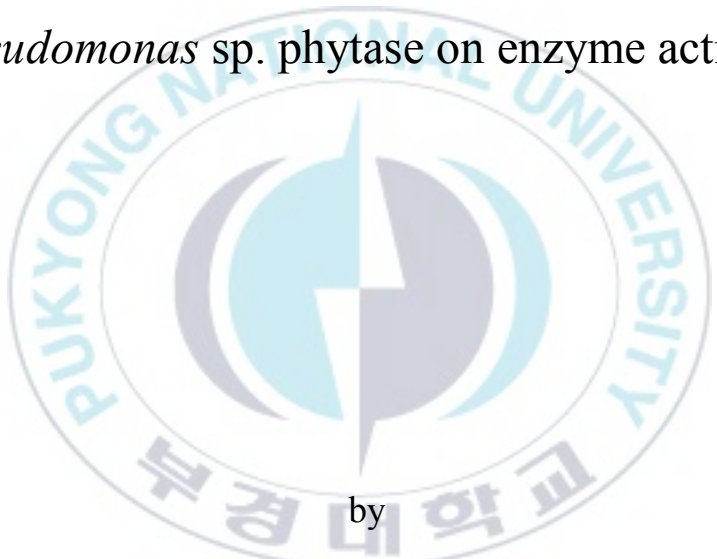
Thesis for the Degree of Master of Engineering

Effect of polar amino acid residue substitution by site-directed mutagenesis in the N-terminal domain of *Pseudomonas* sp. phytase on enzyme activity



by

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School of Marine Fisheries Life Science

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August 2020

Effect of polar amino acid residue substitution by site-directed mutagenesis in the N-terminal domain of *Pseudomonas* sp. phytase on enzyme activity

(*Pseudomonas* sp.로부터 유래된 Phytase의 N-terminal domain에서 부위지정 돌연변이에 의한 극성 아미노산 잔기의 치환이 효소 활성에 미치는 영향)

Advisor: Prof. In-Soo Kong

by

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A dissertation

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Effect of polar amino acid residue substitution by site-directed mutagenesis in the N-terminal domain of *Pseudomonas* sp. phytase on enzyme activity

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Abstract

In aquatic environments with a neutral pH and low temperature, phytase used for decomposition of phytate should maintain high activity even at low temperatures. The N-terminal domain of the *Pseudomonas* sp. FB15 phytase which maintains high activity even at 25 °C increases low-temperature activity and catalytic efficiency. In this study, the three-dimensional structure of the N-terminal domain was predicted and substitutions for the amino acid residues of the region assumed to be the active site were made. The activity of mutants, in which alanine (A) was substituted for the original residue, was investigated at various temperatures and pH values. Significant differences in enzymatic activity were observed only in mutant E263A, suggesting that the amino acid residue at position 263 of the N-terminal domain is important in enzyme activity.

1. Introduction

Phytic acid, myo-inositol 1,2,3,4,5,6- *hexakis*phosphate, is the main storage form of phosphorus in many plant tissues. It is a polyanionic molecule with six phosphate groups that combines with important minerals such as cobalt, copper, iron, manganese, zinc and calcium to form insoluble phytate salts [1, 2]. Phytate salts are not available in monogastric animals which have lack of intestinal phytase required for phytate hydrolysis during digestion and cause reduced bioavailability of minerals in humans, pigs, poultry and fish [3].

Phytase, known as myo-inositol-*hexakis*phosphate phosphohydrolase, hydrolyzes indigestible phytate to liberate inorganic phosphorus. This enzyme has been used to enhance the nutritional value of phytate-rich, plant-based foods. Due to these characteristics, it is used as an animal feed additive. Phytase is divided into four types according to its catalytic and structural characteristics: Beta-propeller phytases (BPPs), purple acid phosphatases (PAPs), histidine acid phytases (HAPs), and cysteine phytases (CPs) [4]. PAPs, HAPs and CPs have acidic pH optima, range of 2.5–5.5 but BPPs have high thermal stability, substrate specificity, and show high activity over the neutral pH (6-8) [5], which makes it suitable for industrial use in aquatic industry field. Enzymes that have high activity at low temperatures and neutral pH are suitable to be used in aquatic environment such as lakes (pH slightly below 7.0) and seawater (pH 8.0), whose temperature is below 25 °C

Unlike BPPs produced by *Bacillus* which have optimum temperature range in 50–70 °C, phytase of *Pseudomonas* sp. FB15 (PSphy) has optimum temperature at 40 °C and an additional N-terminal domain (residue 37-275). Our previous studies have reported that PSphy maintains high activity even at low temperatures, which is suitable for aquatic industry and deletion of N-terminal domain from PSphy showed decreased catalytic efficiency and activity at low temperatures [6]. In other hand, fusion of the N-terminal domain with other *Bacillus*-derived BPP increased the catalytic efficiency and enzyme activity at low temperatures [7]. These results

suggest that the N-terminal domain of PSphy played an important role in catalytic efficiency and activity at low temperatures.

According to these results, it's important to understand the characteristics of the N-terminal domain of PSphy. This experiment was performed to identify amino acid residues that affect enzyme activity in the N-terminal domain of PSphy. Four amino acid residues (E140, Q172, E217, and E263) found in the region predicted as the active site were chosen for testing. Selected amino acid residues were substituted using site-directed mutations and investigated for altered enzyme activity.

2. Materials and Methods

2.1 Three dimensional structural modeling

The three-dimensional structure of PSphy was predicted using SWISS-MODEL (https://swissmodel.expasy.org/) to select amino acid residues in the propeller top (PT) region of the N-terminal domain as substitution residues.

2.2 Bacterial strains, plasmids, media

In this study, *Escherichia coli* DH5α and BL21 (DE3) were the host for gene cloning and protein overexpression. Recombinant *E. coli* was incubated at 37 °C and overexpressed at 25 °C in Luria-Bertani (LB) medium supplemented with ampicillin at 100 μg/ml if necessary. pET-22b(+) was used as the vector for cloning.

2.3 Overlap PCR for site-directed mutagenesis

The primers were designed for mutagenesis (Table 1). The pET-22b(+) vector carrying the phytase gene of *pseudomonas* sp. FB15 was used as a templates. PCR conditions for forward fragment were pre-denaturation at 95 °C for 3min followed 25 cycles of denaturation at 95 °C for 30s, annealing at 70 °C for 30s and extension at 72 °C for 50s. For reverse fragment, PCR was performed in the same situation except annealing at 68 °C and extension at 72 °C for 2min. Then, overlap PCR was conducted using the mixture of forward and reverse fragment as templates. The overlap extension PCR products were cut with restriction enzyme and then ligated with pET-22b(+) vector. *E. coli* DH5α was transformed and cultured in LB agar medium containing ampicillin. Colonies were randomly selected and identified for mutation by DNA sequencing. Recombinant plasmids were extracted and transfected into *E. coli* BL21 (DE3).

2.4 Overexpression and purification of recombinant proteins

E. coli BL21(DE3) containing recombinant plasmid were cultured in 500 ml LB medium containing ampicillin at 37 °C. When optical density at 600 nm reached 0.6, isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added so that the final concentration was 1 mM. After 18h at 25 °C, cells were collected by centrifugation and resuspended in 20 mM Tris-HCl buffer (pH 7.9). The re-suspended cells were disrupted by a sonicator and centrifuged to load the supernatant on a Ni-NTA His-Tag column. The purified enzyme was dialyzed in 20 mM Tis-HCl buffer (pH 6.5) for 18h. The purified enzyme was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue. Bradford assay was conducted for measuring a concentration of the purified phytase.

2.5 Mutant phytases activity assay

Phytase activity was measured by Molybdate-Blue Method with some modification [8]. 50 μL of enzyme solution was mixed with 200 μL of substrate solution (1 mM sodium phytate, Sigma-Aldrich, USA) and incubated at 40 °C for 30min. The reaction was terminated by adding 250 μL of 5%(w/v) trichloroacetic acid. The concentration of released inorganic phosphate was determined by adding 250 μL of coloring reagent (1.2% ammonium molybdate, 0.54% ferrous sulfate and 3.5% sulfuric acid). The absorbance was measured at 700 nm (Optizen POP, Korea).

2.6 Effects of temperature and pH on mutant phytase

The relative activity of phytase was measured at various temperatures and pH. In all experiments, the amount of substrate was 1 mM and $CaCl_2$ concentration was 4 mM. The temperature range was from 25 °C to 50 °C and the pH values was from 3 to 7.

3. Results

3.1 Three dimensional structural modeling

The three dimensional structure of the N-terminal domain was predicted (Fig. 1). N-terminal domain consists of beta sheets and is shaped propeller (Fig. 1A). The polar residue E140, E217, E263 and the hydrophilic residue Q172 are located in the top of the propeller (Fig. 1B).

3.2 Site-directed mutagenesis and sequencing

All PCR products were analyzed by agarose gel electrophoresis (Fig. 2). Forward fragments and revers fragments were identified from 400bp to 800bp (Fig. 2A) and from 1400 to 1000bp (Fig. 2B) respectively. Overlap PCR products were shown around 1800bp (Fig. 2C). Plasmids carrying mutant gene were sequenced and confirmed that all four codons were converted to alanine codons (Fig. 3).

3.3 Expression and purification of recombinant proteins

The supernatant obtained by centrifugation after overexpression at 25 °C for 18h was purified by Ni-NTA His tag column and SDS-PAGE showed that all purified enzyme place near 70 kDa (Fig. 4).

3.4 Effects of temperature and pH on mutant phytase

The relative activity of PSphy was measured at various temperatures and pH values. In all experiments, the substrate concentration was 1 mM and $CaCl_2$ concentration was 4 mM. The relative activity of PSphy was measured at various temperature (25–50 °C) (Fig. 5A). E263A showed the highest comparative activity at all temperatures. Significant differences in enzyme activity at various temperatures (25–50 °C) were observed only at E263A, in which alanine was substituted for the original residue at position 263. At the optimum temperature of 40 °C, enzyme

activity increased by 25.89%. The largest change occurred at 45 °C, at which enzyme activity increased by 39.22%. Activity measurement at various pH resulted in similar results to temperature (Fig. 5B). Significant differences in enzyme activity at various pH values (3–7) were observed only at E263A. At the optimum pH 6, enzyme activity increased by 29.58%. The largest change occurred at pH 7, at which enzyme activity increased by 36.84%.

4. Discussion

Phytic acid and phytate is a major storage form of phosphorus of plants and found in lots of plant derived foods. But mineral chelating property of phytic acid makes it act like an anti-nutritional factor that results in indigestion due to the formation of salts with cations. Phytase is an important enzyme that degrade this phytate salt and is used in many food relative industries for enhancing nutritional value of feed [9]. In our previous experiments [6], phytase from *Pseudomonas* sp. FB15 were found to have C-terminal domain and additional N-terminal domain consisting of 275 amino acids which does not exist in most of BPP from *Bacillus* sp. The additional N-terminal domain improves the catalytic efficiency and activity at low-temperature [6, 7]. These suggest that the N-terminal domain may play an important role in phytate degradation and catalytic efficiency. Until now, studies on which amino acid residues are key to activity by replacing amino acid residues in a catalytic domain of phytase have been conducted [10-12]. However, little research has been carried out to determine the properties of residues in the additional N-terminal domain of PSphy, which is crucial for further development.

In the previous study, it was confirmed that calcium binding site and the phosphate binding site of BPP are polar amino acid residues such as D, K, R, E, and Q. In this study, four residues (E140, Q172, E217, E263) located in the PT region that is generally the active site of BPP in the N-terminal domain were substituted with alanine by using site directed mutagenesis to assay mutant phytase activity. Altering amino acid residues affects the property of the enzyme such as optimum pH and activity [13]. When the negative charge is reduced by replacing the negatively charged residues distributed on the catalytic surface, the specific activity and stability of BPP is affected [14]. Significant differences occurred only in E263A at temperature and pH condition, despite the substitution of four charged residues, similarly charged. The prediction of intramolecular interactions of mutant E263A resulted in the formation of new hydrophobic interactions (Fig. 6). Increased hydrophobic interactions in protein have been reported to increase enzyme stability and

activity [15, 16]. Due to this increased interaction, a significant difference in relative activity would have occurred in E263A, which means that role of amino acid residue at position 263 on the activity of the enzyme is important. It may be more important to reduce the polar residue content of the N-terminal domain and increase the hydrophobic residue portion in order to have a positive effect on the PSphy activity.

In summary, the increased activity of PSphy mutant, E263A, at various temperature and pH through site-directed mutagenesis and the importance of the amino acid residue at position 263 in the N-terminal domain with formation of new hydrophobic interaction were observed. Since much research has not been done on the N-terminal domain of BPP so far, the results of this study will help in understanding BPP deeper.

Table 1 Primer used for site-directed mutagenesis

Primer name	Primer sequence	Size (bases)
PSphy FP(Nde I)	5`-GGCCCATATGAAGATTTCCAGGCTGTAC-3`	28
PSphy RP(Xho I)	5`-GGCCCTCGAGGGGCAACTTCAGCGCGCG-3`	28
E140A FP	5`-GGTGGGC**GCC**GAGGGCAAGG-3`	20
E140A RP	5`-CCTTGCCCTC**GGC**GCCCACC-3`	20
Q172A FP	5`-CGTCGGCG**GCC**TTCTGCCAG-3`	20
Q172A RP	5`-CTGGCAGAA**GGC**CGCCGACG-3`	20
E217A FP	5`-CCAAGCGT**GCC**GCGGGCGCC-3`	20
E217A RP	5`-GGCGCCCGC**GGC**ACGCTTGG-3`	20
E263A FP	5`-GGAGCCG**GCC**CAACTCAGCG-3`	20
E263A RP	5`-CGCTGAGTTG**GGC**CGGCTCC-3`	20

Restriction enzyme sites are underlined.
Nucleotides in bold are mutated sites.

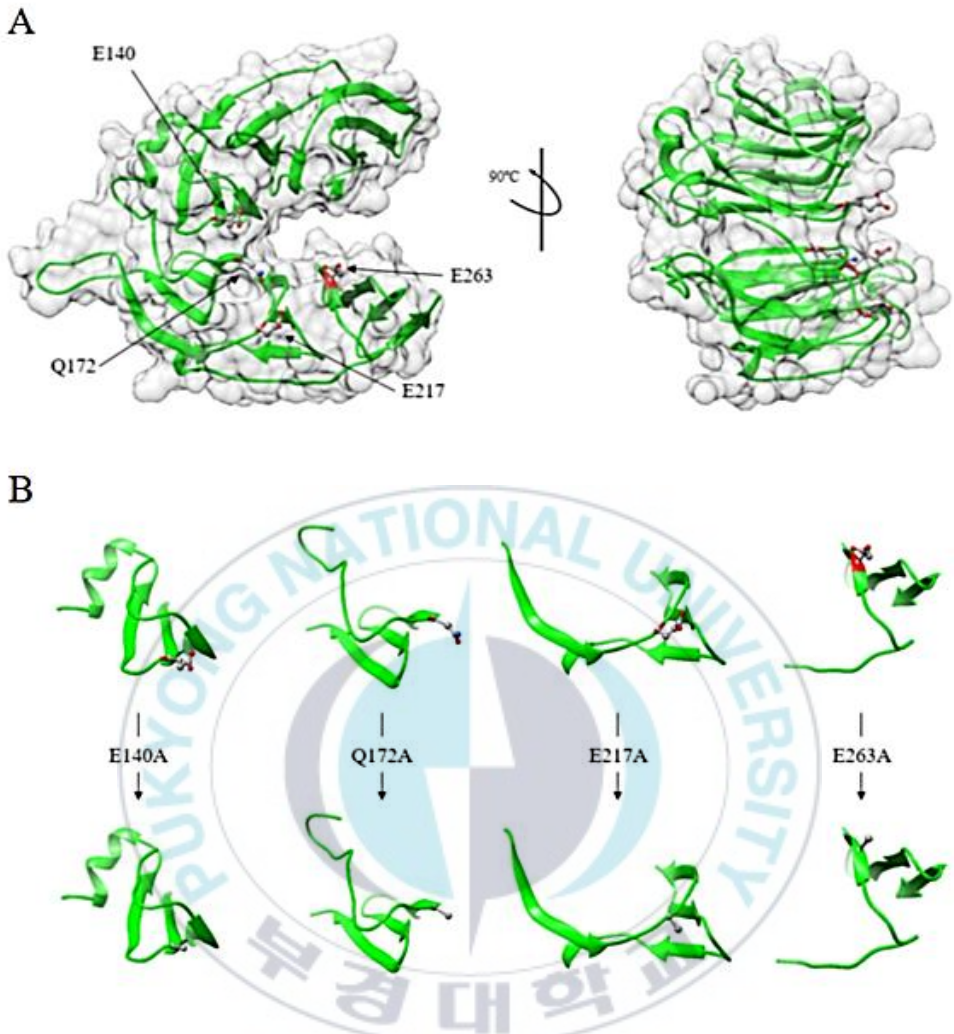

Fig. 1. Prediction of three-dimensional structure of PSphy. (A) N-terminal domain and (B) amino acid substitution sites of PSphy.

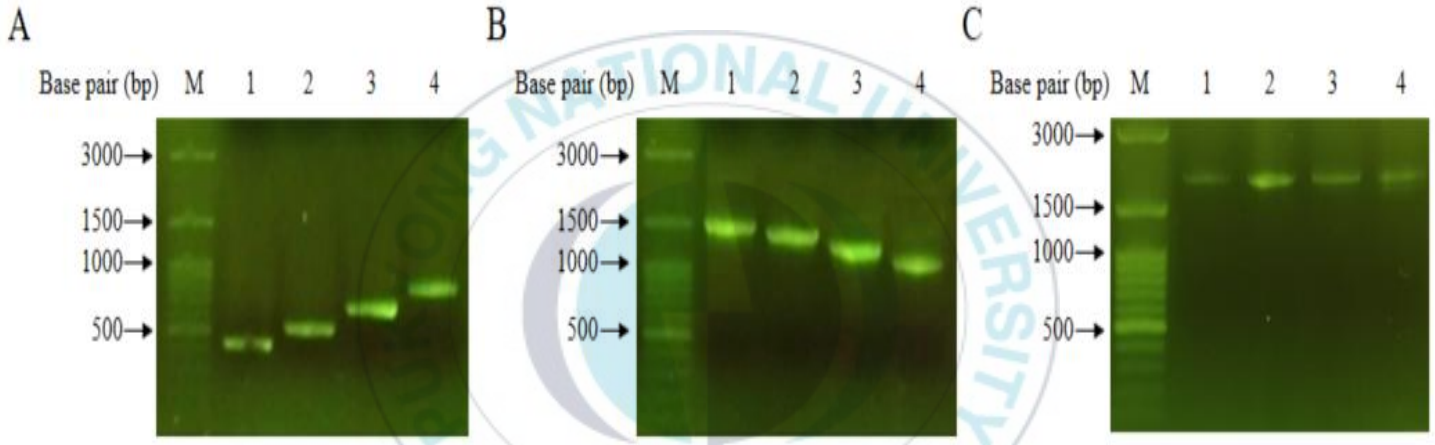

Fig. 2. Agarose gel electrophoresis of PCR products. (A) forward fragment; (B) reverse fragment; and (C) overlap extension PCR products. Lane M. DNA molecular marker; Lane 1. E140A; Lane 2. Q172A; Lane 3. E217A; Lane 4. E263A.

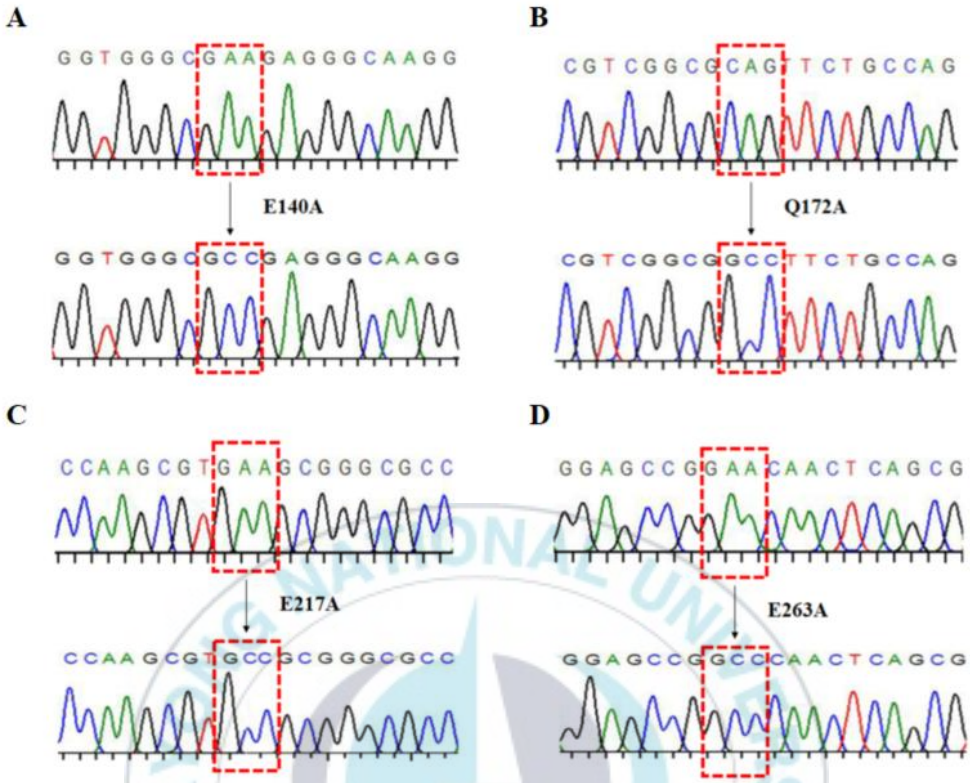

Fig. 3. Sequence of substituted nucleotides by site-directed mutagenesis. (A) E140A, (B) Q172A, (C) E217A, and (D) E263A.

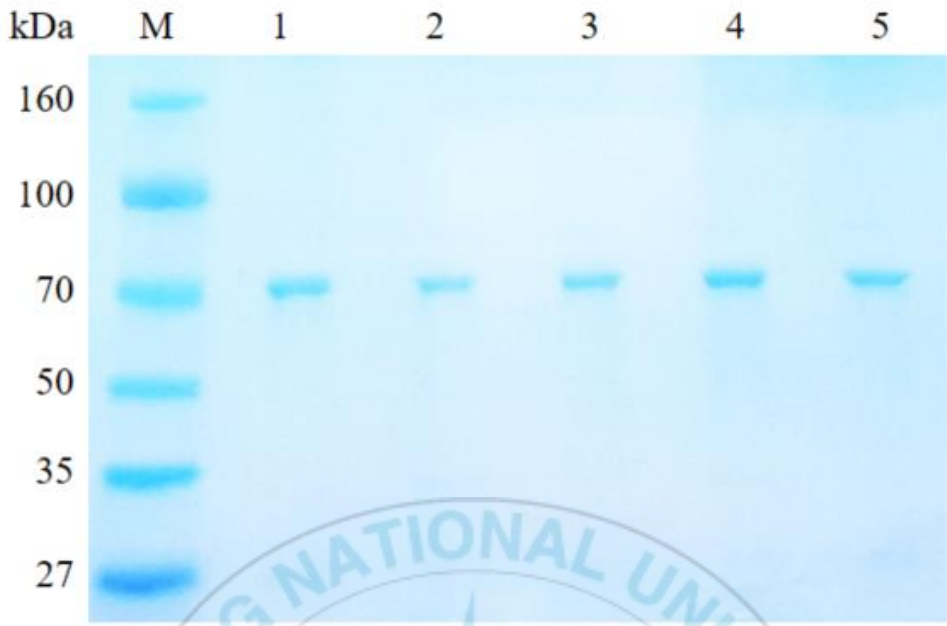

Fig. 4. SDS-PAGE of the purified recombinant phytase. Lane M, molecular weight marker; Lane 1, wild-type; Lane 2, E140A; Lane 3, Q172A; Lane 4, E217A; and Lane 5, E263A.

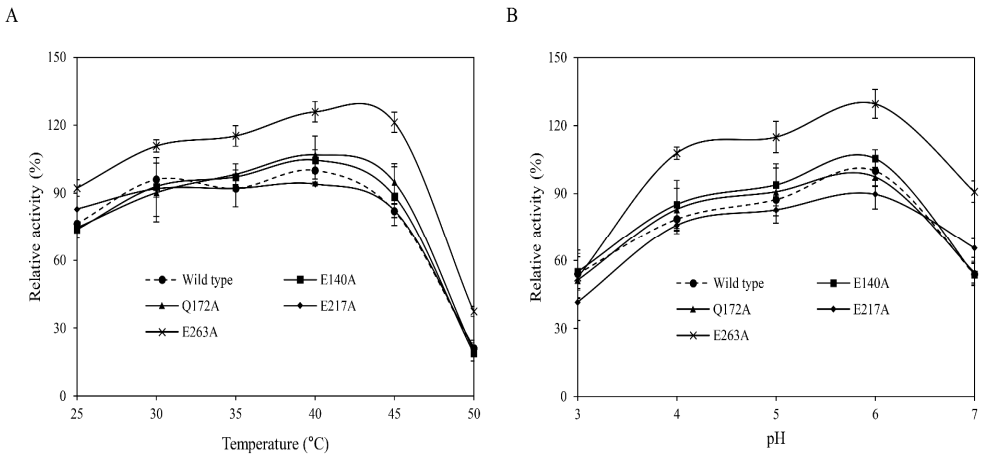

Fig. 5. Effects of temperature and pH on wild-type and mutants phytase activity. Enzyme activity was assayed at various (A) temperatures and (B) pH using 1 mM sodium phytate as a substrate in the presence of 4 mM $CaCl_2$. Relative activity was expressed based on the activity of the wild-type under optimal conditions (40 °C, pH 6).

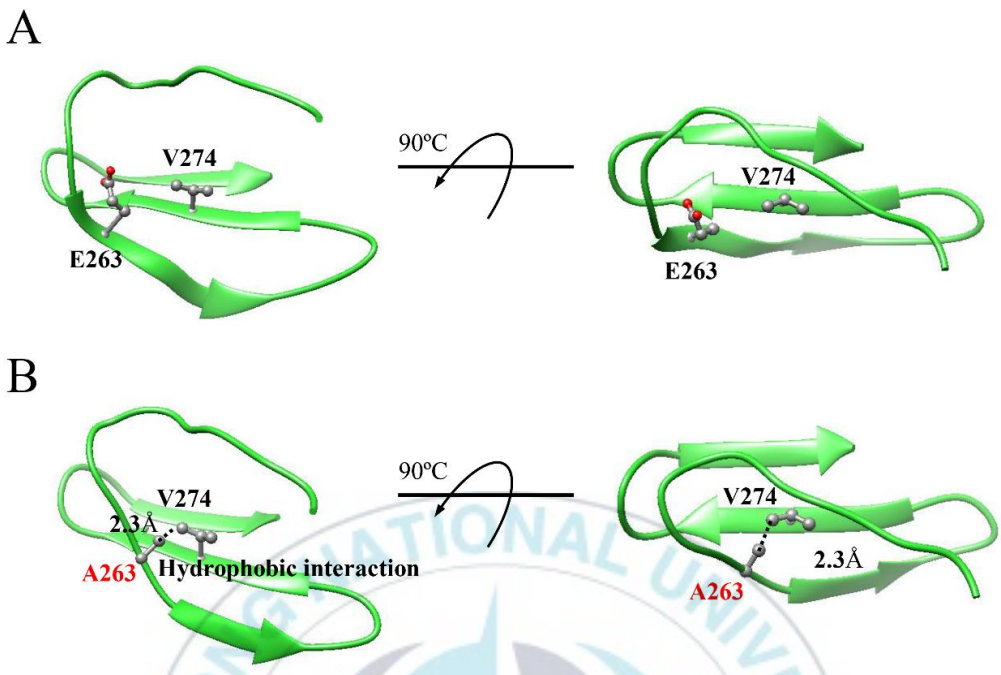

Fig. 6. Schematic diagram of intramolecular interactions of wild-type and E263A. (A) There is no interaction with E263 and V274 in wild-type. (B) The prediction of intramolecular interactions of mutant E263A resulted in the formation of new hydrophobic interactions.

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Abstract (in Korean)

Pseudomonas sp.로부터 유래된 Phytase 의 N-terminal domain 에서 부위지정 돌연변이에 의한 극성 아미노산 잔기의 치환이 효소 활성에 미치는 영향

이가혜

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요 약

Pseudomonas sp. FB15 phytase의 N-terminal domain은 저온 활성 및 그것의 촉매 효율을 증가시킨다. 효소의 활성에 영향을 미치는 N-terminal domain의 중요성은 분명하지만 그것에 대해 많은 연구가 수행되지 않았다. 따라서 본 연구는 부위 지정 돌연변이 유발을 이용하여 N-terminal domain에서 아미노산 잔기의 치환을 통한 효소 활성의 변화를 알아 내기 위해 수행되었다. N-terminal domain의 3차 구조를 예측했고 활성 부위로 추정되는 부위에서 4개의 극성 아미노산 잔기 (E140, Q172, E217, E263)를 부위지정 돌연변이 방법을 이용하여 치환하였다. 알라닌(A) 잔기로 치환된 돌연변이 효소의 활성을 다양한 온도 및 pH에서 조사하였다. 효소 활성의 유의적인 차이는 E263A 재조합 단백질에서만 관찰되었다. E263A 재조합 단백질의 3차 구조를 예측하여 단백질 분자 내 상호 작용을 확인한 결과, 발린 잔기와 치환 된 알라닌 잔기 사이에서 새로운 소수성 상호 작용이 형성되는 것으로 나타났다. 이러한 분자 내 상호 작용은 효소 활성에 영향을 미칠 수 있으며, 이는 N-terminal domain에 263번째 아미노산 잔기가 효소 활성에 중요하다는 것을 보여준다.

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마지막으로 항상 든든한 버팀목이 되어주는 가족들 모두 고맙고 사랑합니다.