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Improving the anti-oxidation of glucose oxidase with computer-aided structure optimization

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Abstract

Glucose oxidase (GOD) can be inactivated by hydrogen peroxide (H_2O_2) produced during glucose oxidation; thus, H_2O_2 is a competitive inhibitor of GOD. In this mechanism, methionine (Met), a sulfur-containing amino acid, can be oxidized by H_2O_2 and converted into methionine sulfoxide, thereby inactivating GOD. In this study, to block GOD oxidation, the two-dimensional structures of three mutants were designed with computer-aided analysis, and the complex structures of GOD mutants and their substrate glucose were determined using the CDOCKER algorithm. Three mutant GODs and the wild-type GOD were expressed in *Pichia pastoris*. After purification, the activities and anti-oxidation capacities were evaluated. The activity of GOD was decreased substantially from 3.24 U/μg in the wild-type protein to 0.05 U/μg in the GOD-M523L-M524L mutant; in contrast, the activity of GOD-M524L-M528L was not different from that of the wild-type protein. Because the activity of GOD-M523L-M524L was negligible, we did not evaluate its anti-oxidative effects. However, the mutant GOD-M524L-M528L had better anti-oxidation capacity than the wild-type GOD. These results were consistent with the results of computer-aided analysis, suggesting that this method may be useful for enzyme structure optimization.

Keywords: Glucose oxidase; Methionine; Rational design; Site-directed mutation; Anti-oxidation.

1. Introduction

GOD (EC1.1.3.4) is one of the most interested enzymes in industrial applications, which exists in plants, animals, and microorganisms. Although the expression of GOD is not high in its native state, engineering technologies have improved our capacity for large-scale GOD production [1]. Recently, GOD has become important in the food industry ,GOD and catalase synergistically to remove dissolved oxygen in food, keeping food original flavor and extending shelf life. The final product is glucose lactone, So it has the advantage of stability, non-oxidizing, odor-free. Such as a novel food packaging material which prolongs the shelf life of food was obtained by immobilizing glucose oxidase in PVA/CS/tea extract electrospun nanofibrous membrane [2], and has been shown to have applications in clinical tests [3], as a food additive [4], and as a biological sensor [5,6].GOD can catalyze glucose oxidation, producing gluconic acid and H_2O_2; moreover, GOD can then be inactivated by H_2O_2 produced during this process [7], suggesting that H_2O_2 is a competitive inhibitor of GOD [8], with inhibition constants similar to the apparent Michaelis constants. To improve the stability of GOD, catalase-conjugated liposomes encapsulating glucose oxidase (CLGs) [9] have been prepared for glucose oxidation with controllable enzyme activities using a novel liposomal catalyst [10]. This facilitated prolonged oxidation of glucose with simultaneous decomposition of H_2O_2, indicating that enzyme inhibition occurred because the H_2O_2 produced during oxidation was effectively suppressed in the liposomal system. However, the mechanisms mediating this phenomenon are unclear. Other protein denaturant such as guanidine hydrochloride, Urea, SDS can also influence the activity of GOD, high concentration of protein denaturant can inhibit the activity of GOD. EDTA and some metal ion such as Zn2+、Fe2+、Mg2+ can activate the GOD activity [11].

Methionine (Met) is one of the α-amino acid, which contains an an α-amino group, an α-carboxylic acid group, and an S-methyl thioether side chain, Met is a non-polar, aliphatic amino acid, it has an Asymmetric carbon atoms, it exhibited optical rotation property and has a couple of antimers. Met is a sulfur-containing amino acid that can be oxidized by H_2O_2 and converted into methionine sulfoxide in an irreversible process [12,13]. Thus, oxidization of Met in GOD may alter the three-dimensional structure of GOD, leading to the inactivation of GOD. Accordingly, in this study, we aimed to improve the oxidation of GOD by designing mutants in which leucine (Leu) was replaced with Met using homology modeling with the CDOCKER algorithm.

2. Materials and methods

2.1 Strains, vectors, enzymes, culture media, and chemicals

Escherichia coli DH5α, used as the recipient strain for recombinant plasmids, was grown in LB medium (1% tryptone, 0.5%

yeast extract, 1% NaCl, pH 7.0) at 37°C. *Pichia pastoris* GS115 and pPIC9k were used as the fungal host and expression vector, respectively. The sequence of the *GOD* gene was obtained from GenBank (accession number X16061.1). The pPic9k plasmid carrying GOD and the pPIC9k plasmid carrying GOD-8 were synthesized by Invitrogen (Carlsbad, CA, USA). *Pfu* polymerase, used for polymerase chain reaction (PCR), T4 DNA ligase, a mixture of dNTPs, and all restriction enzymes were purchased from TaKaRa Biotechnology (Dalian, China). All the other chemicals used in this experiment were purchased from China National Medicines Corporation Ltd.

2.2 Homology modeling and docking

The X-ray structure coordinates of *Aspergillus niger* GOD (PDB entry 1CF3), refined to 1.8- and 1.9-Å resolution by Wohlfahrt [14], was used as a template to generate homology models of GOD-8, GOD-M523L-M524L, and GOD-M524L-M528L. Docking simulations with GOD or the three mutants and β-D-glucose were carried out with the CDOCKER algorithm of Discovery Studio 3.5 [15,16], applying the CHARMm force field. The active sites of the enzyme were chosen as R512, N514, H516, and H559, and the radius of the active pocket was 20 Å.

2.3 Site-directed mutagenesis

The GOD-M523L-M524L and GOD-M523L-M524L genes were amplified by splicing overlap extension (SOE)-PCR [17-19] using pPIC9K-GOD as a template. Primers for GOD-M523L-M524L (**Table 1**) and GOD-M524L-M528L (**Table 2**) were synthesized by Invitrogen. PCR products encoding GOD-M523L-M524L and GOD-524L-528L were digested with *Eco*RI and *Not*I and then cloned into the expression vector pPIC9k. Mutations were confirmed by DNA sequencing (Invitrogen).

2.4 Yeast transformation and screening of the recombinant strains

The recombinant vectors pPIC9K-GOD, pPIC9K-GOD-8, pPIC9K-GOD-M523L-M524L, and pPIC9K-GOD-M524L-M528L were linearized by *Sac*I and transformed into corresponding *P. pastoris* GS115 competent cells by electroporation. The cells were pulsed in a 2-mm electroporation cuvette at 1.5 kV and 200 Ω and then spread and cultured on MD plates. After culture, cells were induced with methanol for 2 days. The plates were then covered with color solution, prepared as follows: 10 mL of 1% agarose was melted, cooled to below 60°C, mixed with 2 mL 18% D-glucose and 200 μL of 0.01 g/mL N-acetyl-D-glucosamine, and stored in methanol solution containing 400 μL of 100 U/mL horseradish peroxidase; the solution was used immediately after preparation. After incubation for 30 min at room temperature, the brown circular area around the colony was measured, and recombinant clones with high enzyme activity were chosen according to the size of the brown circle.

2.5 Protein expression and purification

Recombinant vector expression in *P. pastoris* was carried out as previously described [20,21]. GOD was purified by ion-exchange chromatography (DEAE-52-cellulose), and culture supernatants were desalted with a desalting column (10× 150 mm) in buffer A (50 mM Tris-HCl, pH 7.0) at 10 mL/min, as recommended. The culture supernatants were then loaded onto a 5-mL DEAE-52-cellulose column. The column was washed with buffer A to remove unbound proteins. A ladder gradient of NaCl (from 100 to 500 mM) in buffer A was performed at 4 mL/min for 30 min.

2.6 Analysis of enzymatic activity and soluble proteins

GOD activity was measured using the coupled o-dianisidine–peroxidase reaction [22,23]. Different concentrations (0.8, 1.2, 1.6, 2.0, 2.4, and 2.8 U/mL) of GOD standard were used as standard curve to measure the enzymatic activity of the GOD solution. Soluble proteins in the culture filtrate were determined by the method of Bradford, with the bovine serum albumin fraction as the standard [24,25], and absorbance was measured at 595 nm. Different concentrations (10, 20, 40, 60 80, and 100 μg/mL) of BSA solution were used as the standard curve to quantify the soluble proteins in the GOD solution.

2.7 Analysis of anti-oxidation

One milliliter of H_2O_2 solution was added to 4 mL GOD solution and incubated at 37°C for 3.5 h. Every 30 min, 0.5 mL of the solution was collected, and excess horseradish peroxidase (400 U/mL) was added immediately to remove redundant hydrogen peroxide. The remaining enzyme activity was measured.

3. Results

3.1 Cdocker simulations

We performed simulations of GOD and GOD mutants binding with D-glucose using CDOCKER and found that GOD (**Fig. 1a**) and GOD-8 (**Fig. 1b**) had better associativity than GOD-M523L-M524L (**Fig. 1c**) and GOD-M524L-M528L (**Fig. 1d**). These data suggested that GOD-8 had higher activity than GOD-M523L-M524L and GOD-M524L-M528L. The CDOCKER energy of best pose between GOD-8 and D-glucose is the highest, which is 6.77139, GOD and D-glucose compound take second place, which is 5.07234, but GOD-M523L-M524L or GOD-M524L-M528L and D-glucose compound is lower than GOD and GOD-8 , they have only 0.242621 and 0.877411 of each other.And the -CDOCKER interaction energy is same whith -CDOCKER energy, GOD-8 and D-glucose compound has the highest score, each of GOD-M523L-M524L or GOD-M524L-M528L and D-glucose compound is lower than other two compound.The CDOCKER simulations implies that all the compounds have activity.

3.2 Plate screening

The actual activities of wild-type and mutant GOD proteins are presented in **Fig. 2**. Mutant GOD-8 **(Fig. 2a)** had no activity

through plate screening, whereas GOD-M523L-M524L had lower activity than wild-type GOD **(Fig. 2b)**, and GOD-M524L-M528L had slightly higher activity than GOD **(Fig. 2c)**. Because of the inactivation of GOD-8, we focused on determining the enzymatic activity and soluble protein levels of GOD-M533L-M524L and GOD-M524L-M528L.

3.3 Analysis of enzymatic activity and soluble protein content

The soluble protein contents of GOD, GOD-M523L-M524L, and GOD-M524L-M528L, along with their specific activities, are listed in **Table 3**. Because the specific activity of GOD-M523L-M524L was much lower than that of GOD, we did not compare the anti-oxidation capacities of GOD-M523L-M524L and GOD. However, the specific activity of GOD-M524L-M528L was higher than that of GOD; therefore, we compared the anti-oxidation capacities of GOD and GOD-M524L-M528L.

3.4 Analysis of anti-oxidation capacity

The anti-oxidation capacities of GOD and GOD-M524L-M528L were compared (**Fig. 3**). Interestingly, when H_2O_2 was added to the enzyme solution, the residual enzyme activities of both GOD and GOD-M524L-M528L decreased gradually over the 3.5-h incubation period. Notably, GOD-M524L-M528L showed better anti-oxidation than GOD, and the final activity of GOD was lower than that of the mutant (20% versus 30%, respectively).

Figure Legends

Fig. 1. Interaction between GOD (mutant GODs) and D-glucose. (a):Ten structures were simulated for the docking between GOD and D-glucose. The CDOCKER energy of the best structure was 5.07234, with an interaction energy of 26.6475; (b):Ten structures were simulated for the docking between GOD-8 and D-glucose. The CDOCKER energy of the best structure was 6.77139, with an interaction energy of 30.6795; (c):Ten structures were simulated for the docking between GOD-M523L-M524L and D-glucose, The CDOCKER energy of the best structure was 0.242621, with an interaction energy of 25.579; (d):Ten structures were simulated for the docking between GOD-M524L-M528L and D-glucose, The CDOCKER energy of the best structure was 0.877411, with an interaction energy of 23.5475. Hydrogen bonds with amino acid main chains are represented by blue dashed arrows directed towards the electron donor.

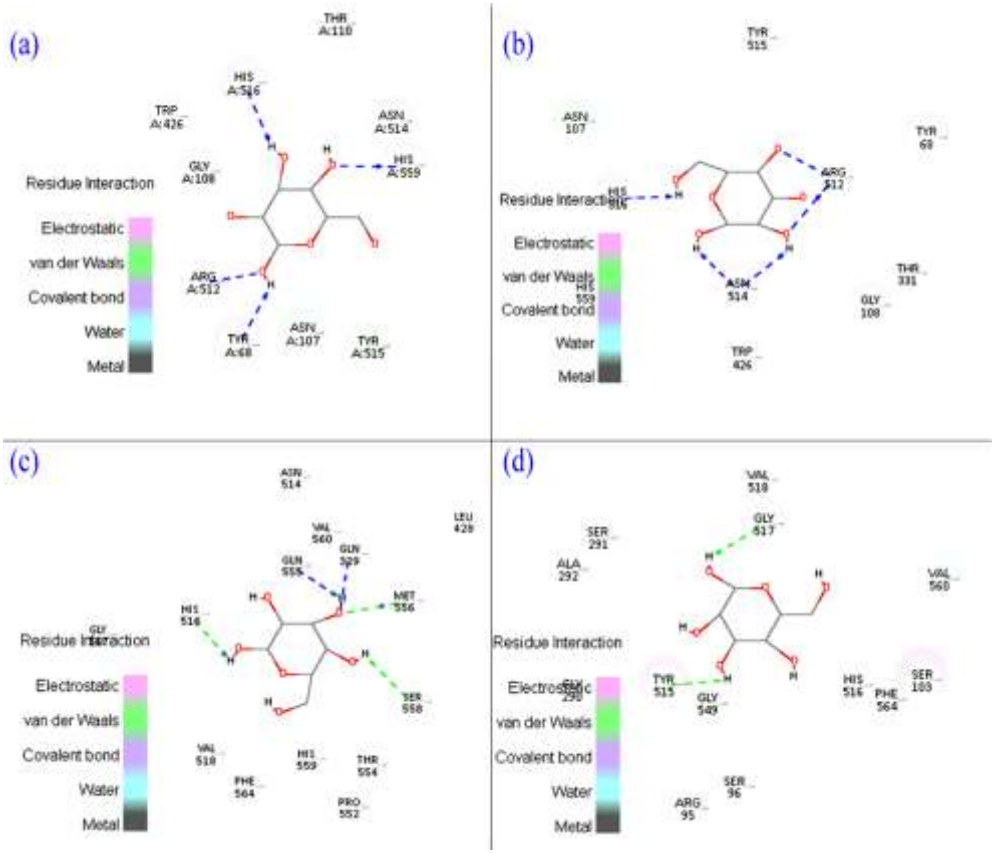

Fig. 2. The activity of GOD and mutant GODs screened on MM. (a): Mutant GOD-8 had no activity through plate screening; (b): GOD-M523L-M524L had lower activity than wild-type GOD; (c): GOD-M524L-M528L had slightly higher activity than the wild-type GOD.

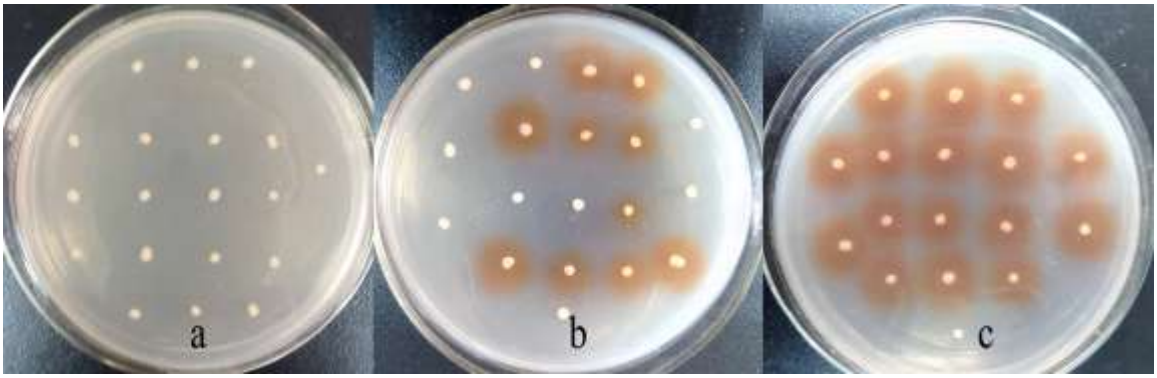

Fig. 3. Comparison of the anti-oxidation capacities of GOD and GOD-M524L-M528L. GOD-M524L-M528L showed better anti-oxidation than the wild-type GOD.

Table

Table 1 Primers for GOD-M523L-M524L

Primer name	Sequence (5′→3′)
GOD-F-*EcoR*I	AAAGAATTCAGCAATGGCATTGAAGCC
GOD-R-*Not*I	TTTGCGGCCGCTCACTGCAT
GOD-M1-F	TGCTCCTTGTTGCCGAAGGAGAT
GOD-M1-R	TCGGCAACAAGGAGCAAGTACCC

Table 2 Primers for GOD-M524L-M528L

Primer name	Sequence (5′→3′)
GOD-F-*EcoR*I	AAAGAATTCAGCAATGGCATTGAAGCC
GOD-R-*Not*I	TTTGCGGCCGCTCACTGCAT
GOD-M2-F	CTTGCTCCATGTTGCCGAAGGAGTTGGGC
GOD-M2-R	CAACACCGCCCAACTCCTTCGGCAACATG

Table 3 The soluble protein contents of GOD, GOD-M523L-M524L, and GOD-M524L-M528L

	Activity (U/mL)	Soluble protein (μg/mL)	Specific activity (U/μg)
GOD	442.755	136.476	3.24
GOD-M523L-M524L	1.21	26.476	0.0457
GOD-M524L-M528L	245.843	68.476	3.59

4. Discussion and conclusion

H_2O_2 is a competitive inhibitor for GOD and can dramatically affect the activity of GOD. Moreover, liposomal glucose oxidase had been shown to inhibit the production of H_2O_2, thereby improving the anti-oxidation capacity of GOD [7,10]. In this study, we designed three GOD mutants with computer-aided and expressed these proteins in *P. pastoris*. Docking analysis of the three mutants revealed that all proteins could bind with D-glucose. However, all proteins had varying activities; for example, GOD-8 had no activity, GOD-M523L-M524L had lower activity than GOD, and GOD-M524L-M528L had higher activity than GOD. Moreover, the GOD-M524L-M528L mutant had higher anti-oxidation capacity than GOD.

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