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Enhancing the Thermal Resistance of a Novel Acidobacteria-Derived Phytase by Engineering of Disulfide Bridges S

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A novel phytase of Acidobacteria was identified from a soil metagenome, cloned, overexpressed, and purified. It has low sequence similarity (<44%) to all the known phytases. At the optimum pH (2.5), the phytase shows an activity level of 1,792 μmol/min/mg at physiological temperature (37°C) and could retain 92% residual activity after 30 min, indicating the phytase is acidophilic and acidostable. However the phytase shows poor stability at high temperatures. To improve its thermal resistance, the enzyme was redesigned using Disulfide by Design 2.0, introducing four additional disulfide bridges. The half-life time of the engineered phytase at 60°C and 80°C, respectively, is 3.0× and 2.8× longer than the wild-type, and its activity and acidostability are not significantly affected.

Keywords: Phytase, acidophilic, acidostable, site-directed mutagenesis, disulfide bridge, thermostable

Phytase is supplemented as an additive enzyme for degrading phytates present in animal feed [5, 25]. For the acidic condition present in livestock and poultry stomach [29], phytase showing high activity and stability at low pH is favored. Moreover, the steaming and pelleting process in feed manufacturing uses high temperatures of 60–80°C [18], and thus the supplemented phytase could suffer from heat denaturation. Therefore, the resistance of phytase to high temperature is a necessary property. To improve its thermal resistance, an enzyme could be modified by enhancing glycosylation [16, 44, 47], or adding disulfide bridge [35, 38], salt-bridges [21, 49], or hydrogen bonds [44, 45, 49, 50], as well as other rational design strategies [3, 41, 46]. Directed evolution of enzymes is also utilized [22, 36, 37, 51]. Among all these strategies, adding disulfide bridges is usually prefered for its highly predictable effect as well as convenience [9, 10]. In this study, a novel phytase with acidophilic and acidostable characteristics was identified from a soil metagenome, and the protein was further engineered to elevate its thermal resistance, by introducing additional disulfide bridges using site-directed mutagenesis.

To screen for novel phytases, proteins of the histidine acid phosphatase (HAP) family were searched in the metagenomic dataset derived from the microbial community of wetland surface sediment in Twitchell Island of Sacramento Delta (CA, USA) [17, 23, 30], which is publicly available in the IMG database [27, 28]. The specific Pfam-identifier of HAP phytase (pfam00328) was used for searching [40]. After discarding incomplete open reading frames (ORFs), only one HAP phytase ORF (IMG gene Accession ID: WSSedL1C_100018107) was identified in this metagenome, which was named PhyA. This phytase consists of 431 amino acids (Fig. S1), including a 22-amino-acid signal peptide region predicted by SignalP 4.1 [11, 34]. Compared by BLASTp, PhyA shows 44% similarity to the predicted phytase of *Granulicella mallensis*, which belongs to Acidobacteria. The 11,806 bp scaffold (IMG Scaffold ID: WSSedL1C_c10001810) where the *phyA* gene locates seems

also to be from Acidobacteria, as estimated by BLASTn based on sequence similarity as well as PhylopythiaS+ based on the compositional profile of the oligonucleotide string [33]. It means that PhyA is possibly an Acidobacteria phytase, which has rarely been reported in previous studies.

Using the procedures previously described [39], the *phyA* gene fragment excluding the signal peptide region was optimized for codon usage in *Escherichia coli*, chemically synthesized, and cloned into a pET26b(+) vector between the NcoI and XhoI restriction sites (Fig. S1). The recombinant rPhyA was overexpressed in *E. coli* BL21(DE3). The protein was purified by Ni-affinity chromatography and further refined twice by gel filtration (Table S1). Protein purity was examined by reversed-phase HPLC with an Agilent Poroshell 300SB-C8 column using the procedures described by Guo *et al*. [15]. The purity of the refined enzyme was higher than 95% (Table 1). The molecular weight (Mw) of the protein was determined by MALDI-TOF analysis (Fig. S2). The purified rPhyA was characterized for its phytase activity, pH profile, temperature profile, pH stability, temperature stability, kinetic constants, and cation effects, using the methods previously described by Garret *et al*. [13] and Tan *et al*. [39]. The melting temperature (T_m) of the protein was determined with the assay described by Inoue *et al*. [20], in which protein melting was monitored by the change of fluorescence intensity, using a CFX-Connect System (Bio-Rad, USA). One unit (U) of the phytase activity is defined as the catalytic ability to release 1 μmol inorganic phosphate (Pi) from the phytate substrate per minute. The results showed that rPhyA is acidophilic and acidostable. The optimum pH for rPhyA is 2.5, at which the phytase demonstrates an activity level of 1,792 ± 81 U/mg at 37°C (Fig. 1A, Table 1). This activity level is competitive with other acidophilic phytases [39, 43, 48]. Moreover, 92% of residual activity is retained after incubation at pH 2.5 for 30 min and above 50% after 5.2 h (Fig. 1B, Table 1), indicating that rPhyA has a long half-life time ($t_{1/2}$) at acidic pH. The optimum temperature for rPhyA is 40°C (Fig. 1C, Table 1), which means the phytase is mesophilic. The $t_{1/2}$ at 60°C and 80°C, which are common temperatures used for steaming and pelleting feed ingredients, is 5.2 min and 2.3 min, respectively (Fig. 1D, Table 1). This is poor thermal resistance. Altogether, the results indicate that rPhyA has good activity as well as acidostability under gastric pH and temperature conditions, which means the enzyme could have potential to work in livestock and poultry stomach [13, 19, 48, 50], but unfortunately the poor resistance to high temperatures could limit its commercial application as a supplementing enzyme in the feed industry.

In order to improve the thermal resistance of rPhyA, the enzyme was engineered. The three-dimensional structure model of rPhyA was predicted by homology modeling using the SWISS-MODEL server [2, 4] and visualized with PyMOL (ver. 1.7.2.1). The phytase of *E. coli* was determined to be the closest template, which has 40% identity to rPhyA. The predicted structure of rPhyA (Fig. 2) possesses eight major α-helices, as well as a twisted β-sheet consisting of six parallel β-strands. Disulfide bridges in rPhyA were analyzed using Disulfide by Design 2.0 [8]. Two disulfide bridges (Cys85-Cys116 and Cys386-Cys393) were predicted (Fig. 2). The fewer disulfide bridges of rPhyA in comparison with known thermostable phytases [40, 42, 49] might be a reason why rPhyA lacks thermal resistance. The amino acid residues that can be substituted to cysteine to form new disulfide bridges were analyzed by Disulfide by Design 2.0 (Table S2). Four potential disulfide bridges were

Table 1. Summary of enzyme characteristics of rPhyA and the engineered mutant rPhyA6DB.

Enzyme characteristics	rPhyA	rPhyA6DB
Mw	45,741.36 Da monomer	45,704.08 Da monomer
Purity	95.15%	95.95%
Optimum pH	2.5	2.5
Activity at pH 2.5, 37°C	1,792 ± 81 U/mg	1,719 ± 97 U/mg
Optimum temperature	40°C	55°C
Maximum activity level	1,844 ± 90 U/mg at pH 2.5, 40°C	2,917 ± 121 U/mg at pH 2.5, 55°C
T_m	48°C	61°C
$t_{1/2}$ at pH 2.5, 37°C	5.2 h	5.4 h
$t_{1/2}$ at pH 7.0, 60°C	5.2 min	14.5 min
$t_{1/2}$ at pH 7.0, 80°C	2.3 min	7.0 min
K_m (at pH 2.5, 37°C)	0.382 ± 0.016 mM	0.405 ± 0.017 mM
V_{max} (at pH 2.5, 37°C)	2,138 ± 93 U	2,086 ± 89 U

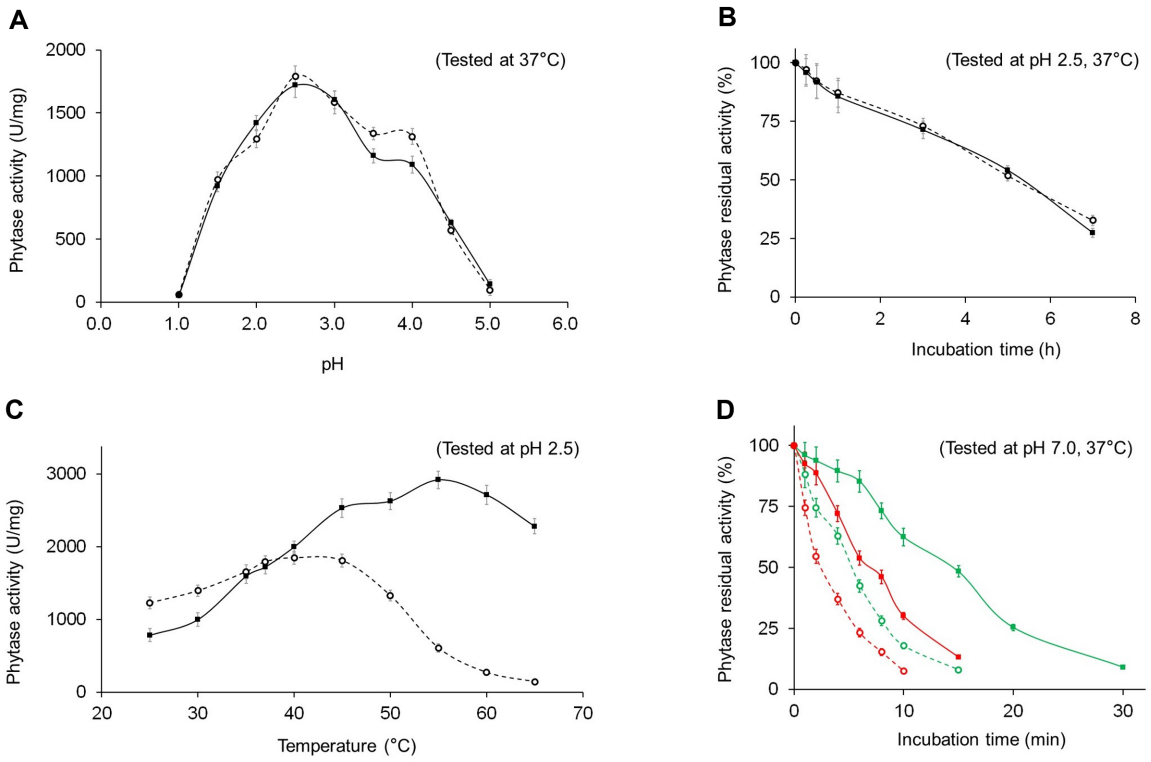

Fig. 1. pH profile (**A**), pH stability (**B**), temperature profile (**C**) and temperature stability (**D**) of the wild-type phytase rPhyA in comparison with the engineered mutant rPhyA6DB.

rPhyA is shown by empty dots with dash lines, while rPhyA6DB is shown by solid squares with solid lines. The stability at 60°C is colored in green and 80°C in red. Each data point is the mean of three individual replications measured.

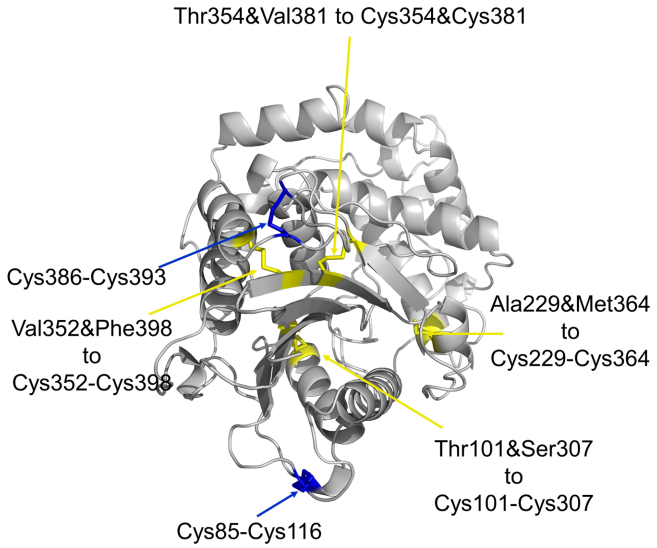

Fig. 2. Engineering of phytase rPhyA shown in a three-dimensional structural model.

The two disulfide bridges natively possessed by the wild-type rPhyA are colored in blue, while the four extra disulfide bridges in the rPhyA6DB mutant added by site-directed mutagenesis are colored in yellow.

selected from all the candidate combinations based on the following criteria: a higher B-factor value is favored, which means good flexibility of the disulfide bridge [24], and meanwhile the distance of two cysteines in the peptide strand should be farther than 20 amino acids in order to avoid disulfide bridges forming between nearby amino acids, which are less effective than connecting different motifs. The amino acid residues selected for constructing the four extra disulfide bridges were Thr101-Ser307, Thr354-Val381, Val352-Phe398, and Ala229-Met364 (Fig. S1, Table S2). Accordingly, the number of disulfide bridges was increased from two in the wild type to six in the engineered mutant (Fig. 2). It is higher than the five disulfide bridges in the thermostable phytases of *Neosartorya spinose* and *Aspergillus ficuum* [32, 42]. To our best knowledge, it is the highest number of disulfide bridges among all the known HAP phytases.

To carry out the amino acid substitutions and construct the mutant, a site-directed mutagenesis approach as previously described [26] was adopted, using a MutanBEST Kit (Takara, Japan). The engineered phytase named

Table 2. Cation effects on the phytase activity.

Cation	Phytase residual activity (%)	
	rPhyA	rPhyA6DB
Na^{+}	107 ± 8	115 ± 9
K^{+}	117 ± 7	104 ± 5
Ca^{2+}	96 ± 3	94 ± 6
Mg^{2+}	102 ± 5	110 ± 6
Al^{3+}	75 ± 6	83 ± 4
Zn^{2+}	97 ± 3	89 ± 5
Fe^{2+}	103 ± 5	93 ± 4
Fe^{3+}	82 ± 4	88 ± 4
Mn^{2+}	89 ± 3	55 ± 2
Cu^{2+}	94 ± 4	63 ± 6
Pb^{2+}	68 ± 5	43 ± 5
Hg^{2+}	46 ± 4	15 ± 3

The phytase residual activity in the presence of each tested cation, measured at pH 2.5 and 37°C, is shown as a percentage compared with the activity without adding the cation (defined as 100%).

rPhyA6DB was cloned, overexpressed, purified (Table S1), and characterized (Table 1) with the same procedures used for rPhyA. Disulfide combinations were verified by peptide mapping using the method described by Chambers *et al*. [7]. The details are described in the supplemental data (see "Verifying disulfide combination" section, Table S3 and Fig. S3).

By introducing the four extra disulfide bridges, the optimum temperature of the phytase was elevated from 40°C to 55°C, along with a large increase in the maximum activity level (Fig. 1C, Table 1). The T_m of rPhyA6DB was elevated by 13°C compared with rPhyA (Table 1), which means the engineered phytase has a higher unfolding temperature. The $t_{1/2}$ of rPhyA6DB at 60°C and 80°C is 14.5 min and 7.0 min, respectively (Fig. 1D, Table 1), which are 3.0 and 2.8 times longer than the wild type. It is a significant improvement in thermal resistance, which is now comparable with several thermostable phytases [1, 12, 14, 46]. Meanwhile, the optimum pH was not altered (Fig. 1A) and the stability in acid was retained (Fig. 1B).

The kinetic constants (K_m and V_{max}) were not significantly changed (Table 1), which means the engineering had rarely any negative effect on the substrate affinity and catalytic velocity of this phytase. rPhyA6DB was more vulnerable to heavy metal cations than rPhyA (Table 2), which could be a side effect caused by the engineering. This might be due to the significant increase of cysteine residues, which are prone to react with heavy metal cations such as Mn^{2+}, Cu^{2+}, Hg^{2+}, and Pb^{2+}. Considering that animal feed should contain a very low content of heavy metal elements [6, 31], the engineering of adding disulfide bridges is overall beneficial to the phytase.

In conclusion, a novel acidophilic and acidostable phytase with low similarity to all the known phytases was identified from a soil metagenome. By adding four extra disulfide bridges, the protein engineering significantly elevated the thermal resistance of this phytase while retaining its activity level as well as acidophilic and acidostable properties. Compared with the wild type, the engineered mutant is more suitable to be utilized as a potential supplementing enzyme in livestock and poultry feeding. This study showed the effectiveness of adding disulfide bridges in enhancing the thermal resistance of an enzyme, and proved Disulfide by Design 2.0 to be an efficient tool for the rational design of enzymes by manipulating disulfide bridges.

The peptide sequence of the engineered mutant rPhyA6DB has been deposited to the GenBank database under the accession number KX096887.

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