Purification and characterization of a novel glycine oxidase from *Bacillus subtilis*

Yoshiaki Nishiya[a,*], Tadayuki Imanaka[b]

[a] *Tsuruga Institute of Biotechnology, Toyobo Co., Ltd., Toyo-cho 10-24, Tsuruga, Fukui 914-0047, Japan*
[b] *Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Kyoto 606-8501, Japan*

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Abstract The open reading frame *yjbR* which had been sequenced as a part of the *Bacillus subtilis* genome project encodes a putative 40.9-kDa protein. The *yjbR*-coding sequence was slightly similar to those of bacterial sarcosine oxidases and possibly compatible with the tertiary structure of the porcine kidney D-amino acid oxidase. The *yjbR* gene product was overproduced in *Escherichia coli*, purified to homogeneity from the recombinant strain, and characterized. This protein effectively catalyzed the oxidation of sarcosine (*N*-methylglycine), *N*-ethylglycine and glycine. Lower activities on D-alanine, D-valine, and D-proline were detected although no activities were shown on L-amino acids and other D-amino acids. Since glycine is a product and not a substrate for sarcosine oxidase, this protein is not a type of demethylating enzymes but a novel deaminating oxidase, named glycine oxidase as a common name. Several enzymatic properties of the *B. subtilis* glycine oxidase were also investigated.

Key words: Glycine oxidase; Sarcosine oxidase; D-Amino acid oxidase; *Bacillus subtilis*

1. Introduction

Bacillus subtilis is important as the source of industrial enzymes such as amylases and proteases. In 1997 the *B. subtilis* genome sequencing project was completed by European and Japanese laboratories [1]. Several informations are being provided from the project, and the sequence data are available through DNA databases. The project has analyzed the data at the transcription and translation level. Among approximately 4100 putative protein-coding genes identified, we had a particular interest in the open reading frame *yjbR*. The *yjbR* gene encodes a putative 40.9-kDa protein, weakly similar to bacterial sarcosine oxidases.

Sarcosine oxidase (sarcosine:oxygen oxidoreductase (demethylating); EC 1.5.3.1) is a flavoprotein and catalyzes the oxidative demethylation of sarcosine (*N*-methylglycine) to form glycine, formaldehyde, and hydrogen peroxide [2]. Although sarcosine oxidase also acts on other amino acid derivatives of the modified amino group, it differs from amino acid oxidases which catalyze the oxidative deamination of amino acids. Accordingly, this enzyme cannot act on glycine and other amino acids, except for proline which has the modified amino group. We have previously screened the sarcosine oxidase from *Arthrobacter* sp. TE 1826, designated SoxA [3]. SoxA has been purified and characterized, and the enzymatic properties were similar to other bacterial sarcosine oxidases. We are intensively analyzing the enzymatic functions of SoxA using the protein engineering techniques [4–7].

In this report, we describe the purification and characterization of the *yjbR* gene product. This protein was not a type of demethylating enzyme but a novel deaminating oxidase, named glycine oxidase. This is the first report about the purification and characterization of glycine oxidase.

2. Materials and methods

2.1. Strains, plasmid, and culture conditions

B. subtilis MT-2, *Escherichia coli* JM109, and pUC18 (Ap^r) were previously described [8]. Strains were grown in Terrific broth [9] or on L-agar (L-broth [3] plus 1.5% agar) at 37°C. The antibiotic used was ampicillin (50 μg/ml).

2.2. Manipulations of DNA

Extraction of chromosomal DNA of *B. subtilis* MT-2, isolation of the plasmid pUC18, cleavage of pUC18 with the restriction enzyme *Sma*I, and ligation of DNA with T4 DNA ligase were done as described previously [3]. Transformation of *E. coli* JM109 was carried out by the competent-cell method [10]. DNA was sequenced by the dideoxy method [11] with Sequencing PRO (Toyobo, Osaka, Japan). Polymerase chain reaction (PCR) was performed under standard conditions [4] with KOD DNA polymerase (Toyobo).

2.3. Construction of the recombinant plasmid

The *yjbR* gene was amplified by PCR using the following primers: 5′-AAAGGAGATGCGCTATGAAAAGGC-3′ (the sense primer, corresponding to the sequence that encodes the ribosomal binding site AAAGGAG and the N-terminal peptide MKR) and 5′-TCATATCTGAACCGCCTCCTTGCG-3′ (the antisense primer, complementary to the sequence that encodes the C-terminal peptide RKEAVQI and the stop codon). The 1124-bp amplified DNA was ligated into the *Sma*I site of pUC18, and the recombinant construct was designated pBSOLR2. The respective nucleotide sequence of *yjbR* was verified after it had been amplified by PCR and cloned in the vector. The pBSOLR2 carrier was induced to produce the *yjbR* gene product by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to the medium.

2.4. Enzyme purification

The recombinant strain *E. coli* JM109(pBSOLR2) was grown to stationary phase, and harvested by centrifugation. Crude extract was prepared by sonication of the cells. Ammonium sulfate was added to the cell-free extract to give 50% saturation. The precipitate collected by centrifugation was dissolved in 50 mM potassium phosphate buffer (pH 8.0) and dialyzed against the same buffer. The dialysate was subjected to ion-exchange chromatography on a DEAE-Sepharose CL-6B column (Pharmacia, Uppsala, Sweden). The enzyme was eluted with a linear gradient (0–0.5 M) of potassium chloride. Active fractions were finally purified to homogeneity by two gel filtration steps.

2.5. Enzyme assay and characterization

The enzyme assay was based on the measurement of hydrogen peroxide produced during the oxidation of substrate. The 4-aminoantipyrine peroxidase system [12] was used for the enzyme assay as described previously [3]. The assay mixture finally contained 10 mM sarcosine, glycine, or one of other substrates, 0.47 mM 4-aminoanti-

*Corresponding author. Fax: (81) (770) 22-7671.
E-mail: yoshiaki_nishiya@bio-toyobo.co.jp

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pyrine, 2.0 mM phenol, 5 U/ml horseradish peroxidase, and 50 mM potassium phosphate (pH 8.0). The appearance of quinoneimine dye formed by coupling with 4-aminoantipyrine, phenol, and horseradish peroxidase was measured at 500 nm by spectrophotometry. One unit of activity was defined as the formation of 1 μmol of hydrogen peroxide (0.5 μmol of quinoneimine dye) per minute at 37°C and pH 8.0. Reaction mixtures containing several concentrations of substrate solution were used to determine the K_m and k_{cat} values. Molecular weights were determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration on a Superdex 200 column (Pharmacia). Isoelectric point was measured by isoelectric forcasting with the Phast system (Pharmacia).

2.6. Analysis of sequence and structure homologies

Amino acid sequence homologies were analyzed with the GENETYX software system (Software Development, Tokyo, Japan). The sequence database used was SWISS-PROT. Sequence-structure compatibilities were analyzed with the software COMPASS [13,14]. The tertiary structure database used was PDB.

3. Results and discussion

3.1. Homology search analysis of the yjbR-coding sequence

Amino acid homology analysis revealed that the *yjbR*-coding sequence was most homologous to those of bacterial sarcosine oxidases [3,15,16]. Although the overall similarities to sarcosine oxidases were very low (17.2–18.4% identical amino acid residues), 50 residues of the N-terminal region and 65 residues of the C-terminal region showed relatively higher similarities (28.0–32.0% and 23.1–26.2% identical amino acid residues, respectively). The N-terminal region of sarcosine oxidase contains the flavin adenine dinucleotide-binding site [6]. This suggests that the *yjbR* gene product is a flavoprotein. On the other hand, the substrate-binding site is in the C-terminal region of sarcosine oxidase [7].

Compatibilities of the *yjbR*-coding sequence with known tertiary structures of several proteins were also analyzed (Table 1). The *yjbR*-coding sequence was possibly compatible with the tertiary structure of the porcine kidney D-amino acid oxidase (PDB entry name: 1AA8; [17]), because of the lower level of a compatibility score (−2.69). In fact, pairs with scores of −2.5 or lower may be compatible, and scores of −3.0 or lower often indicate good compatibility [14]. However, the sequence homology between the *yjbR* gene product and the porcine kidney D-amino acid oxidase was very low (11.3% identical amino acid residues, Table 1). The homology of 50 residues of the N-terminal regions was relatively higher (22.0% identical amino acid residues). D-Amino acid oxidase (EC 1.4.3.3) is also a flavoprotein and catalyzes the oxidative deamination of D-amino acids to form α-ketoacids, ammonium, and hydrogen peroxide [18]. The enzymatic functions of the porcine kidney D-amino acid oxidase have been analyzed [19,20].

Table 1
Compatibility of the *yjbR*-coding sequence with known structures

A	B	C	Sc
1AA8	326	11.3	−2.69
3GRS	351	10.3	−2.26
2SIL	335	8.0	−2.17
1POXA	362	8.2	−2.16
2CSTA	360	10.1	−2.11

A: Structure (PDB entry name: 1AA8, D-amino acid oxidase; 3GRS, glutathione reductase; 2SIL, sialidase; 1POXA, pyruvate oxidase; 2CSTA, aspartate aminotransferase). B: Number of residues mounted on the structure. C: % sequence identity. Sc: Compatibility score.

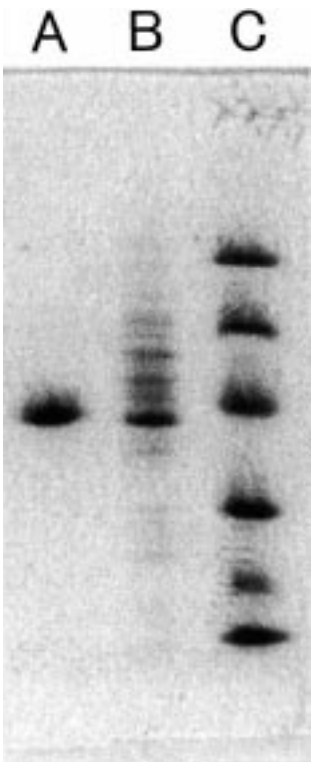

Fig. 1. SDS-PAGE of the *yjbR* gene product. A: The purified protein; B: crude extract of *E. coli* JM109(pBSOLR2); C: molecular weight marker (phosphorylase *b*, 94.0 kDa; bovine serum albumin, 67.0 kDa; ovalbumin, 43.0 kDa; carbonic anhydrase, 30.0 kDa; soybean trypsin inhibitor, 20.1 kDa; α-lactalbumin, 14.4 kDa).

From these data, we expected that the *yjbR* gene product may be a novel type of flavin-containing oxidases and may recognize the common glycine backbone of sarcosine and D-amino acids as substrates.

3.2. Overproduction and purification of the yjbR gene product

The recombinant plasmid pBSOLR2 containing *yjbR* was constructed as described in Section 2. When *E. coli* JM109(pBSOLR2) was grown in 100 ml of Terrific broth

Table 2
Substrate specificity of the *yjbR* gene product

Substrate	Relative activity (%)
Sarcosine	100
Glycine	77.4
N-Ethylglycine	85.3
N,*N*-Dimethylglycine	ND
L-Alanine	ND
D-Alanine	7.4
D-2-Aminobutyrate	2.2
D-Valine	4.8
D-Leucine	ND
D-Serine	ND
D-Aspartate	ND
L-Proline	ND
D-Proline	15.1
N-Methyl-L-alanine	ND
N-Methyl-D-alanine	16.9

At 10 mM substrate in 50 mM potassium phosphate buffer (pH 8.0). ND: not detected.

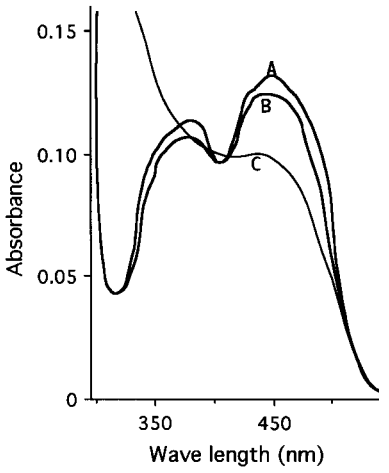

Fig. 2. Absorption spectra of the purified *yjbR* gene product. The spectra were recorded with approximately 1.0 mg/ml of the protein in 50 mM potassium phosphate buffer (pH 8.0). Curve A: The native protein; curve B: the protein heated at 100°C for 5 min; curve C: the protein treated with 10 mM sarcosine under anaerobic conditions.

containing 1.0 mM IPTG at 37°C, 0.45 U/ml (0.027 U/OD_{660}) of the maximal sarcosine oxidase activity was detected. The activities of the parental *B. subtilis* strain and the host strain were not detected. Thus, overproduction of the *yjbR* gene product was achieved by constructing the plasmid pBSOLR2.

Purification of the *yjbR* gene product was performed as described in Section 2. Finally, the purified protein gave a single band on SDS-PAGE (Fig. 1) and exhibited an absorption spectrum characteristic of flavoprotein (Fig. 2). The enzyme effectively catalyzed the oxidation of sarcosine (*N*-methylglycine), *N*-ethylglycine, and glycine (Table 2). Lower activities on D-alanine, D-valine, and D-proline were detected although no activities were shown on L-amino acids and other D-amino acids. Sarcosine oxidase is not an amino acid oxidase and cannot recognize glycine as substrate [2]. On the other hand, D-amino acid oxidase poorly recognizes glycine and amino acid derivatives of the modified amino group as substrates although it effectively oxidizes D-alanine [18]. Therefore, this protein (*yjbR* gene product) is not a type of sarcosine oxidase (demethylating) but a novel type of amino acid oxidase. This protein was regarded as a novel sarcosine:oxygen oxidoreductase (deaminating) and named glycine oxidase as a common name. We designated the *B. subtilis* glycine oxidase as GoxB.

Table 3
Kinetic parameters of GoxB

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
Sarcosine	0.22	1.6	7.3
Glycine	0.99	1.3	1.3
N-Ethylglycine	0.66	1.4	2.1
D-Alanine	81	1.1	0.014
D-Proline	46	1.3	0.028

At 50 mM potassium phosphate buffer (pH 8.0). ND: not detected.

GoxB showed relatively higher activities on substrates of the modified amino group, such as *N*-methyl-D-alanine and D-proline, than D-alanine and D-2-aminobutyrate (Table 2). Sarcosine oxidase also recognizes *N*-ethylglycine, *N*-methylalanine, and proline as substrates [4,21,22]. It appears that the substrate recognition of GoxB is close to that of sarcosine oxidases although the enzyme reaction catalyzed was different. This similarity of the substrate specificities may be related to the homology with the sequences of GoxB and sarcosine oxidases.

3.3. Properties and kinetic parameters of GoxB

The molecular weight of the GoxB subunit was estimated as 42.0 kDa from SDS-PAGE (Fig. 1), which was consistent with the result of the sequence data (40.9 kDa). The molecular weight of the native enzyme was estimated to be 159 kDa by gel filtration. It appears that GoxB acts as a tetramer of identical subunits. The isoelectric point was estimated to be

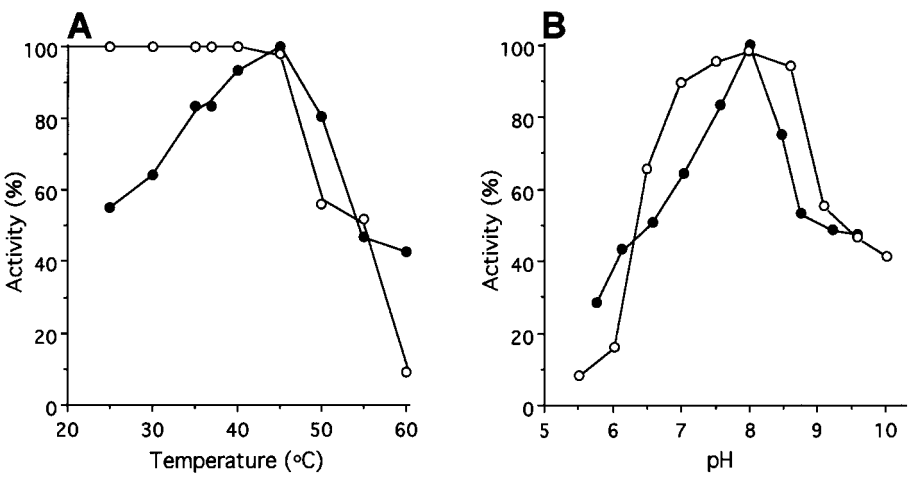

Fig. 3. The effects of temperature and pH on the activity and stability of GoxB. A: Effects of temperature. The enzyme activities at various temperatures were assayed. To examine the thermal stability, the enzyme was incubated at various temperatures for 10 min and immediately cooled. The remaining activities were assayed at 37°C. Symbols: ●, activity; ○, thermal stability. B: Effects of pH. For the pH test, 50 mM potassium phosphate buffer (pH 5.5–8.0), Tris-HCl buffer (pH 8.5), and glycine-NaCl-NaOH buffer (pH 9.0–10.0) were used. The enzyme activities at various pHs were assayed. To examine pH stability, the enzyme was incubated at 25°C for 5 h, and the remaining activities were assayed at pH 8.0. Symbols: ●, activity; ○, pH stability.

5.8 ± 0.2, which was consistent with the value 5.9 calculated from pK_a of amino acid residues [23]. The specific activity of the purified GoxB was 0.59 and 0.46 U/mg for sarcosine and glycine, respectively. By the enzymatic assay of ammonium with the glutamate dehydrogenase-NADH system [24], it was verified that GoxB deaminated glycine. It was also verified that GoxB did not form formaldehyde from sarcosine by the formaldehyde dehydrogenase-NAD system [25], although sarcosine oxidase formed formaldehyde (data not shown). The effects of metal ions and some inhibitors on the enzyme activity were examined, and 2.0 mM of Cd^{2+}, Cu^{2+}, Ag^{2+}, Hg^{2+}, and *p*-chloromercuribenzoate were markedly inhibitory to the enzyme activity (data not shown). The effects of temperature and pH on the activity and stability were also examined, and the results are shown in Fig. 3. The optimum temperature and the optimum pH were 45°C and 8.0, respectively. GoxB was stable up to 45°C after incubation for 10 min. During incubation at 25°C for 5 h, the enzyme was most stable between pH 7.5 and 8.5, where a loss of less than 10% activity was observed.

The kinetic parameters of GoxB for sarcosine, glycine, *N*-ethylglycine, D-alanine, and D-proline were compared (Table 3). While the k_{cat} values of GoxB were nearly the same level in spite of changes in substrate, the K_m values were markedly altered. Therefore, the substrate specificity is mainly dependent on the substrate affinity.

The specific activity and k_{cat} values of GoxB for glycine were approximately less than 1/10 of those of SoxA for sarcosine [3]. As in our previous experiments, when the SoxA-producing recombinant strain was cultivated in the presence of sarcosine, growth was inhibited by excess hydrogen peroxides which were formed by the enzyme reaction. Since glycine oxidase acts on glycine and oxygen molecules in the cell resulting in the production of excess hydrogen peroxides, the low activity might be needed not to be hazardous. We think that the finding of glycine oxidase was probably hard because of the low activity until the success of the genome project.

Analysis of the enzymatic functions of GoxB using the protein engineering techniques is now in progress.

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