Thesis for the Degree of Master of Engineering

A new beta-propeller phytase with optimal activity at low temperature produced by marine microorganism

by

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Abstract

The aim of this study was to isolate phytase, which shows high activity at low temperatures. For this purpose, marine microorganism, producing phytase with high activity at low incubation temperature, was isolated from foreshore soil. The results of 16s rRNA sequence analysis showed that selected marine microorganism was similar to *Pseudomonas* sp. The phytase gene from the isolated *Pseudomonas* sp. was cloned and sequenced. Sequence analysis showed a 1,863 bp fragment encoding 620 amino acid residues. The molecular weight was found to be about 70 kDa by SDS-PAGE. Molecular modeling predictions with the obtained amino acid sequence seemed to belong to the group of beta-propeller phytase (BPP). The characterization of recombinant BPP from *Pseudomonas* sp. (PSphy) showed a highest activity at pH 6 and 40°C. However, our study found that 80% of optimal activity was shown even at the relatively low temperature of 25°C. In addition, $CaCl_2$ was required to show activity and the optimal concentration of $CaCl_2$ was 4 mM. The PSphy activity was maintained at 60 g/l of salt concentration and it was confirmed that the catalytic efficiency was 0.024 $\mu M^{-1} \cdot s^{-1}$ in the optimal conditions. Based on these characteristics, found in this study, it can be suggested that PSphy is a candidate for feed additives in the aquaculture industry and can be used to study low temperature activation mechanisms.

1. Introduction

Phytic acid also known as inositol hexakisphosphate (IP6) is the major storage form of phosphorus in many plant tissues. The six phosphates bound to phytic acid have a strong negative charge and bind strongly to important minerals such as calcium, iron and zinc in the body to form indigestible compound, phytate-mineral salt [1, 2]. Phytate is discovered in potentially usable plant derived ingredients of fish and animal feed such as canola meal and soybean [3, 4]. However, one of the main problems related with the use of plant in fish feed is the existence of anti-nutritional factors, such as phytate-mineral salt and is actually not available for agastric or monogastric aquatic animals [4].

Phytase (myo-inositol hexakisphosphate phosphohydrolase) is an enzyme that hydrolysis from the phytate to frees inorganic phosphorous. This enzyme is widely used to upgrade the nutritional quality of phytate-rich foods and feed additives, ultimately improve the bioavailability of minerals and phosphorus. For these reasons, it has been used as feed additive of swine and poultry. Hence, it can also be applicable to the aquaculture industry and the human food industry.

Phytases are classified into two classes, based on the optimal pH: acid phytases and alkaline phytase. The acid phytases are further divided into three different types of enzymes according to the catalytic mechanism, such as histidine acid phytase, cysteine phytase (purple acid phytase) and protein tyrosine phosphatase. Alkaline phytase is beta-propeller phytase (BPP) by another name [5]. Among the four types, BPP is the main phytate hydrolytic enzymes, and may play a significant role in phosphorus cycling in aquatic environment [6].

Previous reports stated that the optimal pH of most BPPs was neutral pH (pH 6-8). It is appropriate in aquatic environments with a close neutral pH, such as lake water (pH slightly below 7.0) and seawater (pH 8.0) [5]. When used as a feed additive in the aquaculture industry, high activity is required in neutral gut pH of fish. Moreover, the seawater temperature should also be taken into consideration along with the pH and other factors. The optimal temperature of BPP is very diverse

and phytase which show high activity at low temperature is very uncommon. For these reasons, BPPs with high activity at low temperature are potential candidates as feed additives in aquaculture industries [7]. In this context, it is necessary to search for novel phytase or modify them into suitable enzymes through enzyme modification for applying to the aquaculture industry.

Therefore, the present study was aimed to isolate and characterize the marine microorganism which produce phytase, having high activity at low temperature, and to analyze the enzyme kinetics of the phytase for optimal activity and catalytic efficiency.

2. Materials and Methods

2.1. Bacterial strains, plasmids, media and culture conditions

Bacteria isolated from foreshore soil of coast in Busan, Korea (35°02'43.8"N 128°57'50.7"E) were cultivated at 25°C on phytase screening medium (PSM) (2% glucose, 0.5% NH_4NO_3, 0.4% sodium phytate, 0.2% $CaCl_2 \cdot 2H_2O$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.001% $MnSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, pH 6.5 with 1.5% agar). The colony with clear zones around was selected and streaked onto fresh PSM plates [8]. *Escherichia coli* DH5α and BL21 (DE3) were used as hosts for gene cloning and protein expression, respectively. The *E. coli* strains were grown in Luria-Bertani medium at 25°C for overexpression, supplemented, when necessary, with ampicillin at 100μg/ml. pET-22b (+) was used for cloning and nucleotide sequencing.

2.2. Cloning and sequencing of phytase gene

We designed two primers based on alignment of several phytase from the reported strain for amplify the phytase gene. The oligonucleotide sequences were phy FP (Nde1): 5′-GGC CCA TAT GAA GAT TTC CAG GCT GTA C-3′ and phy RP (Xho1): 5′-GGC CCT CGA GGG GCA ACT TCA GCG CGC G-3′. The PCR conditions were pre-denaturation at 94°C for 10 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 2 min, followed by a final extension at 72°C for 7 min. DNA fragment of amplified was ligated into pET-22b(+) using a ligase. The recombinant plasmid was used to transform competent *E. coli* DH5α. It was again inserted into *E. coli* BL21 (DE3) competent cells. The complete nucleotide sequence of the insert was determined.

2.3. Overexpression and purification of recombinant phytase

Cells containing the recombinant plasmid were cultured in 500 ml LB medium containing ampicillin (100 μg/ml) at 37°C. When the optical density at 600 nm reached 0.5, Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 1 mM. After 16 h, the cells were harvested by centrifugation (7000 rpm for 10 min) and resuspended in 40 ml of Tris–

HCl buffer (pH 8.0). The resuspended cells were sonicated and centrifuged (12,000 rpm for 10 min). The supernatant was loaded into a Ni–NTA His-tag affinity chromatography. The purified purified enzyme was identified by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were stained with Coomassie blue. Determination of the purified phytase was measured using a Bradford assay, with bovine serum albumin as the standard.

2.4. Phytase activity assay

Phytase activity was measured by modified Molybdate-Blue Method [9]. It is based on the colorimetrical quantification at 700 nm of free phosphorus released by the hydrolysis of phytate using ammonium molybdate as color reagent. A reaction mixture containing 200 μl of enzyme preparation, 800 μl of 1 mM sodium phytate in 100 mM Tris-HCl buffer pH 6, and 4 mM $CaCl_2$ was incubated at 40°C for 30 min. The reaction was stopped by adding 1 ml of 5% (w/v) trichloroacetic acid. The released inorganic phosphate was determined by adding 1 ml coloring reagent (1.2% (w/v) ammonium molybdate, 0.54% (w/v) ferrous sulfate and 3.5% (v/v) sulfuric acid), and the absorbance was measured at OD_{700}.

2.5. Amino acid sequence analysis and homology

The homology of the protein sequence was determined using BLAST program. The signal peptide was predicted by the signalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). A multiple protein sequence alignment was carried out using the Clustal W program. The phylogenetic tree was constructed by the neighbor joining method using MEGA 6 with 1,000 bootstrap repetitions. Structure of PSphy were predicted using SWISS-MODEL (https://swissmodel.expasy.org/). Interactions involved phosphate binding site were predicted using PDBsum (http://www.ebi.ac.uk/thornton-srv/databases/pdbsum).

2.6. Characterization of purified recombinant phytase

2.6.1. Optimal pH and temperature

The optimal pH and temperature for phytase activity was determine. The relative activity of the enzyme against 1mM sodium phytate was measured at pH values of 3-10. The substrates were dissolved in 100 mM Tris–HCl. The optimal temperature was determined by measuring phytase activity in optimal pH and Ca^{2+} concentration at temperatures ranging from 20-60°C.

2.6.2. Thermal stability

The thermal stability of phytase at 20-100°C was determined by measuring the residual phytase activity after incubation for various periods of time with 5 mM $CaCl_2$ and without $CaCl_2$.

2.6.3. Effects of metal ions and various phytate

The effect of several metal ion was determined by measuring the phytase activity in the presence of 1, 5 and 10 mM Na^+, K^+, Mg^{2+} or Ca^{2+} under the optimal conditions. The effect of calcium concentration was investigated by measuring phytase activity between 0 and 5 mM $CaCl_2$ under the optimal conditions. Phytase activity was determined by measuring after incubation in 100 mM Tris-HCl (pH 6) supplemented with 4 mM $CaCl_2$ and 1mM of tested various phytate (sodium phytate, calcium phytate and dipotassium phytate) at 40°C for 30 min.

2.6.4. Kinetic parameters

To determine kinetic parameters, phytase activity was measured at 40°C in 100 mM Tris-HCl (pH 6) include various concentrations of sodium phytate (0.1-2 mM). The V_{max}, K_m and K_{cat} values were calculated from Lineweaver-Burk plots.

2.6.5. Thermodynamic parameters

Thermal inactivation of phytase was determined by incubating the enzyme with 5 mM $CaCl_2$ and without $CaCl_2$ at various temperatures in the absence of substrate. Sample were withdrawn at different time intervals, cooled on ice for 10 min and residual phytase activity was determined at optimal conditions. The kinetics for irreversible thermal denaturation (K_d) of phytase was

determined and Arrhenius plot was applied to determine the activation energy for denaturation (E_d). Other thermodynamic parameters were calculated using the following equations. $\Delta H = E_d - RT$, $\Delta G = -RT \cdot \ln (K_d \cdot h / K_b \cdot T)$, $\Delta S = (\Delta H - \Delta G) / T$. Where h, K_b, ΔH, ΔG and ΔS are Planck's constant (11.04 · 10^-36J min), Boltzmann's constant (1.38 · 10^-23JK^{-1}), enthalpy change, free energy and entropy change, respectively.

3. Results

3.1. Screening of phytase producing bacteria

Bacteria strains isolated from marine environment were screened on PSM including the sodium phytic acid. Among these strains, Bacteria forming the largest clear zone around the medium was selected. (Fig.1a). 16S rRNA sequence of selected bacteria showed 99% homologous with *Pseudomonas extremorientalis* and *P. fluorescens*. The growth curve of the selected bacteria showed the highest growth rate at 25°C (Fig.1b).

3.2. Gene cloning and sequence analysis of phytase

Phytase gene was amplified by PCR using universal primers designed based on the nucleotide sequences from related *Pseudomonas* species. The sequence analysis showed 1,863 bp fragment was contained which encoded a 620 amino acid residue. Using a signalP 4.1 server confirmed the signal peptide composed of 20 amino acid. 275 amino acids constituted N-terminal domain containing the signal peptide and the remaining amino acids constituted C-terminal domain. Results of the SMART database and structure-based sequence alignments were similar to *Pseudomonas* sp. 10-3-11, it was shown that the N-terminal repeat domain of full amino acid does not contain active-site residues, whereas C-terminal domain provide a catalytic activity [10]. The amino acid sequence was compared with the BPPs containing the N-terminal domain of which the characteristic were revealed. The full amino acid sequence showed 64%, 49%, 39% and 33% homologous with those of *Pseudomonas* sp. 10-3-11 [10], *Janthinobacterium* sp. TN115 [11], *Bacillus* sp. HJB17 [12] and *Shewanella oneidensis* MR-1 [5] respectively (Fig.1c). But N-terminal domain sequence showed 54%, 35%, 25% and 19% homologous with the same bacteria, respectively.

Compared to the reported phytase containing the N-terminal domain. Active site involved phosphate and calcium binding site of conserved in beta-propeller phytase were exist [7, 13].

3.3. Expression and purification of recombinant phytase

The recombinant plasmid for overexpression of PSphy was constructed to pET-22b (+) vector. Recombinant protein was expressed in *E. coli* BL21 and purified by Ni-NTA His-tag affinity chromatography. Enzymes were overexpressed in 1mM of IPTG and present in a soluble protein. Results using the SDS-PAGE confirmed the molecular weight was approximately 70 kDa (Fig.2).

3.4. Effect of temperature and pH on purified recombinant phytase

The optimal temperature for PSphy was 40°C. It has an optimal activity at a lower temperature than the *Pseudomonas* sp. 10-3-11 (50°C) [10], *Bacillus* subtilis (55-60°C) [3] and *Bacillus amyloliquefaciens* (70°C) [3]. Thermal stability remained more than 80% at 40°C with $CaCl_2$. The remaining activity at higher temperature decreased sharply. However, without $CaCl_2$, more than 80% activity remained at higher temperature than 40°C (Fig.3a). This result is different from the result that the thermal stability increases with increasing concentration in Ca^{2+} other BPP reported [14]. The activity remaining at 10 minute intervals from 60-100°C were also measured in the absence of $CaCl_2$ (Fig.3c). The optimal pH for PSphy was pH 6.0. It showed a more than 60% activity at pH 4-7 range (Fig.3b).

3.5. Effect of substrate and metal ions

Table 1 shows that sodium-phytate is more specific than other phytates. Metal ions results showed a high activity under the presence Ca^{2+} like other reported BPP (Table 2) [15]. However, the measurement results of Ca^{2+} concentrations of other BPP activity was reported highest activity at concentrations of 1~2mM of Ca^{2+} ions whereas PSphy was observed the highest activity in the 4mM of Ca^{2+} ions (Fig.3d). In *Shewanella oneidensis* MR-1, it is expected that this result is due to the alternation of four amino acid residues that contain high affinity calcium binding sites [5]. PSphy also had altered amino acid residues at the same position.

3.6. Kinetic and thermodynamic parameters

The kinetic parameters of the enzyme measure to sodium phytate were calculated from Lineweaver-Burk plots. The K_m, V_{max}, K_{cat} and K_{cat}/K_m values of PSphy were 0.858 mM, 18.18 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, 20.39 s^{-1} and 0.024 $\mu M^{-1} \cdot s^{-1}$ respectively (Table 3).

The enzyme thermal stability of PSphy was sharply reduced above 40°C in the presence of $CaCl_2$ while it was stable up to 60°C in the absence of $CaCl_2$, for this reason thermal inactivation of the PSphy was investigated at three temperatures (50, 55 and 60°C). The thermodynamics parameters of enzyme thermo inactivation at different temperatures was calculated (Table 4). The activation energy for denaturation (E_a) was 251.681 kJ/mol in the absence of $CaCl_2$, and 143.3 kJ/mol in 5 mM $CaCl_2$. Therefore, the higher E_a in the absence of $CaCl_2$ indicates that the conformational structure of the enzyme is more stable than presence of $CaCl_2$. The free energy change (ΔG), enthalpy change (ΔH), and entropy change (ΔS) are related with the enzyme stability. Enzyme in the presence of $CaCl_2$ showed smaller ΔG in all of the measured temperatures. A smaller ΔG is indicative of lower enzyme stability [14]. ΔH parameter showed smaller value in the presence of $CaCl_2$. This result also indicate of lower enzyme stability. Both ΔG and ΔH showed a tendency to decrease with increase temperature. This results suggest that PSphy was more stable under the absence of $CaCl_2$ than in the presence of $CaCl_2$.

3.7. Nucleotide sequence accession numbers

The nucleotide sequence for 16S ribosomal RNA gene of *Pseudomonas* sp. was deposited in GenBank under the accession number KY458639. The accession number of the phytase gene (PSphy) was KY471463.

4. Discussion

In feed additive industry, phytase is one of very important enzymes, because it hydrolyzes the indigestible phytate. For industrial applications, higher catalytic efficiency and thermal stability are important factors [7]. In addition, not only high activity at neutral pH such as fish gut but also high activity at low temperature such as aquatic environment is important [5, 13]. Among various phytases, BPP is most suitable under these conditions. Other phytases are classified as acid phytases and the optimal pH is acidic. BPP with high activity at neutral pH is the most beneficial phytase in the aquatic environment as it is known to play a role of phosphorus cycling in soil and aquatic environment.

In the present study, bacterial strain, showing phytase activity at low temperature, was selected from the marine environment. Result of 16s rRNA showed 99% homologous with *P. extremorientalis* and *P. fluorescens*. The phytase produced by the strain belonged to BPP. Its sequence was more similar to *P. extremorientalis* than *P. fluorescens*, and the BPP gene was clonned from *Pseudomonas* sp. and then characterization of the catalytic activity was accomplished.

The characterization results revealed that the optimal temperature of PSphy was 40°C. However, at 25°C, PSphy exhibited more than 80% of the maximum activity. It is better to mention that the growth curves of *Pseudomonas* sp. at various temperatures showed the highest growth rate was at 25°C (Fig.1b). The reason that PSphy can maintain its activity at low temperature is thought to be related to the growth temperature. Bacteria that grow in cold environments, such as *Pseudomonas* sp., are considered as the possible sources of enzymes that can be activated at low temperatures [17].

Sequence analysis showed that PSphy contains two domains (Fig.4). N-terminal domain does not contain active site residues. For this reason it does not degrade IP6 efficiently. The same result was reported in the *Shewanella oneidensis* MR-1 [5] and *Bacillus* sp. HJB17 [12]. On the other hand C-terminal domain contains conserved active site and calcium binding site, and obviously

responsible for the catalytic activity. This region was located on the top of the propeller. Negative charge of the active site provides electrostatic environment for the positive charge of the substrate [18].

Thermal stability of phytase is a very important factor in industrial processes. It was found in most studies that calcium ions are required for thermal stability; that means the effect on the enzyme stability in thermal denaturation [15]. In the present study, PSphy had a high thermal stability in the absence of calcium ion. The result we found was different from the previous study. We investigated the stability of the enzyme with and without calcium ion through thermodynamics parameters. As a result, it was confirmed that the enzyme was stable when calcium was not present. PSphy has altered amino acid residues in high affinity calcium binding sites. Also, the V346 residue was reported to increase the thermal stability by reducing the flexibility of the loop from *Bacillus amyloliquefaciens* US573 [19] was also changed to Q601. Therefore, PSphy seems to maintain thermal stability independently of calcium ion.

Each domain comprises two cysteine residues (124, 174 / 421, 470), which appear to form a disulfide bond. Result of alignment of BPP of *Bacillus* sp. HJB17, *Janthinobacterium* sp. TN115 and *Shewanella oneidensis* MR-1 also demonstrated a disulfide bond in each domain. BPP of gram-negative bacteria has been reported to contain a disulfide bond [20]. However, BPP containing N-terminal domain which was found in both Gram-positive and Gram-negative bacteria included a disulfide bond.

While most BPPs show the characteristics of having highest activity at neutral pH, so classified as alkaline phosphatase, the optimal temperature is variable which ranges between 50°C to 70°C in majority cases. In the present study, the optimal temperature of PSphy was relatively lower than that of the other BPPs and it maintained more than 80% activity even under salt condition of 60g/l (Fig.5). The observation of the low temperature activity to salt tolerance is suggesting the PSphy as suitable for use for aquatic environments. Moreover, in aquaculture, Agastric fishes have a

digestive system with pH of 6.8-7.3. For this reason, it could be concluded that the PSphy, which showed salt tolerance and high activity at neutral pH and at low temperature, is a viable candidate for feed additives in the aquaculture industry.

Table 1. Substrate specificity of PSphy

Substrate	Relative activity (%)
Phytic acid sodium salt	100.0
Phytic acid calcium salt	52.2±1.6
Phytic acid dipotassium salt	9.8±1.1

Table 2. Effects of metal ions on different concentrations

Metal ion	Relative activity (%)		
	1 mM	5 mM	10 mM
Ca^{2+}	100.0	100.0	100.0
Mg^{2+}	23.8±1.6	3.0±0.2	8.7±1.3
Na^{+}	19.8±3.4	5.6±1.1	17.1±2.9
K^{+}	31.8±3.5	3.6±0.5	7.2±0.9

Table 3. Kinetic parameters of beta-propeller phytase

Enzyme	K_m (μM)	V_{max} (μmol·min^{-1}·mg^{-1})	K_{cat} (s^{-1})	K_{cat}/K_m ($\mu M^{-1}\cdot s^{-1}$)	Reference
Pseudomonas sp. PSphy	858	18.18	20.39	0.024	This study
Shewanella oneidensis MR-1 FLPhyS	83	-	2.931	0.035	[5]
Bacillus sp. HJB17 PhyH	500	24.82	27.72	0.054	[11]
Janthinobacterium sp. TN115 PhyA115	280	17.33	22.4	0.08	[10]
Pedobacter nyackensis MJ11 r-PhyP	1280	71.9	45.1	0.035	[12]
Bacillus amyloliquefaciens US573 PHY US573	1125	27.71	28.122	0.024	[18]

- Not reported

Table 4. Thermodynamic parameters of PSphy

a. Without $CaCl_2$

Ea ($kJmol^{-1}$)	Temperature (K)	K_d (min^{-1})	ΔH ($kJmol^{-1}$)	ΔG ($kJmol^{-1}$)	ΔS ($kJmol^{-1}$)
251.681	323.15	0.005	248.993	104.488	0.447
	328.15	0.016	248.952	103.149	0.444
	333.15	0.031	248.910	102.843	0.438

b. 5mM $CaCl_2$

Ea ($kJmol^{-1}$)	Temperature (K)	K_d (min^{-1})	ΔH ($kJmol^{-1}$)	ΔG ($kJmol^{-1}$)	ΔS ($kJmol^{-1}$)
143.300	323.15	0.006	140.613	104.337	0.112
	328.15	0.126	140.572	97.456	0.131
	333.15	0.277	140.530	96.800	0.131

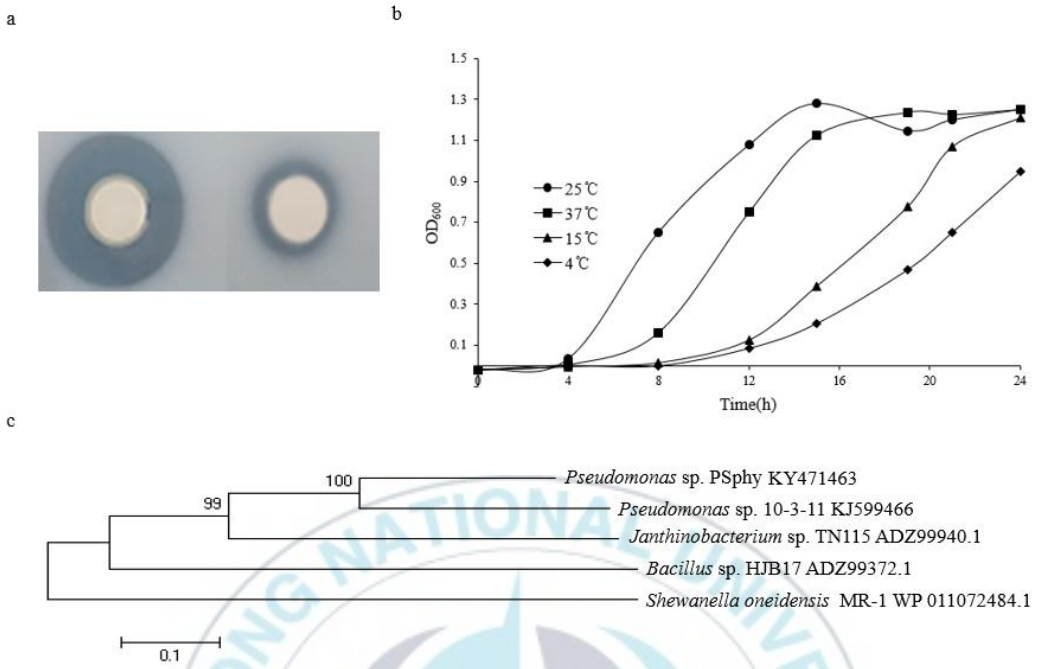

Fig. 1. Characteristics of selected strain. (a) Comparison of phytase activity of phytase producing bacteria. Bacteria forming large clear zone was selected. (b) Growth curve of selected strain. Growth curves of the bacteria were examined at various temperatures. (c) Phylogenetic tree analysis of phytase sequences deduced from amino acid sequences. The phylogenetic tree was constructed by the neighbor joining method using MEGA 6 with 1,000 bootstrap repetitions, after which the amino acid sequences were aligned by the Clustal W program.

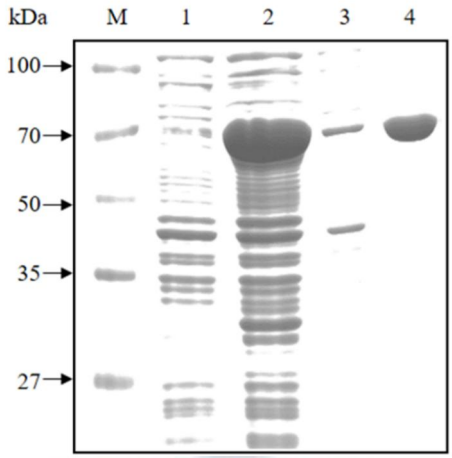

Fig. 2. SDS-PAGE of the purified PSphy. Lane M. molecular weight marker; Lane 1. Crude *E. coli* extract transformed with pET-22 (+) comprising gene; Lane 2. Cell lysates from 16h of culture at 25℃ after IPTG induction; Lane 3. The soluble fraction after induction; Lane 4. Purified recombinant PSphy from Ni-NTA chromatography.

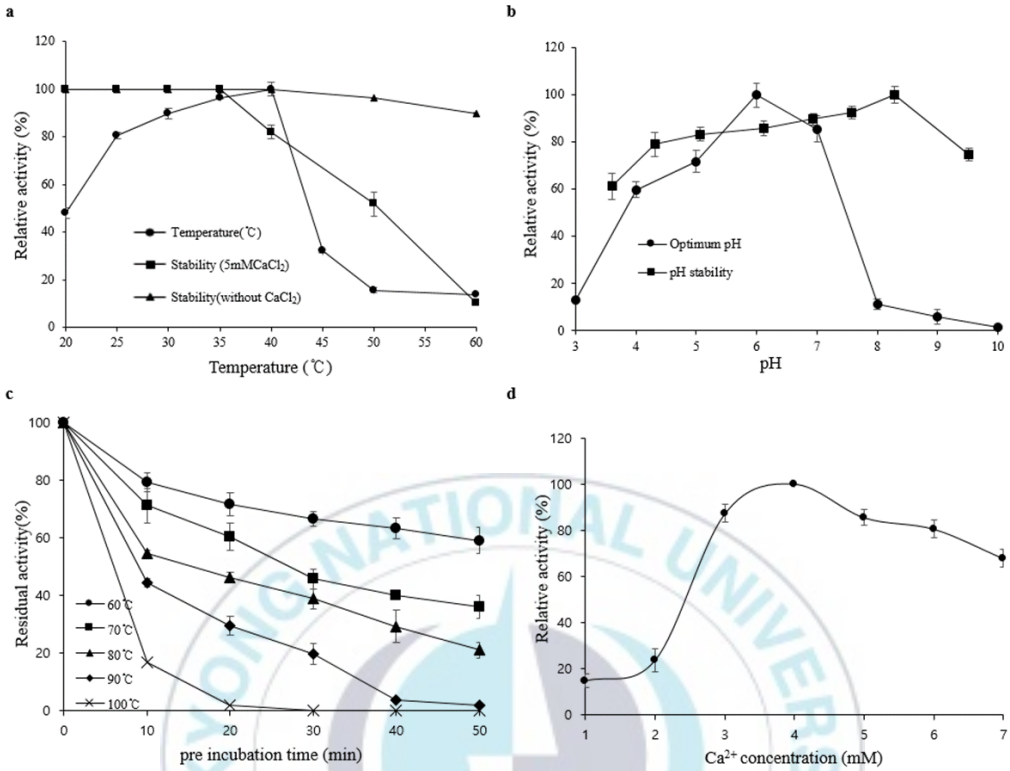

Fig. 3 Characterization of the purified PSphy. (a) The enzyme was assayed at various temperatures (●) using a substrate prepared by mixing 1mM Na-phytate and 4mM Ca2+. PSphy was pre-incubated various temperatures for 10min without (▲) and with (■) 5 mM Ca^{2+}, and residual activity was measured at 40°C. (b) Optimum pH was assayed at various pH. PSphy was pre-incubated various pH for 10min and residual activity was measured at 40°C. (c) Residual phytase activities were determined after incubating the enzymes at various temperatures from 60-100°C. (d) Effects of Ca^{2+} concentrations on the activity of PSphy. The assay was performed at different Ca^{2+} concentrations ranging from 1 to 7 mM.

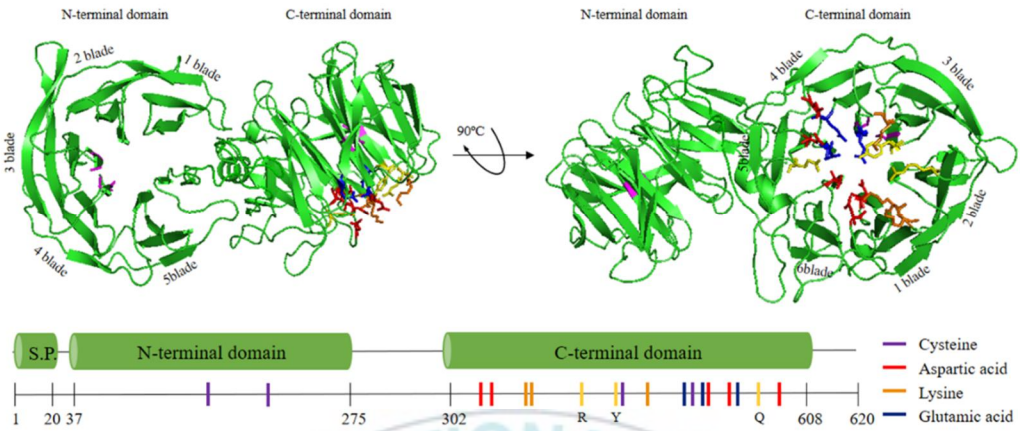

Fig. 4. Prediction of three-dimensional structure of PSphy. N-terminal domain is five bladed beta-propeller. It contains a disulfide bond. C-terminal domain is six bladed beta-propeller. It contains substrate binding site. The amino acid residues involved are indicated as sticks. S.P. indicate signal peptide.

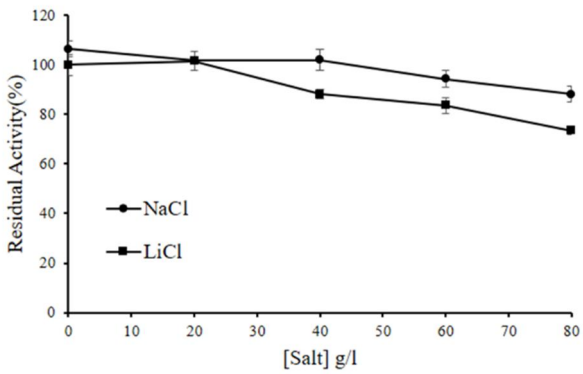

Fig. 5. Effect of salts LiCl and NaCl on the activity of PSphy. The enzyme was assayed at various salts concentrations using a substrate prepared by mixing 1mM Na-phytate and 0-80g/l salts.

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Abstract (in Korean)

해양미생물이 생산하는 신규 저온성 beta-propeller phytase에 관한 연구

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요약

본 연구는 겨울철 해양환경에서 분리한 미생물이 생산하는 beta-propeller phytase(BPP)의 특성에 대한 연구이다. Phytase 선별 배지에서 cleare zone을 형성하는 미생물을 선별하고 16s rRNA를 분석한 결과 *Pseudomonas* sp.와 가장 유사하였다. BPP 유전자를 cloning하여 분자생화학적 특성과 효소학적 특성을 조사하였다. 서열분석결과 1,863개의 nucleotide로 구성된 620개의 아미노산이 존재하였다. 아미노산 서열을 기반으로 분자모델링을 수행한 결과 BPP가 가지는 프로펠러 모양의 구조를 예측할 수 있었고, 보존적인 아미노산 서열을 확인 할 수 있었다. SDS-PAGE를 통해 재조합 단백질이 70kDa의 분자량을 가지고 있음을 확인하였고, 활성 측정을 통해 4mM의 $CaCl_2$가 존재할 때 가장 높은 활성이 나타남을 확인하였다. 최적 pH는 6이었고, 40℃에서 가장 높은 활성을 보였다. 또한 60g/l의 염 농도에서 80%이상의 활성이 있음을 확인하였다. Kinetic parameter를 조사한 결과 K_m, V_{max}, K_{cat}, K_{cat}/K_m은 각각 0.858mM, 18.18μmol・min^{-1}・mg^{-1}, 20.39s^{-1}, 0.024μM^{-1}・s^{-1}임을 확인하였다. 높은 염 농도에서의 활성과 다른 보고된 BPP보다 비교적 낮은 최적 온도의 특성은 양식산업에서 사료 첨가제로서의 응용가능성을 보여준다.

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