SHORT REPORT

A method for high-throughput screening hydrolase of lignin β-aryl ether linkage from directed evolution by glutathione (GSH) assay

Jiaxi Wang1,2, Kun Yan1, Wenya Wang1,2* and Yan Zhou1

Abstract

Background: Lignin's valorization plays a critical role in refining the bioresource. Considering that the β-aryl ether linkage (β-O-4 bond) accounts for 50–70% of lignin chemical linkage between aromatic rings, the hydrolase of lignin β-aryl ether linkage, especially the β-etherase, provided a promising way for the lignin depolymerization and valorization. As a result, it is essential to develop the effective high-throughput methods for screening the mutant library of β-etherase from directed evolution.

Results: Based on the enzymatic mechanism of β-O-4 bond's cleavage by β-etherase, the LigF was selected as the model to study high-throughput method by GSH assay for screening the mutant library of β-etherase from directed evolution. After the primary study with purified LigF and cell lysate, the GSH assay was used to screen mutant library of β-etherase. The study on screening the mutant library with about 600 colonies indicated that the selected transformants all have one or two mutated sites in the gene sequence of LigF, and the activities from GSH assay of most selected transformants were the same as their activities from HPLC assay.

Conclusions: The results from the high-throughput screening of mutant library demonstrated that GSH assay could be applied to screen β-etherase mutant from directed evolution.

Keywords: Lignin valorization, β-etherase, Directed evolution, High-throughput screening

Introduction

Lignin, the second most abundant terrestrial renewable polymer in nature, consists of highly branched three-dimensional poly-phenolic structure that includes three phenylpropane units, p-coumaryl, coniferyl and sinapyl (An et al. 2017; Ragauskas et al. 2014). The annual yield of lignin was more than 50 million tons, and most of them (> 95%) was burned as an energy resource (Gharehkhani et al. 2018). Concerns about the sustainable availability of fossil fuel resources and related environmental problems, and the demands for a green sustainable chemical industry have drawn attention to the valorization of lignin for producing value-added chemicals, especially aromatic compounds (Ragauskas et al. 2014; Linger et al. 2014). Consequently, it plays a critical role for lignin valorization by depolymerizing lignin into aromatics of low-molecular weight. During the past two decades, the depolymerization of lignin has resulted in increasing studies in lignin chemistry (Ragauskas et al. 2014; Rahimi et al. 2014; Shuai et al. 2016). The methods of lignin depolymerization could simply be classified into physical, chemical or biological one, in which the biological way was characterized with efficient, environmentally friendly and specific properties (Wang et al. 2017). Considering that the β-aryl ether linkage (β-O-4 bond) accounts for 50–70% of lignin...
chemical linkage between aromatic rings (Masai et al. 2007), the brokenness of β-O-4 linkage attracts studies of lignin depolymerization over the past two decades. The hydrolase of lignin β-aryl ether linkage is the kind of non-radical-dependent enzyme that could specifically cleave the β-O-4 linkage in lignin model compounds, considering to be the promising biocatalyst in lignin valorization (Picart et al. 2015). The first-reported hydrolases of lignin β-aryl ether linkage were discovered in S. paucimobilis SYK-6 (Masai et al. 1991), whose system includes three enzymes: dehydrogenase (LigD), etherase (LigF) and glutathione-lyase (LigG). The LigDFG system includes three enzymatic reactions: (1) LigD oxidizes the hydroxyl group of Cα in the substrate to form the ketone group in the presence of NAD⁺; (2) LigF breaks the ether bonds by attacking the Cβ with reduced glutathione; (3) LigG, a glutathione transferase, releases an oxidized dimer glutathione and an aromatic monomer from the substrate with the aid of another reduced glutathione (Fig. 1) (Masai et al. 1991; Wang et al. 2017). Among the system described above, etherase (LigF) plays a critical role since it contributes to the brokenness of β-O-4 linkage. During the past several years, the homologous genes of LigDFG system have been explored extensively (Wang et al. 2017; Kamimura et al. 2017). Furthermore, the discovered enzymes also have been applied to degrading the natural lignin, which produced certain number of lignin-derived aromatics (Reiter et al. 2013; Ohta et al. 2017; Picart et al. 2017; Gall et al. 2018). These results facilitate lignin valorization by the way of enzymatic catalysis or metabolic engineering (Wang et al. 2017). However, the hydrolyzing efficiency of β-etherase systems toward natural lignin or lignin-like polymers was far lower than toward lignin model dimers. This partially resulted from the problems of enzymatic enantioselectivity and the structural complexity of lignin (Wang et al. 2016). As a result, it is essential to extensively mine the LigDFG homologous members or screen the effective mutants from the existed LigDFG enzymes by the directed evolution method (Arnold and Volkov 1999), and then the resultant enzymes from gene-mining or selected mutants were subjected to structural analysis for the further structural design and mutant screening, which aimed to screen the effective mutants.

In the case of directed evolution of an enzyme, it is essential to develop an efficient high-throughput screening method for getting target strains from a large number of mutants. Weinstein (1979) used the synthesized lignin model substrates to analyze the activity of etherases, in which the guaiacyl was connected with a fluorescent group by β-O-4 linkage. From then on, the lignin model compounds or fluorescently labeled lignin model compound was used to screen β-etherases for lignin degradation. It should be notable that if the lignin models were not labeled by fluorescent group, the β-etherase activity was only analyzed by HPLC, which cannot afford the high-throughput screening for mutants; while the lignin models were labeled by fluorescent group, the structure of fluorescent group was not actually existed in natural lignin. Recently, the nanostructure-initiator mass spectrometry (NIMS) was developed with the aim to screening lignin-modifying enzymes by high-throughput way (Deng et al. 2018); however, the cost consumption should be considered before its application. Here, we describe a high-throughput method for screening the mutant library of β-etherase from directed evolution, with the characteristics of high efficiency, economy and easy operation. In the present study, β-etherase of LigF was selected as the model to explore the high-throughput screening method, and the GSH content analysis was used to determine the activity of β-etherase based on the β-etherase catalyzing mechanism. We studied the feasibility of this method and confirmed that it is a favorable tool for high-throughput screening of β-etherase from directed evolution.

![Fig. 1 Reaction procedure of the LigDFG enzyme system. GSH, reduced glutathione; GSSG, oxidized dimer glutathione; GVL, guaiacyl-α-veratrylglycerol; GVG, β-guaiacyl-α-veratrylglycerone; GS-VG, β-glutathionyl-α-veratrylglycerol; VG, β-deoxy-α-veratrylglycerone](image-url)
Methods

Bacterial strains, plasmids and chemicals
The bacterial strains and plasmids employed are listed in Table 1. Guaiacyl-α-veratrylglycerol (GVL) was purchased from Shanghai Jianchao Chemical Co., Ltd. All the other chemicals were purchased from Beijing Chemical and Reagent Co., Ltd.

The determination of glutathione (GSH) content
The GSH content was determined according to the previous report with a colorimetric micro-method (Owens and Belcher 1965). 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB) (39.6 mg) and NaHCO₃ (15 mg) were dissolved in 10 mL of 0.01 M phosphate buffer (pH 7.0), and the DTNB reagent was kept at −20 °C in the darkness for the further experiment. The determination of GSH content was carried out in microplates with a total volume of 200 μL, including 50 μL of sample solution, 50 μL of DTNB reagent and 100 μL of 0.01 mol/L phosphate buffer (pH 7.0). And then, the reaction mixture was put at 25 °C for 2 min. The optical density of the reaction mixture was measured at 405 nm using a Thermo scientific microplate absorbance reader. The GSH content was calculated based on the early-prepared standard curve.

The preparation of GVG
The preparation of GVG was according to Wang (Wang et al. 2016) and Sato (Sato et al. 2009). GVG was prepared from GVL by LigD. The reaction system includes 1 mg/mL LigD, 1 mM GVL, 1 mM of NAD⁺, and 25 mM Tris/HCl (pH 7.0), which was carried out at 30 °C for 12 h. The resultant GVG was extracted from the reaction mixture with acidified ethyl acetate and purified by the thin-layer chromatography. The purity of prepared GVG was analyzed with high-performance liquid chromatography (HPLC) system (Shimadzu LC-20AT, Japan) equipped with C₁₈ column (5 μm, 250 × 4.6 mm, Shimadzu, Japan) and UV detector. The mobile phase was a mixture of water (49.5%), acetonitrile (49.5%) and phosphate (1.0%), and the flow rate was 1.0 mL/min.

The induction and purification of LigD and LigF
The protein of LigF and LigD was induced and purified according to Wang et al. (2016). *E. coli* BL21(DE3) containing plasmid with *lig*D or *lig*F gene was cultured in LB medium with ampicillin (100 μg/mL) from OD₆₀₀ 0.1 to OD₆₀₀ 0.6 at 37 °C, then it was induced overnight with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 16 °C. In the case of enzyme purification, the over-expressed bacterial cells were lysed by ultrasonic treatment, and the recombinant protein was purified with a Ni²⁺-NTA column system. The final elution fractions were analyzed by SDS-PAGE to test the protein purity. The protein concentration of each enzyme was determined by BCA method.

Construction of LigF mutant library
The construction of LigF mutant library was according to QuikChange™ site-directed mutant method by error-prone PCR (Kunkel 1985; www.genomics.agilent.com). The plasmid of pET-23a-ligF was used as a template. The forward primer (5′-GGGTCTGAGGCTAGTTGG AAGC-3′) and the reverse primer (5′-CGTTGACT GAGCGTCAGACCC-3′) were used to produce random error in *lig*F gene. The Easy Taq PCR system (TransGen, China) contained 10 ng template plasmid (pET-23a-ligF), 0.1 mM Mn²⁺, 1 × Easy Taq buffer, 0.2 mM dNTPs, 0.2 μM of each primer, and 5 U of Easy Taq DNA polymerase in a total volume of 50 μL, following operating conditions of 94 °C denaturation for 5 min, 35 cycles of 94 °C denaturation for 30 s, 58 °C annealing for 30 s and 72 °C extension for 5 min, final extension for 10 min. The amplified PCR products were then digested with *Dpn* I for 1 h, and then transformed into the strain *E. coli* BL21(DE3).

The assay of LigF activity
The activity of LigF was analyzed according to Wang (Wang et al. 2016) and Masai (Masai et al. 2003). The reaction mixture, if it was not specifically described, was consisted of a certain volume of GVG (20 mM), GSH

Table 1 Strains and plasmids used in this study

| Name         | Description                          | Reference or source       |
|--------------|--------------------------------------|---------------------------|
| Strains      |                                       |                           |
| DH5α         | Plasmids storage strain              | Taihe Biotechnology CO., Ltd. |
| BL21(DE3)    | Protein expression strain            | Taihe Biotechnology CO., Ltd. |
| Plasmids     |                                       |                           |
| pET-23a      | Protein expression vector            | Novegan                   |
| pET-23a-ligD | The expression vector of LigD protein | Wang et al. (2016)         |
| pET-23a-ligF | The expression vector of LigF protein | Wang et al. (2016)         |
(3 mg/mL), cell lysate or LigF and Tris–Cl buffer (50 mM, pH 8.0). The specified total volume of reaction mixture and volume of different components were described in the main text.

**The definition of the activity and specific activity of LigF**
The one unit of LigF activity was described as the amount of LigF protein (mg), which consumes 1 μmol GVG within a certain time. The specific activity of LigF was described as the LigF activity (U) per LigF protein mass (mg).

**High-throughput screening of mutant library**
As described in Additional file 1: Fig. S1, the colonies in mutant library were inoculated in 96-Well Microplate, and the bacterial cells in Microplate were cultured overnight at 37 °C. Then, 100 μL of the cells were inoculated in 96-Well Deep Well Plate with 2 ml of each well, containing 1 mL LB medium with ampicillin (100 μg/mL). The 96-Well Deep Well Plate with 2 ml of each well was set at 230 rpm, 37 °C for 4–6 h until the OD₆₀₀ of cells reached 0.6–0.8. 0.5 mM IPTG was added to each well to induce the protein expression and the 96-Well Deep Well Plate was cultured overnight at 16 °C, 180 rpm. The induced cells were collected by centrifuge for 10 min at 4 °C with 4000 rpm. 10 mM phosphate buffer (pH 7.0) was used to resuspend the pellet, adjusting OD₆₀₀ as 0.6. 200 μL of resuspending cells were transferred to 96-Well Microplate. 50 μL 10 mg/mL lysozyme was added to the resuspending cells and the mixture was cultured for 30 min at 37 °C, 80 rpm. The cell lysate was collected by centrifuge at 4 °C. The mutant activity was analyzed with the reaction mixture, including 60 μL 20 mM GVG, 40 μL 3 mg/mL GSH, 140 μL cell lysate, and 760 μL 50 mM Tris–Cl (pH 8.0). The reaction in 2-ml 96-Well Deep Well Plate was carried out for 30 min at 27 °C, 80 rpm, and then the 96-Well Deep Well Plate was transferred to the boiling water bath for 10 min to kill the enzyme. The 96-Well Deep Well Plate was quickly transferred to the ice for the decrease of temperature. 50 μL of reaction mixture was taken for the GSH content analysis to describe the LigF activity.

![GSH changes with time in the presence of purified LigF](image)

**Fig. 2** GSH changes with time in the presence of purified LigF. In this part, the total volume of reaction mixture was 5 mL, the test group included 100 μL 20 mM GVG, 100 μL 3 mg/mL GSH, 4300 μL 50 mM Tris–Cl (pH 8.0), 500 μL 10 μg/mL LigF. While in the control group, LigF was replaced by 500 μL 50 mM Tris–Cl buffer (pH 8.0). The reaction was carried out at 27 °C and 80 rpm. And then the equal volume of ethyl acetate was added to the reaction mixture to stop the enzymatic reaction. The mixture was centrifuged for 2 min at 13000 rpm and 50 μL water phase was taken for the GSH content analysis to describe the LigF activity.
Results and discussion

The principle of high-throughput screening β-etherase mutants

Figure 1 indicates that the β-O-4 linkage in GVG could be cleaved by β-etherase with the existence of GSH as a cofactor; the GVG was divided into two molecules: a guaiacol and a GSH conjugate, during which the GSH was transformed from the reduced to the oxidized. As a result, we postulated that the variation of reduced GSH could be used to analyze the activity of β-etherases based on the reaction mechanism in Fig. 1, i.e., in the final reaction system the lower GSH content, the higher β-etherase activity. Glutathione (GSH) existed widely in living organisms or cells, participating in various physiological or metabolic activities (Hilf et al. 1976). For the purpose of studying the biological functions of GSH, many methods have been developed to determine the GSH content by analyzing sulphydryl groups in the reduced GSH since 1950s (Hilf et al. 1976). To investigate the feasibility of above hypothesis, a colorimetric micro-method for the determination of glutathione was applied to screening the β-etherase mutants from directed evolution, and LigF was selected as the model enzyme for directed evolution of β-etherase.

The feasible investigation and optimization of β-etherase activity analysis by GSH assay

To investigate the feasibility of β-etherase activity analysis by GSH assay, the purified LigF was first used to catalyze the cleavage of GVG with the aid of reduced GSH (Fig. 1), during which the GSH was transformed from the reduced to the oxidized form. Since the reduce GSH was sensitive to the oxidant agents, there must be a significant difference between the oxidized GSH from LigF-induced reaction and those from the other resources. So, we set up a control group. Figure 2 indicates that in the test group the content of reduced GSH decreased obviously at the initial 10 min, and then the change of reduced GSH became stable, after 30 min there is no significant change to be observed; while in the control group there is no significant change of reduced GSH content to be observed during the whole experiment procedure. Based on the above results, it indicated that the GSH assay could be applied to analyze β-etherase activity.

There existed various resources of sulphydryl group in vivo, such as cysteine, reduced GSH, et al. And, if the directed evolution method wanted to be applied in screening the mutant library, it is essential to use the bacterial lysate containing the target enzyme as the enzyme resource. Consequently, the effect of bacterial cell lysate on GSH content analysis in LigF reaction system was investigated further. Figure 3a indicates that there was no significantly difference of GSH content between No. 2 group (without bacterial cell lysate) and No. 3 group (with the bacterial cell lysate from BL21(DE3)), while the GSH content of No. 1 group (with the bacterial cell lysate from BL21(DE3) containing ligF plasmid) was lower than that of No. 2 group and No. 3 group, indicating that the bacterial cell lysate carrying the ligF could be used as the enzyme resource to screen mutant library from directed evolution. However, the reaction system was still needed to be optimized for effectively screening. After the optimization of the volume of different components in the reaction mixture, including GVG, GSH and cell lysate (Additional files 2, 3, 4, 5, 6: Fig. S2–S5, Table S1), the suitable volume of components in the reaction mixture was determined as GVG (20 mM) 300 μL, GSH (3 mg/mL) 200 μL, cell lysate (protein content 0.4–0.5 mg/mL) 700 μL in 5 mL reaction mixture. Figure 4 indicates that the OD value of the test group has a clear difference from that of the control group after 20 min, i.e., OD value of the former was significantly lower than that of the latter, which indicated the obvious downward
As the result, the GSH content assay was applied to screen the $\beta$-etherase mutant library from directed evolution.

High-throughput screening $\beta$-etherase mutants from direct evolution by GSH assay

After the optimization of the PCR system for the LigF-directed evolution, the resultant PCR products were cleaved by restricted enzymes and ligated into the pET-23a. And then, the ligation products were transformed into the BL21(DE3) to construct the mutant library. The transforming results indicated that only few colonies emerged on the LB plates, possibly due to the lower ligation rate (Additional file 7: Fig. S6). Then, the circular PCR was used to construct the LigF mutant library according to QuikChange™ method, resulting in a lot of transformants (Additional file 7: Fig. S6). The resultant transformants were subjected to high-throughput screening of $\beta$-etherase mutants with GSH assay. About 600 transformants were screened, in which 14 transformants indicated the obvious change of enzymatic activity using GSH assay. To further verify these selected transformants, they were cultured to purify the plasmids for the sequence analysis. The results of sequence analysis trend over time. As the result, the GSH content assay was applied to screen the $\beta$-etherase mutant library from directed evolution.

### Table 2 List of the screened mutants from directed evolution using high-throughput screening method of GSH assay

| Mutants | Mutated site | AA* in the original LigF | AA in the mutant | Activityb |
|---------|--------------|--------------------------|-----------------|-----------|
| B7      | 10           | P                        | L               | −         |
| C1      | 29           | E                        | G               | +         |
| C3      | 23           | E                        | G               | −         |
| E1      | 99           | W                        | R               | −         |
| F1      | 145          | W                        | G               | −         |
| F6      | 125          | Q                        | H               | +         |
| H11     | 109          | W                        | R               | −         |
| AA4     | 87           | P                        | S               | +         |
| AE4     | 123          | I                        | V               | −         |
| AF1     | 181          | K                        | E               | −         |
| AH8     | 83           | P                        | S               | −         |
| 155     | G            | D                        |                |           |
| BE1     | 10           | P                        | R               | −         |
| BG5     | 129          | D                        | G               | +         |
| BG11    | 72           | E                        | G               | −         |

* AA indicates the abbreviation of amino acid. ** +/− indicates that the mutant activity was higher than that of the original LigF; − indicates that the mutant activity was lower than that of the original LigF.
indicated that all these transformants have one or two mutated sites in the gene sequence of LigF (Table 2), which confirmed the feasibility of the application of circular PCR for the mutant library construction, and also demonstrated the effectiveness of directed evolution system for LigF mutation. Then, the 14 proteins of mutant LigF were induced and purified for enzymatic activity assay by HPLC (Fig. 5).

The enzymatic activity of mutants was calculated by the amount of substrate (GVG). The results indicated that the specific activities of AA4 and BG5 were about 1.5-folded of the LigF (control), the specific activities of C1, F6, AE4 and AF1 were about 0.5-fold to 0.75-fold of the control, and the remaining mutants showed no obvious activity. The results of HPLC analysis were similar to those of the GSH assay with the exception that C1 and F6 were selected as positive mutants with increased activity by GSH assay, while they were negative mutants with decreased activity by HPLC analysis (Table 2, Fig. 5). Based on these results, the GSH assay could be developed as a high-throughput method for screening β-etherase mutants of directed evolution.

**Conclusion**

Based on the enzymatic mechanism of β-O-4 bond's cleavage by β-etherase, the LigF was selected as the model to study high-throughput method by GSH assay for screening the mutant library of β-etherase from directed evolution. The results from the high-throughput screening mutant library demonstrated that GSH assay could be applied to screen β-etherase mutant from directed evolution.

**Supplementary information**

Supplementary information accompanies this paper at https://doi.org/10.1186/s40643-020-00317-7.

Additional files 1: Fig. S1. Procedure for high-throughput screening of LigF mutant library from directed evolution.

Additional files 2: Fig. S2. GSH variation curve of different substrate amount.

Additional files 3: Fig. S3. GSH variation curve of different GSH amount.

Additional files 4: Fig. S4. GSH variation curve of different cell lysate.

Additional files 5: Fig. S5. GSH curve of different combination systems.

Additional files 6: Table S1. Reaction system components in Fig. S5.

Additional files 7: Fig. S6. Transforming result of circular PCR.
Abbreviations
LigD: Dehydrogenase; LigF: Etherase; LigG: Glutathione-lyase; GSH: Reduced glutathione; GSSG: Oxidized dimer glutathione; GVL: Guaiacyl-α-veratrylglycerol; GVG: β-guaiacyl-α-veratrylglycerone; GS-VO: β-glutathionyl-α-veratrylglycerol; GSGC: β-deoxy-α-veratrylglycerone; NAD⁺: Nicotinamide adenine dinucleotide; DTNB: 5,5′-dithiobis-(2-nitrobenzoic acid); NaHCO₃: Sodium Bicarbonate; IPTG: Isopropyl β-D-1-thiogalactopyranoside; PCR: Polymerase Chain Reaction; HPLC: High-Performance Liquid Chromatography; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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Authors’ contributions
JW and WW designed the experiments. JW conducted the experiments. WW and Y.Z conceived the research. WW finalized the manuscript with assistance from KY. All authors read and approved the final manuscript.

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Availability of data and materials
They are included within the article and its Additional files: 1, 2, 3, 4, 5, 6, 7.

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Not applicable.

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Competing interests
The authors declare no competing financial interests.

Author details
1 College of Life Science and Technology, Beijing University of Chemical Technology, Beijing 100029, China. 2 Amoy-BUCT Industrial Bio-Technovation Institute, Amoy 361022, China.

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