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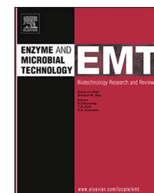

Improved thermostability and enzyme activity of a recombinant phyA mutant phytase from *Aspergillus niger* N25 by directed evolution and site-directed mutagenesis

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We previously constructed three recombinant phyA mutant strains (PP-NP^{m}-8, PP-NP^{ep}-6A and I44E/T252R-PhyA), showing improved catalytic efficiency or thermostability of *Aspergillus niger* N25 phytase, by error-prone PCR or site-directed mutagenesis. In this study, directed evolution and site-directed mutagenesis were further applied to improve the modified phytase properties. After one-round error-prone PCR for phytase gene of PP-NP^{ep}-6A, a single transformant, *T195L/Q368E/F376Y*, was obtained with the significant improvements in catalytic efficiency and thermostability. The phytase gene of *T195L/Q368E/F376Y*, combined with the previous mutant phytase genes of PP-NP^{ep}-6A, PP-NP^{m}-8 and I44E/T252R-PhyA, was then sequentially modified by DNA shuffling. Three genetically engineered strains with desirable properties were then obtained, named*Q172R*, *Q172R/K432R* and*Q368E/K432R*. Among them, *Q172R/K432R* showed the highest thermostability with the longest half-life and the greatest remaining phytase activity after heat treatment, while *Q368E/K432R* showed the highest catalytic activity. Five substitutions (Q172R, T195L, Q368E, F376Y, K432R) identified from random mutagenesis were added sequentially to the phytase gene of PP-NP^{ep}-6A to investigate how the mutant sites influence the properties of phytase. Characterization and structural analysis demonstrated that these mutations could produce cumulative or synergistic improvements in thermostability or catalytic efficiency of phytase.

1. Introduction

Phytase (myo-inositol hexaphosphate phosphohydrolase) catalyzes the hydrolysis of phytate to inositol phosphates, myo-inositol, and inorganic phosphate [1]. Phytase is widely distributed as an additive in industrial feed production for monogastric animals such as poultry and pig, playing a significant role in increasing the bioavailability of nutrients and reducing the accumulation of phosphorus in the environment [2]. However, the feed-pelleting procedure requires high temperatures of ~70–90 °C; at these temperatures, phytase is easily inactivated to 30% or less of its original activity. Thus, recent studies are focused on obtaining phytase with improved thermostability and exploring advanced methods to promote thermostability and enzyme activity.

Directed evolution has led to advances in phytase engineering. Kim and Lei [3] obtained a mutant library of the phytase AppA2 through error-prone PCR; the enzyme activity of AppA2 (K65E/K97M/S209G) increased by 152% compared to the wild-type phytase, and it had a 7 °C higher T_m value. Bei et al. [4] obtained a mutant phytase with improved thermostability, which was adapted to a wider range of pH values and had higher catalytic activity, by recombining the seven phytase fragments that were digested from *Aspergillus niger* NRRL3135 and *Aspergillus fumigatus* ATCC1307. Tian et al. [5] obtained two mutant phytases with higher enzymatic activity by DNA shuffling of the *phyI1s* gene from *A. niger* strain 113.

Nevertheless, site-directed mutagenesis is a much faster and more efficient method for increasing the thermostability or catalytic efficiency of phytase. Kim et al. [6] acquired the mutants D144N/V227A and D144N/V227A/G344D of phytase AppA2 from *Escherichia coli* with 15% higher thermostability than wild type after heating at 80 °C for 10 min. Rodriguez et al. [7] mutated three sites of the phytase gene from *E. coli* (C200N/D207N/S211N); the optimal temperature of this mutant was increased by 10 °C and it had higher enzymatic activity than the wild type after heating at 80 °C and 90 °C for 15 min.

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Hesampour et al. [8] constructed 12 mutants of *A. niger* PhyA phytase with two T314S/Q315R/V62N and S205N/S206A/T151A/T314S/Q315R showed the highest stability with 24% and 22.6% greater retention at 80 °C. Comparing the three-dimensional (3D) structure with other desirable phytases is a much more accurate way toward improving phytase with greater thermostability. For example, Zhang et al. [9] predicted and compared the structural sequences of the non-thermostable phytase PhyA from *A. niger* and the thermostable phytase Afp from *A.fumigatus*, elucidating the key residues related to thermostability (E35, S42, R168, and R248). In addition, the catalytic efficiency of the phytase could be improved by mutating individual amino acids far from the active center [7] or close to the enzyme's active center [10], or involving in binding substrate and product release [11].

Our group has been studying the development and application of phytase for feed for several years [12–16]. Recently, PP-NP^{ep}-6A (E156G/T236A/Q396R) was obtained by modifying the mutant acid phytase (*phyA^m*) gene that was derived from the phytase gene *phyA* (GeneBank ID AF218813) from *A. niger* N25 through error-prone PCR. The catalytic efficiency (k_{cat}/k_m) of the mutant was 84% higher than that of the parental strain [15]. Site-directed mutagenesis was also performed on *phyA^m* to construct two single-site mutants (I44E-PhyA and T252R-PhyA) as well as one double-site mutant (I44E/T252R-PhyA). All three of these mutants showed improved thermostability and catalytic efficiency [16]. In this study, both directed evolution (error-prone PCR and DNA shuffling) and site-directed mutagenesis were performed to improve the previous mutant phytase gene and produce mutant strains with greater thermostability and catalytic efficiency. Structural and characterization analysis of the mutant and parental phytases has enabled us to elucidate the synergistic effects of mutated sites on phytase properties, and establish an experimental basis for the further application of protein-engineering techniques to improve the physicochemical properties of phytase.

2. Materials and methods

2.1. Materials

Oligonucleotides were synthesized by Invitrogen (Carlsbad, CA, USA). The restriction enzyme *BspE*I was purchased from New England Biolabs (Beverly, MA, USA) and all other restriction enzymes, T4 DNA ligase, DNA/protein markers, ampicillin and Multipoints Mutagenesis Kitwere obtained from Takara (Otsu, Japan). Plasmid Mini Kit, Gel Extraction Kit and Fungal DNA Midi Kit were purchased from Omega (Shanghai, China). DIG High Prime DNA Labeling and Detection Starter Kit I was purchased from Roche Applied Science (Mannheim, Germany). In-Fusion™ Advantage PCR Cloning Kit was obtained from Clontech (CA, USA). Phytic acid P-0109 (inositol hexaphosphoricacid, dodecasodium salt from rice) was purchased from Sigma (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade. The bacterial and yeast strains, plasmids, and primers used in this study are listed in Table 1.

2.2. Error-prone PCR and screening

The recombinant plasmid pMDT18-*phyA^{6A}* containing the *phyA^{6A}* gene [15] was used as the template for one round error-prone PCR. EP-P1 and EP-P2 (Table 1) were used for amplification of phytase gene. Error-prone PCR protocol, construction of the recombinant expression vector pPIC9K-*phyA*$^{ep-6A}$, transformation of the expression plasmids into *Pichia pastoris* GS115, and high-throughput screening based on 96-well plates for improved phytase activity or thermostability were carried out as described previously [15].

2.3. DNA shuffling and screening

To increase the gene diversity for DNA shuffling, the desired mutant phytase gene identified from the above random mutant library by error-prone PCR was combined with the previous three mutant phytase genes *phyA^m* [14], *phyA^{6A}* [15], *I44E/T252R-PhyA* [16], and used as the template. The four mutant phytase genes were mixed in equal amounts and then digested with DNase I into random fragments. The small-size fragments (50–100 bp) were recovered using the Small DNA Fragments Extraction Kit from Galaxy Bio (Beijing, China). These small-sized fragments were assembled by PCR without primers. Finally, the full-length genes were constructed by PCR using primers IF-F1 and IF-F2 (Table 1) which both contained 15 bp extensions complementary to the ends of the vector pPIC9K linearized by *Eco*RI/*Not*I double digestion. The resulting fragment was inserted to the vector pPIC9K linearized by *Eco*RI/*Not*I digestion using In-Fushion™ Advantage PCR Cloning Kit (Clontech, CA, USA) according to the manufacturer's instructions. The positive transformants were designated pPIC9K-*phyA^{ds}*, which was linearized using the restriction enzyme *BspE*I and then transformed into *P. pastoris*GS115 by electroporation as described previously [15,16]. The desired mutant transformants were also screened by high-throughput method based on 96-well plates.

2.4. Site-directed mutagenesis

The residue substitutions (Q172R, T195L, Q368E, F376Y, K432R) were identified from the desired mutant phytase genes obtained by error-prone PCR and DNA shuffling. To investigate how these mutant sites influence the properties of phytase and determine whether individual mutations can be sequentially added to produce cumulative or synergistic improvements in thermostability or catalytic efficiency of phytase, *phyA^{6A}* was modified through site-directed mutagenesis using Multipoints Mutagenesis Kit (Takara, Otsu, Japan) according to the manufacturer's instructions. Plasmids derived from pMDT18 harboring the desired mutation(s) were identified by DNA sequence analysis. The recombinant expression plasmids were then constructed by in-fusion cloning methods as described above, and transformed into *P. pastoris* GS115 to generate the mutant strains as described previously [16]. All of the mutants and primers used for site-directed mutagenesis are listed in Table 1 and the flow chart for the modification of the phytase gene is shown in Fig. 1.

2.5. Expression and purification

The desired mutants obtained from directed-evolution mutant libraries were further identified by re-screening in a test tube followed by shaken-flask cultivation as described previously [15,16]. Then, the selected mutant strains and parental strain were cultured in YPD medium, followed by BMGY medium for growth. Phytase induction in BMMY medium was performed for 96 h, with the addition of 1% (v/v) methanol every 24 h, as described previously [15,16]. Phytase activity analysis, determination of the genetic stability of the mutants and Southern blot analysis were performed as described previously [15,16]. The desired phytases were purified by two-step ammonium-sulfate precipitation (30% and 80%, respectively), DEAE-Sepharose chromatography, and Sephadex G-100 gel-filtration chromatography and then visualized by SDS-PAGE, as previously described [15,16].

2.6. Mutant phytase characterization studies

The optimal temperature of the phytase was tested in 0.25 M acetate buffer at pH 5.5 after 30 min of reaction at the following temperatures: 37, 40, 50, 55, 57, 59,60, 61, 63, 65, 70, 75, 80, 85 and 90 °C. The pH activity profile, thermostability and kinetic parameters of phytase were determined as described previously [16]. The half life of phytase was determined by measuring the residual enzymatic activity after incubating at 70 °C for2, 4, 6, 8 and 10 min, followed by immediate cooling. To determine half life ($T_{1/2}$), the remaining phytase activity was first converted to the reaction rate, then the data were fitted to the

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