Thesis for the Degree of master of Science

# Purification and characterization of phytase from mung bean (*Phaseolus radiatus*)

by

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# Purification and characterization of phytase from mung bean (*Phaseolus radiatus*)

# 녹두에서 분리한 phytase의 정제와 그 특성

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## Abstract

Mmaximum phytase activity was reached after 4 day of germination and activity increased to a 7.14-fold compared with dry seed. Phytase was purified to homogeneity using 4 steps of ionic exchange chromatographies from germinated mung bean. Purity of the phytase increased 328-fold with approximately 7.4 % yield. The molecular weight of the enzyme was estimated to be 38 kDa by sodium dodesylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The optimum pH and temperature for hydrolysis of phytic acid were determined to be 5.0 and 60℃, respectively. The enzyme activity was stable in the pH range of 4.5 and 5.5. The enzyme was stable up heat treatment at 45℃ for 30 min. The phytase from mung bean had high activity for β-naphtyl-phosphate in substrate specificity. however, the enzyme did not have activities synthetic substrate such as D-fructose-1,6-diphosphatea and pyridoxal-5-phosphate.

# Introduction

Phytic acid (*myo*-inosotol 1,2,3,4,5,6-hexakisdihydrogen phosphate) is one of the major storage form of phosphorous in the seed of plant, mostly cereal grains, oilseeds, and legumes, which are the principal component of food and feed stuffs. Monogastric animals like pigs and poultry as well as human lack or low phytase activities in their digestive system and most undigested phytic acid was excreted in their manure. The presence of phytic acid in dietary food on feed may works as antinutritional factor in humans on animals, since the phosphate moieties of acid chelate essential minerals such as $Zn^{2+}$, $Ca^{2+}$, $Mg^{2+}$, and $Fe^{2+}$ ion, and their possibly binding to proteins (shown in Fig. 1.). Inorganic phosphate, an essential element for the growth of all organisms, has to be supplemented to the diets to meet the phosphate requirementes of monogastric animals, which causes a higher cost for feed processing. Another problem is the high levels of undigested phytic acid in the fecal waste, which can be discharged in the sewage and become a primary cause of agal blooms in water environmentes.

The degradation of phytic acid in animal feed may increase the absorption of phosphate as well as liberated minerals. Many attempts to enzymatic hydrolysis of phytic acid have been made to improve the nutritional value on food of feed stuffs and to decrease the amount of phosphate excreted by monogastric animals (Liu *et al.*, 1997; Kornegay and Qian, 1996; Adeola, 1995).

Phytase, (*myo*-inositol hexakisphosphate phosphohydrolase), are classified as the family of histidine acid phosphatases and are found primarily in microorganism and plants. These enzyme catalyze the hydrolysis of phosphate groups in phytic acid producing inorganic phosphate and *myo*-inositol phosphate derivatives. There are two classes phytases, such as 3-phytases and 6-phytases. 3-Phytases (EC 3.1.3.8) hydrolyses primarily 3-phosphate group in *myo*-inosotol hexakisphosphate and results in 1,2,4,5,6-pentakisphsphate and free phosphorus. Both enzymes subsequencially hydrolyse phytic acid to *myo*-inosotolpentaphosphate, *myo*-inosotoltetraphosphate, *myo*-inosotoltriphosphate, *myo*-inosotoldiphos-phate, and *myo*-inosotolmonophosphate. 6-Phytase (EC 3.1.3.26) primarily catalyzes on the hydrolysis of 6-phosphate group in *myo*-inosotol hexakisphosphate and results in 1,2,3,4,5-pentakisphosphate and

free phosphorus. 6-Phytase can completely hydrolyses the phosphate groups in phytic acid and produces inositol and free phosphates. However, 3-phytase do not hydrolysed phosphate groups and makes phosphomonoester and free phosphates.

6-Phytase reported in plant such as cereal, regume, and oilseed (Gibson and ullah, 1990). 6-Phytase are distributed in plant seed and their activities were increased on germination (Richardson and Hadobas, 1997) and liberated phosphorus from phytic acid was incorporated into ATP (Gibson and ullah, 1990). Phytases are interesting for producing defined breakdown products and may find aplications in the processing of foods with improved nutritional value and health benefits and retained sensory properties (Greiner *et al.,* 2000, Greiner *et al.,* 2001). These enzyme catalyze the stepwise degradation of phytate, the principle storage form of phposphorus in mature seeds of cereals and legumes (Reddy *et al.*, 1989), to series of lower *myo*-inositol phosphates and orthphosphate. A number of studies have already shown that addition of phytases enhanced phosphate utilization from phytate and drastically reduces orthophosphate excretion. (Cromwell *et al.,* 1995, Simons *et al.*, 1990). As phytate can act as an antinutrient by binding to proteins and by chelating

mineral*s*, such as zinc, iron, ca*l*cium, and magnesium (Cheryan, 1980), additiona*l* of phytases can improve the nutritiona*l* va*l*ue of p*l*ant-based foods by enhancing protein digestibi*l*ity and minera*l* avai*l*abi*l*ity through phytate hydro*l*ysis during digestion in the stomach (Sandberg *et al.*, 1996, Yi *et al.,* 1996) or during food and feed processing (Reddy *et al.*, 1989). Severa*l* p*l*ant phytases have been studied inc*l*uding rice bran (Hayakawa *et al.*, 1989), cano*l*a (Houde et a*l*., 1990), wheat bran (Nagai and Funahashi, 1962), rye (Greiner *et al.*, 1998), wheat (Nakano *et al.*, 1999), spe*l*t (Konietzny *et al.*, 1995), oat (Greiner *et al.,* 1999), bar*l*ey (Greiner *et al.,* 2000), tomato root (Li *et al.*, 1997), *l*upine (Greiner *et al.,* 2001), soy bean (Gi*l*son *et al.,* 1988), sca*ll*ion *l*eaves (Phi*ll*ippy *et al.,* 1998), and faba bean (Greiner *et al.,* 2001),

The major objectives of the present investigation were to estab*l*ish the simp*l*ified purification method of phytases from mung bean and the characterization of the enzymes.

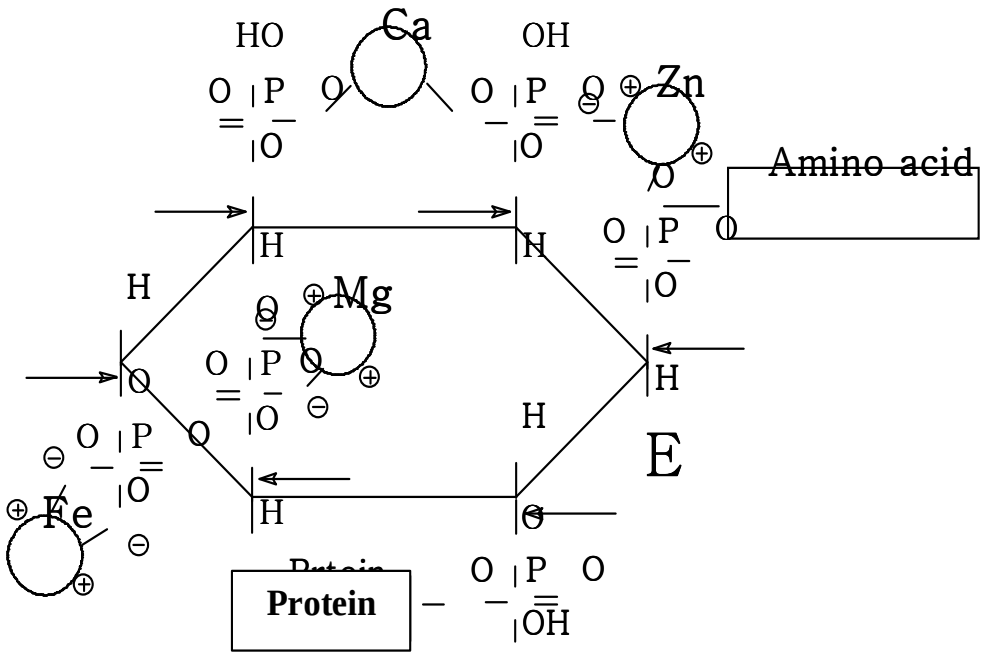



Fig. 1. Structure of phytic acid

# Materials and method

## 1. Materials

Mung bean was obtained from local market. Phytic acid dodecasodium was purchased from Sigma Co. (MO. USA). S-Sepharose, Resourse-S, Mono-S column for FPLC system were purchased from Pharmacia Biotech (Uppsals, Sweden). Synthetic substrates such as ρ-nitrophenyl phosphate, D-fructose-1,6-diphosphate, pyridoxal-5-phosphate, α-naphtyl-phosphate, β-naphtyl-phosphate, and adenosine triphosphate (ATP), adenosine monophosphate (AMP), inosinen nonophosphate (IMP) were purchased from Sigma Co. (MO, USA). Low molecular weight protein markers for SDS-PAGE and standard proteins for gel filtration chromatography were obtained from Bio-Rad (CA, USA). All other reagents were of analytical grade.

## 2. Methods

### Seed germination

For surface sterilization of mung bean were soaked in the following solution; (1) 0.1% Tween-80 for 5 min; (2) 0.5% NaOCl for 2 min; (3) 0.75% $H_2O_2$ for 1 min. The seed were thoroughly rinsed with sterile water after each treatment. They were allowed to germinate on sterile boxes in the dark at 20℃ for up 7 days.

### Enzyme extract

Mung bean was ground by mortar and pestle, and pulverized by the Tissuemizer with 600 ㎖ of 50 mM sodium acetate buffer (pH 5.0). Homogenate was incubated by shaking for 2 h at 4℃ and centrifuged at 20000 x g for 30 min. The supernatant was used for crude enzyme solution.

### Assay of phytase activity

Phytase activity was determined by modified method of Greiner et al. (1999) at 37℃. The reaction mixture containing 10 ㎕ of enzyme solution, 250 ㎕ of 50 mM sodium phytate (pH 5.0), and 1 mM $CaCl_2$ was incubated at 37℃ for 30 min. The reaction was

stopped by adding 1.5 m*l* of a fresh prepared so*l*ution of acetone: 5N $H_2SO_4$ : 10 mM ammonium mo*l*ybdate (2:1:1, v/v/v) and 100 ㎕ of 1.0 M citric acid to the assay mixture. The *l*iberated inorganic phosphate was measured by modification of the ammonium mo*l*ybdate method. Any c*l*oudiness was removed by centrifuging at 3000 x g for 15 min in prior to the measurement of the absorbance at 410 nm.

### Determination of phosphatase activity

Phosphatase activity was determined by modified method of Greiner et a*l*. (2001). The reaction mixture containing 10 ㎕ of enzyme so*l*ution, 250 ㎕ of 50 mM sodium acetate buffer (pH 5.0), contaning 1 mM of *p*-nitropheny*l* phosphate was incubated at 35℃ for 15 min. The reaction was stopped by adding 1 m*l* fo 0.5 N NaOH. Phosphatase activity was determined by measuring the absorbance of the formed *p*-nitropheny*l* phosphate at 410 nm.

### Determination of protein concentration

The protein concentration of fraction from chromatographies was estimated spectophotometrica*ll*y at 280 nm. The protein concentration of poo*l*ed enzyme so*l*ution from each purification

step were determined according to the method of Bradford (1976). Bovine serum albumin was used as a standard protein.

### Purification of phytase

All steps were performed at 4℃. After a 4 day-germination of mung bean, cell debris were removed by centirifugation at 12000 x *g* for 30 min. The supernatant was loaded onto a S-Sepharose column (2.5 x 12 cm) equilibrated with 20 mM sodium acetate buffer (pH 5.0). The enzyme was eluted with 400 ㎖ of liner gradient ranging from 0 to 1.0 M NaCl in the same buffer. Enzyme fractions were collected and diluted with 2 volume of equilibration buffer and loaded onto S-Sepharose column (2.5 x 12 cm) equilibrated with 20 mM sodium acetate buffer (pH 5.0). Enzyme fractions were collected and diluted with 2 volume of equilibration buffer and loaded onto Resoures-S column equilibrated with 20 mM sodium acetate buffer (pH 5.0). The elution was performed with a linear gradient of 0.2 to 0.3 M NaCl in 20 mM sodium acetate buffer (pH 5.0). The flow rate and fraction size were 30 ㎖/h and 1 ㎖, respectively. Enzyme fractions were further purified by Mono-S column chromatography with previous condition. With 3 times of Mono-S

chromatography, the phytase was purified and stored at -20℃ for characterization studies.

### Determination of the molecular weight

The molecular weight of the purified phytase was determined by SDS-PAGE according to the method of Laemmli (1970). For the preparation of protein samples for SDS-PAGE, sample was concentrated by precipitation with trichloroacetic acid (TCA) and cold acetone. Enzyme solutionwas mixed with 2 vol. of 20% TCA and stood for 30 min in 4℃. The solution was centrifuged at 20,000 x g for 20 min and supernatant was removed, and added 200 ㎕ of cold acetone. After stood for 30 min at room temperature, actone was removed by centrifugation at 20000 x g for 20 min. Precipitated protein was dried for short time and disssolved in SDS-PAGE loading buffer. Phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), glutamate dehydrogenase (62 kDa), alcohol dehydrogenase (45 kDa), carbonic anhydrase (31 kDa), and myoglobin (16.9 kDa) were used as the SDS-PAGE marker proteins.

### pH dependence

Buffer solutions for the determination of the enzyme activity were prepared to four sequences as follows; 0.1 M glycine-HCl buffer (pH 2.0 to 3.0), 0.1 M sodim citrate buffer (pH 3.0 to 4.0), 0.1 M sodium acetate buffer (pH 4.0 to 5.0), and 0.1 M N-ethylmorpholine acetate buffer (pH 5.0 to 7.0).

### Temperature dependence

The effects of temperature on phytase activity were examined under the standard assay conditions except that the temperature was varied from 20℃ to 80℃ with the same concentration of enzyme solution used in pH dependence.

### pH stability

For the determination of the pH stability, 2 ㎕ of enzyme solution was incubated with 748 ㎕ of buffer with different pH values for 24 hr at 4℃ and the remaining activity was measured under the standard assay conditions.

### Temperature stability

Thermal stabilities were measured through incubation of the phytase at various temperature for 30 min. The remaining activities were determined using 5 mM sodium phytate solutionas a substrate at pH 5.0 and 37°C.

### Synthetic substrate specificity

For the determination of the subsutrate specificities, the sodium phytate in assay mixture was replaced with different phosphate compounds as a concentration of 5 mM D-fructose-1,6-diphosphate, pyridoxal-5-phosphate, α-naphtyl-phosphate, β-naphtyl-phosphate, ATP (adenosine triphosphate), AMP (adenosine monophosphate), IMP (inosinen nonophosphate).

The reaction mixture containing 10 ㎕ of enzyme solution, 250 ㎕ of 50 mM sodium acetate buffer (pH 5.0), contaning 5 mM of synthetic substrates were incubated at 35℃ for 15 min. The reaction was stopped by adding 1.5 ml of a fresh prepared solution of acetone: 5N $H_2SO_4$ : 10 mM ammonium molybdate (2:1:1, v/v/v) and 100 ㎕ of 1.0 M citric acid to the assay mixture. The liberated inorganic phosphate was measured by

modification of the ammonium molybdate method. Any cloudiness was removed by centrifuging at 3000 x g for 15 min in prior to the measurement of the absorbance at 410 nm.

### Determination of kinetic parameters

Apparent Michaelis-Menten constant ($K_m$) and substrate turnover munber ($V_{max}$) were determined by Lineweaver-Burk plot. Kinetic data with NPP as a substrate for phytase reaction were obtained with substrate concentrations of 0.01, 0.05, 0.1, 0.25, 0.5, 0.75, 1, 1.25, 1.5, and 1.75 mM. The reactions were performed at pH 5.0 and 37℃ for 15 min.

# Result and Discussion

## 1. Purification of phytase from mung bean

### Germination of mung bean

During germination of mung bean, maximum phytase activity was reached after 4 days, which a 7.14-fold increase in phytase activity was observed (Table 1). Phytase activity was reached a maximum level at 4 day germination period then decreased. Therefore. mung bean germinated for 4 days were used as a source of the enzyme purification.

The reduction of phytase activity could be related either to inhibition by the accumulation of released Pi (Sartirana and Bianchetti, 1967) or to degradation of the enzyme by proteases (Kruger et al., 1987). Lupine seeds showed a maximum activity after 4 day germination and a phytase activity increased 5.7-fold (Greiner, 2002), which showed a similar result with this study. Furthermore, phytase activities of 4 kinds of canola seeds were determined during germination, those were miximum after 6 and 8 day germination (Houde et al., 1990). However, phytase and

acid phosphatase activities was not changed during 10 day germination of rye (Greiner et *al.*, 1998). Therefore, activation of phytase during germination might be different with a species of seed.

Table. 1. Phytase Activity of germinated mung bean

| day | Relatively activity (%) |
|---|---|
| 1 day | 14 |
| 2 days | 60 |
| 3 days | 66 |
| 4 days | 100 |
| 5 days | 92 |
| 6 days | 86 |
| 7 days | 72 |

**Purification of phytase from mung bean**

A*ll* step were performed at 4℃. Crude extract of 4 day germinated mung bean was *l*oaded onto a S-Sepharose co*l*umn (2.5 x 12 cm) equi*l*ibrated with 20 mM sodium acetate buffer (pH 5.0). The enzyme was e*l*uted with 400 ㎖ of *l*iner gradient ranging from 0 to 1.0 M NaC*l* in the same buffer. F*l*ow rate was 120 ㎖/ hr and the fraction vo*l*ume was 5 ㎖per tube. The phytase was e*l*uted around sa*l*t concentration of 0.2-0.3 M of NaC*l* in 20 mM sodium acetate buffer (pH 5.0), that is tube number 26 to 48 (Fig. 2). The fractions showing high phytase activity (fraction number 33-45) were poo*l*ed and di*l*uted with 2 vo*l*umes of 20 mM sodium acetate (pH 5.0) for further purification by Resource-S ion exchange chromatography. As shown in Fig. 3, chromatogram of Resource-S co*l*umn had a shou*l*der in protein peak, which suggested a contamination of other protein (Fig. 3). Each fraction having phytase activity contained severa*l* contaminated proteins determined by SDS-PAGE (data not shown). For further purification of phytase, phytase fraction from Resource-S co*l*umn were poo*l*ed and di*l*uted with 2 vo*l*ume of 20 mM sodium acetate buffer (pH 5.0) and *l*oaded onto Mono-S co*l*umn. Fig. 4A shows a first chromatogram

of Mono-S column and enzyme fraction had a minor fraction of contaminated proteins. Phytase fractions from 1'st Mono-S column chromatography were pooled and diluted again with 20 mM sodium acetate buffer (pH 5.0) and loaded onto Mono-S column. Fig. 4B shows a 2'nd chromatogram of Mono-S column, which displayed a sharp protein curve. Aliquots of phytase fractions from 2'nd chromatography were concentrated by TCA precipitation and run on SDS-PAGE. A single protein band having 38 kDa was shown on the SDS-PAGE gel (Fig. 5). With series of ion exchange chromatographies, phytase was purified from germinated mung bean as a homogeniety.

Results of purification of phytase from mung bean are summarized in Table 2. The specific activity and purity increased 155-fold and 36.7-fold, respectively. Yield of purified phytase was estimated to be 7.4% against crude enzyme solution.

Several plant phytases were purified through gel and ionic exchange chromatographies. Purity of phytase from germinated canola showedd 60-fold with 19% recovery (Houde et al., 1990). Purity of phytase from germinated rye increased to 1988-fold and recovery was estimated to be 6% (Greiner et al., 1998). Phytase from germinated faba beans was purified through series of

ammonium sulfate and acetone precipitation, ion exchange and gel chromatographies and the purity were increased to 2190 with 6% recovery (Greiner et al., 2001). Three phytases from germinated lupine seeds were more highly purified in the range of 13105 and 15973-fold with about 10% recoveries (Greiner, 2002).

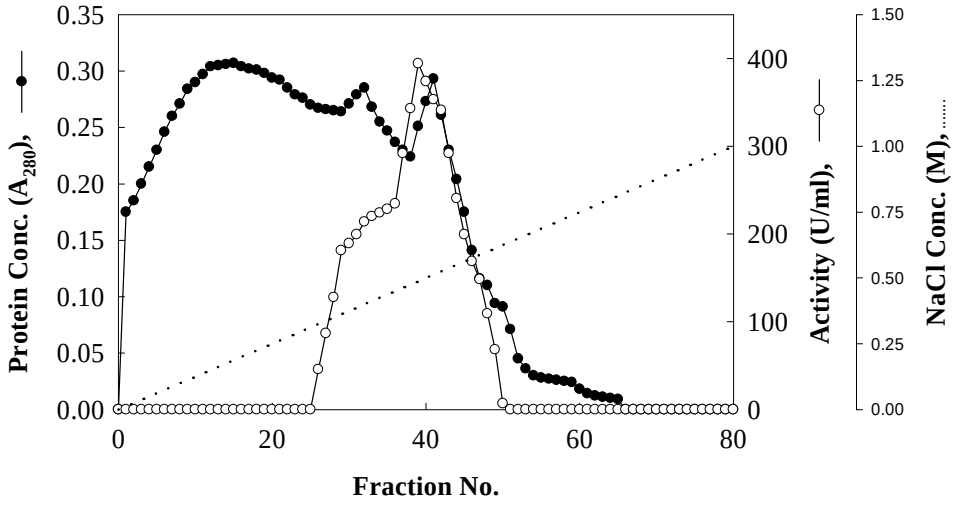
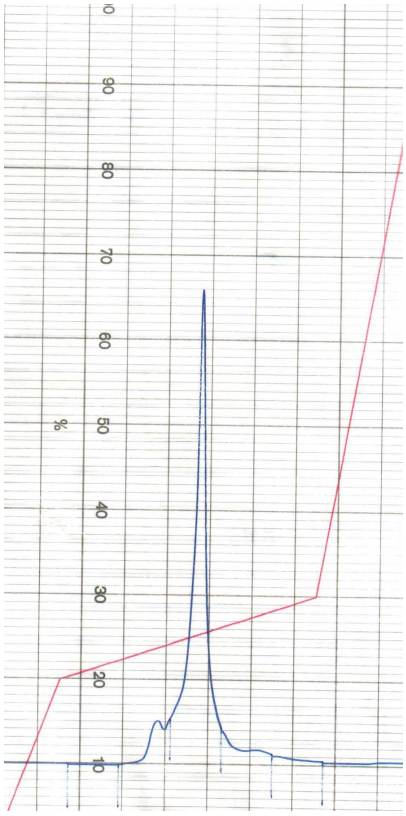
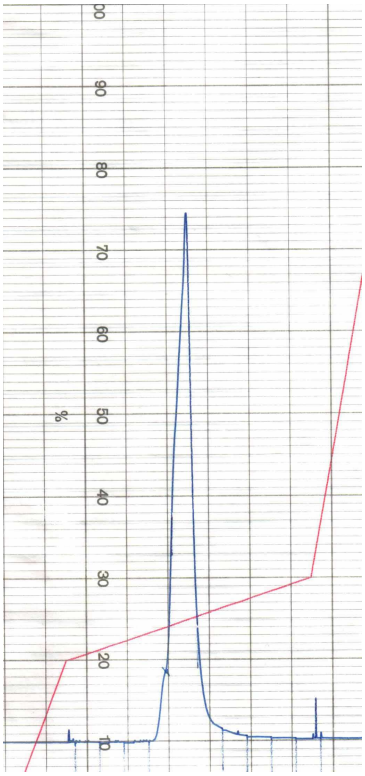



**Fig. 2. Chromatogram of a S-Sepharose ionic exchange chromatography of the flow-through from S-Sepharose column. Flow rate was 120ml/hr and fraction volumn was 5ml/tube. The phytase was eluted with a gradient of 0 - 1.0 M NaCl in 20mM sodium acetate (pH 5.0) The protein concentration was measured at 280 nm.**

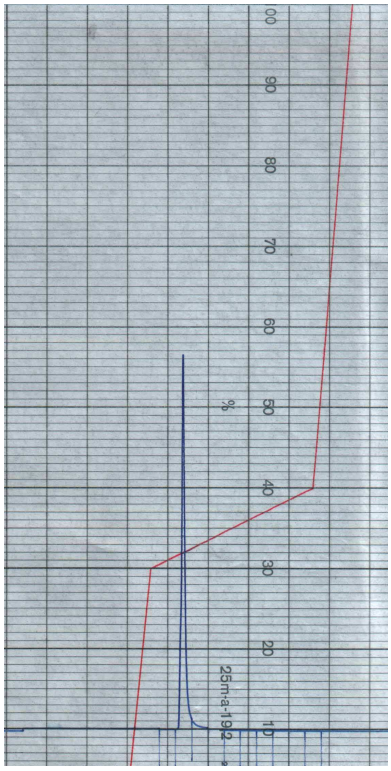



Resource-S (1st)

Fig. 3. Chromatogram of Resource-S co*l*umn chromatography of phytase from the S-sepharose chromatography




A; Mono-S (2nd)




B; Mono-S(3rd)

Fig. 4. Chromatogram of Mono-S column chromatography of phytase from the Resource-S chromatography

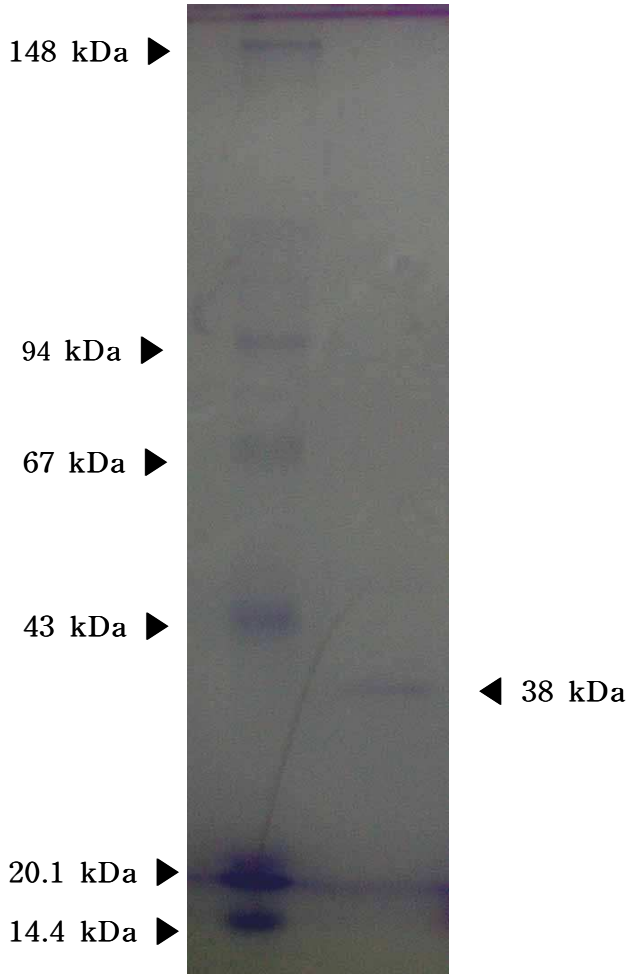



Fig. 5. SDS-PAGE of purified phytase from Mono-S

Lane A, molecular marker

Lane B, purified phytase

Table. 2. Purification of phytase from mung bean

| Fraction | Volume (ml) | Protein conc. (mg/ml) | Activity (U/ml) | Specic activity (U/mg) | Purity (fold) | Yeield (%) |
|---|---|---|---|---|---|---|
| crude | 120 | 4.1 | 17.6 | 4.3 | 1 | 100 |
| S-Sepharose | 80 | 0.480 | 14.6 | 27.5 | 6.4 | 49.9 |
| Resource-S(1st) | 10 | 1.103 | 595.4 | 64.6 | 15.1 | 33.8 |
| Mono-S(2nd) | 5 | 0.712 | 390.2 | 139 | 30.5 | 22.1 |
| Mono-S(3rd) | 5 | 0.198 | 131.8 | 158 | 36.7 | 7.4 |

**Molecular weight**

The molecular weight (MW) of the purified phytase from mung bean was determined to be 38 kDa by SDS-PAGE (Fig. 5). The molecular weight of mung bean phytase in this study was same as reported maize root and seed 38 kDa (Hűbel and Beck 1996, Laboure *et al.,* 1993). However, various molecular weight of phytases were reported as 68 kDa in rye. oat, and spelt (Greiner *et al.,* 1999; 1998; Konietzny *et al.*, 1995), 70-72 kDa in soy bean (Gilson *et al.,* 1988), 67 kDa in faba bean (Greiner *et al.,* 2001), 72 kDa in scallion leaves (Phillippy *et al.,* 1998), 68 kDa of PHY1 and 66 kDa of PHY2 in wheat (Nakano *et al.*, 1999), 66 kDa in barley (Greiner *et al.,* 2000), 82 kDa in tomato root (Li *et al.*, 1997), and 57-64 kDa in lupine (Greiner *et al.,* 2001).

Fungal phytases reported until now were glycosylated and their MWs determined by SDS-PAGE or gel permeation chromatography were higher than those calculated by amino acid sequence analysis. The extent of glycosylation ranged from 20 to 65% of the total MW (Wyss et al., 1999a). The difference between the MW determined by gel filtration and SDS-PAGE were depend on the extent of glycosylation, which was calculated from the difference between the MW obtained by amino acid

sequence analysis. Therefore, the higher MWs obtained by gel filtration are most likely artifacts due to the degree of glycosylation. Similar observations have previously been made for Sephadex columns, in which glycoproteins also eluted earlier than expected (Andrews, 1965). However, MWs of phytases from bacteria were lower than those from fungi such as 40 kDa from *Klebsiella terrigena*(Greiner et al., 1997), 44 kDa from *Bacillus* sp. DS11 (Kim et al., 1998), 38 kDa from *B. subtilis* (Shimizu, 1992), 36.5 kDa from *B. subtilis* (Powar et al., 1982), 42 kDa from *E. coli*(Greiner et al., 1993). Even though MWs of bacterial phytases calculated by amino acid sequence analysis were similar to fungal phytases, MWs of bacterial phytases were lower than fungal phytases. High MWs of fungal phytases was due to glycosylation. There is no evidence that plant phytases were glycosylated.

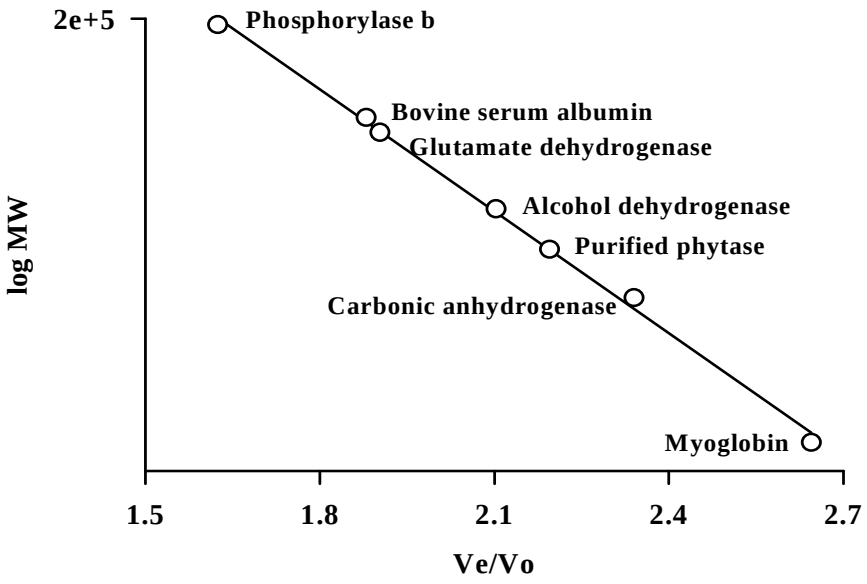



**Fig. 6. Determination of molecular weight of phytase purified from mung bean using SDS-PAGE.**

## 2. Characterization of purified phytase

### pH dependence

The pH dependence phytase activity of the purified phytase was shown in Fig. 7. The highest activity was appeared at pH 5.0 and phytase activity at pH 4.0 and pH 6.0 showed about 50% of its maximum activity. The purified enzyme had a broad optimum pH range from pH 4.5 to pH 5.5. Enzyme activity was abruptly decreased at alkaline and acid pH range. Activity was not shown at pH 2.0 and 7.0. The pH dependence of the phosphatase was shown in Fig. 7. The highest activity appeared at pH 5.0 and phosphatase activity at pH 4.0 and pH 6.0 showed about 50% of its maximum activity like a phytase activity. The pH dependence of phosphatase activity of the purified phytase was displayed in Fig. 8. As shown in Fig. 8. pH profile of the phoosphatase was similar to that of phytase activity.

Most plant phytases are active within the pH range of 4.5-5.5. The pH optima for plant phytases were reported as pH 5.2 for dwarf bean (Gibbins and Norris, 1963), pH 5.0 for lupine (Greiner *et al.,* 2002) and faba bean (Greiner *et al.,* 2001), pH 4.8 for maize (Laboure *et al.,* 1993), and pH 5.5 for scallion leaves (Phillippy *et al.,* 1998). However, a fraction of wheat phytase was

reported to have a pH optimum of pH 7.2 (Lim and Tate, 1973) and partially purified phytase from germinated mung bean showed maximal activity at pH 7.5 (Loras and Markakis, 1977). Two distinct pH optima could be commonly identified in the phytase from *Aspergille* (Pasamontes et al., 1997; Ullah and Gibson, 1987). The highest activity was displayed at pH 5.0-5.5 and the second activity peak occurs at pH 2.5, and the phytase is 40% less active at pH 2.5 than at pH 5.5 (Ullah and Gibson, 1987). Also, pH 2.5 acid phytase showed one distinct pH optimum at pH 2.5 (Ullah and Cummins, 1987). However, phytases from *A. terreus*, *Emericella nidulans*, and *Myceliophthora thermophila* displayed only one distinct pH optima (Wyss et al., 1999b). However, phytases from plant source had their pH optima arounf weak acid pH range.

### Temperature dependence

As shown in Fig. 9, the enzyme activity was gradually increased in the temperature range of 25 - 80℃ and the optimum temperature was found to be 60℃. The enzyme showed 20% of its maximum activity at 25℃. The enzyme lost most of activity at the temperature over 75℃ possibly due to thermal denaturation of the enzyme. As shown in Fig. 10, the phosphatase activity

was gradually increased in the temperature range of 25 - 80℃ and the optimum temperature was found to be 60℃ as shown in phytase activity pattern.

The phytase from mung bean showed its maximal activity at 60℃, and this temperature is slightly higher than the optimal temperature of lupine seed which is the most activity at 50℃ (Greiner *et al.,* 2002), faba bean at 50℃ (Greiner *et al.,* 2001), rye at 45℃ (Greiner *et al.,* 1997), scallion leaves at 51℃ (Phillippy *et al.,* 1998), canola seed at 50℃ (Houde *et al.,* 1990), and maize 55℃ (Laboure *et al.,* 1993). The optimal temperature of the enzyme activity is 60℃ which is different from other phytase. It means that phytase from mung bean have thermal stability than other plant phytases.

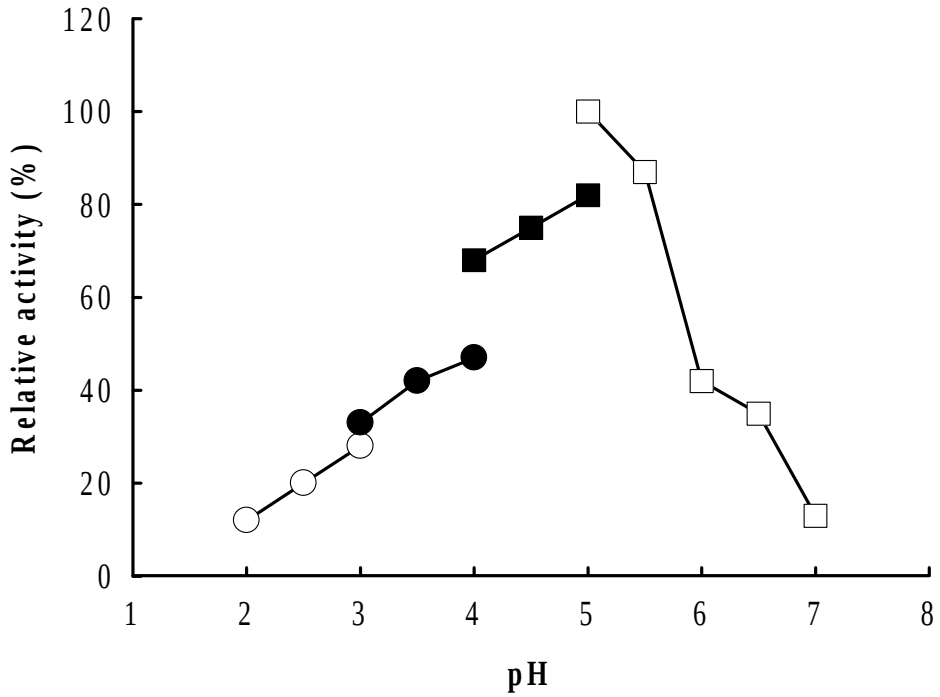



**Fig. 7. pH stability of the activity of phtase with various buffer solution. The buffers used: open circles, 10mM glycine-HCl buffer (pH 2.0 to 3.0); solid circles, 10mM sodim citrate buffer (pH 3.0 to 4.0); sold squares, 10mM sodium acetate buffer (pH 4.0 to 5.0); open squares, 10mM N-ethylmorpholine acetate buffer (pH5.0 to 7.0).**

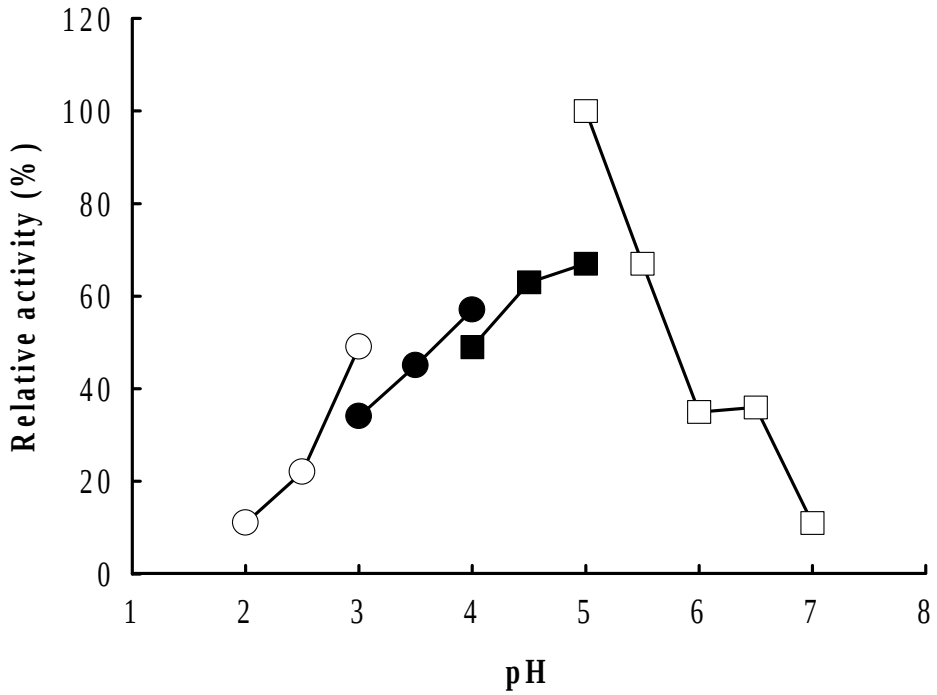



**Fig. 8. pH dependnece of the activity of phosphatase with various buffer solution. The buffers used: open circles, 10mM glycine-HCl buffer (pH 2.0 to 3.0); solid circles, 10mM sodim citrate buffer (pH 3.0 to 4.0); sold squares, 10mM sodium acetate buffer (pH 4.0 to 5.0); open squares, 10mM N-ethylmorpholine acetate buffer (pH5.0 to 7.0).**

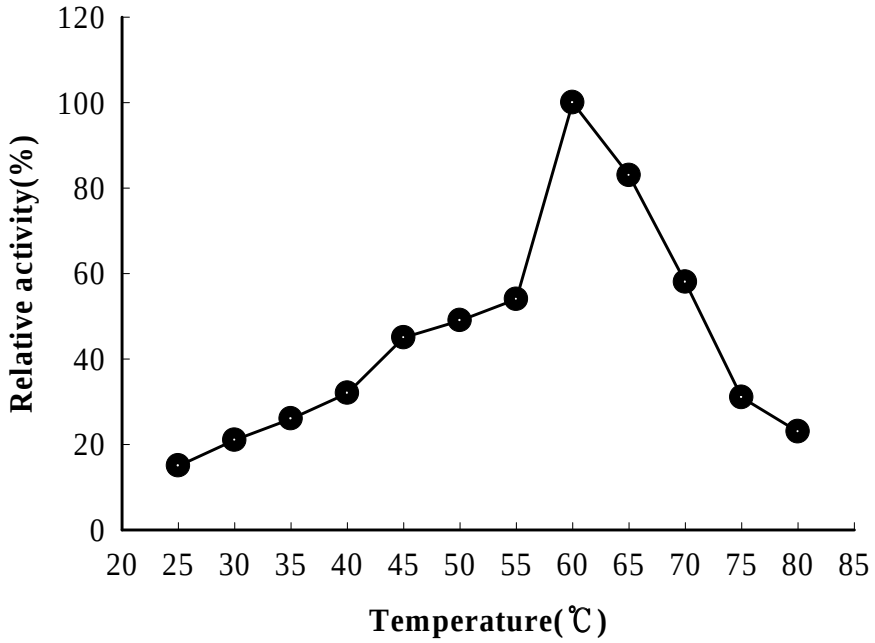



**Fig. 9. Temperature stability of phytase activity**
**Phytasee activity was measured at various temperatures in 10mM N-ethylmorpholine acetate buffer, pH 5.0 for 15 min, respectively.**

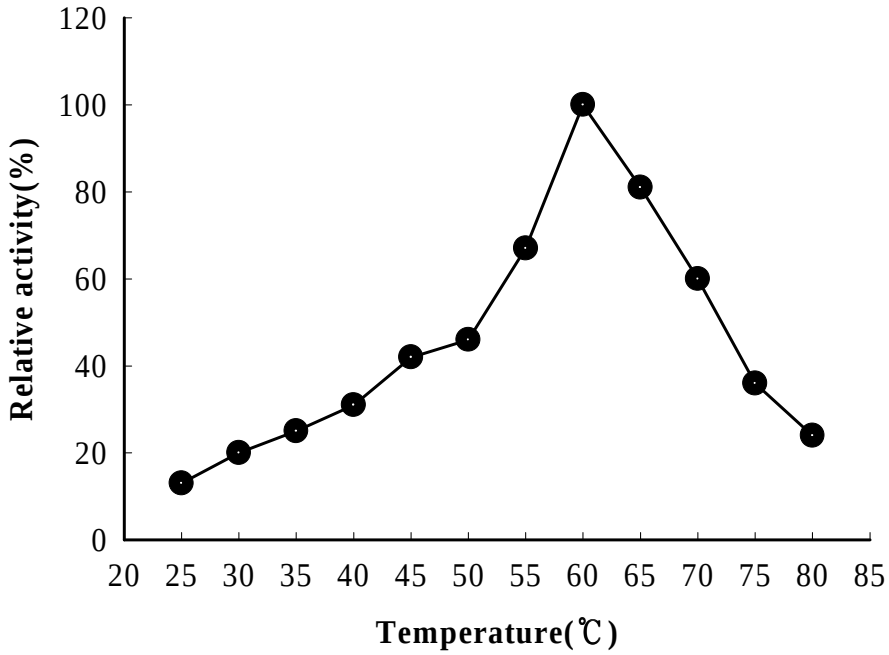



**Fig. 10. Temperature stability of phosphatase activity**
**Phosphatase activity was measured at various temperatures in 10mM N-ethylmorpholine acetate buffer, pH 5.0 for 15 min, respectively.**

### pH stability

To confirm pH stability of the phytase, enzyme was incubated 4℃ for 24 hr at various pH value of buffers and the remaining activity was measured at pH 5.0 at 37℃. As shown in Fig. 11, the enzyme activity was stable over the pH range from 4.5 to 5.5. To confirm pH stability of the phosphatase, the enzyme activity was stable at pH range 5.0 in Fig. 12.

Phytase from faba bean were stable after incubation in the pH ranges of 4.0 to 7.5 (Greiner *et al.,* 2001). Furthermore, phytases from lupine seed (Greiner *et al.,* 2002) and rye (Greiner *et al.,* 1997) were stable after incubation in the pH ranges of 3.5 to 7.5 and 3.0 to 7.5, respectively. Aforementioned, pH stability of phytase from mung bean had a narrow pH range than other phyatses.

### Temperature stability

To confirm thermostability of the purified phytase, enzyme was preincubated for 30 min at different temperature range from 25 to 60℃ and immediately cooled on ice. Residual activity was determined at 37℃ at pH 5.0. As shown in Fig. 13, the activity was stable up to heat treatment at 25℃ since the relative activity

was 80 % after 30 min incubation at this temperature range. The enzyme was rapidly inactivated at temperature above 35℃. As shown in Fig. 14, the phosphatase activity was stable up to heat treatment at 25℃ since the relative activity was 88 % after 30 min incubation at this temperature range. The enzyme was rapidly inactivated at temperature above 30℃.

In contrast to the phytase from faba bean (Greiner *et al.,* 2001), lupine seed (Greiner *et al.,* 2002), rye (Greiner *et al.,* 1997), and scallion leaves (Phillippy *et al.,* 1998), the enzyme was rapidly inactivated at temperature above 30℃.

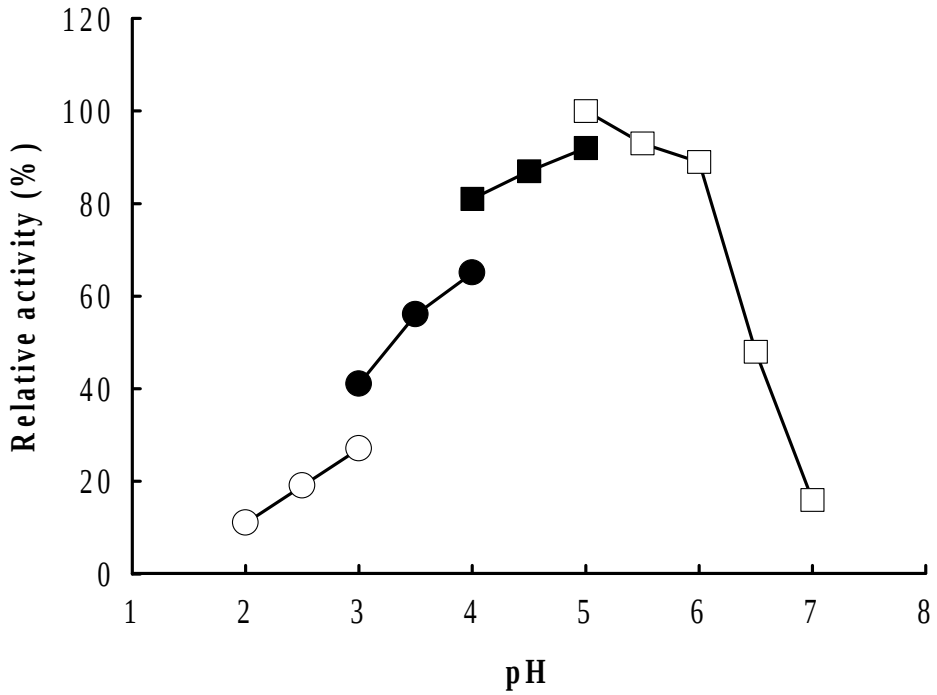



**Fig. 11. pH stability of the activity of phytase with various buffer solution. The buffers used: open circles, 10mM glycine-HCl buffer (pH 2.0 to 3.0); solid circles, 10mM sodim citrate buffer (pH 3.0 to 4.0); sold squares, 10mM sodium acetate buffer (pH 4.0 to 5.0); open squares, 10mM N-ethylmorpholine acetate buffer (pH5.0 to 7.0).**

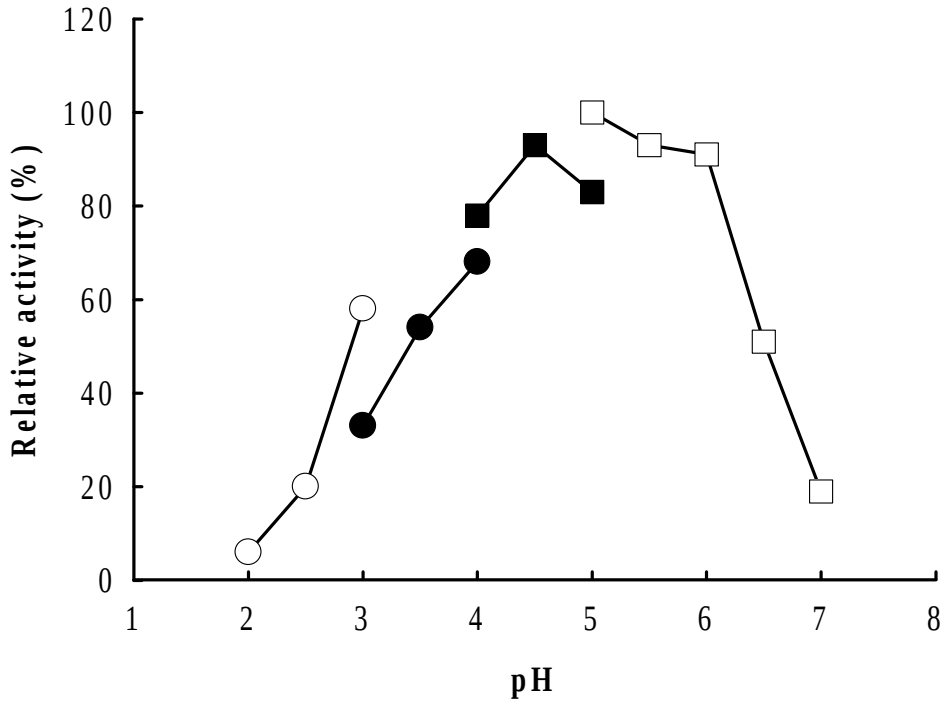



**Fig. 12. pH stability of the activity of phosphatase with various buffer solution. The buffers used: open circles, 10mM glycine-HCl buffer (pH 2.0 to 3.0); solid circles, 10mM sodim citrate buffer (pH 3.0 to 4.0); sold squares, 10mM sodium acetate buffer (pH 4.0 to 5.0); open squares, 10mM N-ethylmorpholine acetate buffer (pH5.0 to 7.0).**

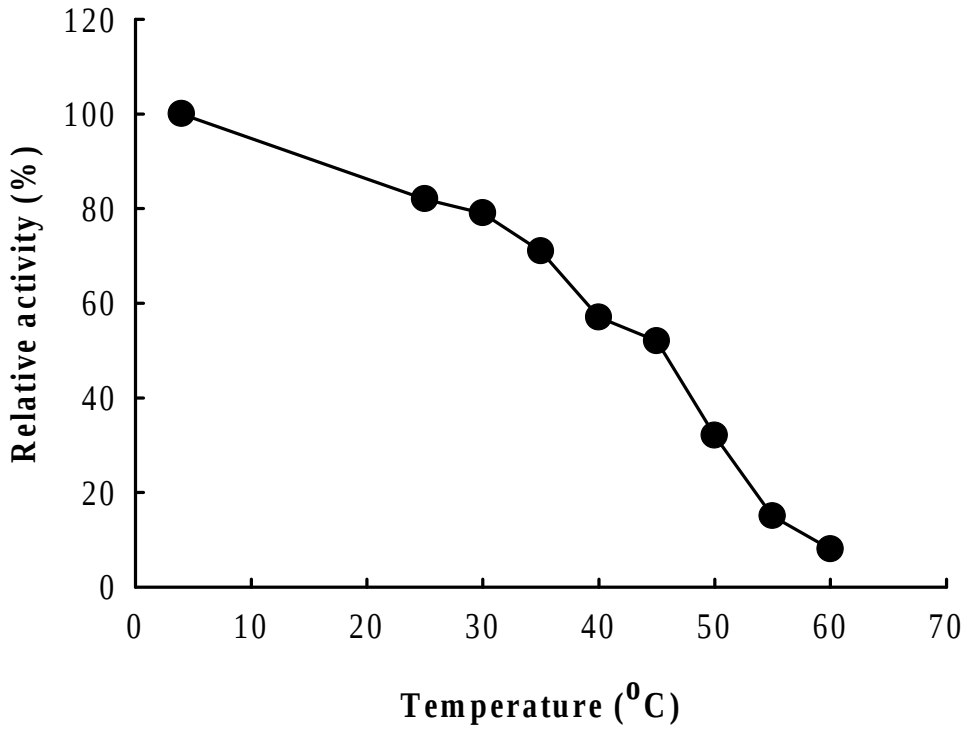



**Fig. 13. Stability of the phytase activity at various temperature. Enzyme activity was measured at various temperature in N-ethylmorpholine acetate buffer, pH 5.0 for 30min, respectively**

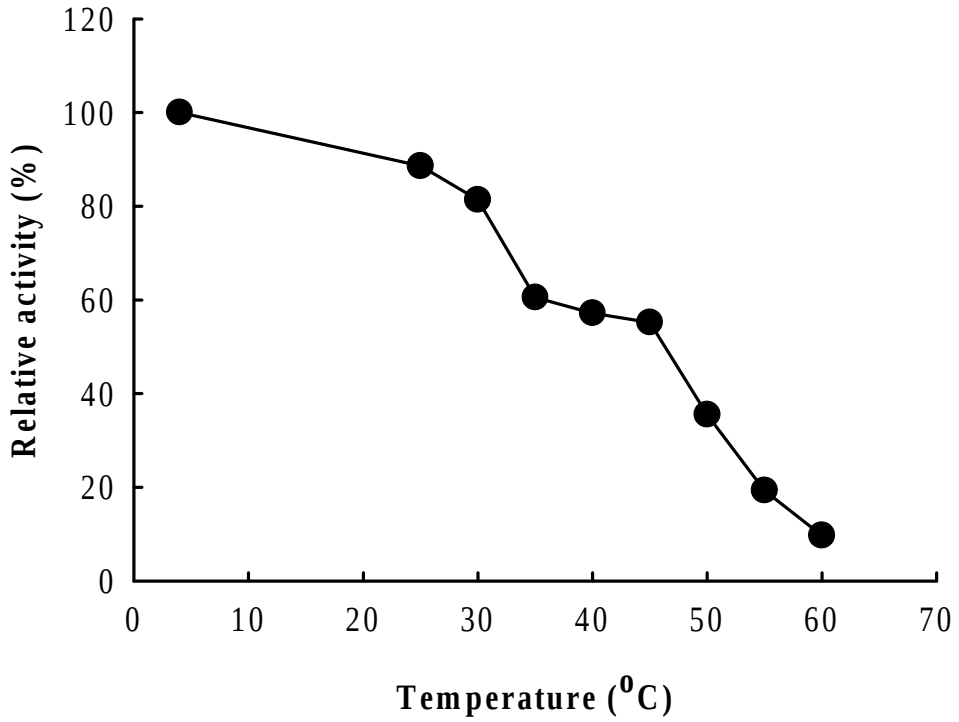



**Fig. 14. Stability of the phosphatase activity at various temperature. Enzyme activity was measured at various temperature in N-ethylmorpholine acetate buffer, pH 5.0 for 30min, respectively**

**Substrate specificity on synthetic substrate**

The purified phytase was reacted with 5 mM of synthetic substrate such as D-fructose-1,6-diphosphate, pyridoxal-5-phosphate, α-naphtyl-phosphate, β-naphtyl-phosphate, ATP (adenosine triphosphate), AMP (adenosine monophosphate), IMP (inosinen nonophosphate). As shown in Table 3, the highest activity was found with β-naphtyl-phosphate.

Bacterial phytases isolated from *E. coli* (Greiner et al., 1993), *Klebsiella terrigena* (Greiner et al., 1977) and *Bacillus* sp. DS11 (Kim et al., 1998) shown a particularly high specific activity for sodium phytate. Phytases from *A. fumigatus*, *Emericella nidulans*, and *Myceliophthora thermophila* had broad substrate specificities, however, those from *A. niger* and *A. terreus* had rather specificity for phytate (Wyss et al., 1999a).

The phytase with broad substrate specificity could excert significant levels of hydrolytic activity with a range of phosphate compounds that were not necessarily structurally similar to phytate (e.g., phenyl phosphate, pNPP, and phosphoenolpyruvate) and quickly hydrolyzed phytate to *myo*-inositol 2-monophosphate without accumulation of any intermediates. The phytases with narrow substrate specificity were rather specific for *myo*-inositol

phosphates and resu*l*ted in *myo*-inosito*l* tris- and bisphosphate accumu*l*ation during phytic acid degradation. Hydro*l*ysis of phytic acid was coup*l*ed with a progressive decrease in the rate of phosphate re*l*ease, which suggested that *l*ow inosito*l* phosphates are worse substrates than phytic acid. Therefore, The phytases with broad substrate specificity intrinsica*ll*y have rather *l*ow specific activity for phytate, whi*l*ethose with narrow substrate specificity have high specific activity. The difference in the substrate specificities of the two c*l*asses of phytases, therefore, ref*l*ects to a se*l*ective difference in the specific activities with phytate (Wyss et a*l*., 1999a).

Table. 3. Substrate specificity of the purified phytase on synthetic substrate.

| Synthetic sbstrate | Relatively activity (%) |
|---|---|
| phytic acid | 100 |
| D-fructose-1,6-diphosphate | N/D |
| pyridoxal-5-phosphate | N/D |
| α-naphtyl-phosphate | 111.5 |
| β-naphtyl-phosphate | 369.7 |
| ATP (adenosine triphosphate) | 224.5 |
| AMP (adenosine monophosphate) | 29.2 |
| IMP (inosinen nonophosphate) | 24.5 |

**Determination of kinetic parameters**

Michaelis-Menten constant (Km) and substrate turnover number (Vmax) of the enzyme were determined by liner regression of plotting the inverses of enzyme activity against the inverse of substrate concentration (Fig. 15.). The apparent Km and Vmax of phytase were estimated to be 0.7 mM and 305,744 $sec^{-1}$, respectively.

The apparent Km values of phytase from faba bean (Greiner *et al.,* 2001), lupine seeds L11, L12, L2 (Greiner, R. 2002), maize (Laboure *et al.,* 1993), and rye (Greiner *et al.,* 1998) were determined to be 148 μM, 80 μM, 300 μM, 130 μM, 117 μM, 300 μM, respectively.

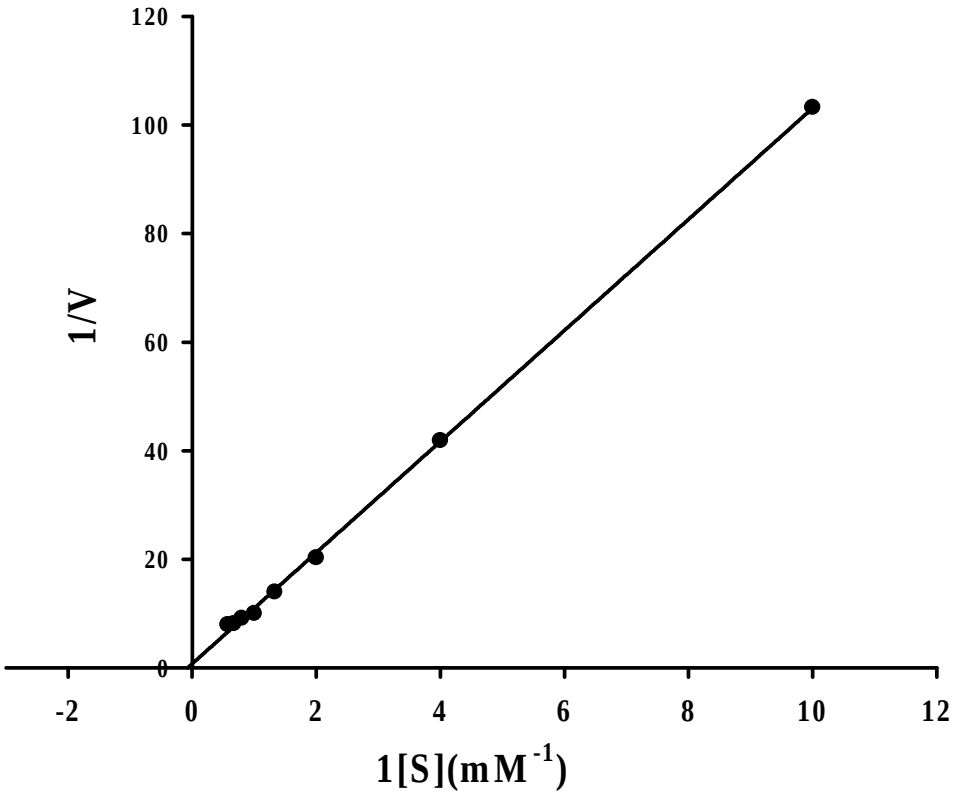



Fig. 15. Kinetic data of phytase for determination of $K_m$ and $V_{max.}$

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# 녹두에서 분리한 phytase의 정제와 그 특성

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## 요 약

Phytic acid는 곡류 또는 종자 식물의 씨앗이 성장하는 중에 생성되는 화합물로서 곡류와 콩과 식물의 씨앗중 인과 inositol의 주요한 저장형태이다. 곡류중 총 인의 70-80%가 phytic acid 상태로 존재하며, 구조적으로는 단백질과 결합된 마그네슘염 또는 칼슘염의 형태로 존재한다. 이러한 phytic acid를 분해하는 phytase를 녹두로부터 추출, 정제 및 그 특성을 알아보기 위해서 수행되었다. 다른 미생물의 번식을 막기 위해 멸균된 암상자에서 4일 동안 발아시켜 파쇄한 후 50mM sodium acetate buffer pH 5.0에 4℃에서 2시간 동안 조효소를 추출하였다. 이렇게 추출된 효소를 이온교환 크로마토그래피를 통하여 효소를 분리되었는데 S-sepharose 이온교환 크로마토그래피에서 49%의 수율과 14배의 정제도를 나타내었으며, Resource-S 이온교환 크로마토 그래피에서 33%의 수율과 132배의 정제도를 나타내었으며, 최종적으로 Mono-S 이온교환 크로마토 그래피에서는 7.4%의 수율을 가지고, 325배의 정제도를 나타내었다. 이 효소는 SDS-PAGE에 의해서 분자량이 38KDa으로 나타내었다. 활성에 대한 최적온도는 60℃ 이고, 최적 pH는 5.0으로 나타났다. 그리고 40℃ 이상의 온도에서는 효소의 활성이 점차 감소하는 것으로 나타났다. 가장 안정한 pH로는 pH 5.0에서 안정한 것으로 나타났다.