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Engineering of protease-resistant phytase from *Penicillium* sp.: High thermal stability, low optimal temperature and pH

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Phytase is widely used as a feed additive in industry. It is important to investigate the thermal stability, optimal pH and temperature and protease resistance of phytases in application. We introduced random mutations in a protease-resistance phytase gene of *Penicillium* sp. using Mn^{2+}-dITP random mutation method, and identified two mutants 2-28 (T11A, G56E, L65F, Q144H and L151S) and 2-249 (T11A, H37Y, G56E, L65F, Q144H, L151S and N354D) with improved thermal stability and optimal temperature and pH. The mutants retained their high resistance to pepsin. The catalytic activity at 37 °C was up to 133.3 U and 136.6 U per mg protein with broad optimal temperature ranges of 37–55 °C and 37–50 °C, respectively. After a heat treatment at 100 °C for 5 min, the two mutant proteins retained about 72.81% and 92.43% of the initial activity, respectively. In addition, the optimal pH of mutant 2-249 was reduced to 4.8. All these improved properties made them more suitable to be used as feed additive in the feed industry than the present commercial phytases. Structure analysis suggested that the replacements of G56E, L65F, Q144H, and L151S improved the thermal stability of the protein by increasing new hydrogen bonds among the adjacent secondary structures. Moreover, the mutation of L151S enhanced the activity in the range of 37–70 °C and pH 2.5–7.0 by facilitating the interaction between the substrate and the catalytic centre. The substitution of N354D influenced the pH profile by weakening the bondage with the side chain of D353, which caused a pKa shift of the catalytic centre.

[**Key words:** *Penicillium* sp.; Phytase engineering; Protease resistance; Thermal stability]

Phytate (myo-inositol hexakisphosphate) is the major storage form of phosphorus in animal feed such as soybean meals, oilseeds and cereals (1). The monogastric animals (e.g., poultry and pig) cannot use the phosphorus in phytate because they have little or no phytate-degrading enzymes in their gastrointestinal tracts (2). Therefore, inorganic phosphorus has to be added to the commercial animal feed, which increases the feed cost. Moreover, adding excess inorganic phosphorus to feed causes environmental pollution (3). Unabsorbed phytate is antinutritive because it chelates important minerals such as copper, iron, magnesium, calcium and zinc (4–8). The addition of phytase (myo-inositol hexakisphosphate phosphohydrolase, EC 3.1.3.8 and 3.1.3.26) may solve this problem because it can hydrolyze phytate into myo-inositol and inorganic phosphate (9) in the gastrointestinal tracts of monogastric animals. Many phytases from animals, plants and microbes have been isolated and employed as feed additives (10,11). Among them, the most widely used phytases are isolated from *Aspergillus niger* (12), *Aspergillus fumigatus* (13), *Aspergillus ficcum* (14), *Escherichia coli* (15) and so on, and they are usually over-expressed by *Saccharomyces cerevisiae, Pichia pastoris* or other hosts.

Several factors affect the application of phytase. Firstly, a good thermal stability is required for phytase to sustain the heat denaturation step in the regular feed pelleting process in which the temperature is up to 65–90 °C for several seconds or minutes. The thermal stability can be defined as an ability to resist heat denaturation or appropriately refold into the native conformation after heat treatment. Secondly, the catalysis of phytate hydrolysis occurs in the animal stomach and small intestine at the body temperature (39 °C) and pH (about 2.0–7.5), in the presence of proteases like pepsin and trypsin (10). Therefore, the low catalytic temperature and pH and the presence of pepsin and trypsin seriously affect the activity of phytase, thereby limiting its application. Thus, most of the fungal phytases available in nature should be modified in order to improve their commercial usage. Previous studies on phytase have focused on single property improvement, such as increasing the expression level (12), improving thermal stability or/and pH profile (16–18), or modifying protease resistance (19). Unfortunately, no phytase protein can satisfy all these properties required in practice after modification to date. Thus, it is still seriously necessary to obtain a phytase that is thermostable and highly active under the conditions of digestive tract in monogastric animals.

Many approaches have been used to improve phytase properties, and these approaches can be generally divided into two types. One is rational protein engineering in which point mutations were inserted

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based on the information of protein structure, and the other is directed evolution in which the natural evolutionary process is mimicked in laboratory. The optimizing results of phytases from different species suggest strong connections between the property improvements and the structure changes. For example, when an α-helix in the surface of *Aspergillius terreus* phytase was replaced by the corresponding stretch of *A. niger* (20), the hybrid protein exhibited unchanged activity but higher thermal stability compared with the former. It suggested that the new secondary structure stretch from the more thermal stable parent enzyme played an important role in improving the structural stability, although it only had a short length of 31 amino acids. Moreover, the increase of the covalent or non-covalent interactions or the electric charge shifts at critical locations (such as the molecular surface or the activity centre), can change enzyme properties. Kim and Lei enhanced about 20% of the thermal stability of *E. coli* phytase AppA2 by increasing hydrogen bonds between adjacent secondary structures (21). Kim et al. reported that the optimal pH of *A. niger* phytase decreases from 5.5 to 3.8 by a single-site mutation of K228E (17), and structure analysis suggested that the positively charged lysine residue substitution at the position 228 influenced the pK_a of the catalysis centre.

We cloned a new phytase gene from *Penicillium* sp. in soil utilizing consensus-degenerate hybrid oligonucleotide primer polymerase chain reaction (CODEHOP PCR) and thermal asymmetric interlaced (TAIL) PCR (22). The phytase gene which had a length of 1503 bp, showed a similarity of 99% with PJ3 phytase gene (accession number AY071824 in GenBank) from *Penicillium oxalicum*. The similarity with phytase gene from *A. niger* (accession number EF197825 in GenBank) was 65%. Similar to several published phytase genes, it contained the RHDXRXP and HD sequences which exist typically in the family of histidine acid phosphatases (HAPs). The cDNA sequence (1386 bp) was integrated into the genome of *P. pastoris* GS115. Under the control of the alcohol oxidase I promoter and facilitated by α-factor in the yeast host, the cDNA sequence produced a highly-expressed protein with phytase activity in the culture supernatant. The recombinant protein showed strong resistance to pepsin, which made it particularly suitable for working in the stomach of monogastric animals. In addition, it also maintained similar properties of most fungal phytases (13,14): optimal temperature at 50 °C, optimal pH 5.5, molecular size of 67–75 kDa from sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and 50 kDa after deglycosylation by endoglycosidase H_f. Considering of the acidic and low-temperature conditions in the stomach of monogastric animals and the high temperature in the pellet process, here we modified the phytase protein by the Mn^{2+}-dITP random mutation method to reduce its optimal temperature and pH as well as to improve its thermal stability.

MATERIALS AND METHODS

Strains, plasmids and chemicals Strains and plasmids used in this study were listed in Table 1. All restriction enzymes, *Taq* polymerase and PFU were purchased from Fermentas (Vilnius, Lithuania). Diethylaminoethyl (DEAE)-Sepharose Fast flow Column and Sephacryl S-100HR Column were purchased from Pharmacia (Stockholm, Sweden). The sodium phytate, pepsin and trypsin were purchased from Sigma (St. Louis, USA). All chemicals used in this study were commercially available and were of analytical grade unless stated otherwise.

Random mutation of the phytase gene The phytase gene was mutated by two PCR steps with Mn^{2+} and dITP added respectively. The template used in the first PCR system was the mature phytase coding sequence inserted in the expression plasmid pPIC9. The system contained 1 × PCR buffer (with $MgSO_4$), 0.2 mM dNTP, 0.1 μM each primer, 1 ng template, 5 units (U) *Taq* polymerase, and 30 μM $MnCl_2$ in a total volume of 50 μl. The primer sequences were as follows: Po1 (5′-CCACCACTCGAGAAAAGA-GAGGCTGAAGCTA-3′), and Po2 (5′-CCGGAATTCTCACTTTGAAGAAACGCCACAT-3′). The PCR program contained a first denaturation step at 94 °C for 4 min, then 20 cycles of 1 min at 94 °C, 1 min at 48 °C, 2 min at 72 °C, and a last elongation step at 72 °C for 10 min. The second PCR system used the DNA product of the first PCR step as template. A total of 100 μl volume contained 1 × buffer (with $MgSO_4$), 0.2 mM dNTP, 4 μl template, 30 μM dITP, 10 U *Taq* polymerase, and the same primers as the first PCR system. The program contained 30 cycles of 1 min at 94 °C, 1 min at 48 °C, 2 min at 72 °C, and the other conditions were the same as the first PCR program. In order to obtain different mutations as many as possible, 5 parallel tubes, each with 100 μl reaction mixture, were used in the second PCR system.

The aforementioned product was double digested by *Xho* I and *Eco*R I, and then ligated with the expression vector pPIC9 that was digested by the same restriction enzymes and transformed into *E. coli* DH5a by the MOPs method. The positive clones containing recombinant plasmid pPIC9-mut*phy*A were screened on the Luria Bertani (LB) medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl) with 1 mg/ml Ampicillin. All the *E. coli* transformants on one plate (total 10 plates, each with about 500 clones) were mixed and cultivated in 100 ml LB medium. The recombinant plasmid pPIC9-mutphyA was extracted by conventional method and stored at −20 °C for later use.

The construction of mutant library in *P. pastoris* The recombinant plasmid pPIC9-mutphyA was linearized by *Stu* I and transformed into *P. pastoris* GS115 with the PEG1000 transformation method according to the 'Manual of Methods for Expression of Recombinant Proteins in *Pichia pastoris*' (Invitrogen, USA). Positive transformants were screened on minimal dextrose (MD) medium (1.34% (w/v) yeast nitrogen base (YNB), 4×10^{-5}% (w/v) biotin and 2% (w/v) dextrose). The recombinant plasmid pPIC9-mutphyA DNA extracted from 100 ml LB culture (with DNA content of 77–98 μg) was transformed to one tube of competent yeast cells and spread on one selective plate. In total, 10 recombinant plasmid samples were transformed, and as a result, over 5 thousand clones were obtained on the 10 MD plates after 3 days. Single positive clones of transformants were first inoculated into modified buffered glycerol-complex (BMGY) medium (1% (w/v) yeast extract, 2% (w/v) peptone, 1.34% (w/v) YNB, 4×10^{-5}% (w/v) biotin, 1% (v/v) glycerol, pH 6.0) and cultivated at 30 °C, 200 rpm until the cultures reached an optical density $(O.D.)_{600}$ of 2–6. *P. pastoris* cells were harvested (5000 × *g*, 5 min) and then diluted with modified buffered methanol-complex (BMMY) medium (1% (w/v) yeast extract, 2% (w/v) peptone, 1.34% (w/v) YNB, 4×10^{-5}% (w/v) biotin, 1% (v/v) methanol, pH 6.0) to a density of $O.D._{600}$ of 1.0. Methanol (1%, v/v) was added every 24 h to induce phytase expression. The culture supernatant with the recombinant phytase was prepared by centrifugation (8000 × *g* at 4 °C for 5 min) after 72 h. The *P. pastoris* GS115 strain containing the wild type mature phytase cDNA GS115-pPIC9-WT*phy*A was also induced by methanol (1%, v/v).

Screening the mutant library Phytase activity of the culture supernatant was detected, and the transformants with distinct phytase activity were screened. Then, a heat treatment of the culture supernatants of the screened transformants at 80 °C for 5 min was applied to evaluate the thermal stability of the phytase. Clones with a residual activity higher than 70% were selected and purified to further determine their pH profiles, optimal temperatures and thermal stabilities.

Purification and analysis of the recombinant phytase protein The culture supernatant was first concentrated about 10-fold by ultrafiltration and then dialyzed followed by passed through a DEAE-Sepharose Fast flow Column using 0.02 M Tris–HCl buffer (pH 7.4) as equilibrium buffer and NaCl buffers with solubility from 0 to 0.8 M dissolved in 0.02 M Tris–HCl buffer (pH 7.4) as elution buffer. Phytase activity and $O.D._{280}$ were checked to identify the desired protein peak. After dialysis and ultrafiltration, target protein was further purified by Sephacryl S-100HR Column using 0.02 M Tris–HCl buffer (pH 7.4) with 0.15 M NaCl. Purified phytase enzyme was then detected by SDS-PAGE.

Phytase activity was assayed as previously described (24), with 0.05 M sodium phytate dissolved in the buffer as substrate. The pH profile was determined at 37 °C with different buffers: 0.05 M Glycine–HCl buffer of pH 2.2, 2.6, 3.0 and 3.4; 0.05 M NaAc–HAc buffer of pH 4.0, 4.4, 4.8, 5.2 and 5.8; 0.05 M Imidazole–HCl buffer of pH 6.2, 6.6 and 7.0; 0.05 M Tris–HCl buffer of pH 7.5, 8.0, 8.5 and 9.0. The optimal temperatures were determined at each optimal pH and at different temperatures of 37 °C, 45 °C, 50 °C, 55 °C, 60 °C, 65 °C and 70 °C. To determine the thermal stability, the enzyme samples were incubated at different temperatures of 50 °C, 60 °C, 70 °C, 80 °C, 90 °C, 95 °C and 100 °C for 5 min, respectively. After the heat treatments, the samples were immediately placed on ice for 30 min. The residual activity was measured at 37 °C under optimal pH, and then compared with the samples without heat treatment. One unit was defined as the amount of enzyme that released 1 μmol of inorganic phosphorus from substrate per min.

The protein contents were determined by the Bradford method according to the manufacturer's instructions (25). In order to determine the protease resistance, pepsin (3000–3500 NFU/mg) and trypsin (1500 BAEE unit) were dissolved into 10 mM HCl, pH 2.0 and 80 mM ammonium bicarbonate, pH 7.5 to a final concentration of 0.1 mg/ml, respectively (26). In a total volume of 100 μl, 10 μg of purified phytase protein was incubated with pepsin at different protease/phytase ratios (w/w) of 0.001, 0.002, 0.005 and 0.01, or with trypsin at the ratios of 0.001, 0.005, 0.01 and 0.025 at 37 °C for 2 h. The protease/phytase mixtures were then incubated on ice to terminate the reaction, and adjusted to pH 8.0 for SDS-PAGE analysis. The residual activity was measured at 37 °C, pH 5.5 with 2 μl of protease/phytase mixture (containing 0.2 μg phytase), with 0.2 μg untreated purified phytase as 100% standard.

Structure prediction of the phytase protein The amino acid sequence of the phytase from *Penicillium* sp. was submitted into SWISS MODEL (http://swissmodel.expasy.org) (27–29) web server, and a project containing the structure information was then sent to the appointed e-mail address. The structure was observed and analyzed with a PDB viewer.