

Short Communication

Functional screening of phosphatase-encoding genes from bacterial sources

Mohammad Reza Sarikhani^{1,2}, Mohammad Ali Malboobi^{1*}, Nasser Aliasgharzad², Ralf Greiner³, Bagher Yakhchali¹

¹National Institute of Genetic Engineering and Biotechnology, P.O. Box 14965/161, Tehran, I.R. Iran ²University of Tabriz, Faculty of Agriculture, Department of Soil Science, P.O. Box 5166616471, Tabriz, I.R. Iran ³Federal Research Institute of Nutrition and Food, Haid-und-Neu-Straße 9, 76131 Karlsruhe, Germany

Abstract

Phosphatase (APase) enzymes including phytases have broad applications in diagnostic kits, poultry feeds, biofertilizers and plant nutrition. Because of high levels of sequence diversity among phosphatases, an efficient functional screening method is a crucial requirement for the isolation of the encoding genes. This study reports a functional cloning screening method for the isolation of APase-encoding genes from bacterial genomic libraries in a medium containing a chromogenic substrate. The method was optimized to distinguish the desired signal from the background chromosomal APase activity. This screening method led to the isolation of two novel APase-encoding genes from *Pseudomonas putida* with no similarities to the known genes in the databases, indicating successful implementation of the developed method.

Keywords: *Pseudomonas putida*; Phosphatase; Phytase; Phytate; BCIP

Bacterial phosphatases have been of interest in recent decades because they play multiple vital roles in cell signaling, regulation of molecular activities and phosphate homeostasis as well as being exploited as (i) investigative tools in enzymology and regulation of gene expression (ii) plant nutrition, (iii) poultry feeds, (iv) markers for bacterial taxonomy and identification, (v) reporter in diagnostic kits, and (v) tools for biore-

mediation in environmental microbiology (Rossolini *et al.*, 1998).

Thaller and colleagues (1998) placed prokaryotic non-specific APases in three distantly related families A, B and C on the basis of shared conserved motifs within each family despite lacking overall sequence similarities. As the sequence diversity is much higher in eukaryotic APases, (Feizi and Malboobi, unpublished data) classified plant APases into five distinct groups with almost no similarities among them, even between the family motifs.

With respect to the important industrial and agricultural applications of APases, isolation of relevant genes has been of great interest. However, because of high levels of sequence diversity among phosphatases (APases), an efficient functional screening method is still a crucial requirement for the isolation of the encoding genes.

So far, various methods have been employed for the purpose of gene cloning. For instance, PCR and RT-PCR are the simplest procedures for the isolation of homologous genes from genomes or transcriptomes. In fact, there are several reports on the isolation of the APase-encoding gene similar to the known ones by the use of RT-PCR techniques in eukaryotic organisms (Bei and Xiang-Ning, 2008; Celler *et al.*, 1998). However, the short half-life of mRNA and the absence of a poly (A) tail make it difficult for prokaryotic genes to make cDNA templates for the RT-PCR procedure (Saleh-Lakha *et al.*, 2005). Also, due to the high sequence diversity of APases, regular RT/PCR is of limited use. This is more pronounced when novel APase-encoding genes are being explored.

*Correspondence to: Mohammad Ali Malboobi, Ph.D.

Tel: +98 21 44580369; Fax: +98 21 44580369

E-mail: malboobi@nigeb.ac.ir

Another widely used method is the plate screening method resulting in clear zones around microorganisms expressing extracellular APases (Malboobi *et al.*, 2009; Gargova *et al.*, 1997). However, the clear zones could be related to organic acids hydrolyzing mineral phosphate compounds rather than APase activity (Malboobi *et al.*, 2009; Mehta and Nautiyal, 2001; Bae *et al.*, 1999). Also, the desired genes could be cloned by designing degenerate primers based on the partial sequences of the purified enzyme, in order to find the corresponding gene in the genome (Cho *et al.*, 2005; Kerovuo *et al.*, 1998). However, this method requires identification and purification of the enzyme which is not an easy task. Again, none or little sequence similarity among these enzymes would constrain this approach to the same APase family.

More specifically, certain screening methods have been developed for the isolation of microorganisms-not their genes- producing strong APases (Mehta and Nautiyal, 2001; Van Ommen Kloeke *et al.*, 1999). For instance, Gibson and coworkers (1988) reported the direct staining of microbial colonies by using 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Others used chromogenic substrates, such as para-nitrophenyl phosphate (pNPP) (Pradel and Boquet, 1988) and phenolphthalein diphosphate/methyl green (Riccio *et al.*, 1997), which produce a yellow or green color, respectively, to screen for APase activity. In addition, a precipitating fluorescent dye was used to monitor APase activity in bacterial colonies (Van Ommen Kloeke *et al.*, 1999). Also, a method for phytase screening in a genomic library has been described by Senn and Wolosiuk (2005), which involves the detection of the released phosphate ion (Pi) following hydrolytic reaction in bacterial colonies.

In this research, attempts were made to develop a rapid and reliable screening method to isolate genes responsible for APase activity by using minimal medium containing phytate, as the only source of phosphorus, and BCIP, as an indicator. Most of the studies have shown that APases, and especially phytases are induced in absence of Pi or in the presence of phytate (Greiner, 2004; Kerovuo *et al.*, 1998). This study describes the utility of this new methodology for screening of the *Pseudomonas putida* DSM 23335 genomic DNA libraries to isolate genes coding for novel APases.

Libraries were constructed by complete or partial digestions of *P. putida* strain P13 (DSM 23335) genomic DNA with *EcoRI*, *SalI*, *HindIII* or *Sau3AI* (Fermentas, Lithuania) as described by Sambrook and

Russell (2001). The DNA fragments were ligated into pBluescript KS⁻ vector using T4 DNA ligase (Fermentas, Lithuania) following incubation at 22°C overnight. The ligation products were used to transform *Escherichia coli* DH5 α through electroporation carried out by the Bio-Rad Gene Pulser (Bio-Rad laboratories, Richmond, CA) set at 1.8 kV, 25 μ F and 500 Ω .

Screening of the genomic library was performed on solid Sperber medium containing 16 g/l of agar, 10 g/l of glucose, 2.5 g/l of sodium phytate, 0.5 g/l of yeast extract, 0.1 g/l of CaCl₂, 0.25 g/l of MgSO₄, pH 7.2 supplemented with 0.025 g/l of BCIP and 100 μ g/ml of ampicillin. The presence of APase activity was judged by the intensity of the blue color as well as hollow zone diameters formed around the bacterial colonies after 24 to 48 h. In a series of preliminary experiments, two bacterial species, *P. putida* and *E. coli*, were cultured on LB or Sperber agar medium containing BCIP. *E. coli* strain DH5 α produced pale blue colonies on both media, while *P. putida* strain P13, a bacteria with strong APase activity, produced intense blue colonies only in the latter medium. This shows that strain P13 carries one or more inducible APase-encoding genes. Regarding the presence of minimal nutrients in the Sperber medium, sodium phytate was used as the only source of phosphate such that the APase-encoding genes were induced via their own endogenous promoter in absence of free phosphate. Although blue color intensity produced by *P. putida* P13 and *E. coli* DH5 α contrasted sufficiently, a time-coursed experiment using the Sperber medium containing BCIP at concentrations of 10, 25 and 50 mg/l was designed. Twenty

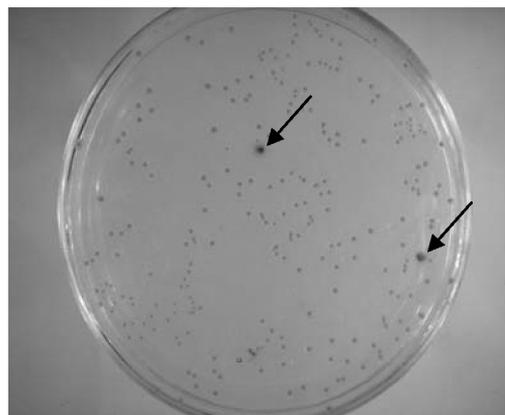


Figure 1. Screening for strong APase-expressing clones. Arrows show two intensely blue-stained colonies grown on medium containing BCIP, which appeared to carry the same APase-encoding genes later.

five mg/l of BCIP was determined to be the optimum concentration for plate monitoring for monitoring differential blue staining within 36 h (data not shown). Nevertheless, the clear-zone diameters did not differ considerably between the bacterial species.

After construction of the *P. putida* P13 genomic library in *E. coli*, more than thirty positive clones were screened from approximately 750000 colonies (Fig. 1). Based on restriction maps as well as sequence data, all the isolated clones were found to be derived from two distinct loci. Two intensely blue-stained clones carrying recombinant plasmids, named clones A and B, with 2.4 and 2.7 kb DNA inserts, respectively, showed inducible APase activity in a medium containing insoluble phytate as the sole source of phosphate (Figs. 2 and 3). The restricted DNA fragments or PCR products were subcloned in order to find the open reading frame responsible for strong APase activity.

Figure 2 demonstrates the qualitative assessment of the blue phenotype related to APase activity of the isolated clones versus the control ones, *P. putida* strain P13 and *E. coli* carrying pBluescript with no insert, have been shown in this figure. To determine phosphatase activity in a crude extract of blue clones, enzyme extraction and assay were performed as described by Greiner, 2004. In order to check the APase and phytase activities encoded by the isolated clones, some phosphorylated substrates, such as glucose-6-phosphate, fructose-6-phosphate, pNPP and sodium phytate, were used. The released inorganic phosphate was measured by the modified ammonium molybdate method (Heinonen and Lahti, 1981). The unit of activity was expressed as 1 μ mol of released phosphate per min. Total protein contents were determined by the Bradford method (1976) using bovine serum albumin (BSA) as the standard. Phytase activities in crude extracts of the isolated clones were at least 50 times higher than those of the controls (Fig. 4). Comparison of the activities of enzymes encoded by the isolated genes showed substrate specificities of clones A and B for phytate and sugar phosphate,



Figure 2. Comparison of the phosphatase activities of native bacterium *P. putida* P13 (left), clone B as one of the positive clones (middle) and control (*E. coli* carrying pBluescript KS⁻; right), in minimal medium containing BCIP.

respectively (Fig. 5). Phytase specific activities in the crude extracts were 0.95 U/mg and 0.46 U/mg for clones A and clone B, respectively. In fact, these findings confirmed the robustness of the method to overcome problems with cloning of the APase genes being overlooked due to none or little sequence similarities. Further characterizations of the isolated genes are currently underway.

BCIP has widely been employed for the detection of alkaline phosphatases in cytochemical staining as well as serological procedures. Gibson *et al.* (1988) have demonstrated the potential usefulness of BCIP-containing medium for visualization of APase activity in both fungal and plant cell cultures. Jacobs and colleagues (2003) used *phoA* as a reporter gene to follow up the construction of a comprehensive transposon mutant library. The transfer of the transposon was traced by the expression of the reporter gene and, thus, the appearance of a blue colony phenotype on LB medium containing 40 mg/l of BCIP. Potts *et al.* (1993) used either *p*-nitrophenylphosphatase or 5-

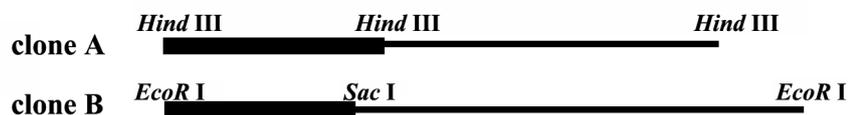


Figure 3. Restriction maps of the genomic DNA inserts of the selected clones. Initial size of clone A and clone B were 7 and 8 kb, respectively. Thick bars show the subcloned fragments carrying APase-encoding genes. The open reading frames within the subcloned fragments, 2.4 and 2.7 Kb, encode proteins with high APase activities.

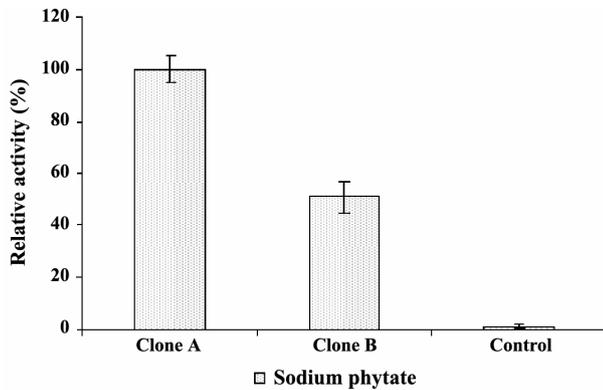


Figure 4. Relative APase activity of enzymes encoded by clone A, clone B and control (*E. Coli* DH5 α carrying pBluescript KS⁻) in the presence of sodium phytate. The crude protein extracts from each clone and control were incubated in 100 mM acetate buffer (pH 5.0) containing 5 mM sodium phytate at 37°C for 30 min, and the released Pi was measured. The highest percentage of activity was taken as 100. Data points represent the mean \pm SD of duplicated assays.

bromo-4-chloro-3-indolyl phosphatase activity of an APase-encoding gene, *iphP*, isolated from the Cyanobacterium, *Nostoc commune*, and the BCIP-containing medium allowed direct visualization of APase activity. However, using BCIP for screening of genomic/cDNA libraries has not been considered by researchers as it could be masked by endogenous APase activity of host cells and/or repression by phosphorus present in the medium. Having optimized the technique, we were able to readily distinguish the APase activities related to the cloned-DNA fragments from the background activity. The BCIP-containing medium allows a rapid and direct visualization of APase production. Besides, the intensity of blue color could be qualitatively used to simply assess the levels of gene expression and/or enzyme activity.

Acknowledgments

We would like to acknowledge the National Institute of Genetic Engineering and Biotechnology and the Federal Research Institute of Nutrition and Food for facilities available to conduct this research. We also grateful to Green Biotechnology for biomaterial and fund providing services and supports.

References

- Bae HD, Yanke LJ, Cheng KJ, Selinger LB (1999). A novel staining method for detecting phytase activity. *J Microbiol Methods*. 39: 17-22.
- Bei G, Xiang-Ning J (2008). Cloning of Trehalose-6-Phosphate Phosphatase and Transformation to Tobacco. The 2nd International Conference on Bioinformatics and Biomedical

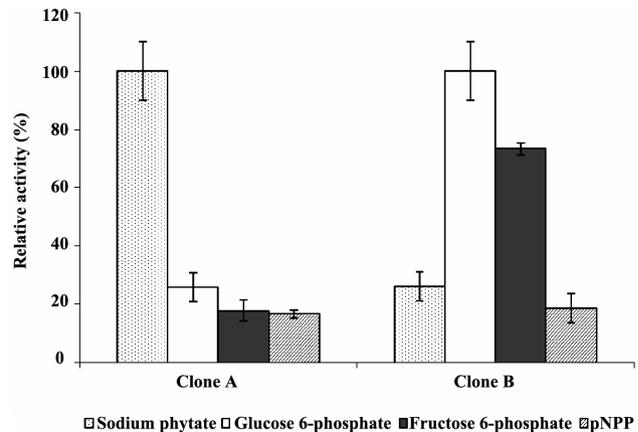


Figure 5. Relative APase activity of enzymes encoded by the cloned genes in the presence of various substrates. The bacterial crude protein extract of each clone was incubated in 100 mM acetate buffer (pH 5.0) containing 5 mM of each substrate at 37°C for 30 min, and the released Pi was measured. The highest percentage of activity was taken as 100. Data points represent the mean \pm SD of duplicated assays.

Engineering 441-444, Shanghai, China.

- Bradford M (1976). A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal Biochem*. 72: 248-254.
- Celler JW, Luo X, Böhmer FD (1998). Protein tyrosine APase gene expression analysis in Swiss 3T3 fibroblasts. *Mol Cell Biochem*. 178: 157-162.
- Cho JS, Lee CW, Kang SH, Lee JC, Bok JD, Moon YS, Lee HG, Woo J, Choi YJ (2005). Molecular cloning of a phytase gene (*phy M*) from *Pseudomonas syringae* MOK1. *Curr Microbiol*. 47: 290-294.
- Gargova S, Roshkova Z, Vancheva G (1997). Screening of fungi for phytase production. *Biotechnol Tech*. 11: 221-224.
- Gibson DM, Christen AA, Mullaney EJ (1988). Direct screening for acid phosphatase production on BCIP-Agar plates. *Biotechnol Tech*. 2: 63-68.
- Greiner R (2004). Purification and properties of a phytate-degrading enzyme from *Pantoea agglomerans*. *Protein J*. 23: 567-576
- Heinonen JK, Lahti RJ (1981). A new and convenient colorimetric determination of inorganic orthophosphate and its application to assay inorganic pyrophosphatase. *Analytical Biochem*. 113: 313-317.
- Jacobs MA, Alwood A, Thaipisuttikul I, Spencer D, Haugen E, Ernst S, Will O, Kaul R, Raymond C, Levy R, Chun-Rong L, Guenther D, Bovee D, Olson MV, Manoil C (2003). Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proceedings of the national academy of science of the united states of america*. 100: 14339-14344.
- Kerovuo J, Lauraeus M, Nurminen P, Kalkkinen N, Apajalahti J (1998). Isolation, Characterization, Molecular Gene Cloning and Sequencing of a Novel Phytase from *Bacillus subtilis*. *Appl Environ Microbiol*. 64: 2079-2085.
- Malboobi MA, Owlia P, Behbahani M, Sarokhani E, Moradi S, Yakhchali B, Deljou A, Morabbi K (2009). Solubilization of

- organic and inorganic phosphates by three efficient soil bacterial isolates. *World J Microbiol Biotechnol.* 25: 1471-1477.
- Mehta S, Nautiyal CS (2001). An efficient method for qualitative screening of phosphate-solubilizing bacteria. *Current Microbiol.* 43: 51-56.
- Potts M, Sun H, Mockaitis K, Kennelly PJ, Reed D, Tonks NK (1993). A protein-tyrosine/serine phosphatase encoded by the genome of the cyanobacterium *Nostoc commune* UTEX 584. *The J of biological chemistry.* 268: 7632-7635.
- Pradel E, Boquet PL (1988). Acid phosphatases of *Escherichia coli*: molecular cloning and analysis of *agp*, the structural gene for a periplasmic acid glucose phosphatase. *J Bacteriol.* 170: 4916-23.
- Riccio ML, Rossolini GM, Lombardi G, Chiesurin A, Satta G (1997). Expression cloning of different bacterial phosphatase-encoding by histochemical screening of genomic libraries onto an indicator medium containing phenolphthalein diphosphate and methyl green. *J Appl Microbiol.* 82: 177-185.
- Rossolini GM, Schippa S, Riccio ML, Berlutti F, Macaskie LE, Thaller MC (1998). Bacterial nonspecific acid phosphohydro-
lases: physiology, evolution and use as tools in microbial biotechnology. *Cell Mol Life Sci.* 54: 833-850.
- Saleh-Lakha S, Miller M, Campbell RG, Schneider K, Elahimanesh P, Hart MM, Trevors JT (2005). Microbial gene expression in soil: methods, applications and challenges. *J Microbiol Methods.* 63: 1-19.
- Sambrook J, Russell DW (2001). Molecular cloning a laboratory manual. Third edition. Cold Spring Harbor laboratory Press, Cold Spring Harbor, New York.
- Senn AM, Wolosiuk RA (2005). A high-throughout screening for phosphatases using specific substrates. *Analytical Biochem.* 339: 150-156.
- Thaller MC, Schippa S, Rossolini GM (1998). Conserved sequence motifs among bacterial, eukaryotic, and archaeal phosphatases that define a new phosphohydrolase superfamily. *Protein Sci.* 7: 1647-1652.
- Van Ommen Kloeke F, Baty AM, Eastburn CC, Diwu Z, Geesey GG (1999). Novel method for screening bacterial clones for phosphatase activity. *J Microbiol Methods.* 38: 25-31.

