# Purification and Characterization of Extracellular Recombinant Phytase from *Escherichia coli*

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In the previous work, we have established the extracellular expression system of *Escherichia coli appA* gene by introducing signal peptides[1], and found out the proper condition of cultivation and induction to increase the extracellular phytase activity in media up to 91.5U/mL[1]. In this paper, we are reporting the research results of characterization of extracellular recombinant phytase from *E. coli*.

## Materials and Methods

The induced culture of the recombinant strain was centrifuged (4 800r/min, 5min), and the phytase in supernatant was purified by ammonium sulfate salting out separation at 25%, 70% of saturation, and by gel chromatography of Sephadex G-150.

The values of $K_m$, $V_{max}$ were estimated by measuring the reaction velocity of phytase in various concentrations of sodium phytase ranging from 0.1 to 10mmol/L.

SDS-PAGE was carried out on 12% gel according to the protocol[5],and the phytase activity was measured by the method based on formation of phosphomolybdate[3]. One unit of phytase activity was defined as the amount of enzyme releasing 1 μmol of phosphate per minute under the assay conditions.

## Results and discussion

### 1. Purification of extracellular recombinant phytase

The phytase enzyme was separated by ammonium salting out at 25%, 70% of saturation, desalinized in 20mmol/L Tris-HCl buffer(pH 8.0), and fractionized in the column of Sephadex G-150 gel.(Fig. 1)

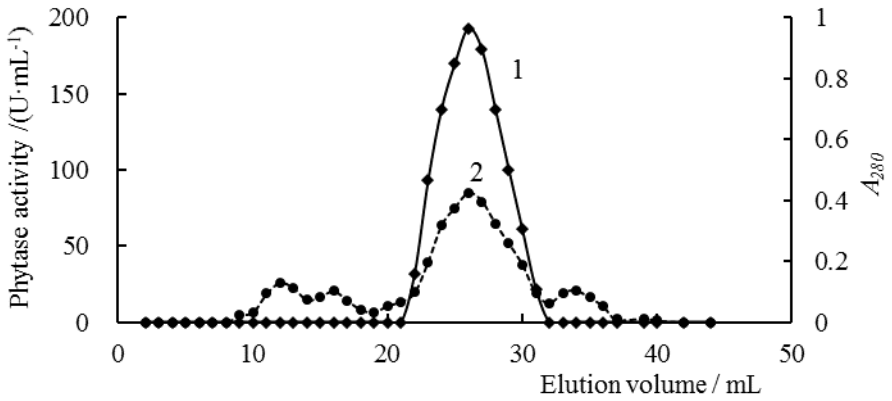


Fig. 1. Sephadex G-150 gel filtration chromatogram of salted out enzyme

1-phytase activity, 2-protein concentration

gel column: $\Phi$ 1.6×22cm, elution speed: 8mL/h, enzyme volume: 1mL

The phytase activities and protein contents of different purification steps are listed in Table.

Table. Phytase activities and protein contents of different purification steps

| Purification step | Protein /mg | Phytase activity /U | Specific activity /(U/mg$^{-1}$) | Purity /times | Recovery /% |
|---|---|---|---|---|---|
| Crude enzyme | 6.0 | 4 500 | 750 | 1.0 | 100 |
| Salting out | 3.7 | 3 285 | 900 | 1.2 | 73 |
| Gel filtration | 1.3 | 1 708 | 1 314 | 1.8 | 38 |

As shown in Table, the extracellular phytase was purified from induced culture supernatant of the recombinant strain by 1.8 times with 38% of recovery rate by two-step purification of ammonium sulfate salting out and Sephadex G-150 chromatography.

The crude enzyme and purified one were analyzed by SDS-PAGE to ensure the

purity of the extracellular recombinant phytase. (Fig. 2)

*E. coli* does not normally excrete large amount of proteins, and so the specific extracellular expression of target gene would simplify the purification process.

Since we used the supernatant of induced culture as a crude enzyme solution, major protein of which was phytase (50% in SDS-PAGE), relatively pure phytase was recovered during salting out and chromatography steps with 1.8 times of purity.

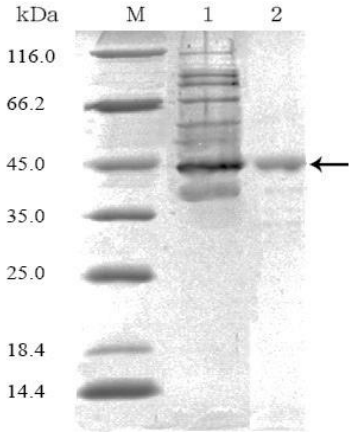


Fig. 2. SDS-PAGE of purified extracellular phytase
1-supernatant of 16h induced culture, 2-purified extracellular phytase, M-protein marker

## 2. Characterization of extracellular recombinant phytase

### Molecular weight

The molecular weight of purified extracellular phytase was determined by SDS-PAGE.

The value was calculated as about 45kDa from the relationship curve between relative migration lengths and log values of molecular weight of the standard protein marker bands and phytase band. And this is consistent with that of other extracellular phytase excreted by AppA native signal peptides and *kil* coexpression[7].(Fig. 3)

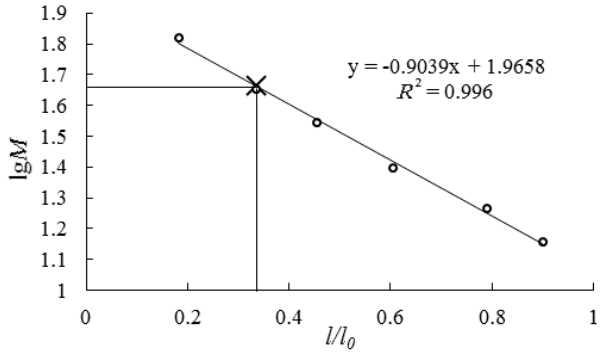


Fig. 3. Relationship curve between relative migration lengths and log values of molecular weight of the standard protein marker bands

**Substrate concentration dependence**

The values of $K_m$, $V_{max}$ on sodium phytate were estimated as 0.361mmol/L, 1314μmol/(min mg), respectively, by Lineweaver–Burk graph.

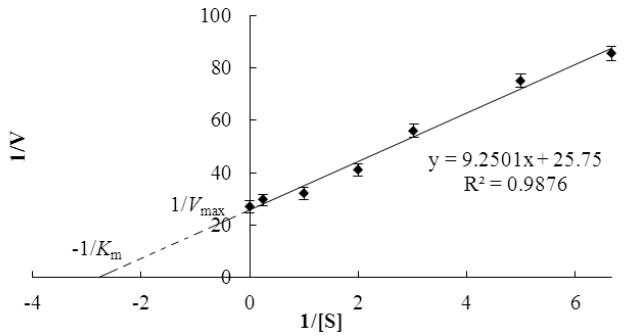


Fig.4. Lineweaver–Burk graph of extracellular phytase.

**Optimal temperature and pH**

The changes of activity of extracellular phytase in various temperature and pH were shown in Fig. 5 and 6, respectively.

The optimal temperature is 55℃, and the optimal pH is 4.5. At 37℃ and pH 2.0, the phytase displayed 47% and 58% of the highest activity at the optimal temperature and pH,

respectively. This means that the enzyme can effectively hydrolyze phytate in animal stomach.

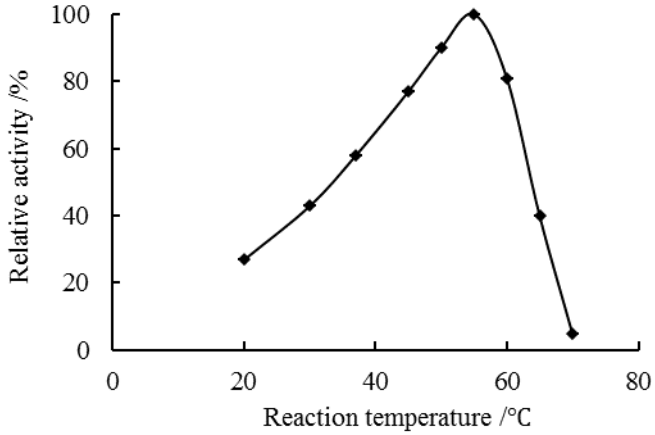


Fig. 5. Activity change according to temperature

: pH 4.5, reaction time 15min

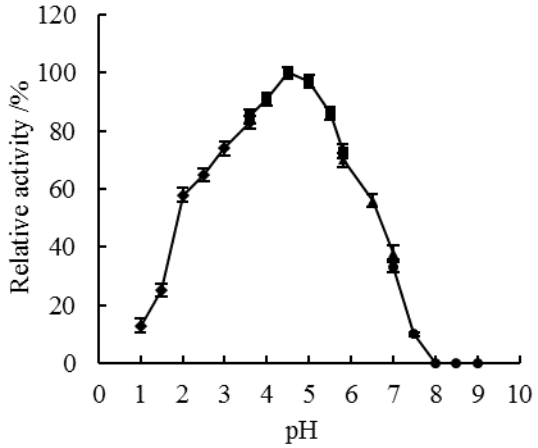


Fig. 6. Activity change according to reaction pH.

pH 1~ 3.6 (0.2mmol/L Gly-HCl),

pH 3.6~5.8: 0.2mmol/L acetic acid-sodium acetate,

pH 5.8~7.0 (0.2mmol/L Tris-acetic acid),

pH 7.0~9.0 (0.2mmol/L Tris-HCl),

reaction temperature 37℃, reaction time 15min

**Thermal and pH stability**

The thermal stability was estimated by treating enzyme solution at 40, 50, 60℃ for 0~120min before activity assay. (Fig.7)

As shown in Fig. 7, the enzyme was highly stable at 40℃ for 2h, but the activity decreased to 43% at 50℃, and to about 40% at 60℃ within 10min.

In general, AppA has an advantage of high specific activity and proteolytic resistance, but on the contrast, it lacks of thermal stability[6].

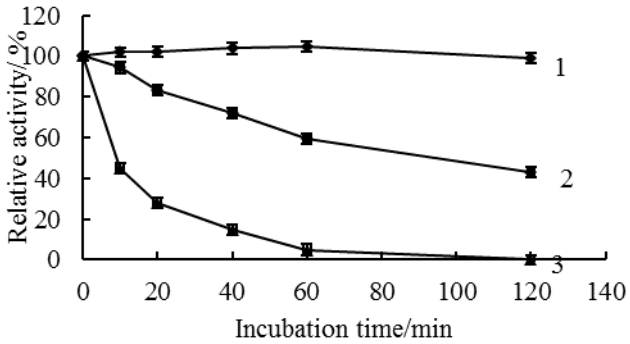


Fig. 7. Thermal stability of extracellular phytase.

1- 40℃, 2-50℃, 3-60℃

reaction temperature 37℃, pH 4.5, reaction time 15min

When the extracellular phytase is used as an additive for fish fodder, it should withstand high temperature during pelleting process. To this end, it is desirable to increase the thermal stability of the enzyme.

The pH stability was investigated by measuring the remaining activity of phytase after incubating at various pH buffers for 1h. (Fig.8)

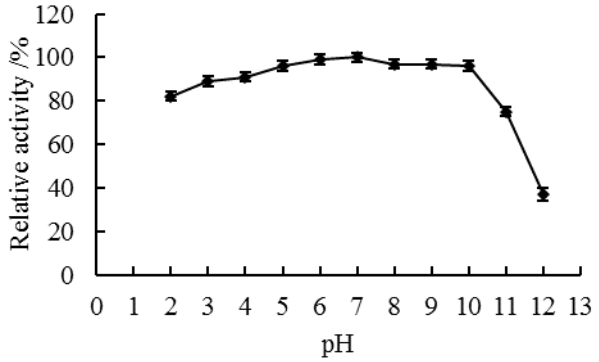


Fig. 8. pH stability of extracellular phytase

same buffers of Fig. 6 were used for pH 2.0~9.0,

pH 9.0~10.0 (0.2mmol/L Gly-NaOH)

pH 11.0~12.0 (0.2mmol/L NaOH)

pH 4.5, temperature 37℃, reaction time 15min

The extracellular phytase maintained enzymatic activity within wide range of pH from 3.0 to 10.0. In pH 2.0, very acidic buffer, it maintains about 70% of activity, and in pH 12.0 it loses 70% of its activity. And this proves that the extracellular phytase will safely maintain its activity within the pH range of digestive tract of animals.

**Effect of metal ions**

The effect of metal ions was estimated by comparing the phytase activities within reaction solutions containing various metal ions with the activity of control. (Fig. 9)

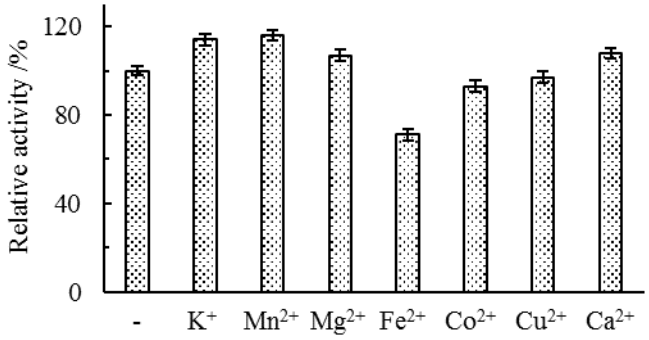


Fig. 9. Effect of metal ions on phytase activity

Buffer:100mmol/L Tris-HCl(pH 7.0) containing

0.5mmol/L of KCl, $MnSO_4$, $MgSO_4$, $FeSO_4$, $CoCl_2$, $CuSO_4$, $CaCl_2$

The extracellular phytase was activated by $K^+$, $Mn^{2+}$, $Mg^{2+}$, $Ca^{2+}$, among which the $Mn^{2+}$ has the highest effect, and the enzyme was strongly inhibited by $Fe^{2+}$.

**Proteolytic resistance**

1mL of extracellular phytase (20U/mL) was mixed with 1mL of pepsin solution (40U/mL, pH 2.0) and pancreatin solution (40U/mL, pH 8.0), respectively, and incubated for 0~60min. The mixture was diluted with 0.1mmol/L acetate buffer (pH 4.5) and the activities were measured and compared. (Fig. 10)

As shown in Fig. 10, the enzyme is stable on pepsin proteolysis, but it gradually loses its activity by trypsin proteolysis.

And the proteolytic resistance of extracellular phytase is higher than those of phytases of *Bacillus* or *Aspergillus* species.

Phytase from *Bacillus* species is stable against trypsin treatment, but it severely loses its activity by pepsin. Phytase from *Aspergillus* species has a little proteolytic resistance[6].

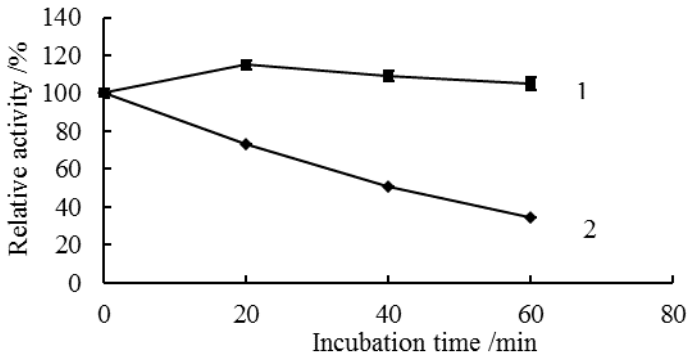


Fig. 10. Proteolytic resistance of extracellular phytase

1-pepsin, 2-pancreatin

pH 4.5, reaction temperature 37℃, reaction time 15min

The high pepsin proteolytic resistance is a great advantage of extracellular AppA, for it should be active in animal stomach as a feed additive.

## Conclusion

The extracellular phytase is purified by 1.8 times with 38% of recovery rate by two-step purification of ammonium sulfate salting out and Sephadex G-150 chromatography.

The molecular weight of purified extracellular phytase is determined by SDS-PAGE as 45KDa. It displays the maximal activity at pH 4.5 and remains stable in the broad range of pH 3.0~10.0 at 40~50℃. The optimum temperature of extracellular recombinant phytase is 55℃ at reaction time of 15min.

The values of $K_m$, $V_{max}$ on sodium phytate are 0.361mmol/L, 1 314μmol/(min mg), respectively.

The extracellular phytase is stable against pepsin and pancreatin treatment.

## References

[1]. Journal of **Kim Il Sung** University (Natural Science), 62, 8, 110, 2016.
[2]. Journal of **Kim Il Sung** University (Natural Science), 62, 11, 119, 2016.
[3]. Journal of **Kim Il Sung** University (Natural Science), 61, 4, 98, 2015.
[4]. Journal of **Kim Il Sung** University (Natural Science), 62, 6, 110, 2016.
[5]. J. Sambrook et al.; Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press, 1~300, 2001.
[6]. X.G. Lei et al.; Annu. Rev. Anim. Biosci., 1, 283, 2013.
[7]. G. Miksch et al.; Appl. Microbiol. Biotechnol., 59, 685, 2002.

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