Advanced Studies in Biology, Vol. 3, 2011, no. 3, 103 – 110

Molecular Analysis of Phytase Gene Cloned from *Bacillus subtilis*

R. Bawane[1], K. Tantwai[1*], L.P.S. Rajput[1], M. Kadam-Bedekar[2]

S. Kumar[1], I. Gontia[1] and S. Tiwari[1]

[1]Biotechnology Centre, J.N. Agricultural University
Jabalpur 482 004, India

[2]Animal Biotechnology Centre, M.P. Veterinary University
Jabalpur 482 001, India

tantwaik@yahoo.com

Abstract

Phytases are enzymes capable of hydrolyzing phytic acid to less-phosphorylated *myo*-inositol derivates. Phytases are becoming essential supplement to animal feeds. *Bacillus* sp. produces a thermostable phytase which renders it suitable for commercial production. Two strains of *Bacillus subtilis* NCDC-070 and NCIM-2712 were selected for cloning and sequencing of *phy* gene. Published *phy* gene gene sequences were used to design specific primers for amplification. *B. subtilis* strains amplified 1059bp product through PCR. The *phy* gene from *B. subtilis* NCDC-070 and NCIM-2712 were cloned in InsT/A vector and sequenced. The 928bp sequence from *Bacillus* strains NCIM-2712 and 826bp from NCDC-070 were compared with *phy* gene reference sequence. Comparison revealed 6 nucleotide and 3 amino acids variations. The *phy* gene from *B. subtilis* NCIM-2712 exhibited 99% similarity with one from NCDC-070 at nucleotide level.

Keywords: *Bacillus, phy* gene, Cloning, Sequencing, Phylogenetic analysis

Introduction

Phytic acid (also known as *myo*-inositol hexakis phosphate or phytate when in salt form) is the principal storage form of phosphorus in plants, particularly in cereals, grains and legumes and it represents approximately 75-80% of the total phosphorus found in nature. Cereals, legumes and oilseed crops grown over 90% of the world's harvested area serve as a major source of phosphorus for humans and animals. Phytic acid does not convert into available phosphorus in monogastric animals, such as pig, poultry and fish since they are unable to metabolize it. This compels us to supplement inorganic phosphate in their diets. In addition, undigested phytic acid released in feces contributes to phosphorus pollution in the livestock intensive areas (Nayini and Markakis, 1983; Common, 1989; Nasi, 1990). Phytic acid also acts as an antinutritional agent in monogastric animals by chelating various metal ions such as calcium, copper and zinc required for the growth of animals (Graf, 1983; Lee et al., 1988; Lei et al., 1993).

Phytic acid can be reduced through supplementing phytase enzyme in animal feeds as it is capable of hydrolyzing phytic acid to less-phosphorylated *myo*-inositol derivates. Phytases also reduce the antinutritional effect of food having high phytate content. Phytase is widely present in nature, occurring in microorganisms, plants as well as in some animal tissues. Phytase genes have been isolated from plants (Reddy et al., 1989), bacteria (Rodriguez et al., 1999) and fungi (Pasamontes et al., 1997a, b). Several phytases have been identified and cloned from *Pseudomonas* sp., *Escherichia coli, Klebsiella* sp. Phytase appA from *E. coli* is known to have the greatest specific activity but it lacks desired thermostability. In order to produce a more stable enzyme based on the *E. coli* phytase, it is necessary to have a set of homology to *E. coli* phytase sequence. However, this protein has only a few homologies with low sequence identity not exceeding beyond 30% to other known phytases. The phytase (*phy*C) from *Bacillus* sp. are beta propeller phytase which exhibit an optimum pH from 6.0-9.0, suitable for neutral animal tracts such as trout and cyprinids with more thermostability.

Although number of phytase producing organisms and their types have been reported, a thermostable and acid tolerant phytase with broad substrate specificity and high specific activity is highly desirable for animal nutrition purposes as it will be of great commercial importance.

Materials and method

Bacterial strains: Two *Bacillus subtilis* strains namely NCDC-070 and NCIM-2712 were procured to isolate phytase (*phy*) gene from National Chemical Laboratory (NCL), Pune. These strains were grown by incubating at 37°C for 16 h at 180 rpm in nutrient medium (Beef Extract 10g, NaCl 5g, Peptone10g, Agar 20g, Distilled water 1.0L, pH 7.0-7.5).

DNA isolation: Genomic DNA was isolated from *B. subtilis* strains grown overnight in nutrient broth. Approximately 2 ml of grown bacterial culture was centrifuged for 2 min at 10,000 rpm. Pellet was re-suspended in 467µl TE buffer and 30 µl of 10% SDS and 3 µl of 20 mg/ml Proteinase K were added to above mixture followed by 1hr incubation at 37°C. The suspension was extracted with equal volume of phenol/chloroform by centrifugation at 12,000 rpm for 10 min. Subsequently 1/10 volume of sodium acetate was mixed, and DNA was precipitated using equal volume of isopropanol. The DNA was recovered by centrifugation at 10,000 rpm for 10 min at 4°C. DNA pellet was washed with 70% ethanol, air dried, re-suspended in 50 µl TE buffer and stored in 10mM Tris-Cl and 1mM EDTA solution with 8.0 pH at -20°C.

Cloning of phy gene and sequencing: The *phy* gene specific primers were designed with 'Gene Tool' using the published sequence of *phy* gene in NCBI Genbank Account AF298179 (*Phy*F 5'CTGTCTGATCCTTATCATTT3' and *Phy*R 5'TCCGCTTCTGTCGGTCA3'). Amplification of the phy gene region was carried out using the primer set with initial denaturation on 95°C for 5 min followed by 35 cycles of denaturation, annealing and extension at 95°C for 1 min, 52°C for 1 min and 72°C for 1 min, respectively and the final extension was carried out at 72°C for 10 min. Using standard PCR protocol, about 1 Kb DNA fragment from *B. subtilis* NCDC-070 and NCIM-2712 were amplified. The PCR products were purified using Zymoclean gel DNA recovery kit (Zymo Research, USA), ligated into InsT/A cloning vector pTZ57R/T as per the manufactures recommendations (Fermentas®). The ligation product was transferred to *E. coli* JM-109. The recombinants were selected on the LB agar plate containing ampicillin (75µg/ml). The presence of the gene in the selected clone was confirmed by PCR amplification using specific primer and restriction analysis. The presence of the insert in the recombinant clones was confirmed by sequencing using M13 primer.

Nucleotide and phylogenetic sequence analysis: The nucleotide sequencing of the *phy* gene was done by out sourcing at Bangalore Genie (India). Nucleotide and amino acid sequence homology searches were performed on the NCBI database by BLAST search was done using the CLUSTAL W program. For phylogenetic analysis, gene sequences obtained in this study and *phy* gene sequences from

different *Bacillus* sp. available in NCBI database were used. Pair wise sequence alignment was done by CLUSTAL W algorithm. Phylogenetic tree was created using neighbour-joining method with 100 replicates.

Results and discussion

The *phy* gene isolate from two *B. subtilis* strains of NCDC-070 and NCIM-2712 gave an amplification of 1059 bp amplicon (Fig.1).

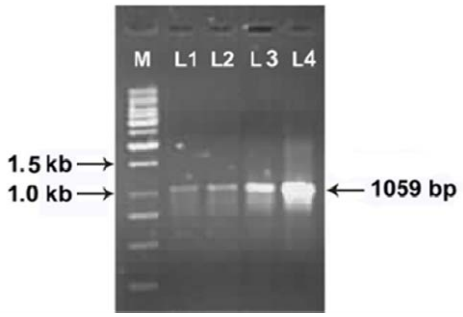

Fig. 1. PCR amplification of phytase gene from *Bacillus* sp. showing amplicon of 1059bp on 1% agarose gel. M- Molecular weight marker; L1, L2- *Bacillus subtilis* NCDC-070; L3, L4- *Bacillus subtilis* NCIM-2712

Cloning and sequence alignment *phy* gene: The PCR amplified 1059 bp DNA fragment corresponding to *phy* gene from *B. subtilis* NCDC-070 and NCIM-2712 was cloned using InsT/A cloning vector. Successful cloning was achieved in transformed *E. coli* JM-109 host selected on ampicillin containing media. The clones were confirmed using colony PCR and restriction digestion of positive clones with *Sac*-I and *Hind*-III restriction enzymes. The nucleotide sequence of *phy* gene from *B. subtilis* strains NCIM-2712 and NCDC-070 were processed to remove vector sequence through Gene Tool and submitted to NCBI, Genbank. The assigned accession numbers are GU475116 for *B. subtilis* NCDC-070 and GU475117 for *B. subtilis* NCIM- 2712, respectively. The available sequence information from cloned fragment was subjected to analysis using BLAST algorithm available at http://www.ncbi.nlm.nih.gov. The phytase gene from strain NCIM-2712 had 99% similarity with NCDC-070 at nucleotide level. This indicates close relation between these two strains. The nucleotide sequence of NCDC-070 compared with reference sequence at positions no. 6, 591, 752, 787,

789 and 818 showed variations and insertion or deletion at position of 839 and 918 were also found. The deduced amino acid sequence of the strain NCDC-070 compared with reference sequence, showed variation at positions *viz*. 97, 143, 144, 180, 188, 189, 195, 196 and 200. There were deletion or insertion at amino acid position 143 to 144 and 188 to 189, insertion 4 amino acid *viz.* 143-D (Aspartic acid), 144-D, 188-K (Lysine) and 189-K in reference sequence and NCIM-2712. The variation between NCDC-070 and reference sequence was similar at position 97 but previously cloned NCIM-2712 was different at position 97 that formed 'Y' (Tyrosine). At position 180 and 195 NCDC-070 and reference sequence were different that formed 'V' (Valine) and 'R' (Arginine) but NCIM-2712 were similar to reference sequence, at position 200 NCDC-070 showed insertion but reference sequence and NCIM-2712 were differing that formed 'A' (Alanine). At position 196 NCDC-070 and reference sequence were similar but NCIM-2712 formed 'N' (Asparagine). The two of the strains showed close relationship with earlier reports from Indian strains (AF298179, EF092835 and ARRMK33), Asian strains (AY220075 WHNB02 China, AY518208 SD01N China, EU624118 B9601-Y2 China, FJ986327 WYCQ02 China and AJ584664 Vietnam) Korean strains (BSU85968 DS11 Korea and DQ346197 IDCC1102 Korea). The *phy* gene sequence of 1724 bp from Bacillus sp. DS11 has been known previously (Kim et al., 1998). Similarly, *phy* gene sequence of 1290 bp from *B. subtilis* strain VTT E-68013 has been known (Kerovuo et al., 1998).

The phytase gene (*phyC*) of 1300 bp from *Bacillus subtilis* VTT E-68013 was cloned and inserted into *Escherichia coli-Bacillus* sp. shuttle vector pMK3 and then transferred into *E. coli* by electrotransformation. pMK3P plasmid isolated from *E. coli* was used to introduce into *B. coagulans* DSM1 by electrotransformation (Ozusaglam and Ozcan, 2009). The *phy* gene sequence from both the strains showed 98% homology with *Bacillus* spp. DS11 (U85966) and only 91% homology with the *B. subtilis* strain VTT E-68013. Phylogenetic tree showing the relationship between *phy* gene from Bacillus strain (NCDC-070 and NCIM-2712) and the other *phy* gene sequences from the database is presented in Fig. 2. Phylogenetic analysis based on nucleotide sequence reconfirmed that both isolates were grouped together and were closely related to other Asian strains. From present investigation, it can be concluded that *phy* gene amplified and cloned from *B. subtilis* NCDC-070 and NCIM-2712 are almost similar with few residue changes and might have originated from common ancestral DNA sequence.

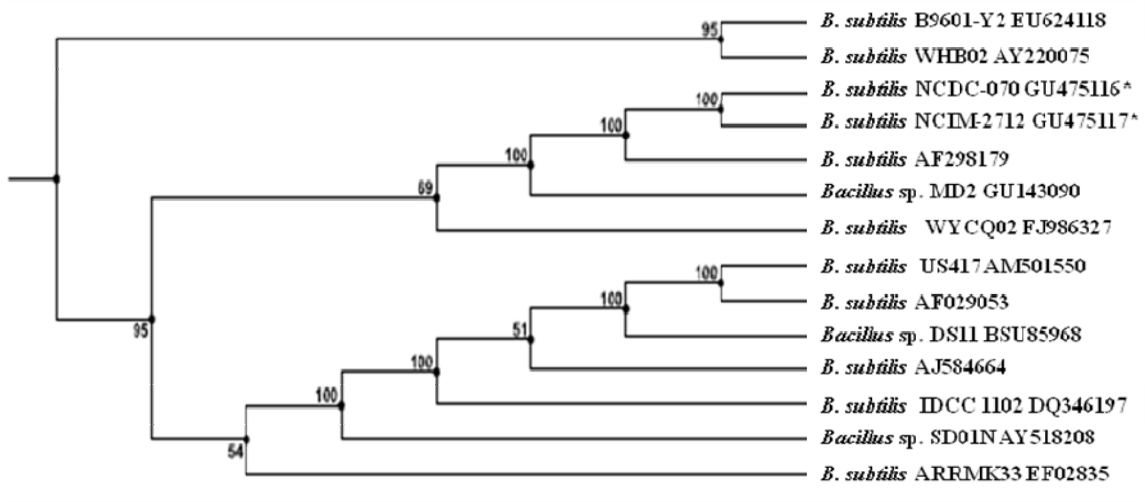

Fig. 2. Phylogenetic tree showing relationship of *phy* gene from *Bacillus subtilis* strain NCDC-070 and strain NCIM-2712 with other related *phy* gene sequences.

References

[1] F.H. Common, Biological availability of phosphorus for pigs, Nature 143 (1989), 370–380.

[2] E. Graf, Calcium binding to phytic acid, J. Agric. Food Chem. 31 (1983), 851–855.

[3] Y.O. Kim, J.K. Lee, H.K. Kim, J.H. Yu and T.K. Oh, Cloning of the thermostable phytase gene (*phy*) from Bacillus sp. DS11 and its overexpression in *Escherichia coli*, FEMS Microbiol. Lett. 162 (1998), 185-191.

[4] J. Kerovuo, M. Lauraeus, P. Nurminen, N. Kalkkinen and J. Apajalahti, Isolation, characterization, molecular gene cloning, and sequencing of a novel phytase from *Bacillus subtilis,* Appl. Environ. Microbiol. 64 (1998), 2079-2085.

[5] D. Lee, J. Schroeder and D.T. Gordon, Enhancement of Cu bioavailability in the rat by phytic acid, J. Nutri. 118 (1988), 712–717.

[6] X. Lei, K. Pao, R.M. Elwyn, D.E. Ullrey and M.T. Yokoyama, Supplemental microbial phytase improves bioavailability of dietary Zinc to weanling pigs, J. Nutr. 123 (1993), 1117–1123.

[7] M. Nasi, Microbial phytase supplementation for improving availability of plants phosphorus in the diets of growing pigs, J. Agric. Sci. 62 (1990), 435-442.

[8] N.R. Nayini and P. Markakis, Effects of inositol phosphates on mineral utilization, Fed. Proc. 45 (1983), 819–826.

[9] M.A. Ozusaglam and N. Ozcan, Cloning of phytase gene in probiotic bacterium *Bacillus coagulans,* Adv. Studies Biol. 1 (2009), 15-24.

[10] L. Pasamontes, M. Haiker, M. Henriquez-huecas, D.B. Mitchell and A.P. Van-Loon, Cloning of the thermostable phytases from *Emericella nidulans* and the thermophilic fungus *Talaromyces thermophilus*, Biochem. Biophys. Acta. 1353 (1997), 217-223.

[11] L. Pasamontes, M. Haiker, M. Wyss, M. Tessier and A.P. Van Loon, Gene cloning, purification and characterization of a heat-stable phytase from the fungus *Aspergillus fumigates*, Appl. Environ. Micorbiol. 63 (1997b), 1696-1700.

[12] N.R. Reddy, M.D. Pierson, S.K. Sathe and D.K. Salunkhe, Phytates in cereals and legumes, (1989) CRC Press, Boca Raton, Fl.

[13] E. Rodriguez, Y. Han and X.G. Lei, Cloning, sequencing and expression of an *Escherichia coli* acid phosphatase, phytase gene *(app*A2*)* isolated from pig colon, Biochem. Biophy. Res. Comm. 257 (1999), 117-123.

Received: March, 2011