Construction of Glycine Oxidase Mutant Libraries by Random Mutagenesis, Site Directed Mutagenesis and DNA Shuffling

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[Abstract] Glyphosate, a broad spectrum herbicide widely used in agriculture all over the world, inhibits 5-enolpyruvylshikimate-3-phosphate synthase in the shikimate pathway, and glycine oxidase (GO) has been reported to be able to catalyze the oxidative deamination of various amines and cleave the C-N bond in glyphosate (Pedotti et al., 2009). Here, in an effort to improve the catalytic activity of the glycine oxidase that was cloned from a glyphosate-degrading marine strain of Bacillus cereus (BceGO), we used a bacteriophage T7 lysis-based method for high-throughput screening of oxidase activity and engineered the gene encoding BceGO by directed evolution.

Materials and Reagents

1. Bacillus cereus HYC-7
2. Escherichia coli (E.coli) DH5α strain, bacteriophage T7
3. Glyphosate (Sigma-Aldrich, catalog number: PS1051)
4. Tryptone (Difco)
5. Yeast extract (Difco)
6. Ampicillin
7. o-Dianisidine dihydrochloride (Sigma-Aldrich, catalog number: D3252)
8. Horseradish peroxidase (Sigma-Aldrich, catalog number: P6782)
9. Protein expression vector of pGEX-6P-1 (the plasmid full length 4,984 bp) (GE Healthcare, catalog number: 28-9546-48; Genbank accession number: U78872.1)
10. Recombinant plasmid pGEX-GO contains encoding gene of glycine oxidase from Bacillus cereus HYC-7
   The nucleotide sequence (1,110 bp) was submitted to the NCBI Genbank and gained the accession number (KC203486.1).
11. Taq DNA polymerase (Takara, catalog number: R500A)
12. DpnI restriction enzyme (Takara, catalog number: 1235A)
13. dATP, dTTP, dCTP, dGTP (Takara, catalog numbers: 4026Q, 4029Q, 4028Q, 4027Q)
14. TransStart® FastPfu DNA polymerase (TransGen Biotech, catalog number: AP221-01)
15. High Pure dNTPs (TransGen Biotech, catalog number: AD101-01)
16. Luria-Bertani medium (see Recipes)

Equipment

1. 96 deep-well plates (Axygen, catalog number: P-DW-20-C-S)
2. Gel purification column (Axygen)
3. Thermo Multiskan spectrum plate reader (Thermo Scientific, catalog number: 51118600)
4. Thermal cyclers (Bio-Rad Laboratories, catalog number: 186-1096)
5. Ultrasonic processor (Sigma-Aldrich, catalog number: Z412619-1EA)

Procedure

A. Random mutagenesis

1. Prepare the amplification mixture (100 µl) as follows:
   - 10 µl of 10x Taq buffer (Mg²⁺ plus)
   - 5 µl of 10 mM Mn²⁺
   - 2 µl of 10 mM dGTP and dCTP
   - 1 µl of 10 mM dATP and dTTP
   - 2 µl of 100 nM oligonucleotide primer F
   - 2 µl of 100 nM oligonucleotide primer R
   - 1 µl of recombinant plasmid pGEX-GO as template
   - 2 µl of Taq DNA polymerase
   - Add ddH₂O to a final volume of 100 µl

2. The error-prone PCR procedure was performed using the following parameters:

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3. Check product by electrophoresis of 5 µl of error-prone PCR product on 1% agarose gel.
4. Error-prone PCR products were purified, digested with BamHI and XhoI, cloned into pGEX-6P-1, and transformed into E.coli DH5α to construct the random mutant library.
B. Site directed mutagenesis

1. Prepare the amplification mixture (50 µl) as follows:
   - 10 µl of 10x FastPfu buffer (Mg²⁺ plus)
   - 1 µl of 10 mM high pure dNTPs
   - 1 µl of 100 nM oligonucleotide primer F
   - 1 µl of 100 nM oligonucleotide primer R
   - 1 µl of dsDNA template
   - 1 µl of FastPfu DNA polymerase
   Add ddH₂O to a final volume of 50 µl
2. The site directed mutagenesis PCR procedure was performed as the following parameters:

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<td>72 °C</td>
<td>1 min/kb of plasmid length</td>
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<tr>
<td>3</td>
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3. Check product by electrophoresis of 5 µl of the site directed mutagenesis PCR product on 1% agarose gel.
4. The site directed mutagenesis PCR products were purified, digested with DpnI, and
transformed into *E. coli* DH5α.

**Figure 2** Agarose gel electrophoresis analysis of cycled PCR product and mutated plasmid. A. PCR product. Lane 1: Wide Range DNA Marker (500~12,000 bp); Lane 2: Cycled PCR product. B. Mutated plasmid. Lane 1: The empty vector pGEX-6P-1; Lane 2: Mutated plasmid.

C. DNA shuffling

1. Obtaining DNA fragments for shuffling.
   a. Prepare the parental genes by PCR amplification (100 µl) as follows:
      1. 10 µl of 10x *Taq* buffer (Mg²⁺ plus)
      2. 2 µl of 10 mM High Pure dNTPs
      3. 2 µl of 100 nM oligonucleotide nested primer F1
      4. 2 µl of 100 nM oligonucleotide nested primer R1
      5. 1 µl of beneficial mutatant as template
      6. 2 µl of *Taq* DNA polymerase
         Add ddH₂O to a final volume of 100 µl
   b. The DNA shuffling PCR procedure was performed as the following parameters:

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c. Check product by electrophoresis of 5 μl of product on 1% agarose gel.

d. Mix ~1 μg of each purified PCR products.

e. Prepared for DNA fragmentation by ultrasonic treatment at 0 °C for 40 min to generate a pool of fragments, then the DNA fragments between 100~200 bp were purified using gel purification column.

f. Check the DNA fragments by electrophoresis of 10 μl of product on 3% agarose gel.

![Figure 3. Schematic of parental genes with the nested primers in the process of DNA shuffling](image)

2. Reassembled by primerless PCR.
   a. Prepare the amplification mixture (50 μl) as follows:
      10 μl of 10x Taq buffer (Mg²⁺ plus)
      2 μl of 10 mM high pure dNTPs
      42 μl of purified fragment DNA
      1 μl of Taq DNA polymerase
      Add ddH₂O to a final volume of 50 μl

   b. The primerless PCR procedure was performed as the following parameters:

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<td>40 °C</td>
<td>30 sec</td>
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<td></td>
<td></td>
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<td>20 sec + 1 sec per cycle</td>
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<tr>
<td>3</td>
<td>1</td>
<td>72 °C</td>
<td>7 min</td>
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c. Check product by electrophoresis of 5 μl of PCR product on 1% agarose gel. A smear of reassembled product that extends above the molecular weight of the parent gene should be visible.

3. Amplification of full-length sequences.
   a. Prepare the amplification mixture (50 μl) as follows:
      10 μl of 10x Taq buffer (Mg²⁺ plus)
2 µl of 10 mM high pure dNTPs  
1 µl of 100 nM oligonucleotide nested primer F2  
1 µl of 100 nM oligonucleotide nested primer R2  
5 µl of unpurified reassembly reaction mixture as template  
1 µl of Taq DNA polymerase  
Add ddH2O to a final volume of 50 µl  
b. The PCR procedure was performed as the following parameters:

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c. Check product by electrophoresis of 5 µl of product on 1% agarose gel.  
d. PCR products were purified, digested with BamHI and XhoI, cloned into pGEX-6P-1, and transformed into E.coli DH5α to create the DNA shuffling library.

Figure 4. The schematic of glyphosate oxidase gene by DNA shuffling.  
A. Amplification of six variants GO. Lane 1: Wide Range DNA Marker (500~12,000 bp); Lanes 2-7: DNA fragments encoding variants GO. B. DNA fragmentation. Lanes 1-8: DNA fragments were treated by ultrasonic at 0 °C with a time gradient of 5 min from 0 to 35 min; Lane 9: 20 bp DNA Ladder Marker (20~500 bp). C. Purification of DNA fragments. Lane 1: 20 bp DNA Ladder Marker (20~500 bp); Lane 2: DNA fragments of 100~200 bp were purified from an agarose gel. D. Fragments were reassembled without primers. Lane 1: Wide Range DNA Marker (500~12,000 bp); Lane 2: DNA fragments were reassembled into a full-length gene by 60 cycles without primers. E. The reassembled full-length products were amplified by the standard PCR. Lane 1: Wide Range DNA Marker (500~12,000 bp); Lane 2: PCR product with primers.
D. Screening

1. The resulting library of BceGO mutants were expressed into 96 deep-well plates (containing 0.6 ml Luria-Bertani medium) and transferred onto Luria-Bertani agar plates as corresponding copies, followed by an overnight growth (37 °C, 300 rpm).

2. When the cultures grew to saturation, both IPTG (at a final concentration of 0.1 mM) and the bacteriophage T7 (above 100 particles per cell) were added into 96 deep-well plates to synchronize the induction of recombinant mutants with the release of the lysis of the host *E.coli* DH5α at 37 °C with shaking for 6 h.

3. The enzyme-coupled colorimetric assay (200 µl) was performed as follows:
   - 159 µl of lysis cell extracts
   - 20 µl of 50 mM glyphosate (at a decreasing substrate concentration gradient in sequential rounds of screening system)
   - 20 µl of 0.32 mg/ml o-dianisidine dihydrochloride
   - 1 µl of 5 unit/ml horseradish peroxidase
   - Then incubated at 25 °C for 8 h

4. The absorbance change at 450 nm for each well in the microtiter plates was measured and compared with the control (harboring wild-type BceGO or containing the empty vector pGEX-6P-1). Mutants that outperformed the wild-type were chosen for further activity analysis (Figure 1).

![Figure 5. The screening process of glycine oxidase mutant library](http://www.bio-protocol.org/e1252)
Table 1. The apparent kinetic parameters on glycine and glyphosate measured for wild-type BceGO and variants obtained by random mutagenesis, site saturation mutagenesis and DNA shuffling

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<thead>
<tr>
<th></th>
<th>Glycine</th>
<th>Glyphosate</th>
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<tr>
<td></td>
<td>$k_{cat,app}$ (min$^{-1}$)</td>
<td>$K_{m,app}$ (mM)</td>
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<tr>
<td>Wild-type</td>
<td>8.17 ± 0.31</td>
<td>1.04 ± 0.17</td>
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<tr>
<td>22D11</td>
<td>1.16 ± 0.05</td>
<td>54.6 ± 3.47</td>
</tr>
<tr>
<td>23B1</td>
<td>4.56 ± 0.38</td>
<td>0.99 ± 0.04</td>
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<tr>
<td>B1R</td>
<td>0.44 ± 0.03</td>
<td>58.5 ± 5.26</td>
</tr>
<tr>
<td>B2R11</td>
<td>1.86 ± 0.09</td>
<td>105.6 ± 7.31</td>
</tr>
<tr>
<td>B2R14</td>
<td>1.35 ± 0.12</td>
<td>92.5 ± 7.43</td>
</tr>
<tr>
<td>B2R23</td>
<td>13.02 ± 0.96</td>
<td>101.8 ± 8.29</td>
</tr>
<tr>
<td>B2R81</td>
<td>5.41 ± 0.83</td>
<td>134.4 ± 10.33</td>
</tr>
<tr>
<td>B3S1</td>
<td>5.43 ± 0.79</td>
<td>41.55 ± 3.32</td>
</tr>
<tr>
<td>B3S4</td>
<td>5.68 ± 0.64</td>
<td>80.43 ± 5.01</td>
</tr>
<tr>
<td>B3S6</td>
<td>10.14 ± 1.32</td>
<td>138.1 ± 12.16</td>
</tr>
<tr>
<td>B3S7</td>
<td>2.30 ± 0.31</td>
<td>41.64 ± 2.10</td>
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Especially, B3S1 demonstrated a 160-fold increase in substrate affinity for glyphosate, a 326-fold increase in catalytic efficiency towards glyphosate and a significant enhancement in the specificity constant over the wild-type BceGO, achieving the goal of efficient oxidation of glyphosate by evolution of glycine oxidase.

Notes

1. The error-rate is controlled by varying the amount of MnCl$_2$ and unbalanced dNTPs added to the error-prone PCR reaction.
2. Design nested primer to amplify the parental genes for the DNA fragmentation and
full-length sequences from reassembly products.

**Recipes**

1. Luria-Bertani medium
   - 10 g/L tryptone
   - 5 g/L yeast extract
   - 10 g/L NaCl
   - Ampicillin (50 mg/L) was added as needed.

**Acknowledgments**

We thank Drs. Ziduo Liu and Dexin Kong for valuable discussions about this article.

**References**