토양세균 Fluorescent *Pseudomonas* sp. BUN 1 균주 유래의 파이테이즈(Phytase)의 일반적 특성규명

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General Properties of Phytase Produced by Fluorescent *Pseudomonas* sp. BUN1

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요 약

우사주변 경작지 토양으로부터 세포내 파이테이즈(phytase) 생산능력이 우수한 Fluorescent *Pseudomonas* sp. BUN1 세균성 균주를 분리, 동정하였다. 그 균주로부터 유래한 BUN1 파이테이즈(phytase) 효소를 각각 양이온, 음이온 크로마토그래피 기법을 이용하여 부분정제하여 효소적 특성을 규명한 결과, 각각 40℃와 pH 5.5에서 최적의 효소활성을 나타내었다. BUN1 파이테이즈(phytase)는 효소의 기질특이성 측면에서 다른 유기인산화합물에 비해 특히 피틴태인(phytate)의 분해이용성이 매우 우수한 반면, 구리(Cu^{2+}), 카드뮴(Cd^{2+}), 아연(Zn^{2+})과 같은 금속 2 가이온에 대하여 그 효소활성이 강하게 억제되었다. 또한 BUN1 균주의 효소생산 배지(PSM) [0.5% sodium phytate, 0.5% $(NH_4)_2SO_4$, 0.5% KCl, 0.01% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCl_2 \cdot 2H_2O$, 0.01% NaCl, 0.001% $FeSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot 4H_2O$; pH 6.5]에 탄소원으로서 옥수수전분(corn starch)의 첨가는 조사된 다른 탄소 배지원에 비하여 현저하게 파이테이즈(phytase) 생산을 촉진시켰다.

(**주요어 :** Bacterial, Intracellular, *Pseudomonas*, Phytase, Phytate)

Ⅰ. INTRODUCTION

Phytase (myo-inositol hexakisphosphate phosphohydrolase, EC 3.1.3.8 and EC 3.1.3.28) belongs to a member of histidine acid phosphatases and initiates the hydrolysis of phosphate from phytate (myo-inositol hexakisphosphate), which is the major phosphate reserve in cereal grains, legumes, and oilseeds industrially used for manufacturing animal feeds (Haefner et al., 2005; Reddy et al., 1982). Since monogastric animals, like swine and poultry have very low or no phytase activities in their digestive tracts, most of the ingested phytate is not metabolized and discharged in their feces, contributing to environmental concerns in areas with concentrated livestock production (Comon, 1989; Wodzinski and Ullah, 1996). Phytate also forms insoluble complexes with nutritionally important metals such as iron, zinc, calcium, magnesium, and proteins, thereby showing anti-nutritional effects (Rojas and Scott, 1968). Supplemental phytase to diets for these animals effectively improves the bioavailability of phytate-phosphorus, thus alleviating phosphorus pollution.

So far, a variety of phytases have been widely found in plants and microorganisms (Oh et al., 2004). Although two filamentous fungal-derived strains, *Aspergillus niger* and *Aspergillus ficuum* are currently available for the commercial production, studies are ongoing to identify more desirable phytases and to induce their over-expression in industrially attractive host, such as yeast or *E. coli* through recombinant DNA work for animal feed application so as to overcome the defects of the fungal enzyme with regard to substrate specificity and catalytic efficiency (Chi et al., 2009; Huang et al., 2009; Hussin et al., 2007).

In this study, an intracellular phytase produced by a bacterial isolate, fluorescent *Pseudomonas* sp. BUN1 was partially characterized.

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Ⅱ. MATERIALS & METHODS

1. Screening of a phytase-producing bacterium

The screening of a phytase-producing bacterium was performed by a modified method of Richardson and Hadobas (1997). Various soil samples were collected from cultivable fields near cowsheds. Approximately fifty grams of each soil were suspended in 100 ml of sterile 0.9% (w/v) NaCl, and subjected to reciprocal shaking (200 rpm, 2h). After 20 min settling period, each aliquot (1 ml) of the soil suspension was inoculated into 100 ml of phytase-synthetic medium (PSM) in 500 ml Erlenmeyer flasks. This synthetic medium was composed of (g/l) : [sodium phytate, 5; $(NH_4)2SO_4$, 5; KCl, 5; $MgSO_4 \cdot 7H_2O$, 0.1; $CaCl_2 \cdot 2H_2O$, 0.1; NaCl, 0.1; $FeSO_4 \cdot 7H_2O$, 0.01; $MnSO_4 \cdot 4H_2O$, 0.01; pH 6.5], using sodium phytate as a sole source of carbon and phosphate, and each flask was incubated at 28℃ for 48h with vigorously shaking. 1 ml of the cultures was appropriately diluted, plated on PSM, and incubated at 28℃ for 3~4 days. Diversified morphological types of bacterial colonies were selected and purified. Each of the isolated strains was grown in 10 ml of PSM broth at 28℃ for 48 h on a reciprocal shaker (200 rpm). Cells were harvested by centrifugation at 5,000 g for 10 min, resuspended in 1 ml of 50 mM acetate buffer (pH 5.5) and ground with 0.2 g of glass beads (425~600 microns; Sigma) using Bead-Beater (Biospect Products). The supernatant fluids were collected by centrifugation, and used for the phytase activity assay.

2. Identification of a phytase-producing bacterium

The bacterial strain showing the highest phytase activity was identified by Gram staining, as well as biochemical, and physiological tests. The morphology of the isolated strain was observed by a scanning electron microscopy (SEM). The metabolic characteristics of the isolated strain were examined by the API kit (Bio-Merieux). The cellular fatty acid composition of the isolated strain was analyzed using gas chromatography (Hewlet-Packard 6890A) as described by Ikemoto et al. (1978).

3. Phytase assay

Phytase assay was done as duplicates in 1 ml volume at 40℃ for 30 min in 50 mM acetate buffer (pH 5.5) containing 1 mM sodium phytate by the modified method of Heinonen and Lahti (1980) unless otherwise stated. The liberated inorganic phosphates were spectrophotometrically quantitated using a freshly prepared acetone-acid-molybdate (AAM) reagent consisting of acetone, 5 N sulfuric acid, and 10 mM ammonium molybdate (2:1:1, v/v). The assay was stopped by adding 2 ml AAM solution per assay tube. After 30 sec, 0.2 ml of 1 M citric acid was admixed to each tube, and absorbance was read at 405 nm. One unit of phytase activity was defined as the amount of enzyme required to liberate 1 μmol of phosphate per minute under the assay condition. The enzyme activity assay (n=2) with other phosphorylated compounds was performed as described above using 1 mM of each substrate. Acid phosphatase activity (n=2) was assayed using *p*-nitrophenylphosphate as described by Greiner et al. (1993). The release of *p*-nitrophenol was measured at absorbance of 410 nm. The pH profile of the phytase activity (n=2) was determined at 40℃ with a pH range from 2.5 to 8.5 using glycine-HCl (2.5-3.5), sodium-acetate (4.5-5.5), bis-tris (6.5), and tris-HCl (7.5-8.5) buffers at 50 mM each. The optimum temperature of the phytase activity (n=2) was also measured at various temperatures ranging from 20 to 80℃ with a pH 5.5.

4. Protein measurement

Protein concentration was measured by the method of Bradford (1976) using a protein assay kit (Bio-Rad) with bovine serum albumin as the standard.

5. Partial purification of BUN1 phytase

The partial purification of BUN1 phytase was done through a modified method previously described by Ullah et al. (2002). BUN1 strain was inoculated into 1000 ml of PSM and aerobically incubated at 28℃ for 72 h with reciprocal shaking (200 rpm). The cells were harvested by centrifugation at 10,000 g for 15 min at 4℃, resuspended in 50 mM Tris buffer (pH 8.0), and homogenized with 10 g of glass beads (425-600 microns; Sigma) in a pre-chilled vortexing mixer (Scientific Industries). The homogenate was then centrifuged at 10,000 g for 20 min at 4℃. After filtering the supernatant fluid with disposable syringe filter unit (0.45 μm; ADVANTEC), the crude extract was used for enzyme purification. A BioLogic HR LC system (Bio Rad) was used to perform chromatography. The crude enzyme was loaded onto an anion-exchange UNO Q-6 column (12 by 53 mm; Bio Rad) that had previously been equilibrated with buffer A (50 mM Tris buffer; pH 8.0). The column was washed with the same buffer and developed by running a linear salt gradient of 0 to 1 M NaCl in the buffer at a flow rate of 2.0 ml/min. The peak fractions of phytase activity were pooled and diluted

ten-fold with buffer B (25 mM acetate buffer; pH 5.5). Subsequently, the dilute was applied to a cation-exchange UNO S-6 column (12 by 53 mm; Bio Rad) that had previously been equilibrated with the acetate buffer, and the phytase was eluted with a linear salt gradient of 0 to 1 M NaCl in the same buffer at a flow rate of 2.0 ml/min. The active fractions were pooled, dialyzed in 10 mM Tris buffer (pH 7.5), and stored at −80℃ for further analysis.

6. Effect of additional carbon sources and nitrogen sources on phytase production

BUN1 strain was inoculated into 50 ml of PSM in a 500 ml Erlenmeyer flask as a basal medium, and incubated at 28 C° for 36 h on a reciprocal shaker (200 rpm). The parameters tested consist of carbon sources including glucose, glycerol, galactose, raffinose, maltose, sucrose, cellobiose, lactose, and corn starch at 0.5% (w/v), and nitrogen sources including ammonium chloride, ammonium nitrate, urea, casein hydrolysate, tryptone, peptone, casamino acid, beef extract, and yeast extract at 0.5%(w/v). After cultivation, the crude enzyme extracts were prepared as described above and the phytase assay done. The data was expressed as means from three experiments.

Ⅲ. RESULTS & DISCUSSION

A number of bacterial colonies were isolated, and the strains, which can grow on PSM containing sodium phytate as a sole source of phosphate and carbon, were selected. Among about three hundred strains isolated, BUN1 strain showed the highest phytase activity in the cell pellet (data not shown). The colony morphology of BUN1 strain was round with cream color. The strain was a motile, gram-negative, and rod-shaped bacterium (Fig. 1) especially producing fluorescent pigment in King's B medium (King et al., 1954). The basic biochemical and physiological properties of BUN1 strain are shown in Table 1. A cellular fatty acid analysis of the strain revealed the presence of C16:1w7c/C15 iso 2OH (34.3%), C16:0 (26.6%), and C18:w7c (15.4%) as the major constituents, with a minor presence of C17:0 cyclo (2.2%). Based on these biochemical and physiological characteristics, BUN1 strain was assigned to a fluorescent *Pseudomonas* sp. Generally, *Pseudomonas* strains which are ubiquitous group of microorganisms found in soil, seem to play a crucial role in utilizing inositol phosphates which are considered to be recalcitrant in soils and waterbodies, even though a clear mechanism of their behavior in the environment still remains elusive (Turner et al., 2007). In previous study, fluorescent

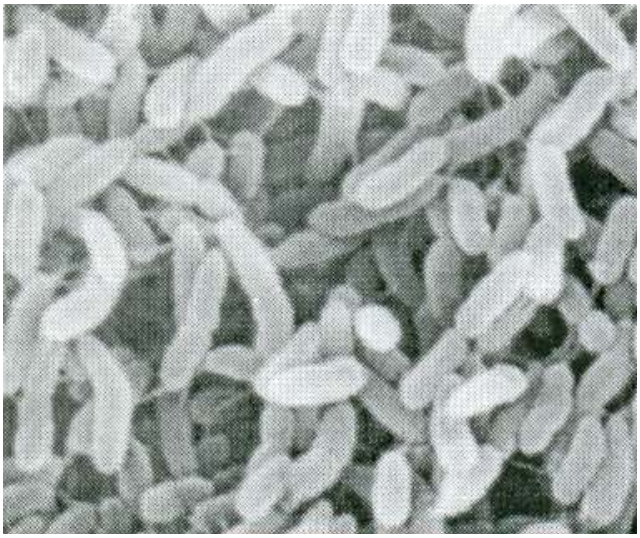

Fig. 1. Scanning electron microscope (SEM) of *Pseudomonas* sp. BUN1.

Pseudomonas strains markedly exhibited phytase activity in comparison with nonfluorescent *Pseudomonas* strains (Richardson and Hadobas, 1997). When BUN1 strain was cultured in PSM, no phytase activity was detected in the culture medium. During the osmotic shock, no release of the phytase was observed. Therefore, BUN1 phytase was regarded as a

Table 1. Basic biochemical and physiological properties of strain BUN1

Characteristics	Strain BUN1
Enzyme activity of	
Cytochrome oxidase	+
Arginine dihydrolase	+
Lysine decarboxylase	−
Ornithine decarboxylase	−
β-galactosidase	−
Production of	
Indole	−
Acetoin	+
Urease	−
H_2S	−
Gelatin liquefaction	+
Nitrate reduction	+
Denitrification	−
Glucose fermentation	−
Citrate utilization	+
Assimilation of	
Glucose	+
Arabinose	+
Mannose	+
Mannitol	+
N-acetylglucosamine	+
Gluconate	+
Carprate	+
Malate	+
Citrate	+

+ : Positive −: Negative.

cytoplasmatic enzyme. On the other hand, several *Bacillus* species (Choi et al., 2001; Kerovuo et al., 1998; Kim et al., 1998), *Enterobacter* sp. (Yoon et al., 1996) and *Lactobacillus amylovorus* (Hussin et al., 2007) phytase exist as extracellular forms.

The intracellular BUN1 phytase was partially purified by cation and anion chromatography with a yield of 22% and its specific activity was 50.15 U/mg of protein at pH 5.5 and 40℃. The enzyme showed a pH optimum of 5.5 (Fig. 2A) and more than 80% of the maximal activity was observed at pH values between 4.5-6.5, which closely matches the pH range at potential sites of action, like the salivary glands (pH 5.0) and upper part of the small intestine (pH 4.0-6.0) (Casey and Walsh, 2003). Most of fungal and yeast phytases were largely active at acidic pH (Dvorakova et al., 1997; Han and Lei., 1999; Han et al., 1999; Liu et al., 1999; Segueilha et al., 1992), while some bacterial phytases, such as *Bacillus* sp. (Choi et al., 2001; Kerovuo et al., 1998; Kim et al., 1998), *Enterobacter* sp. (Yoon et al., 1996) and *Aeromonas* sp. (Seo et al., 2005) exhibited optimum activity at neutral or alkaline pH of 6.5-8.5. The temperature optimum of the enzyme was found at 40℃ (Fig. 2B), as compared that the well-known *Aspergillus* sp. and *Bacillus* sp. DS11 phytase showed the optimal activity at higher temperature of 60℃ and 70℃, respectively, but their percentage optimal activity retained at the physiological temperature (37-40℃) was no more than 40% (Dvorakova et al., 1997; Liu et al., 1999; Kim et al., 1998).

The effects of inhibitors and metal ions on BUN 1 phytase activity were examined using sodium phytate as a substrate (Table 2). The enzyme activity was strongly inhibited by Cu^{2+}, Cd^{2+}, and Zn^{2+}, and moderately inhibited by Ba^{2+}, Co^{2+}, Mn^{2+}, and EDTA at 5 mM each. These cations may form insoluble complexes with phytate and therefore decrease net concentration of the active substrate in the activity assay. This may interrelate with previous report that the stability of metal ion-phytate complexes at pH 7.4 was found to decrease in the following order: $Cu^{2+} > Zn^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+} > Fe^{3+} > Ca^{2+}$ (Wyss et al., 1999). Meanwhile, Ca^{2+}, Mg^{2+}, and PMSF did not have a significant impact on the enzyme activity. To date, most of phytases have not been described as metalloenzymes, except that some *Bacillus* sp. phytase required calcium for active conformation and thermostability, and readily lost the enzyme activity through its removal by EDTA (Choi et al., 2001; Kerovuo et al., 2000). BUN1 phytase had high activity for phytate, but none or low activity on other phosphorylated compounds

Table 2. Effects of metal ions and inhibitors on partially purified BUN1 phytase

Reagents (5 mM)	[a]Relative activity(%)
None	100
$MgCl_2$	81
$CdCl_2$	1.7
$AlCl_3$	108
$BaCl_2$	22.6
LiCl	114
$ZnCl_2$	1.7
$CaCl_2$	95
$CuCl_2$	1.9
$CoCl_3$	39
$MnCl_2$	32.3
EDTA	47.4
PMSF	77

[a] 100% activity was taken when phytase assay was performed at 40℃ and pH 5.5 in the absence of metal ions and inhibitors.

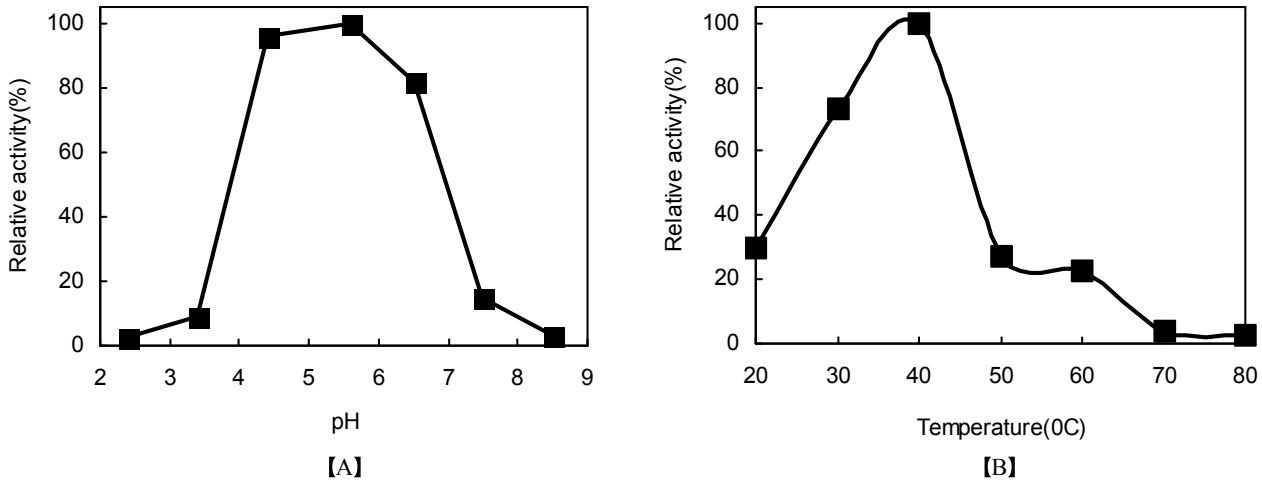

Fig. 2. Effect of pH (A) and temperature (B) on the activity of partially purified BUN1 phytase. The relative activity was expressed as a percentage of the maximum activity taken as 100%.

including p-nitrophenylphosphate, a representative substrate for acid phosphatase (Table 3). These results indicate that the enzyme is very specific for phytate. In contrast, many fungal phytases have broad specific activity for phosphorylated compounds such as glucose-6-phosphate and fructose 1,6-bis phosphate (Wyss et al., 1999).

In fluorescent *Pseudomonas* sp. BUN1, the phytase seems to be inducible under carbon limitation only in the presence of phytate, like other phytase-producing soil isolates, *Klebsiella* species (Greiner et al., 1997). There was no difference in the phytase activities when inorganic nitrogen sources were added to the basal medium, PSM. However, incorporation of organic nitrogen sources into the medium which contains enough inorganic phosphate increased the growth rate of the strain, bearing no detectable phytase activity (data not shown). As shown in Table 4, BUN1 strain exhibited a high growth rate when glucose or glycerol was used as the additional carbon source, whereas its specific phytase activity per mg of protein was low. It is not clear whether this effect is due to repression by glucose or catabolite, though catabolic repression on the enzyme production occurs frequently in microorganism when the cell is grown in a medium containing more than one utilizable growth substrate (Lan et al., 2002). BUN1 strain may utilize glucose and glycerol more rapidly, resulting in an accumulation of large amount of organic acid such as acetic acid, which not only reduces pH of the medium, but also does harm to the bacterial cells, thus suppressing the enzyme synthesis. On the other hand, the addition of corn starch was found to significantly induce the phytase activity in comparison with other carbon sources tried. This is compatible with an earlier study by Shieh and Ware (1968) that the phytase production of fungal strain, *Aspergillus niger* NRRL 3135 was also greatly influenced by the type of corn starch used in the culture medium. It may be supposed that a less soluble phytate enclosed by corn starch in the medium was hydrolyzed slowly, which triggers to the continuous induction of phytase production by preventing of the end-product inhibition.

From an applied perspective, BUN1 phytase can be an interesting candidate due to its strict substrate specificity, and physiologically-relevant temperature and pH profile. Further recombinant DNA technology including gene cloning will support an efficient strategy for mass production of the enzyme as an environmental-friendly feed additive.

Table 3. Substrate specificity of partially purified BUN1 phytase

Substrate (1mM)	[a]Relative activity(%)
Sodium phytate	100
Sodium pyrophosphate	11
ATP	14
ADP	11
AMP	0
Fructose-1-phosphate	0
Fructose-6-phosphate	0
Glucose-1-phosphate	0
α-glycerophosphate	2.8
β-glycerophosphate	3.6
α-naphtylphosphate	2.0
ρ-nitrophenylphosphate	2.9

[a] 100% activity was taken when the phytase was assayed with sodium phytate at 40℃ and pH 5.5.

Table 4. Effects of additional carbon sources on BUN1 phytase activity and growth

Carbon sources (0.5%)	[b]Growth (OD_{600})	Specific phytase activity (U/mg of protein)
[a]No addition	0.519	0.59
Raffinose	0.669	1.37
Glucose	1.471	0.13
Glycerol	1.386	0.53
Galactose	1.256	0.78
Sucrose	1.212	0.84
Lactose	0.628	1.33
Cellobiose	0.651	1.27
Maltose	0.550	1.39
Corn starch	0.780	4.05

[a] Cultivation was carried out at 28℃ for 36 h in PSM [0.5% sodium phytate, 0.5% $(NH_4)_2SO_4$, 0.5% KCl, 0.01% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCl_2 \cdot 2H_2O$, 0.01% NaCl, 0.001% $FeSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot 4H_2O$; pH 6.5] as a basal medium.

[b] Bacterial growth was determined by measuring the turbidity at OD 600 nm.

Ⅳ. ABSTRACT

A bacterial strain producing intracellular phytase was isolated from cultivable soil near cowsheds and identified as a fluorescent *Pseudomonas* sp. BUN1. The BUN1 phytase, partially purified by cation and anion exchange chromatography, exhibited its optimal activity at 40℃ and pH 5.5. As for substrate specificity, it was very specific for phytate and showed little activity on other phosphorylated conjugates. Its activity was greatly inhibited by metal ions such as Cu^{2+}, Cd^{2+}, and Zn^{2+}. Addition of corn starch to PSM (phytase-synthetic medium) [0.5% sodium phytate, 0.5% $(NH_4)_2SO_4$, 0.5% KCl, 0.01% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCl_2 \cdot 2H_2O$, 0.01% NaCl, 0.001% $FeSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot 4H_2O$; pH 6.5] for the phytase production significantly induced its enzyme activity in comparison with other carbon sources tested.

(**Key words** : Bacterial, Intracellular, *Pseudomonas*, Phytase, Phytate)

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