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Isolation and Characterization of Microbial Phytases

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Introduction

Phytase catalyze the hydrolysis of phytate (myo-inositol hexakisphosphate) which is the main form of phosphorus predominantly occuring in cereal grains, legumes and oilseeds. The phytate is the primary source of inositol (and inositol phosphates) and the storage form of phosphorus in plant seeds. In plant-derived dietary food and feed, the phytate acts as an antinutritional factor because of its chelation of various metals and binding of protein, therefore diminishing the bioavailablility of proteins and nutritionally important minerals in feeding of monogastric animals that lack suitable phosphohydrolyzing enzymes in their gut(1). As a result, the intake of large amounts of foods rich in phytate may cause several mineral deficiency symptoms and phosphorus pollution problems in areas of intensive livestock production. The poor digestibility of phytate-phosphorus increases also the cost of production since additional sources of available phosphorus are needed in ration formulation to meet the nutritional requirement of the animal. Consequently, adding phytase to monogastric animal diets such as pig, poultry and fish can not only improve feed efficiency but also reduce environmental pollution.

The phytase is a kind of phosphohydrolases or phosphatases. Classification of phosphatases is initially based on the biochemical and biophysical properties of the enzyme such as optimal pH range, substrate specificity and enzyme molecular size. The phytase, considered as a special type of phosphatase, is capable of accepting phytate as a substrate and coincides with the hydrolyzing properties of nonspecific acid phosphatases (2). Accordingly, by testing with phytate as substrate, it is possible to distinguish phytase from acid phosphatase, which is incapable of degrading phytate. Especially, phytases from *A. niger*, *Bacillus* and *E. coli* was fairly specific for phytate than other diversified phosphorylated compounds. However, the substrate specificity for the phytase may vary because of the differences in the molecular characteristics of enzymes purified from different sources. To date, considerable amount of research has been reported for phytase as an animal feed additive and many phytases were isolated from fungi, bacteria, animal, and plants. Among them, microbial sources are promising for their commercial application. The earliest attempts to develop phytases began with a microorganism of the *Aspergillus* family found in the soil (3). Development of molecular biology enabled several phytase gene be cloned and overexpressed.

Currently, many microbial phytases have been used as feed enzyme to compensate for the lack of phytase activity in gut of animals. Phytases from fungi have most commonly been employed for its commercial production. There have been several reports on the cloning of the fungal phytases from

Aspergillus niger (4. 5), *A. fumigatus*, *A. terreus*, *Myceliophthora thermophila*, *Emericella nidulans*, *Talaromyces thermophilus*, *Thermomyces lanuginosus*, *Peniophora lycii*, *Agrocybe pediades*, *Trametes pubescens*, and a *Ceriporia* sp. Only few studies have been reported on the phytase of the yeasts such as *Saccharomyces cerevisiae* and *Schwanniomyces castellii* (6).

Phytases from bacterial sources have been found in *Aerobacter aerogenes*, *Peudomonas* sp., *Bacillus subtilis*, *Klebsiella* sp., *E. coli*, *Enterobacter* sp. 4, and *Bacillus* sp. DS11 (later designated as *B. amyloliquefaciens*) (7). Among these bacterial phytases, the extracellular phytase have been only known as that produced in *Bacillus* and *Enterobacter* (8). The most of phytases produced from gram negative bacteria was interacellular phytase reported as acid phosphatase. *Bacillus* and *E. coli* phytase have been investigated for the industrial application as feed enzyme because of the excellent characterization such as a heat-tolerance (in the case of *Bacillus*), a high specific activity to protein quantity, and a acidic pH optimum close to the physiological pH of animal stomach where may occur the phytate hydrolysis (in the case of *E. coli*).

As above the mentioned, recent research has shown that a variety of microorganisms have been screened for the better enzyme with distinct aspects in molecular features as well as catalytic properties depending on the source of origin (9, 10). In the present study, we isolated a high phytase activity-possessing bacteria strain from seawater nearby Busan, which was identified to be *Citrobacter braakii* YH-15, purified and characterized phytase from the strain. We have also cloned the phytase gene from YH-15 strain.

Screening and Identification of Phytase Producing Bacteria

We isolated 108 bacterial strains, showing clear zones (phytate degradation zones) around them on the screening plates. Among the isolated bacterial strains, five strains were selected by the phytase assay of cell culture as the candidates of phytase producing bacteria. Based on the results of physiological characteristics and 16S rDNA sequence analysis, five strains were identified as *Enterobacter cloacae, Escherichia coli, Citrobacter braakii, Enterobcter amnigenus, Enterobacter cloacae*, respectively. *Citrobacter braakii* called the strain YH-15 was found to have the highest level of phytase activity in the cell lysate and was therefore chosen for phytase enzyme production. We designated the strain YH-15 as *Citrobacter braakii* YH-15.

Production and Purification

Optimal culture conditions to produce phytase from *Citrobacter braakii* YH-15 were studied and evaluated. The highest phytase activity was detected when Luria broth was used at pH 7.0 and the activity markedly increased when the cells ran into the stationary phase at 30℃ culture condition. Furthermore, phytase production was very stable until 24hr incubation and remained constant up to 36hr.

The phytase was purified to homogeneity in five steps by ammonium sulphate fractionation, hydrophobic interaction chromatography, and ion-exchange chromatography (three types). A 12,950-fold purification was achieved from cell free extract with an overall recovery of 28 %. Among other phytases reported to date, *E. coli* phytase appA is known to have the highest specific

activity (1,800 U/mg protein) (11). However, in the present study, the specific acitivity from the purified *C. braakii* YH-15 phytase was 3,457 U/mg, which is 1.92 times higher than appA phytase of *E. coli*.

Biochemical and Molecular Properties

The molecular mass of the *C. braakii* YH-15 phytase was 47 kDa as determined by SDS-PAGE analysis. The purified enzyme exhibited a sharp optimal activity at around pH 4.0. and at least 50% of the maximal acitivity was obtained at pH 2.5. Within 7 days, the enzyme lost only a little activity at pH levels ranging from 3.0-4.5. However, the enzyme was completely inactivated at pH values below 3 after 4 hr preincubation. The optimal temperature for the enzyme activity was 50℃, and sharp decrease in activity was observed at 55℃. The enzyme was stable up to 50℃ for 1 hr. It retained 75% of its activity at 55℃, when exposed for 10min. In the study the effects of metal ions and reagents, the Fe^{3+}, Zn^{2+}, and Cu^{2+} had strongly inhibitive effects at lower concentrations on the phytase activity. 1 % Triton X-100 treatment enhanced the enzyme activity by 150 % (as relative activities. The purified enzyme looks fairly specific for sodium phytate and had little activity on other substrates. The kinetic parameters for the hydrolysis of sodium phytate has a *Km* of 0.46mM and a *Vmax* of 6027 U/mg as determined from a Lineweaver-Burke plot. The purified phytase was highly resistant to proteolysis. The phytase activity was not significantly changed when the purified phytase digested with either pepsin or trypsin. Other proteases (papain, elastase and pancreatin) slightly decreased the phytase activity to 85, 80 and 70% of original activity, respectively.

Cloning and Nucleotide sequence of the phytase gene

A 7.5 kb DNA fragment digested with *Pst I* from chromosomal DNA was detected by southern blot using DIG-labeled oligonucleotide probe degenerated based on the N-terminal sequence of the purified phytase. The released 7.5 kb DNA fragment containing the phytase coding gene, designated as *phyCB*, was cloned into a pBluescript vector treated with *Pst I*. This gene comprised 1302 bp nucleotides and encodes a polypeptide of 433 amino acids with a calculated mass of 47 kDa. The amino acid sequence corresponding to *phyCB* shows the presence of a 22-amono acid N-terminal hydrophobic signal peptide. The deduced N-terminal amino acid sequence of the mature phytass confirmed by N-terminal sequence of purified enzyme to homogeneity.

No significant nucleotide sequence homologies were found in the GeneBank using BLAST program. However, the deduced peptide sequence had 60% identity with *E. coli* appA. The most of histidine acid phosphatase known as phytase have been presented in the N-terminal RHG motif, and shown to possess another histidine that is located more than 250 amino acids further along. Analogues of all previously identified conserved residues were identified as being present in *phyCB*.

In order to increase the production level, we constructed an expression plasmid and overexpressed it in *E. coli* BL21(DE3) using the pET22b vector with the inducible T7 promoter.

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