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Exchanging the active site between phytases for altering the functional properties of the enzyme

MARTIN LEHMANN, RUAL LOPEZ-ULIBARRI, CLAUDIA LOCH, CÉLINE VIAROUGE, MARKUS WYSS, AND ADOLPHUS P.G.M. VAN LOON

F. Hoffmann-La Roche Ltd., Vitamins and Fine Chemicals Division, 4070 Basel, Switzerland

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Abstract

By using a novel consensus approach, we have previously managed to generate a fully synthetic phytase, consensus phytase-1, that was 15–26 °C more thermostable than the parent fungal phytases used in its design (Lehmann et al., 2000). We now sought to use the backbone of consensus phytase-1 and to modify its catalytic properties. This was done by replacing a considerable part of the active site (i.e., all the divergent residues) with the corresponding residues of *Aspergillus niger* NRRL 3135 phytase, which displays pronounced differences in specific activity, substrate specificity, and pH-activity profile. For the new protein termed consensus phytase-7, a major—although not complete—shift in catalytic properties was observed, demonstrating that rational transfer of favorable catalytic properties from one phytase to another is possible by using this approach. Although the exchange of the active site was associated with a 7.6 °C decrease in unfolding temperature (T_m) as measured by differential scanning calorimetry, consensus phytase-7 still was >7 °C more thermostable than all wild-type ascomycete phytases known to date. Thus, combination of the consensus approach with the selection of a "preferred" active site allows the design of a thermostabilized variant of an enzyme family of interest that (most closely) matches the most favorable catalytic properties found among its family members.

Keywords: acid phosphatase; active site; consensus protein; expression system; *Hansenula polymorpha*; heterologous expression; phytase; protein engineering; yeast

Phytase (= *myo*-inositol hexakisphosphate phosphohydrolase, EC 3.1.3.8 and 3.1.3.26) catalyzes phosphomonoester cleavage of phytic acid (= *myo*-inositol hexakisphosphate), the major storage form of phosphorus in plant seeds (Wodzinski & Ullah, 1996), and thereby liberates inorganic phosphate. Since monogastric animals virtually lack phytase activity in the digestive tract, and since plant phytases are normally inactivated during feed processing (in particular, due to the high temperatures of 65–95 °C reached in feed pelleting), phytic acid phosphorus is largely unavailable to these animals. This problem may be circumvented by addition of a thermostable phytase to pig or poultry feed.

Recently, we have used a novel consensus approach to increase the intrinsic thermostability of fungal phytases (Lehmann et al., 2000). The sequences of 13 wild-type ascomycete phytases were aligned to calculate a consensus amino acid sequence, which was back-translated into a DNA sequence. The consensus phytase-1 gene was generated subsequently from overlapping, synthetic 85 bp-oligonucleotides using the method of extensive overlap extension by polymerase chain reaction (PCR). Surprisingly, consensus phytase-1 was 15–26 °C more thermostable than the parent phytases used in its design.

Aspergillus niger NRRL 3135 phytase has catalytic properties that are distinctly different from those of consensus phytase-1, and it is the only phytase known so far that displays two pH optima (2.5 and 5.0–5.5, respectively; Howson & Davis, 1983; Ullah & Gibson, 1987; Sandberg et al., 1996; Wyss et al., 1999a). In the present work, we attempted to combine the higher thermostability of consensus phytase-1 with the particular catalytic properties of *A. niger* NRRL 3135 phytase.

Engineering of catalytic properties is still an enigmatic task, since no easily applicable concepts are available on how to *rationally* modify a given catalytic property of interest (e.g., pH-activity dependence, substrate specificity, specific activity, K_m, or enantioselectivity). Consequently, optimization of catalytic properties has been approached in the past mostly on a trial-and-error basis: (1) random mutagenesis does not require information on the three-dimensional (3D) structure or the reaction mechanism of an enzyme; however, the chance of success is rather low, so that many mutants have to be screened in order to find the (rare) improved ones. More recently, technological advances have paved the way for several "directed evolution" approaches, i.e., rapid, iterative processes of mutation and/or recombination of genes and selection or screening for improved protein variants (Stemmer, 1994a, 1994b;

Reprint requests to: Martin Lehmann, Roche Vitamins Inc., Bldg. 102, 340 Kingsland Street, Nutley, New Jersey 07110; e-mail: martin.lehmann@roche.com.

Crameri et al., 1998; Shao et al., 1998; Zhao et al., 1998). Although these directed evolution strategies (a) access a much larger proportion of the sequence space than the more conventional mutagenesis approaches; (b) may therefore be more powerful for the systematic engineering of catalytic properties (e.g., Kuchner & Arnold, 1997; Zhang et al., 1997); and (c) allow, in a single step, optimization of two or more properties of an enzyme (Ness et al., 1999), they heavily depend on appropriate equipment and on efficient screening assays that are not necessarily available or not even feasible for all enzymes and all catalytic properties of interest. (2) Site-directed mutagenesis of amino acid residues of the active site that are involved in substrate binding and/or catalysis has resulted in some impressive alterations and/or improvements in catalytic properties (e.g., Wilks et al., 1988; Hayashi et al., 1989; Harford-Cross et al., 2000), but still, the accuracy of prediction of a successful amino acid exchange is modest, and the approach depends on a more detailed knowledge of enzyme structure and function.

In this paper, to our knowledge, we describe for the first time the simultaneous exchange of *all* divergent active site residues of an enzyme with the respective residues of a homologous enzyme with distinctly different catalytic properties. By doing so, the catalytic properties of the "donor" enzyme (i.e., *A. niger* NRRL 3135 phytase) were almost entirely transferred to the "recipient" enzyme (i.e., consensus phytase-1).

Results and discussion

By exchanging solely amino acids within or immediately adjacent to the active site (Fig. 1), we intended to preserve the high intrinsic thermostability of the parent consensus phytase-1 that was used as scaffold, and to combine it with the catalytic properties (in particular its activity at low pH) of *A. niger* NRRL 3135 phytase from which the active site residues of consensus phytase-7 were derived.

As shown by the pH-activity profiles (Figs. 2, 3), substrate specificities (Fig. 4), as well as the specific activities, at pH 5.0, with phytic acid as substrate of 102.5 ± 19.9 U (mg protein)$^{-1}$ for *A. niger* NRRL 3135 phytase ($n = 8$), 44.1 ± 4.1 U (mg protein)$^{-1}$ for consensus phytase-1 ($n = 12$), and 63.7 ± 7.2 U (mg protein)$^{-1}$ for consensus phytase-7 ($n = 11$), our approach was largely successful. Although the shape of the pH-activity profiles of consensus phytase-7 and *A. niger* NRRL 3135 phytase is almost identical, the specific activity is lower over the entire pH range for consensus phytase-7 (Figs. 2, 3). This may suggest that (1) not all divergent residues (between *A. niger* NRRL 3135 and consensus phytase-1) that have an influence on the catalytic properties have been exchanged, or that (2) the higher intrinsic thermostability of consensus phytase-7 as compared to *A. niger* NRRL 3135 phytase (see below) is associated with a higher rigidity of the active site, thereby decreasing the specific activity. While the pH-activity profiles favor option (2), the substrate specificities—with higher specific activities of consensus phytase-7 as compared to *A. niger* NRRL 3135 phytase with most of the other phosphate compounds tested (Fig. 4)—are more in line with option (1). Clearly, more detailed investigations will be required to discriminate between these two possibilities.

Several researchers, including our own group, found two pH optima at 2.2–2.5 and 5.0–5.5 for *A. niger* phytase (see Howson & Davis, 1983; Ullah & Gibson, 1987; Sandberg et al., 1996; Wyss et al., 1999a; Tomschy et al., 2000; Figs. 2, 3). In all these studies, acetate, borax-succinate, or no buffers have been used in the pH range 3.5–4.5 where a local minimum in specific activity is seen. When citrate was used as buffer substance (Fig. 3), the local activity minimum at pH 4.0 was no longer observed. Instead, the two peaks fused to give a single pH optimum. One obvious interpretation might be inhibition of *A. niger* NRRL 3135 phytase and consensus phytase-7 around pH 4.0 by acetic acid or acetate ions. Very much to the contrary, however, acetate in the concentration range 10–400 mM had only a relatively minor influence on the specific activity of these proteins at pH 4.0 (consensus phytase-7: 25.5 U/mg at 10 mM, 27.4 U/mg at 400 mM; *A. niger* NRRL 3135 phytase: 44.1 U/mg at 10 mM, 44.4 U/mg at 400 mM), while citrate strongly increased the specific activity of both *A. niger* NRRL 3135 phytase and consensus phytase-7 at pH 4.0 (from 56.6 and 32.5 U/mg at 10 mM to 85.3 and 67.8 U/mg at 300 mM, respectively). In comparison, stimulation of activity of consensus phytase-1 around pH 4.0 was less pronounced and seemed to have no impact on the location of the single pH optimum (Fig. 3). As a working hypothesis, these results might indicate that (1) in both *A. niger* NRRL 3135 phytase and consensus phytase-7, a reaction step becomes rate-limiting around pH 4.0 that is different from the rate-limiting step(s) below pH 2.5 and above pH 4.5–5.0, and that

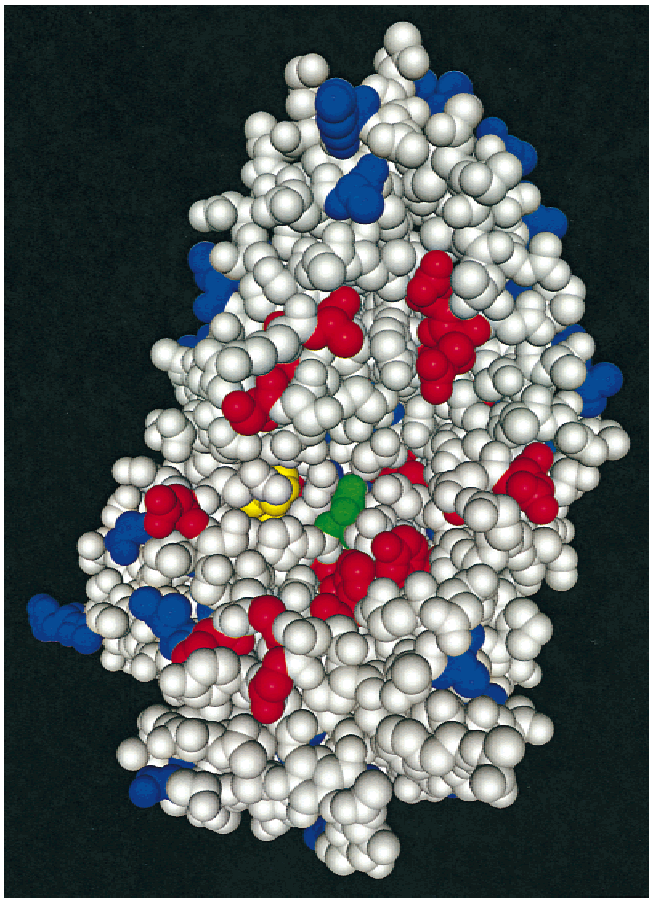

Fig. 1. Space-filling structural model of consensus phytase-1, showing the location of the active site. Amino acids that are identical between consensus phytase-1 and *A. niger* NRRL 3135 phytase are shown in white; amino acids that differ between the two phytases but were not exchanged in consensus phytase-7 are shown in blue; and amino acids that differ and were exchanged are shown in red. The catalytically active His59 is shown in yellow, and Glu339 is green.

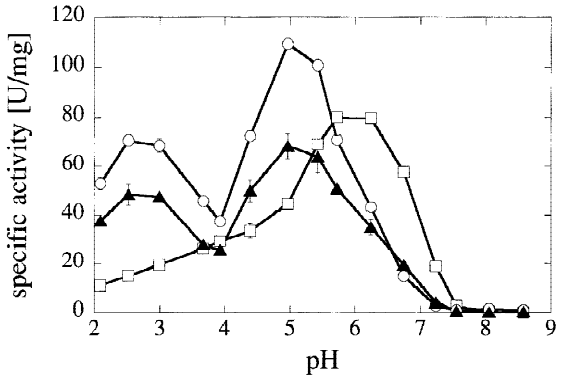

Fig. 2. pH-activity profiles of *A. niger* NRRL 3135 phytase (○), consensus phytase-1 (□), and consensus phytase-7 (▲). The activity was measured at the respective pH values at a phytic acid concentration of 5 mM as previously described (Wyss et al., 1999a). The data represent the means ± SD of 3–4 measurements.

(2) citrate somehow offsets this rate limitation. Possibly, as postulated for *A. niger* T213 phytase (Tomschy et al., 2000), release of the highly negatively charged product, *myo*-inositol pentakisphosphate, becomes rate-limiting, and citrate, in contrast to the less negatively charged acetate, helps to displace the product from the active site. Only more careful analysis of the reaction mechanism(s) of several phytases will allow definite conclusions on this issue.

Although the exchange of the active site was associated with a decrease in intrinsic thermostability relative to consensus phytase-1, consensus phytase-7 still was >7 °C more thermostable than all fungal phytases used in the design of consensus phytase-1 (Lehmann et al., 2000). Differential scanning calorimetry yielded an unfolding temperature (T_m) of 63.3 °C for *A. niger* NRRL 3135 phytase, 78.0 °C for consensus phytase-1 and 70.4 °C for consensus phytase-7 (Fig. 5). These findings are not unexpected, since in the design of consensus phytase-7, possible impacts of the amino acid exchanges on favorable, stabilizing interactions were not taken into account. Since (1) not only "true" active site residues, but also amino acids next to active site residues were exchanged (because they might have an indirect effect on the catalytic properties of the enzyme; see Fig. 1), and since (2) even in the active site, some amino acid replacements may have decreased thermostability while having no effect on the catalytic properties, more site-directed mutagenesis work will be required to more clearly define the contributions of individual residues that are located in or close to the active site to phytase thermostability and activity.

For production of phytase on an industrial scale, we have optimized an alternative yeast expression system, *Hansenula polymorpha*, for heterologous protein production, yielding expression levels of up to 8.5 g per liter culture supernatant of *Aspergillus fumigatus* phytase, and 13.5 g per liter culture supernatant of consensus phytase-1 within a fermentation time of 160–168 h (Mayer et al., 1999). With the new consensus phytase-7 construct, expression levels on a gram per liter scale were reached within 124 h by using a faster feed profile (not shown), thus confirming the potential of this expression system for heterologous protein production. Densitometric analysis of SDS-polyacrylamide gels showed that consensus phytase-7 corresponds to about 97% of the total protein present in the culture supernatant.

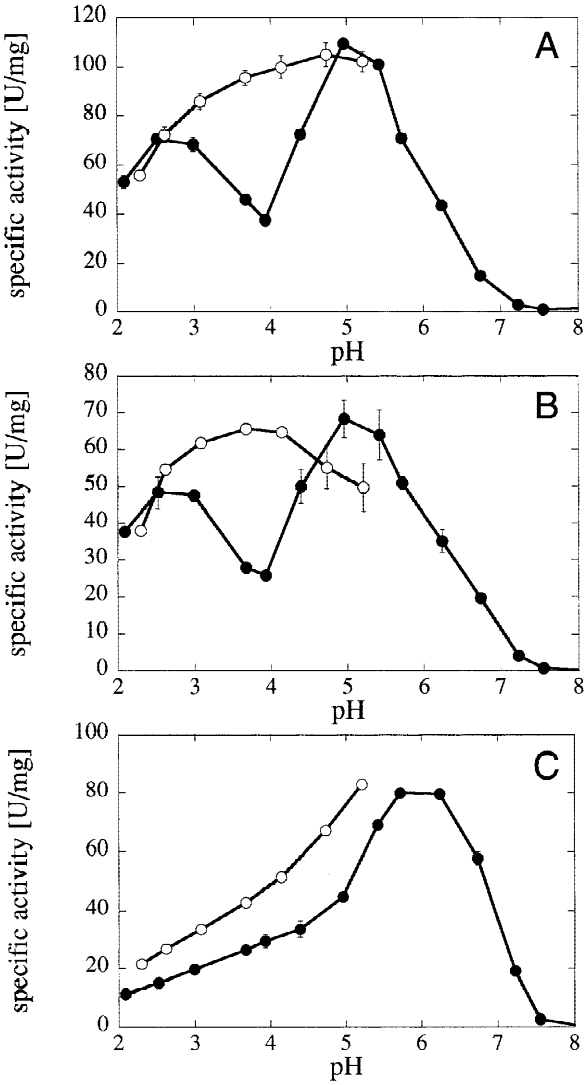

Fig. 3. Impact of buffer composition on the pH-activity profiles of **(A)** *A. niger* NRRL 3135 phytase, **(B)** consensus phytase-7, and **(C)** consensus phytase-1. (●) pH-Activity profiles determined by using the standard buffers (see Wyss et al., 1999a): 0.2 M (end concentration) glycine for pH 2.5; 0.2 M acetate for pH 3.0–5.5; 0.2 M imidazole for pH 6.0–7.0; 0.2 M Tris for pH 7.5–9.0. (○) pH-Activity profiles determined in 0.2 M citrate buffer, pH 2.3–5.2. The data represent the means ± SD of 3–9 measurements.

In conclusion, the results obtained show that the catalytic properties of consensus phytase-1 can be changed to a large extent to those of *A. niger* NRRL 3135 phytase by exchanging all divergent active site residues of consensus phytase-1 with the respective amino acids of *A. niger* phytase. More generally, it now seems possible to increase the intrinsic thermostability of an enzyme of interest having favorable catalytic properties by leaving the entire active site unchanged, and by applying the consensus approach for thermostability engineering of proteins (Lehmann et al., 2000) to the remaining parts of the molecule. For this approach to be applicable, a set of homologous amino acid sequences must be known, together with—among this set of homologous enzymes—at least one member with favorable catalytic properties, and one member for which the 3D structure is available. Given the steadily increasing amount of information on (wild-type and mutant) enzyme se-

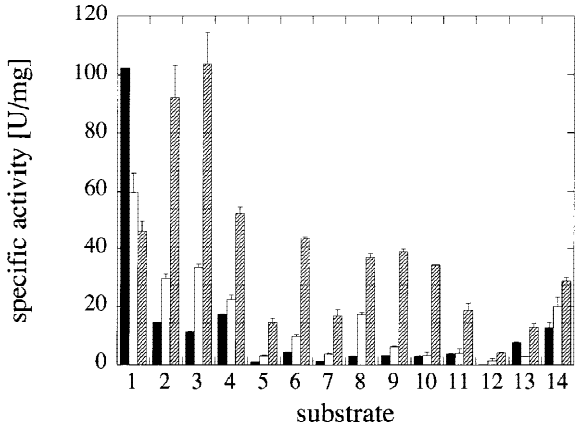

Fig. 4. Substrate specificity of *A. niger* NRRL 3135 phytase (■), consensus phytase-1 (hatched bars) and consensus phytase-7 (□). The specific activities with 5 mM concentrations of a series of phosphate compounds were determined at pH 5.0 as previously described (Wyss et al., 1999a). (1) Phytic acid; (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. The data represent the means ± SD of three measurements.

quences and catalytic properties, as well as the ease of constructing synthetic genes, the present approach may be seen as a complementary tool for designing improved biocatalysts.

Materials and methods

Materials

Phytic acid (dodecasodium salt) was from Sigma and p-nitrophenyl phosphate from Merck. *A. niger* NRRL 3135 phytase, which is sold under the tradename NATUPHOS, was purchased from BASF and purified as previously described (Wyss et al., 1999b). Consensus phytase-1 was designed, overexpressed in *H. polymorpha* and purified as also reported previously (Lehmann et al., 2000).

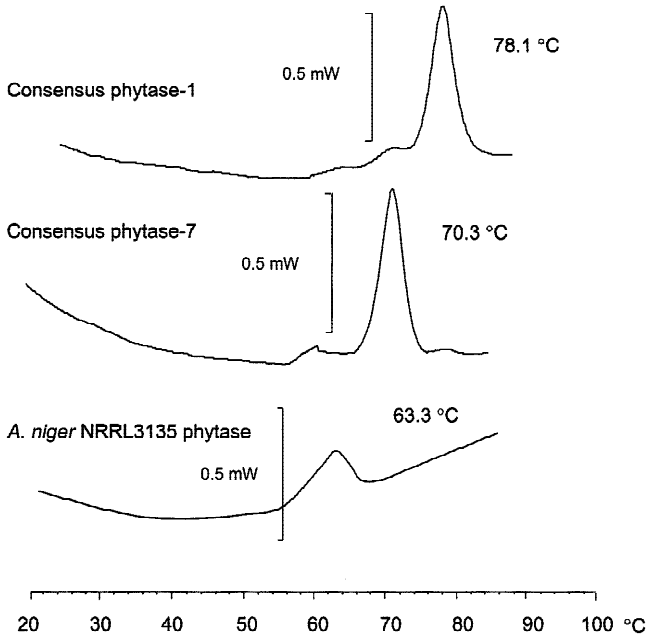

Fig. 5. Differential scanning calorimetry of *A. niger* NRRL 3135 phytase, consensus phytase-7, and consensus phytase-1. Individual, representative scans are shown.

Consensus phytase-7 gene construction and protein purification

Phytase active site residues were identified on the basis of the 3D structures of *A. niger* NRRL 3135 phytase (Kostrewa et al., 1997) and consensus phytase-1 (Lehmann et al., 2000), and have recently been confirmed by the structure of *Escherichia coli* phytase in its complex with phytic acid (Lim et al., 2000). Active site residues of consensus phytase-1 that are supposed to be in direct contact with the substrate, phytic acid, and those immediately adjacent were replaced—if different—by the corresponding amino acids of *A. niger* NRRL 3135 phytase (S89D, S92G, A94K, D164S, P201S, G203A, G205S, H212P, G224A, D226T, E255T, D256E, V258T, P265S, Q292H, G300K, Y305H, A314T, S364G, M365I, A397S, S398A, G404A, and A405S; Fig. 6). In the case of residue 292, we preferred to use a His present in both *A. niger awamori* (Piddington et al., 1993) and *A. niger* T213 phytase (Mitchell et al., 1997) rather than an Asn as occurring in *A. niger* NRRL 3135 phytase (van Hartingsveldt et al., 1993). The codon frequency table of highly expressed *Saccharomyces cerevisiae* genes (GCG package, release 9.0 [Devereux et al., 1984]) was used to design, based on the modified amino acid sequence, the DNA sequence of the mature protein. The DNA sequence for the signal peptide, which was taken from *Aspergillus terreus* CBS phytase (van Loon et al., 2000), was tailored for expression in yeast according to Purvis et al. (1987).

The synthetic consensus phytase-7 gene (*fcp7*) was generated by dividing the calculated DNA sequence into oligonucleotides of 85 bp each (purchased from Microsynth, Balgach, Switzerland). Every oligonucleotide overlapped 20 bp with the previous and the following oligonucleotide of the opposite strand as shown in Figure 7. The PAGE-purified oligonucleotides were assembled to the complete *fcp7* gene by three PCRs using the following oligonucleotide mixes (0.1 μM of each oligonucleotide): Mix 1.7: CP-1, CP-2, CP-3, CP-4.7, CP-5.7, CP-6, CP-7, CP-8.7, CP-9, CP-10.7; Mix 2.7: CP-9, CP-10.7, CP-11.7, CP-12.7, CP-13.7, CP-14.7, CP-15.7, CP-16, CP-17.7, CP-18.7, CP-19.7, CP-20, CP-21, CP-22. In PCRs *a* and *b*, the oligonucleotides of mix 1.7 (1 pmol of each oligonucleotide) and mix 2.7 were each assembled to a DNA fragment using the primer pair CP-a (5′-TATAT*GAATTC*ATGGG CGTGTTCGTC-3′) and CP-c.7 (5′-CATGTCCATCAAGTAAG TAACTTCAG-3′) for PCR *a* and the primer pair CP-b (5′-TGAAAAGTTCATTGAAGGTTTC-3′) and CP-e (5′-TATAT *GAATTC*TTAAGCGAAAC-3′) for PCR *b*, respectively, under the following conditions: step 1: 2 min, 45 °C; step 2: 30 s, 72 °C; step 3: 30 s, 94 °C; step 4: 30 s, 52 °C; step 5: 1 min, 72 °C. Steps 3 to 5 were repeated 40 times. The PCR products (670 and 905 bp in length) were purified by 0.9% agarose gel electrophoresis, followed by gel extraction (QIAEX II Gel Extraction Kit, Qiagen, Hilden, Germany). The purified DNA fragments were used for PCR reaction *c* (step 1: 2 min, 94 °C; step 2: 30 s, 94 °C; step 3: 30 s, 55 °C; step 4: 1 min, 72 °C). Steps 2 to 4 were repeated 31 times. The PCR product was again purified by agarose gel electrophoresis, eluted from the gel, and *Eco*RI-digested. The *fcp7* gene, prepared in this way, was cloned into the *Eco*RI site of the expression vector pFP (Gellissen et al., 1991) downstream of the

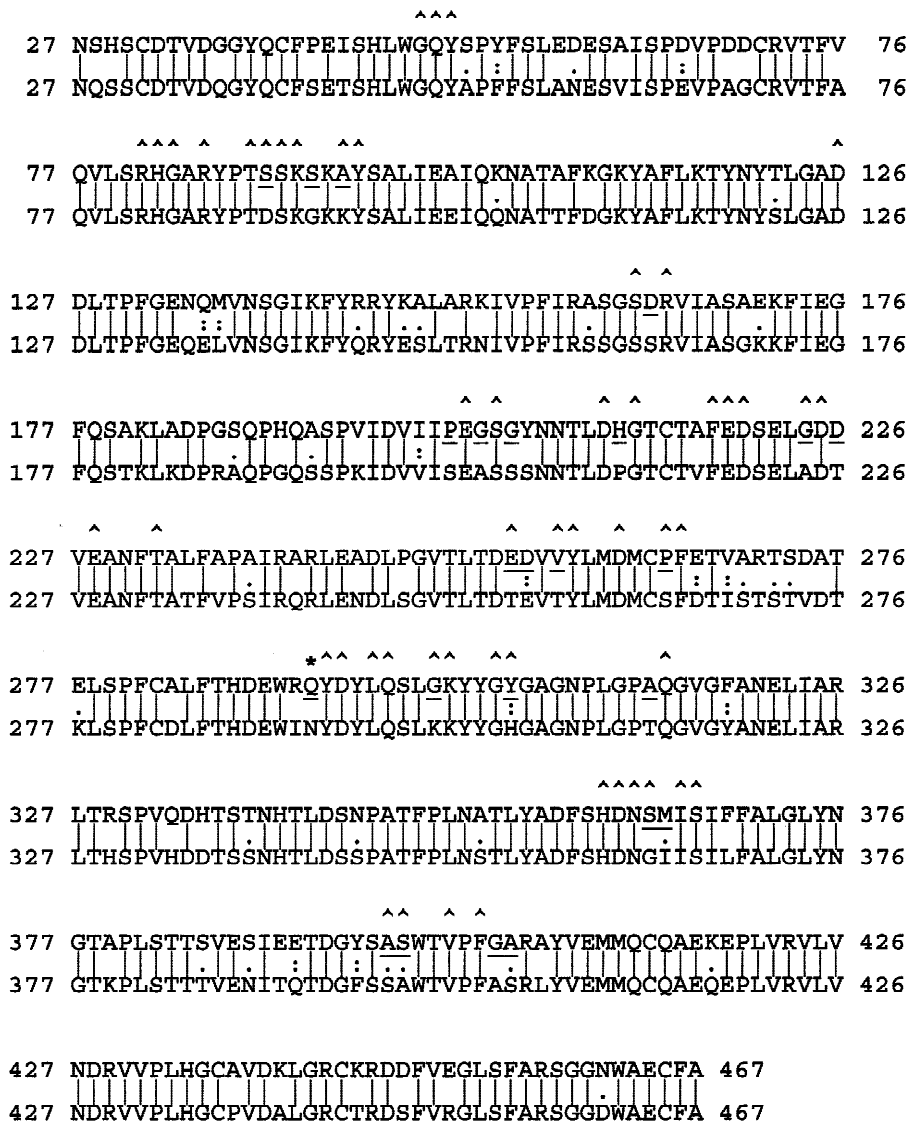

Fig. 6. Alignment of the amino acid sequences of mature consensus phytase-1 and *A. niger* NRRL 3135 phytase using the program GAP (which is part of the program package GCG 9.1) with standard parameters. Identical amino acids are marked by a vertical line in between the two sequences; homologous, but nonidentical residues are marked by a colon or a single dot, depending on the degree of homology of the aligned residues. Amino acids that are underlined in the consensus phytase-1 sequence (upper sequence) were replaced by the corresponding *A. niger* NRRL 3135 phytase residue below. The symbol "^" marks residues that are part of the (presumed) active site of the two phytases. Residue 292, which is marked by a star, was not replaced by Asn as found at this position in *A. niger* NRRL 3135 phytase, but by the corresponding amino acid, His, found in *A. niger* T213 (Mitchell et al., 1997) and *A. niger awamori* phytase (Piddington et al., 1993).

formate dehydrogenase promoter (Hollenberg & Janowicz, 1989). The resulting plasmids were transformed into *H. polymorpha* strain RB11 (*ura*$^-$). Transformation of *H. polymorpha* and expression of consensus phytase-7 were performed as described elsewhere (Gellissen et al., 1996). All other DNA manipulations were performed according to standard procedures (Sambrook et al., 1989). DNA sequencing of double-stranded DNA was done by the dideoxy method (Sanger et al., 1977) using the ABI PRISM Dye Terminator Cycle Sequencing Kit and the ABI PRISM 310 genetic analyzer as recommended by the manufacturer (Applied Biosystems, Warrington, Great Britain).

Consensus phytase-7 fermentation supernatants were sterile-filtered, desalted against 10 mM sodium acetate, pH 5.0, and subjected to anion exchange chromatography as described previously for other phytases expressed in *H. polymorpha* (Wyss et al., 1999ab).

Measurements of enzymatic activity

Measurements of specific activity with 5 mM concentrations of phytic acid or of a series of other phosphate compounds (phenyl phosphate, *p*-nitrophenyl phosphate, sugar phosphates, glycerophosphates, adenine nucleotide phosphates, etc.) and of the pH-dependency of enzymatic activity with phytic acid as substrate were performed as described previously (Wyss et al., 1999a). In a second set of experiments, glycine/HCl and sodium acetate buffers were replaced by 0.2 M sodium citrate buffer, and pH-activity

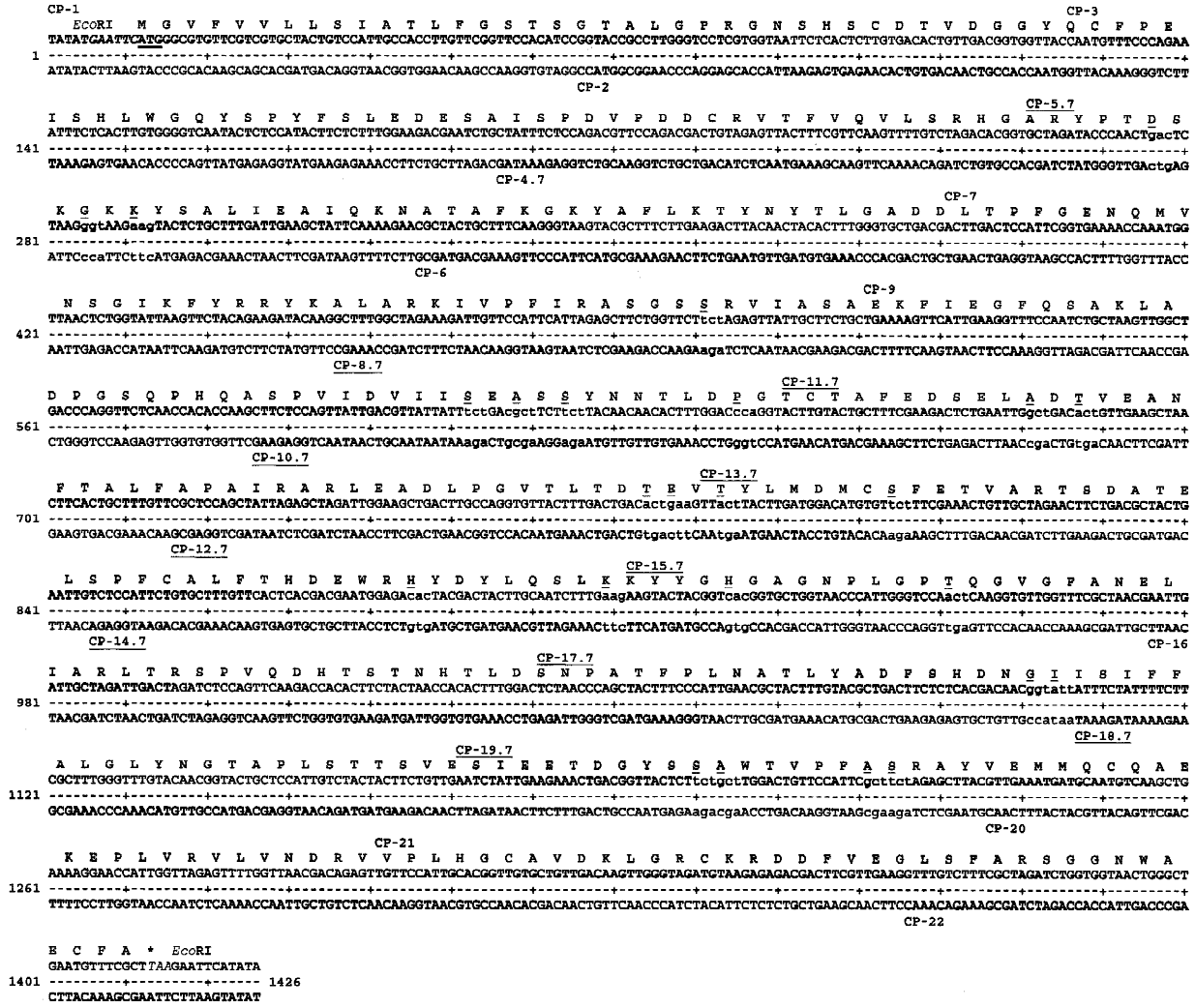

Fig. 7. DNA and amino acid sequence of consensus phytase-7. The amino acids are written above the DNA sequence using the one-letter code. The oligonucleotides used to assemble the gene are in bold. Oligonucleotides and amino acids that were exchanged (relative to the gene construction and amino acid sequence of consensus phytase-1; see Lehmann et al., 2000) are underlined and the corresponding nucleotide triplets highlighted by using small letters. The *fcp7* gene was assembled from the following oligonucleotides: CP-1, CP-2, CP-3, CP-4.7, CP-5.7, CP-6, CP-7, CP-8.7, CP-9, CP-10.7, CP-11.7, CP-12.7, CP-13.7, CP-14.7, CP-15.7, CP-16, CP-17.7, CP-18.7, CP-19.7, CP-20, CP-21, and CP-22. Consensus phytase-7 has 24 amino acids changed in comparison to the original consensus phytase-1 sequence: S89D (first letter for consensus phytase-1, second letter for consensus phytase-7), S92G, A94K, D164S, P201S, G203A, G205S, H212P, G224A, D226T, E255T, D256E, V258T, P265S, Q292H, G300K, Y305H, A314T, S364G, M365I, A397S, S398A, G404A, and A405S. The consensus phytase-7 amino acid sequence shares 94.9 and 81.6% identity with the amino acid sequences of consensus phytase-1 and *A. niger* NRRL 3135 phytase, respectively.

profiles were determined from pH 2.5 to 5.0 according to the procedure mentioned above.

Differential scanning calorimetry

Differential scanning calorimetry was performed as described previously (Brugger et al., 2000; Lehmann et al., 2000). A constant heating rate of 10 °C min^{-1} was applied up to 90 °C.

Other methods

Protein concentrations of purified proteins were calculated from the OD at 280 nm, using theoretical absorption values calculated from the known protein sequences with the DNA* software (DNASTAR, Inc., Madison, Wisconsin). An absorption of 1 A at 280 nm corresponds to 1.03 mg/mL of purified *A. niger* NRRL 3135 phytase, 0.95 mg/mL of consensus phytase-1, and 0.98 mg/mL of consensus phytase-7.

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