A high-throughput screening for phosphatases using specific substrates

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Abstract

A high-throughput screening was developed for the detection of phosphatase activity in bacterial colonies. Unlike other methods, the current procedure can be applied to any phosphatase because it uses physiological substrates and detects the compelled product of all phosphatase reactions, that is, orthophosphate. In this method, substrates diffuse from a filter paper across a nitrocellulose membrane to bacterial colonies situated on the opposite face, and then reaction products flow back to the paper. Finally, a colorimetric reagent discloses the presence of orthophosphate in the filter paper. We validated the performance of this assay with several substrates and experimental conditions and with different phosphatases, including a library of randomly mutagenized rapeseed chloroplast fructose-1,6-bisphosphatase. This procedure could be extended to other enzymatic activities provided that an appropriate detection of reaction products is available.

Keywords: Phosphatases; High-throughput screening; Enzyme activity; Transformed bacteria

Phosphatases participate in multiple physiological processes. Therefore, numerous screening methods have been developed for the isolation of phosphatase-producing bacteria [1], the selection of phosphatase-encoding genes [2-4], the discrimination of microbial taxa [5,6], and the detection of yeast mutants [7]. Screening methods based on this activity have also been applied to detect bacteria transformed with recombinant plasmids as an alternative to the conventional lacZ system. In these studies, vectors harboring different phosphatases have been designed as tools for cloning-dependent insertion inactivation [8,9].

Methods for assaying phosphatase activity of bacterial colonies rely on phosphorylated substrates that are either added to the growth medium or provided by flooding colonies with the catalytic solution. These artificial substrates yield a colored or fluorescent product after orthophosphate (Pi) release. For example, bacterial colonies stain yellow, blue, or deep green when Pi is released from p-nitrophenylphosphate (PNPP) [2,3], 5-bromo-4-chloro-3-indolyl phosphate [8], or phenolphthalein diphasphate/methyl green [4,5,9–11], respectively. Phosphatase activity can also be detected by monitoring the fluorescence that follows the hydrolysis of 2-(5′-chloro-2′-phosphoryloxyphenyl)-4-[3H]-quinazolnolone [1] or 4-methylumbelliferyl phosphate derivatives [12].

Phosphatases differ greatly in substrate specificities. Therefore, successful screenings require sensitive and accurate procedures for estimating a catalytic capacity that reasonably reflects their native activity. Because a
screening method for evaluating specific substrates was not available, we developed a system whose distinguishing feature is the detection of the common product of all phosphatase reactions, that is, Pi. After the hydrolytic reaction takes place in bacterial colonies, the products diffuse into a filter paper where Pi is detected. This procedure does not require complex and expensive handling, and it provides a high-throughput screening (HTS) for the analysis of enzyme libraries. We also validated the suitability of this approach using several substrates and bacteria producing different phosphatases. The method is versatile and could be exploited for detecting other enzymatic reactions.

Materials and methods

Materials

_Escherichia coli_ JM109(DE3) was transformed with pBAD, pBAD18, and pET 22b(+) vectors (Novagen, Madison, WI, USA) harboring genes that coded for alkaline phosphatase (phoA), acid glucose phosphatase (agp), and the mature form of rapeseed chloroplast fructose-1,6-bisphosphatase (CFBPase), respectively [13,14]. Supported nitrocellulose membranes (pore size 0.2 μm, Bio-Rad, Richmond, CA, USA) and Whatman 3MM filter papers were used in all screenings.

Screening of phosphatase activity in bacterial colonies

All procedures were carried out at 37°C. _E. coli_ cells were appropriately diluted and grown directly over a nitrocellulose membrane that lay on Luria–Bertani (LB) agar supplemented with 100 μg/ml ampicillin (Scheme 1). When the diameter of colonies was 1 mm (~24 h), the expression of CFBPase in the pET vector was induced by spreading 0.2 ml of 40 mM isopropyl-β-d-thiogalactopyranoside (IPTG) onto the agar surface, whereas the expression of phoA and agp in pBAD vectors was induced with 0.2 ml of 10% (w/v) L-arabinose. After 3 h, the nitrocellulose membrane was withdrawn from the Petri dish and placed with bacteria facing up for 10 min on a Whatman filter paper soaked in water. After this washing step was repeated twice, the nitrocellulose membrane was similarly placed for 5–10 min on another Whatman filter paper previously soaked in the corresponding solution for catalysis. The catalytic solution employed to detect the CFBPase activity was 50 mM Tris–HCl buffer (pH 7.8), 10 mM MgCl₂, and 3 mM fructose 1,6-bisphosphate (FBP). Alternatively, the assay of other phosphatases was performed in a 50 mM Tris–HCl buffer containing 3 mM of the phosphorylated substrate. The nitrocellulose membrane was withdrawn and stored for further studies. The filter paper was first dipped gently for 2 s into the reagent of Chen and coworkers (1.2 N sulfuric acid, 0.5% (w/v) ammonium molybdate, and 2% (w/v) ascobic acid) for the assay of Pi [15] and subsequently placed onto an absorbent paper to remove the excess of liquid, and kept for color development.

Construction of the CFBPase library by random mutagenesis

Random mutagenesis of the mature form of CFBPase was performed by error-prone polymerase chain reaction (PCR) [16]. The _CFBPase_ gene (1080 bp) cloned in pET 22b(+) was flanked by an _XbaI_ restriction site 37 bp before the start of the open reading frame and by an _EcoRI_ site after the stop codon. PCR primers, T7 promoter (TAATACGACTCACTATAGGG), and T7 terminator (GCTAGTTATTGCTCAGGG) were synthesized to complement regions 30 bp upstream of the _XbaI_ site and 105 bp downstream of the _EcoRI_ site.
Thus, the random mutagenesis took place in a nucleotide sequence of 1250 bp that contained the full open reading frame of mature CFBPase. To promote appropriate rates of mutagenesis, 40 cycles of amplification (15 s at 94 °C, 15 s at 55 °C, 45 s at 72 °C) were carried out in 50 µl of a solution containing 20 mM Tris–HCl buffer (pH 8.5), 50 mM KCl, 2 mM MgCl₂, 0.1 mM MnCl₂, 0.1 mM dNTP, 0.2 µM of the above-mentioned primers, and 2 U of Taq DNA polymerase (Gibco, Gaithersburg, MD, USA). The PCR products were precipitated by adding 50 µl of 13% (w/v) PEG8000 and 1.6 M NaCl, treated with XbaI and EcoRI restriction enzymes (New England BioLabs, Beverly, MA, USA), purified by electrophoresis on 1% agarose gel using the Wizard DNA Clean-up System for the extraction (Promega) and ligated with T4 DNA ligase (Gibco) to the pET 22b(+) vector previously linearized with XbaI and EcoRI and treated with calf intestinal alkaline phosphatase (New England BioLabs). The plasmid library was used for the transformation of E. coli JM109(DE3) by electroporation.

Analysis of selected clones

Protein expression. Selected bacteria were grown to mid-log phase in 40 ml LB medium and subsequently induced with IPTG for 3 h at 37 °C. Cell cultures were successively centrifuged; resuspended in 0.6 ml of 100 mM Na acetate (pH 5.2), 300 mM NaCl, and 20 mM EDTA; disrupted by sonication; and centrifuged again. The supernatant and pellet resuspended in 0.6 ml of 1% (w/v) sodium dodecyl sulfate (SDS) were considered as the soluble and insoluble fractions, respectively.

Western blot. The same volume of soluble and insoluble fractions was loaded onto an SDS–polyacrylamide gel. After electrophoresis, the amount of CFBPase protein was estimated by Western blot [17] using polyclonal antibodies raised in rabbits against the recombinant rapeseed CFBPase [14].

In vitro assay of CFBPase activity. The one-stage assay for measuring CFBPase activity was adopted with minor modifications [14]. Briefly, triplicate samples of soluble proteins from transformed bacteria (typically 3 µl) were incubated at 25 °C in microtiter plates containing a solution of 50 mM Tris–HCl buffer (pH 7.8), 1 mM FBP, and 10 mM MgCl₂. The total reaction volume was 0.13 ml/plate well. After 30 s, the released Pi was determined by adding an equal volume of the reagent of Chen and coworkers [15].

Assay of proteins. The concentration of proteins was determined by the method of Lowry and coworkers using bovine serum albumin as the reference standard [18].

Results

Screening of CFBPase activity

CFBPase, an important enzyme of the Benson–Calvin cycle for photosynthetic CO₂ assimilation, exhibits...
a high specificity for the phosphorylated substrate [19]. Therefore, current-screening methods are not effective with this enzyme. To validate the procedure described in Materials and methods, we analyzed the behavior of the rapeseed CFBPase using FBP as substrate for catalysis (Fig. 1). After induction of the expression with IPTG, colonies harboring the enzyme yielded blue spots on the filter paper, whereas bacteria hosting the empty vector did not produce signal. Parallel experiments showed that the induction with IPTG was not strictly necessary because the leaky expression of the pET plasmid was sufficient for the detection of the phosphatase activity.

We next determined whether the sensitivity of the signal was convenient for screening libraries containing different forms of inactive and active phosphatases. Thus, we introduced mutations in the coding region of the mature form of CFBPase via error-prone PCR. Bacteria transformed with the DNA library of mutated CFBPase were plated on the nitrocellulose membrane and screened using FBP as substrate (Fig. 2). The colorimetric data showed that numerous bacteria expressed functional CFBPase, whereas others did not. At this stage, we explored whether the observed activity was linked to alterations of gene expression. Therefore, we first selected colonies that appeared inactive (colonies 1–6) and active (colonies 7–11) and subsequently assessed the presence of (i) mutations by sequencing the DNA, (ii) the respective protein by Western blot, and (iii) the CFBPase activity by assaying the in vitro catalytic capacity of whole-cell protein preparations (Table 1). The absence of CFBPase activity in colonies 1–6 correlated with mutations that caused low expression (colonies 1 and 2) or null expression (colonies 4 and 6) of the respective protein as well as the formation of insoluble proteins on high expression (colonies 3 and 5). These variants were nearly devoid of phosphatase activity when assayed in vitro, confirming the result of the screening. On the other hand, four colonies (colonies 8–11) harbored plasmids coding for the wild-type CFBPase that, as expected, yielded blue spots in the screening. Notably, colony 7 contained an active CFBPase that bore five mutations in different parts of the primary structure. Although further research will be necessary to establish whether other kinetic features of the latter mutant differ from the wild-type counterpart, these findings evinced the advantage of this approach for the

Fig. 2. Active/Inactive mutants in a library of CFBPase. The superimposed image shows E. coli cells expressing a CFBPase library prepared by random mutagenesis and the filter paper used for catalysis, as described in Fig. 1. Indicated colonies are analyzed in Table 1.

Fig. 3. Differentiation between CFBPase and phoA activity. Bacteria harboring the CFBPase gene, the phoA gene, and the empty vector pET22b(+) were mixed and plated onto a nitrocellulose membrane. The hydrolysis of FBP was assayed as described in Fig. 1. Then the nitrocellulose membrane was placed for 10 min onto a second filter paper soaked in water. This washing step was repeated three times prior to PNPP hydrolysis with a solution containing 100 mM Tris–HCl buffer (pH 8) and 3 mM PNPP. (A) Overlay of FBP hydrolysis (blue spots) and PNPP hydrolysis (yellow spots). Yellow spots of the second filter paper were obtained after color replacement of the original blue spots using Corel Photo-Paint 10. (B) Restriction analysis of plasmids from selected colonies. Plasmids, isolated from selected colonies as indicated, were digested with EcoRI and XbaI and reaction products electrophoresed on 1% agarose gel.
selection of catalytically (in)active mutants with altered structures.

**Evaluation of the screening method with phoA and PNPP**

The successful detection of Pi separated from the catalytic hydrolysis of FBP made plausible the use of the screening for other phosphatases and substrates. To evaluate this possibility, we grew *E. coli* cells hosting phoA in the pBAD vector, whose distinguishing feature is the ability to control the level of protein expression [13]. Subsequently, we performed the screening using a catalytic solution containing 100 mM Tris–HCl buffer (pH 8) and 3 mM PNPP. Finally, we estimated the production of Pi by the reagent of Chen and coworkers [15] in marked contrast to that in most studies that rely on the release of the yellow *p*-nitrophenolate from the colorless PNPP. The sequential appearance of two experimental features gave clear indication of the movement of substrates and reaction products. First, bacteria expressing phoA stained yellow during the course of the reaction. Second, these colonies produced blue spots on the filter paper. At variance, bacteria hosting empty vectors were totally ineffective with the nonphysiological substrate. Hence, this analysis not only validated the method for the detection of phoA activity with PNPP as substrate but also illustrated visually that catalysis took place in bacteria on the nitrocellulose membrane and that, subsequently, reaction products diffused through the membrane to the filter paper.

**Analysis of different phosphatases under various experimental conditions**

Although the experiments described above were performed with CFBPase and phoA using FBP and PNPP as substrates, we foresaw that the screening procedure may be adapted to other phosphatases and phosphorylated substrates. However, under some experimental conditions, the presence of endogenous phosphatases diminished the sensitivity toward the detection of the cloned counterpart (cf. null in Table 2). For example, the determination of phoA was feasible with FBP or PNPP, more difficult with fructose 1-P and fructose 6-P, and impractical with glucose 1-P (Table 2). These results indicated that *E. coli* strains devoid of interfering phosphatases should be used as hosts when particular substrates elicit a low signal-to-background response. In

<table>
<thead>
<tr>
<th>Colony</th>
<th>Mutations</th>
<th>Expression</th>
<th>Activity&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>D81N, F199L, E218G, S267T, R292L</td>
<td>Low</td>
<td>1.70</td>
</tr>
<tr>
<td>2</td>
<td>T43A, F93Y, C157W, L283S, P291R, K352E</td>
<td>Low, insoluble</td>
<td>1.07</td>
</tr>
<tr>
<td>3</td>
<td>G345R</td>
<td>High, insoluble</td>
<td>0.30</td>
</tr>
<tr>
<td>4</td>
<td>C96STOP, S152G</td>
<td>None</td>
<td>0.67</td>
</tr>
<tr>
<td>5</td>
<td>T7S, I38L, M46T, Q82P, E111G, F148Y, I150T, S161Y, L303H, E335D</td>
<td>High, insoluble</td>
<td>0.93</td>
</tr>
<tr>
<td>6</td>
<td>C96G, Y125H, Y216N, K257E, C307S</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>D81E, A140G, E156A, T164I, D330G</td>
<td>High, soluble</td>
<td>11.23</td>
</tr>
<tr>
<td>8</td>
<td>None</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>9</td>
<td>None</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>10</td>
<td>None</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>11</td>
<td>None</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>WT&lt;sup&gt;b&lt;/sup&gt;</td>
<td>None</td>
<td>High, soluble</td>
<td>11.44</td>
</tr>
</tbody>
</table>

Note. The CFBPase of colonies indicated in Fig. 2 was analyzed for mutations, expression level, and catalytic activity as described under Materials and methods. n.d., not determined.

<sup>a</sup> Specific activity: μmol Pi released (min mg protein)<sup>−1</sup>

<sup>b</sup> Wild-type CFBPase used as control.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Buffer</th>
<th>Null</th>
<th>Agp</th>
<th>phoA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNPP</td>
<td>Tris–HCl, pH 8</td>
<td>–</td>
<td>n.d.</td>
<td>++</td>
</tr>
<tr>
<td>FBP</td>
<td>Tris–HCl, pH 8</td>
<td>–</td>
<td>n.d.</td>
<td>++</td>
</tr>
<tr>
<td>Fructose 1-P</td>
<td>Tris–HCl, pH 8</td>
<td>++</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Fructose 6-P</td>
<td>Tris–HCl, pH 8</td>
<td>+</td>
<td>n.d.</td>
<td>++</td>
</tr>
<tr>
<td>Glucose 1-P</td>
<td>Tris–HCl, pH 8</td>
<td>++</td>
<td>n.d.</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Na acetate, pH 4.6</td>
<td>+</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Note. The color intensity of blue spots on the Whatman paper was estimated for *E. coli* JM109(DE3) harboring agp, phoA, and the empty plasmid pET 22b(+) (null). The phoA and agp were assayed as described under Materials and methods using 100 mM Tris–HCl buffer (pH 8) and 50 mM sodium acetate buffer (pH 4.6), respectively. –, no intensity; +, low intensity; ++, high intensity; n.d., not determined.
spite of these limitations, we found that the overall efficacy of the approach was high under many experimental conditions. The activity of agp, a phosphatase that is active at acid pH but inactive at alkaline pH, was successfully monitored using either fructose 6-P or glucose 1-P as substrate. In summary, the overview of Table 2 indicates that it is possible to accommodate a range of cloned phosphatases, phosphorylated substrates, and experimental conditions.

Taken together, these results suggest that the procedure could be an important tool for studies on the directed evolution of enzymes in which variants might be selected for specific features of enzyme catalysis. Therefore, we finally evaluated whether the same bacterial colonies could be successively tested against different catalytic conditions. To this end, we mixed bacteria harboring the empty vector pET 22b(+), the CFBPase gene, and the phoA gene, and we assayed the catalytic activity initially with FBP and, after washing the membrane, with PNPP. Given that FBP can be hydrolyzed by both CFBPase and phoA, the second filter paper displayed a subset of the spots presented in the first one. To visualize all data in a single picture, we used image-processing software to transform the blue color of the second filter paper to yellow. The overlay of colorimetric data showed that bacterial colonies holding the empty vector, the CFBPase gene, and the phoA gene yielded no color, blue spots, and green (blue + yellow) spots, respectively (Fig. 3A). In line with these data, the plasmids isolated from selected clones coded for the expected enzyme (Fig. 3B). In other experiments, we performed the catalytic step more than twice, showing that several experimental conditions can be tested on the same bacterial colonies.

Discussion

The release of Pi is the common feature of all phosphatase reactions. Since 1925 [20], the most sensitive assays for the estimation of Pi have been based on the blue-colored compound that appears on the reduction of the complex originated from Pi and acidified ammonium molybdate [21,22]. Although these procedures are adequate for the detection of phosphatases in zymograms, the strong acid solution required for color development and unspecific reactions with cellular components preclude their use with living organisms. To circumvent this restriction, we developed a screening method for phosphatases that separates in space and time the detection of Pi from its formation in bacteria. While bacteria are retained on the surface of the nitrocellulose membrane, small solutes flow freely from the filter paper, catalysis takes place in the bacterial colony, and reaction products diffuse back to the filter paper, where the assay of Pi is finally performed. We validated this experimental design using not only physiological and nonphysiological substrates for the detection of CFBPase, phoA, and agp activities but also the same bacterial colonies for many catalytic assays.

The procedure is sensitive enough to detect endogenous phosphate activity of bacteria under some experimental conditions. Although this feature appears to be useful for searching bacterial phosphatases, it can be a drawback when studying cloned enzymes. In those cases, E. coli strains with diminished phosphatase activity at the pH of interest should be used. In addition, plasmids of a high number of copies could be employed to reduce the time of catalysis and the background signal generated by chromosomal phosphatases. In most experimental conditions, we were able to differentiate the cloned phosphatase activity from the background activity (Table 2).

Within modern enzymology, considerable efforts have been applied to analyze libraries of enzymes with HTS that are well suited to their ease of detection [23]. Quite often, however, these procedures rely on nonphysiological substrates holding a colored or fluorescent moiety that generally impairs the intrinsic activity [24]. In these studies, our HTS could be adapted to other catalytic reactions provided that an appropriate detection of reaction products is available. The current experimental design has two additional advantages: the ability to screen with specific substrates and the possibility of testing different experimental conditions using the same bacterial lawn. These features not only broaden the range of enzymes to be studied but also facilitate further selections based on the action that different agents have on the enzymatic reaction.

Acknowledgments

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References


