Short Communication

Bacterial overexpression of the human interleukin-2 in insoluble form via the pET Trx fusion system

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Abstract
Selection of a system for successful recombinant protein production is important. The aim of this study was to produce high levels of human interleukin-2 (hIL-2) in soluble form. To this end, the pET32a vector in *Escherichia coli* BL21 (DE3) was used as an expression system, since it was previously used for the production of mouse IL-2 in soluble form. The results indicated that contrary to expectations, the expressed protein was in the form of inclusion bodies and perhaps amino acid differences between human and mouse IL-2 should be determinant. The hIL-2 protein is a small peptide, therefore its recovery as a biologically functional protein by the process of refolding may be feasible and could lead to high yields at the industrial scale.
***Keywords*:** Human IL-2; Soluble protein; Inclusion body; Overexpression; *E. coli*

Human interleukin-2 (IL-2) is a protein which plays a central role in immunological responses (Minami *et al*., 1993). Therefore, soon after its discovery, extensive studies were carried out regarding the cloning (Minami *et al*., 1993), gene structure (Holbrook *et al*., 1984), high level expression (Williams *et al*., 1988) and crystallography of the protein (Bazan, 1992). The genetic locus for the human IL-2 is located on the long arm of chromosome 4 (Holbrook *et al*., 1984), which encodes a human IL-2 precursor, with a molecular weight of approximately 15 kDa (Kashima *et al*., 1985) , consisting of 153 amino acids. The 20 amino acids at the N-terminal of hIL-2 comprise a signal peptide that is excluded from the mature IL-2 (Cerretti *et al*., 1986). Post-translational modifications include glycosylation of Thr3 (Robb *et al*., 1981) and the formation of disulfide bridges between cysteines located on amino acid residues 58 and 105 (Gomez *et al*., 1998). Human IL-2 glycosylation does not affect its biological activity *in vitro* and is probably effective only in increasing the half life and solubility of the protein in biological systems (Sarah and Kathleen, 2004). This protein is postulated to play a role in various cancers especially renal cell cancer (RCC), melanoma as well as AIDS (Sereti *et al*., 2002). Human IL-2 has been cloned in several microorganisms as host cells (Hyung *et al*., 2005), among which in this work, *E. coli* BL21 carrying the pET32a vector was considered for the overexpression of hIL-2 in sol-

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Short Communication

Functional screening of phosphatase-encoding genes from bacterial sources

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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 bioremediation 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.

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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 *Eco*RI, *Sal*I, *Hin*dIII or *Sau*3AI (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* DH5α through electroporation carried out by the Bio-Rad Gene Pulser (Bio-Rad laboratories, Richmond, CA) set at 1.8 kV, 25 μF 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 $CaCl_2$, 0.25 g/l of $MgSO_4$, pH 7.2 supplemented with 0.025 g/l of BCIP and 100 μg/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 DH5α 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* DH5α contrasted sufficiently, a time-coursed experiment using the Sperber medium containing BCIP at concentrations of 10, 25 and 50 mg/l was designed. Twenty

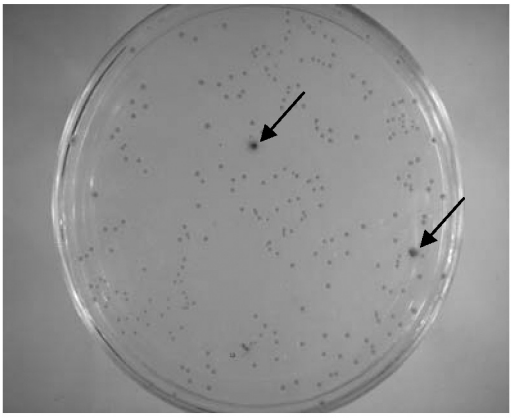

Figure 1. Screening for strong APase-expressing clones. Arrows show two intensely blue-stained colonies grown on medium containing BCIP, which appeared to carry the same APase-encoding genes later.

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 μmol 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 *pho*A 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-

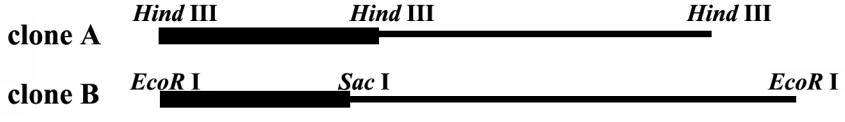

Figure 3. Restriction maps of the genomic DNA inserts of the selected clones. Initial size of clone A and clone B were 7 and 8 kb, respectively. Thick bars show the subcloned fragments carrying APase-encoding genes. The open reading frames within the subcloned fragments, 2.4 and 2.7 Kb, encode proteins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

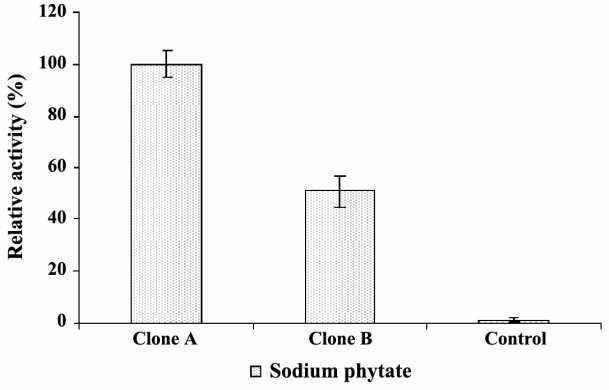

Figure 4. Relative APase activity of enzymes encoded by clone A, clone B and control (*E. Coli* DH5α carrying pBluescipt KS$^-$) in the presence of sodium phytate. The crude protein extracts from each clone and control were incubated in 100 mM acetate buffer (pH 5.0) containing 5 mM sodium phytate at 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 ± SD of duplicated assays.

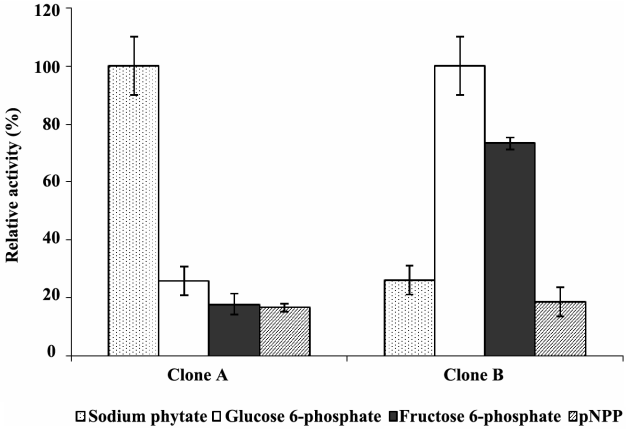

Figure 5. Relative APase activity of enzymes encoded by the cloned genes in the presence of various substrates. The bacterial crude protein extract of each clone was incubated in 100 mM acetate buffer (pH 5.0) containing 5 mM of each substrate at 37°C for 30 min, and the released Pi was measured. The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

bromo-4-chloro-3-indolyl phosphatase activity of an APase-encoding gene, *iph*P, 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.

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