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Research article

Asn336 is involved in the substrate affinity of glycine oxidase from *Bacillus cereus*

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ABSTRACT

Background: Glycine oxidase (GO), a type of D-amino acid oxidase, is of biotechnological interest for its potential in several fields. In our previous study, we have characterized a new glycine oxidase (BceGO) from *Bacillus cereus* HYC-7. Here, a variant of N336K with increased the affinity against all the tested substrate was obtained by screening a random mutant library of BceGO. It is observed that the residue N336 is invariable between its homogeneous enzymes. This work was aimed to explore the role of the residue N336 in glycine oxidase by site-directed mutagenesis, kinetic assay, structure modeling and substrate docking.
Results: The results showed that the affinity of N336H, N336K and N336R increased gradually toward all the substrates, with increase in positive charge on side chain, while N336A and N336G have not shown a little significant effect on substrate affinity. The structure modeling studies indicated that the residue Asn336 is located in a random coil between β-18 and α-10. Also, far-UV CD spectra-analysis showed that the mutations at Asn336 do not affect the secondary structure of enzyme.
Conclusion: Asn336 site was located in a conserved GHYRNG loop which adjoining to substrate and the isoalloxazine ring of FAD, and involved in the substrate affinity of glycine oxidase. This might provide new insight into the structure–function relationship of GO, and valuable clue to redesign its substrate specificity for some biotechnological application.

1. Introduction

Glycine oxidase (GO, EC 1.4.3.19), a homotetrameric flavoenzyme, contains non-covalently attached FAD molecule [1,2]. BceGO catalyzes the oxidative deamination of various amines (glycine, sarcosine, N-ethylglycine) and some D-isomer of amino acids (D-alanine, D-proline, etc.) to yield corresponding α-keto acid(s), ammonia/amine, and hydrogen peroxide. GO appears to be stereo-specific in oxidizing the D-amino acids and its substrate specificity partially similar to D-amino acid oxidase (DAAO, EC 1.4.3.3) and sarcosine oxidase (SOX, EC 1.5.3.1). It plays an important role in the biosynthesis of the thiazole ring of thiamine pyrophosphate cofactors in *Bacillus subtilis* [2]. The broad substrate specificity and stereoselectivity of GO confers it great potential in several biotechnological fields, such as industrial biocatalysis, biosensors and developing glyphosate-resistant crop [3,4,5]. This promotes scientists to search new enzyme, study the structure–function relationship and redesign its application by protein engineering [5,6].

In our previous study, we have reported a new glycine oxidase (BceGO) with glyphosate-oxidative activity from *Bacillus cereus* and, developed a high through screening method for improving its affinity and activity toward glyphosate [7]. Here, we continued to screen new mutant with higher specificity to glyphosate from a random mutation library of BceGO, and obtained a mutant, N336K, whose $K_{m,\ app}$ on glyphosate decreased 3.77-fold. Sequence alignment showed that the residue N336 is highly conserved in BceGO and its homogeneous enzymes. Here, we attempted to investigate the role of N336 residue in the catalytic activity of GO by site-directed mutagenesis, three-dimensional structure modeling and ligand docking assay.

2. Materials and methods

2.1. Reagents, strains, and plasmid

Glyphosate, glycine, sarcosine, D-alanine, *o*-dianisidine dihydrochloride, horseradish peroxidase and FAD were purchased from Sigma (USA). Taq DNA polymerase, T4 DNA ligase and restriction enzymes were purchased from TAKARA (Japan). Fast Pfu polymerase, DNA purifcation kits, GST Binding Resin and Bradford protein assay kits were acquired from TransGen (Beijing, China), Axygen (USA), Novagen (Germany), and

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Table 1
Primers used for gene BceGO mutagenesis. The BamHI and XhoI sites were italic and underlined, and the mutation positions were underlined.

Target sites	Sequence (5′-3′)
BceGO-F	CGC*GGATCC*ATGTGTRAGAAGTATGATGTAGCGAT
BceGO-R	CCG*CTCGAG*CTAAACBSTYYTAGAAAGCAATGAAT
N336H-F	GGCCATTATCGACATGGTATTTTAT
N336H-R	ATGTCGATAATGGCCCGTGCAAGTA
N336R-F	GGCCATTATCGACGTGGTATTTTAT
N336R-R	ACGTCGATAATGGCCCGTGCAAGTA
N336A-F	GGCCATTATCGAGCGGGTATTTTAT
N336A-R	CGCTCGATAATGGCCCGTGCAAGTA
N336G-F	GGCCATTATCGAGGCGGTATTTTAT
N336G-R	GCCTCGATAATGGCCCGTGCAAGTA

Sangon (Shanghai, China), respectively. *Escherichia coli* DH5α and *E. coli* BL21 (DE3) were used as for gene cloning and for protein expression, respectively.

2.2. Construction of mutant library and site-directed mutagenesis

The BceGO random mutant library was generated by error-prone PCR used pGEX-GO as the template. The amplification mixture, which contained 20 nM primers, 0.2 mM dATP and dCTP, 0.1 mM dTTP and dGTP, 2 U Taq DNA polymerase and Taq buffer containing 5 mM MgCl2 and 0.5 mM MnCl2 in 100 μL volume, was cycled in an Bio-rad thermal cycler (California, USA) for 30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 70 s. PCR products were purified, digested with *Bam*HI and *Xho*I, cloned into pGEX-6P-1, and transformed into *E. coli* DH5α to obtain the random mutant library.

PCR-based site-directed mutagenesis was carried out to generate single-mutant [8]. PCR reactions (50 μL) contained 20 ng template (pGEX-GO), 0.2 mM dNTP, 20 nM each primer, 10 μL PCR buffer and 1 unit of *Pfu* DNA polymerase (Transgen, China). The PCR cycling parameters were: 1 cycle of 2 min at 97°C, 20 cycles of 20 s at 95°C, 30 s at 54°C, and 160 s at 72°C, and incubation of 10 min at 72°C. Then the PCR products were treated with *Dpn*I to digest the parental DNA at 37°C for 8 h. Finally, *Dpn*I digestion mixture was transformed into *E. coli* DH5α competent cells, and the transformant was selected on ampicillin plates. The primers used were listed in Table 1. The desired mutants were validated by DNA sequencing.

2.3. Screening for GO mutants

The mutant library was screened by an enzyme-coupled assay using horseradish peroxidase (5 U/mL) and *o*-dianisidine dihydrochloride as described previously [9]. Single colony from random mutation library was cultured in deep-well plates containing 0.6 mL LB medium, and induced by IPTG. Then cell extracts containing target protein were prepared by adding the bacteriophage T7. To screen mutants with higher specificity to glyphosate, 100 μL of each cell lysate was incubated with 20 μL of 50 mM glyphosate, 20 μL of 0.32 mg/mL *o*-dianisidine dihydrochloride, and 1 μL of 5 U/mL horseradish peroxidase in sodium phosphate buffer (50 mM; pH 8.5) followed by measuring the absorbance values at 450 nm. Mutants showed higher absorbance than the wild-type were selected for further activity analysis.

2.4. Enzyme expression and purification

The recombinant BceGO and its mutant were purified by affinity chromatography using the methods described previously [7]. Briefly, the recombinant plasmids were transformed into the host *E. coli* BL21 (DE3). Recombinant cells grew at 37°C in LB medium containing 100 μg/mL ampicillin. Protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.2 Mm, when the OD600 reached 0.6. After an overnight induction at 22°C, 1.5 L culture was collected and disrupted by the high pressure homogenizer (NiroSoavi, Italy). Then, the supernatant of the lysate was mixed with 1.5 mL GST·Bind Resin that had been equilibrated with 50 mM disodium pyrophosphate buffer. The resin was washed with disodium pyrophosphate buffer (50 mM, pH 7.5) to elute the unspecific-binding protein. Finally, the GST-free recombinant protein was prepared by on-column cleavage with PreScission protease [10]. The concentration of the wild-type BceGO and mutants was measured by the method of Bradford assay [11]. The purity of the protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.5. Determination of kinetic parameters

The kinetic parameters of wild-type BceGO and mutants were assayed using a fixed amount of enzyme and various concentration of substrates (glycine, 0–300 mM; glyphosate, 0–600 mM; sarcosine, 0–300 mM; D-alanine, 0–600 mM). The absorbance was measured at 450 nm using a microplate reader (Thermo Scientific, Multiscan spectrum). The initial reaction velocities under various concentrations of each substrate were fitted to the Lineweaver-Burk transformation of the Michaelis–Menten equation to figure out apparent kinetic parameters (*i.e.*, $K_{m,app}$ and V_{max}). Further, the $k_{cat,app}$ was calculated by the equation: $k_{cat,app} = V_{max} / [E]$, in which [E] is the total amount of enzyme in the reaction mixture.

2.6. Circular dichroism and secondary structure prediction

Secondary structure of BceGO was predicted by using the program PSIPRED [12]. Circular dichroism (CD) spectra of BceGO and variants were recorded with a Jasco-810 CD spectrometer (Jasco Corp., Japan). The data were collected at room temperature from 190 to 260 nm using 1 mm quartz cuvette (400 μL). The conversion to the Mol CD (Δε) in each spectrum was performed with the Jasco Standard Analysis software. Estimation of the secondary structure content from far-UV circular dichroism (CD) spectra was performed by using the CDPro

Table 2
Comparison of the apparent kinetics parameters of the wild-type BceGO and the mutants toward different substrates.

		Wild-type	N336H	N336K	N336R	N336A	N336G
Glycine	$k_{cat,app}$ (s^{-1})	0.71 ± 0.03	0.37 ± 0.02	0.25 ± 0.003	0.024 ± 0.0002	0.52 ± 0.02	0.67 ± 0.03
	$K_{m,app}$ (mM)	1.04 ± 0.12	0.95 ± 0.11	0.79 ± 0.12	0.53 ± 0.07	1.41 ± 1.23	2.25 ± 2.52
	k_{cat}/K_m	0.68	0.39	0.32	0.045	0.37	0.3
Glyphosate	$k_{cat,app}$ (s^{-1})	0.87 ± 0.02	0.23 ± 0.05	0.19 ± 0.002	0.021 ± 0.0003	0.67 ± 0.08	0.62 ± 0.02
	$K_{m,app}$ (mM)	84.79 ± 2.34	42.31 ± 1.84	22.45 ± 1.44	10.44 ± 0.33	68.36 ± 3.15	96.73 ± 2.81
	k_{cat}/K_m	0.01	0.0054	0.0084	0.002	0.0098	0.0064
Sarcosine	$k_{cat,app}$ (s^{-1})	0.98 ± 0.01	0.68 ± 0.04	0.28 ± 0.003	0.035 ± 0.0002	0.26 ± 0.03	0.59 ± 0.04
	$K_{m,app}$ (mM)	1.51 ± 0.18	1.39 ± 0.15	0.36 ± 0.02	0.15 ± 0.006	1.56 ± 0.17	2.72 ± 1.35
	k_{cat}/K_m	0.65	0.49	0.78	0.23	0.17	0.22
D-Alanine	$k_{cat,app}$ (s^{-1})	0.81 ± 0.04	0.37 ± 0.03	0.21 ± 0.001	0.028 ± 0.0004	0.65 ± 0.01	0.46 ± 0.15
	$K_{m,app}$ (mM)	34.65 ± 1.22	24.63 ± 1.12	6.81 ± 0.32	1.31 ± 0.04	37.6 ± 0.95	48.85 ± 1.86
	k_{cat}/K_m	0.023	0.015	0.03	0.021	0.017	0.009