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Substitution of the critical methionine residues in *Trigonopsis variabilis* D-amino acid oxidase with leucine enhances its resistance to hydrogen peroxide

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Abstract

Each of the six oxidative-sensitive methionine residues in *Trigonopsis variabilis* D-amino acid oxidase (DAAO) was changed to leucine by site-directed mutagenesis. The wild-type and mutant enzymes with an apparent molecular mass of about 39.3 kDa were expressed in *Escherichia coli*. The specific activity of four mutant DAAOs (Met^{104}Leu, Met^{226}Leu, Met^{245}Leu, and Met^{339}Leu) was decreased by more than 96%, while Met^{156}Leu and Met^{209}Leu showed about 23% and 96% higher activity, respectively, than the wild-type enzyme. The kinetic parameters of the two more active enzymes were determined and a 2.2-fold increase in K_m was observed for Met^{209}Leu. Comparison of Met^{156}Leu and wild-type DAAO revealed a 95% increase in k_{cat}/K_m. Met^{156}Leu, Met^{209}Leu, and Met^{226}Leu were resistant to inactivation by 50 mM H_2O_2. The other three mutant DAAOs were also slightly more resistant than the wild-type enzyme to chemical oxidation. These observations indicate that the oxidative stability in *T. variabilis* DAAO can be improved by substitution of methionine residues with leucine. © 2000 Published by Elsevier Science B.V. All rights reserved.

Keywords: *Trigonopsis variabilis*; D-Amino acid oxidase; Site-directed mutagenesis; H_2O_2 resistance

1. Introduction

D-Amino acid oxidase (DAAO, EC 1.4.3.3) catalyzes the oxidation of D-amino acids to their corresponding imino acids, which are subsequently hydrolyzed to keto acids and ammonia. The reduced enzyme is reoxidized by molecular oxygen with the release of hydrogen peroxide. Because of the strict stereoselectivity of DAAO, it has been exploited for the production of L-amino acids [1], the quantitative analysis of D-amino acids using biosensors [2], and the bioconversion of cephalosporin C to 7-glutarylcephalosporanic acid [3]. The latter process has been industrialized, and *Trigonopsis variabilis* DAAO is used as one of the two biocatalysts that take part in the enzymatic two-step conversion of cephalosporin C to 7-aminocephalosporanic acid [4], which is a key compound for the synthesis of many β-lactam drugs. However, hydrogen peroxide, the byproduct of DAAO, has been found to inhibit the enzyme activity thus resulting in low operational stability [5].

Several enzymes can be inactivated by metal-catalyzed or mixed-function oxidative systems [6,7] in reactions that involve the formation of highly reactive hydroxyl radicals [7]. It has been reported that the common sites for the oxidative reaction are histidine, arginine, lysine, proline, cysteine and methionine residues [7]. Methionine oxidation is a significant form of protein damage caused by endogenous or environmental oxidizing agents [8]. Methionine residues may be oxidized to the sulfoxide form with hydrogen peroxide [9] or to the sulfone form with the other oxidizing agents under harsher conditions [10]. Although oxidation of methionine residues has no effect on the function of some polypeptides [9,11], evidence for severe inhibition of biological function in other proteins has been provided [12,13].

T. variabilis DAAO has been purified to homogeneity and some of its properties have been determined [14]. It consists of two identical subunits and requires one FAD molecule per subunit for catalytic activity [15]. The *daao* gene encodes a protein of 356 amino acids with a calcu-

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lated molecular mass of 39.3 kDa [16]. *T. variabilis* DAAO is inactivated by hydrogen peroxide [17], indicating that the enzyme is susceptible to oxidative inactivation. Such susceptibility could be linked to the presence of six Met residues at positions 104, 156, 209, 226, 245, and 339 in the primary amino acid sequence of the enzyme [17]. It is possible that these residues may be oxidized by endogenous hydrogen peroxide during normal DAAO activity. Recently, we have demonstrated that the conserved Asp^{206} and His^{324} residues of the enzyme might play an important role in FAD binding [18,19]. In this study, we replaced the Met residues by Leu using site-directed mutagenesis in an attempt to improve the resistance of the enzyme to hydrogen peroxide. The results provide evidence that the Met residues sensitize the native DAAO protein of *T. variabilis* to hydrogen peroxide.

2. Materials and methods

2.1. Bacterial strains, growth conditions, and plasmids

Escherichia coli JM109 [*recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi* Δ(*lac-proAB*) F′ (*traD*36 *proAB* $lacI^q$ *lacZ*ΔM15)] was used as host for cloning and expression of recombinant DAAOs. *E. coli* CJ236 [*dut*1 *ung*1 *thi*1 *relA*1/pCJ105(cam^r F′)] was used in the production of uracil-enriched, single-stranded DNA for site-directed mutagenesis. *E. coli* strains were grown in Luria–Bertani (LB) medium with ampicillin (100 μg ml^{-1}) whenever required. Plasmids used were pKm-DAAO [20], pET21b-DAO [18], and pQE30-DAO [18] in which the expression of the *T. variabilis daao* gene is under the control of *tac*, T7, and T5 promoters, respectively.

2.2. DNA methods

Standard methods, as described by Sambrook et al. [21], were used for plasmid isolation, restriction analysis, ligations, and transformations. Site-directed mutagenesis of the *daao* gene was performed on phagemid pM19-DAO [18] using the Muta-Gene M13 in vitro Mutagenesis kit from Bio-Rad Laboratories (Richmond, CA, USA). The oligonucleotides used were synthesized on an Applied Biosystems DNA synthesizer model 380A and are listed in Table 1, and the altered codons are underlined. DNA sequencing was performed by the dideoxy chain termination method using a Sequenase R version 2 sequencing kit (United States Biochemical Co., Ohio, USA) and primers synthesized on the basis of the *daao* gene.

Table 1
Oligonucleotide primers used in site-directed mutagenesis study

Enzyme	Mutagenic primers
Met^{104}Leu	5′-GGAAGGTGCC<u>CTG</u>TCGGCCA-3′
Met^{156}Leu	5′-GAACTGGCTG<u>CTG</u>TCCCAAT-3′
Met^{209}Leu	5′-GGACAAGAAG<u>CTG</u>TACCCTA-3′
Met^{226}Leu	5′-TCTTCCTTTT<u>CTG</u>GCCTCCT-3′
Met^{245}Leu	5′-TCTATATATC<u>CTG</u>ACCCGAT-3′
Met^{339}Leu	5′-CTCTTACGGC<u>CTG</u>GCTGATG-3′

2.3. Expression of wild-type and mutant DAAOs

Expression of recombinant DAAOs was carried out as described previously [18]. Briefly, each bacterial clone was grown in LB medium supplemented with ampicillin (100 μg ml^{-1}) at 28°C with shaking. After incubation to an optical density at 600 nm of 1.0, 1 mM isopropyl-β-thiogalactopyranoside (IPTG) was added to the medium to induce the synthesis of desired proteins. The cultures were further cultivated for another 5 h and then collected by centrifugation and resuspended in the binding buffer (5 mM imidazole, 500 mM NaCl, and 20 mM Tris–HCl; pH 7.9). Cells were disrupted by sonication, and the recombinant proteins were purified with a His-bind affinity column (Novagen, Madison, WI, USA).

2.4. Immunodetection of DAAO

SDS polyacrylamide gel electrophoresis (SDS–PAGE) was carried out with the Protean II minigel system (Bio-Rad Laboratories). After electrophoresis, proteins were subjected to immunoblotting analysis with polyclonal anti-DAAO antibodies as described previously [14].

2.5. Enzyme assay

DAAO activity was assayed as already described by Lee et al. [22]. The reaction mixture contained 100 mM potassium phosphate (pH 8.0), 400 U bovine liver catalase, 10 mM D-alanine, 1 μM FAD, and the appropriately diluted enzyme solution. After 15 min incubation at 37°C, the resulting keto acid was determined by a colorimetric assay using dinitrophenylhydrazine. One unit of DAAO activity is defined as the amount of enzyme producing 1 nmol of pyruvate per minute. Protein was determined by the method of Bradford [23] with bovine serum albumin as a standard. The k_{cat} and K_m values were determined by fitting the initial rates as a function of substrate concentration to the Michaelis–Menten equation using Grafit software (Sigma Chemical Co., St. Louis, MO, USA). k_{cat} represents a direct measure of catalytic production of product under optimum conditions and K_m means the affinity of enzyme for substrate.

3. Results and discussion

Since endogenous hydrogen peroxide could affect *T. variabilis* DAAO activity [24], we replaced six methionine residues of its primary sequence with leucine by site-directed mutagenesis. After the verification of the gene

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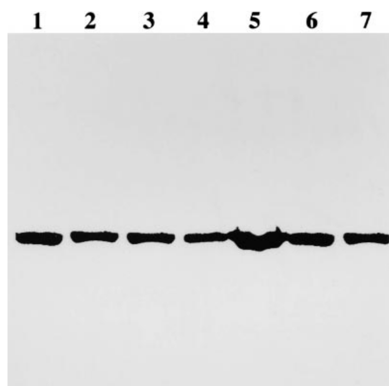

Fig. 1. Western blotting analysis of the wild-type and mutant DAAOs. Lanes: 1, wild-type DAAO; 2, Met[104]Leu; 3, Met[156]Leu; 4, Met[209]Leu; 5, Met[226]Leu; 6, Met[245]Leu; 7, Met[339]Leu.

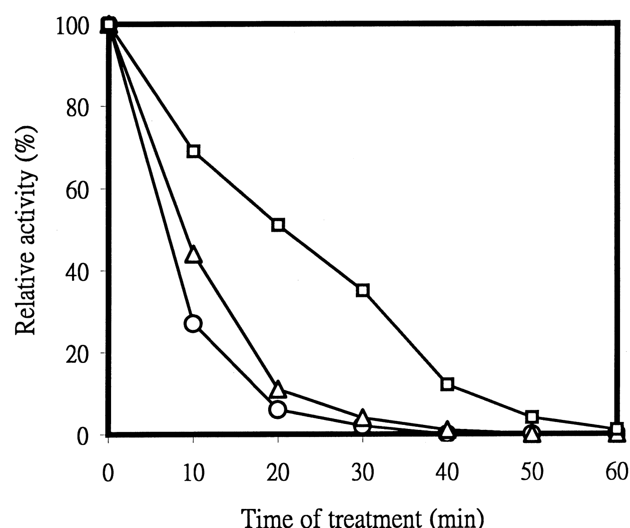

Fig. 2. Inactivation of wild-type DAAOs by hydrogen peroxide. Crude cell extracts from *E. coli* JM109 harboring pKm-DAAO, pET21b-DAO, and pQE30-DAO, respectively, were incubated at 37°C in 100 mM potassium phosphate buffer in the presence of 100 mM H_2O_2. At the indicated times, aliquots were removed and the residual DAAO activity was measured. The specific activities at $t=0$ were 4.9, 4.2, and 5.1 U mg^{-1} of protein for DAAOs from *E. coli* JM109 harboring pKm-DAAO (○), pET21b-DAO (□), and pQE30-DAO (△), respectively. The data represent the average values of three measurements. The standard deviations are estimated to be ±21%.

sequence, the wild-type and mutant *daao* genes were cloned into *Bam*HI and *Hin*dIII sites of pQE-30 and expressed in *E. coli* JM109. Wild-type and mutant enzymes were purified to homogeneity by metal chelate column chromatography. The preparations displayed a single band with an apparent molecular mass of about 39.3 kDa on SDS–PAGE (data not shown). As shown in Fig. 1, the purified enzymes were recognized by the polyclonal anti-*T. variabilis* DAAO, indicating that the mutant proteins are immuno-identical with that of wild-type enzyme. The specific activity is greatly decreased by the mutations introduced at positions 104, 226, 245, and 339 of *T. variabilis* DAAO. Losses of 72, 97, 69, and 75% are observed for Met104/226/245/339Leu mutants, respectively (Table 2), while substitution of Met[209] with Leu caused a 90% increase in specific activity. Met[156]Leu was highly active compared with wild-type DAAO, with increased k_{cat} and K_m values of 200% and 54% (Table 2), respectively. As compared with wild-type DAAO, both Met[156]Leu and Met[209]Leu had an increased K_m value and specific activity. Such apparently contradictory results may be due to the contribution of increased oxidative stability in mutant DAAOs being greater than the negative effect caused by a decreased affinity for substrate. The catalytic efficiency (k_{cat}/K_m) was 195% that of wild-type DAAO. Met[209]Leu displayed a similar catalytic efficiency to that of wild-type DAAO, with a great increase in k_{cat} and K_m values. Since the other mutant DAAOs had only about 3.2–31% of wild-type activity, their kinetic parameters were not determined. Although chemical modification of pig kidney DAAO led to the identification of Met[110] as the catalytic residue [25], it was concluded that this residue was not directly involved in the catalytic activity [26]. Moreover, alignment of amino acid sequences shows that methionine residues are not well conserved in all DAAOs (data not shown). This suggests that Met[104], Met[226], Met[245], and Met[339] are not essential for catalytic function, but they are of structural importance in *T. variabilis* DAAO.

The sensitivity of different native *T. variabilis* DAAOs toward hydrogen peroxide was investigated. The results showed that the inactivation of the enzymes was time-dependent (Fig. 2). After 10 min treatment with hydrogen peroxide, about 73% of the enzyme activity was abolished in DAAO from *E. coli* JM109 harboring pKm-DAAO.

Table 2
Kinetic parameters of wild-type and mutant DAAOs purified by metal chelate column chromatography[a]

Enzyme	Specific activity (U mg^{-1})	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (min^{-1} M^{-1})
Wild-type	6.91	0.34	4.63	73.43
Met[156]Leu	8.51	1.02	7.12	143.26
Met[209]Leu	13.51	1.10	14.76	74.53
Met[104]Leu	1.92	ND[b]	ND	ND
Met[226]Leu	0.22	ND	ND	ND
Met[245]Leu	2.15	ND	ND	ND
Met[339]Leu	1.72	ND	ND	ND

[a]The data represent the average values of three measurements. The standard deviations are estimated to be ±17%. The protein concentration used in the above assays was 1 μg.
[b]ND, not determined.

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Previously, we constructed a plasmid, pET21b-DAO, expressing the enzyme tagged by six histidines at its C-terminal end. The DAAO-His_6 protein was overproduced in pET21b-DAO-harboring cells upon IPTG induction, and only one chromatographic step was required for its almost complete purification. It should be noted that the resistance of the fused protein to hydrogen peroxide was greatly increased (Fig. 2). The reason for the increased resistance is unknown, but it may relate to the His_6 tag at the C-terminal end of the protein. The unexpected influence of a C-terminal His tag on the kinetic parameters of a *Bacillus licheniformis* β-lactamase was recently reported [27]. Consequently, we also constructed a plasmid pQE30-DAO for overproduction of the N-terminally His_6-tagged form of *T. variabilis* DAAO. The result showed that the oxidative stability of the above DAAO appeared to be slightly higher than that of the enzyme without a His tag (Fig. 2). As a control, no significant decline in activity of native enzymes was observed in the absence of exogenous hydrogen peroxide over the duration of the experiment (data not shown). These findings suggested that the His tag may increase the oxidative stability of the enzyme toward hydrogen peroxide.

DAAO can be exposed to oxidative stress during activity due to the formation of hydrogen peroxide and these conditions can lead to a relatively low operational stability of the immobilized enzyme, even in the presence of catalase [28]. Estell et al. [29] reported that the substitution of Met^{222} in *Bacillus subtilis* subtilisin by other amino acids not only improves its resistance to hydrogen peroxide but also increases the specific activity and thermal stability. To evaluate the resistance of wild-type and mutant DAAOs to oxidative inactivation, the enzymes were incubated in 100 mM potassium phosphate buffer containing various concentrations of H_2O_2 at 37°C for 30 min and the residual activities were measured. In the presence of 50 mM H_2O_2, the wild-type DAAO was almost inactivated while about 5–51% of the specific activity was retained in mutant enzymes (Table 3). When the concentration of H_2O_2 was increased to 100 mM, 11, 20, and 21% of the original activity was observed in Met^{209}Leu, Met^{226}Leu and Met^{156}Leu, respectively. These results indicated that substitution of methionine residues by Leu, an oxidatively stable residue, imparts oxidative resistance. Although other sites may be oxidized, it is clear that substitutions at positions 156 and 209 of *T. variabilis* DAAO could improve its catalytic efficiency and oxidative stability. In general, surface-exposed methionine residues are more susceptible to oxidation than buried residues [9]. It is possible that Met^{156} and Met^{209} residues are located at the surface of the enzyme; therefore, they could be more susceptible to endogenous hydrogen peroxide. This work and that of Rosenberg et al. [30] demonstrate that oxidative stability in these proteins can be enhanced by replacement of oxidative-sensitive residues. Even though oxidatively sensitive residues may be of functional importance in *T. variabilis* DAAO, this work demonstrates that it may be possible to find oxidatively stable amino acid substitutions in this enzyme. This could have significant benefit in stabilizing enzymes used in industrial processes.

Table 3
Oxidative stability of wild-type and mutant DAAOs[a]

Enzyme	Specific activity (U mg^{-1}) at H_2O_2 concentration				
	0 mM	10 mM	20 mM	50 mM	100 mM
Wild-type	6.32	4.03	2.06	0.02	0.00
Met^{104}Leu	2.14	2.01	1.06	0.32	0.00
Met^{156}Leu	9.01	8.72	6.01	4.59	1.85
Met^{209}Leu	15.21	12.08	7.37	6.21	1.60
Met^{226}Leu	0.25	0.20	0.18	0.12	0.05
Met^{245}Leu	2.87	2.13	1.02	0.14	0.00
Met^{339}Leu	1.74	1.07	0.65	0.11	0.00

[a]Oxidative stability was determined by preincubated buffered enzyme at different concentrations of H_2O_2 for 30 min. The residual activities were measured under the standard assay conditions. The data represent the average values of three measurements. The standard deviations are estimated to be ±15%.

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