphoenzyme intermediate is rate-limiting, while at alkaline pH, the release of phosphate from the non-

covalent enzyme–phosphate complex becomes rate-limiting (see [18]). These different findings probably reflect the fact that a highly charged, hydrophilic, bulky molecule like phytic acid interacts differently with a given phosphatase than does a smaller, more hydrophobic molecule like *p*-nitrophenyl phosphate.

Except for ATP, virtually no differences in specific activities were observed for the other phosphate compounds tested (Fig. 3), probably because either (i) they are smaller than phytic acid and therefore are unable to interact simultaneously with R297 (or Q297) and the presumed catalytically active H82 (of the RHGXRXP motif that is typical for high molecular weight acid phosphatases [17,19]), or (ii) they lack – besides the phosphate group that binds to the catalytically active H82 – additional charges in a suitable distance to the phosphate group that might allow electrostatic interaction with R297.

Now that position 297 has been identified as critically influencing the catalytic properties, is there further variability at this position in other wild-type phytase sequences? The phytases from *Aspergillus fumigatus*, *Aspergillus nidulans* and *Myceliophthora thermophila*, with specific activities of 23–42 U/mg protein, have a Gln at position 297, while the *Aspergillus terreus* 9A1 and CBS phytases (142 and 196 U/mg protein) have a Leu and *Thermomyces lanoginosus* phytase has a Tyr (110 U/mg protein at pH 6.0) [10,20]. It will therefore be interesting to see what impact the R297L, R297Y or other mutations at this position have on the specific activity of *A. niger* T213 phytase. For comparison, in *A. fumigatus* phytase having a Gln at position 297, the Q297L mutation slightly increased the specific activity, from 23.4 ± 3.6 ($n = 3$) to 31.6 ± 3.9 U/mg protein ($n = 5$) for preparations purified from *S. cerevisiae* culture supernatants.

In conclusion, the combination of (i) correlating amino acid sequence differences with differences in catalytic properties, and (ii) narrowing of the candidate amino acids responsible for these differences by inspection of the 3D structure of the protein has proven successful in the present model example for quickly identifying the amino acid exchange responsible for a three-fold difference in specific activity between two *A. niger* wild-type phytases. This general approach is also applicable to more distantly related proteins. For example, based on a comparison of *A. fumigatus* and *A. terreus* phytase amino acid sequences and catalytic properties, we have recently managed to increase the specific activity of *A. fumigatus* phytase by up to seven-fold [21]. Since the latter was achieved through site-directed mutagenesis at position 50, position 297 is – as expected – not unique in having a critical impact on the specific activity of fungal phytases.

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