A common precursor for the three subunits of L-glutamate oxidase encoded by *gox* gene from *Streptomyces platensis* NTU3304

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Abstract: A segment of DNA containing the L-glutamate oxidase (*gox*) gene from *Streptomyces platensis* NTU3304 was cloned. The entire nucleotide sequence of the protein-coding portion consisting of 2130 bp (710 codons, including AUG and UGA) of the cloned DNA fragment was determined. The *gox* gene contained only one open reading frame (ORF) which coded for a 78-kDa polypeptide, the precursor of active extracellular Gox. Mature Gox is composed of three subunits, designated as α, β, and γ, with molecular masses of 39, 19, and 16 kDa, respectively. Analyses of the N-terminal amino acid sequences of the subunits revealed that the order of subunits in the precursor polypeptide encoded by the ORF, from N-terminus to C-terminus, is α–γ–β. The presence of the flavin adenine dinucleotide (FAD)-binding motif place Gox as a member of the flavoenzyme family. Furthermore, a negative effect of glucose on the biosynthesis of Gox was observed when it was used as carbon source.

Key words: L-glutamate oxidase, *gox* gene, signal peptide, DNA sequence, flavoenzyme, pIJ702 vector.

Résumé : Nous avons cloné un segment d'ADN de *Streptomyces platensis* NTU3304 contenant le gène de la L-glutamate oxidase (*gox*). La séquence complète de nucléotides de la portion codante de l'ADN cloné, représentant 2130 pb (710 codons, incluant les AUG et UGA), fut déterminée. Le gène *gox* contenait seulement un cadre de lecture ouvert (CLO) qui codait un polypeptide de 78-kDa, le précurseur de la protéine Gox extracellulaire. La protéine Gox mature est formée de trois sous-unités, nommées α, β, et γ, ayant des masses moléculaires respectives de 39, 19, et 16 kDa. Les analyses des séquence amino-terminales des acides aminés des sous-unités ont révélé que l'ordre des sous-unités, telle que retrouvée dans le polypeptide précurseur codé par le CLO, est α–γ–β, de la terminaison aminée à la terminaison carboxy. Le présence du motif de liaison à la flavine adénine dinucléotide (FAD) place Gox dans la famille des flavoenzymes. Dans une autre expérience, le glucose a eu un effet négatif sur la biosynthèse de Gox lorsqu'il était utilisé en tant que source de carbone.

Mots clés : L-glutamate oxidase, gène *gox*, peptide de signal, séquence d'ADN, flavoenzyme, vecteur pIJ702.

[Traduit par la Rédaction]

Introduction

L-Glutamate oxidases (EC 1.4.3.11) produced by some *Streptomyces* spp. are flavoenzymes which catalyze the oxidative deamination of L-glutamate to α-ketoglutarate with the reduction of oxygen to hydrogen peroxide (Kusakabe et al. 1983; Matsuzaki et al. 1982; Bohmer et al. 1989; Chen and Su 1991). Based on this unique enzymatic reaction, L-glutamate oxidases make useful analytical tools for the quantitative assay of L-glutamic acid produced in fermentation processes (Chen and Su 1991). This enzyme has also attracted considerable attention due to its potential application in the clinical diagnosis of liver function, where it is used to measure serum glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) levels in the clinical laboratory (Cooper et al. 1991). Furthermore, by taking advantage of its specificity for L-glutamate, this enzyme is also useful in evaluating the quality of foods (Chen and Su 1991; Blankkenstein and Preuschoff 1993). Another remarkable advantage of this enzyme is its potential for use together with an O_2 and H_2O_2 electrode in the construction of an amperometric biosensor for L-glutamic acid or GOT/GPT (Chen and Su 1991; Cooper et al. 1991).

Many microorganisms can produce L-amino acid oxidases (Stumpf and Green 1944; Knight 1948; Burton 1951; Duerre et al. 1975); however, L-glutamate oxidases are produced mainly by *Streptomyces* spp. Although the properties of L-glutamate oxidase have been reported (Matsuzaki et al. 1982; Kusakabe et al. 1983; Bohmer et al. 1989; Chen and

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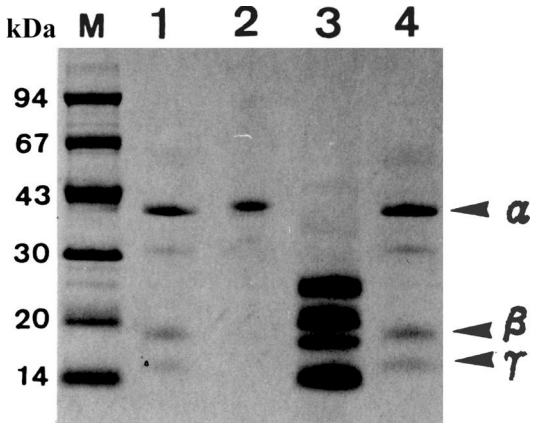
Fig. 1. SDS-PAGE of purified L-glutamate oxidase. The enzyme was purified from the culture broth. Lanes 1 and 4: α, β, and γ subunits of L-glutamate oxidase from *Streptomyces platensis* NTU3304 and *Streptomyces lividans* (pWLC09), respectively, are indicated by arrows on the right. Lane 2: alcohol dehydrogenase (39.8 kDa). Lane 3: trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa), myoglobin (17.6 kDa), α-lactalbumin (14.2 kDa). M indicates standard protein markers including phosphorylase B (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.2 kDa).



Su 1991), the information concerning the *gox* gene encoding this enzyme is not available. In this study, we reported on the cloning, organization, and sequence analyses of the gene encoding L-glutamate oxidase from *S. platensis* NTU3304.

Materials and methods

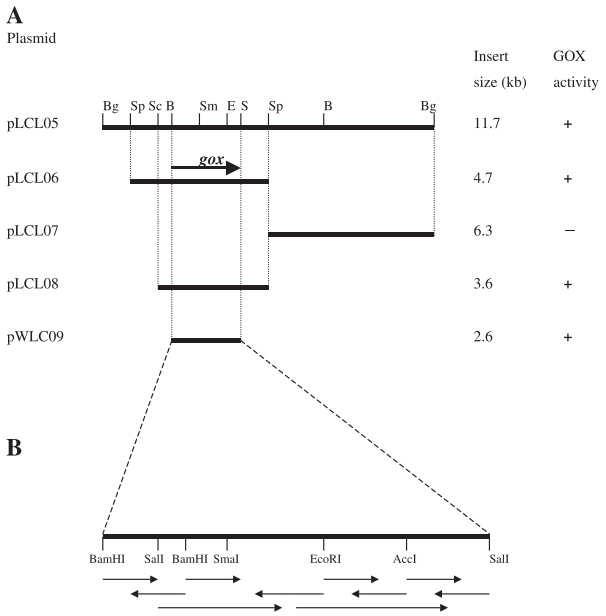
Bacterial strains and plasmids

Streptomyces platensis NTU3304 was isolated from a soil sample collected in Taiwan (Chen and Su 1991) and deposited in the Culture Collection Station at Department of Agricultural Chemistry, National Taiwan University, Taipei, Taiwan, ROC. *Streptomyces lividans* 66, *Escherichia coli* JM109, *Streptomyces* plasmid pIJ702, and *E. coli* plasmid pUC19 were purchased from the Cultural Collection and Research Center (CCRC), Hsinchu, Taiwan, ROC.

Media and production of L-glutamate oxidase

Streptomyces strains were aerobically grown at 28°C in YEME and R2YE media (Kieser 1984). *Streptomyces lividans* 66 carrying pIJ702 and its derivatives were grown in the presence of 50 μg/mL thiostrepton (Hopwood et al. 1985). *Escherichia coli* strains containing pUC19 were grown in Luria-Bertani Broth (LB) or Luria-Bertani Agar (LA) containing 100 μg/mL ampicillin (Maniatis et al. 1989). The medium for production of Gox contained 1% potato starch (Sigma), 2.0% defatted soymeal (Difco), 0.5% yeast extract (Difco), 0.05% KH_2PO_4 (Fisher Scientific Co.), 0.05% $MgSO_4 \cdot 7H_2O$, 0.6% $CaCl_2 \cdot 2H_2O$ (Mallinckrodt Chemical Works), and 0.005% monosodium L-glutamate (Sigma) at pH 8.0. In the experiment for studying the effect of carbon source on the biosynthesis of Gox, potato starch was replaced with different carbon source including monosaccharide, disaccharide, and cellulose. Production of Gox was carried out in a triple-baffled 300 mL Erlenmeyer flask (Bellco Glass, Inc., N.J.) containing 50 mL of the production medium, and capped with a cotton gauze closure. All flasks were incubated on a shaker (Hotech, model 706R, Taiwan) running at 240 rpm and 27°C for 8 days. Cell growth was measured by packed cell volume in mL per 10 mL of fermentation broth. A 10 mL volume of the 8-day-old fermentation broth sample was centrifuged at 3000 × *g* for 10 min. The packed cell volume was measured immediately after the centrifugation.

Fig. 2. (A) A detailed restriction map of pLCL05 and its derivative plasmids. Only the position of relevant restriction enzyme cleavage sites are shown. The thick line represents the cloned DNA fragment from *S. platensis* NTU3304 and the thin one the pIJ702 vector. Gox activity for cells carrying these plasmids is indicated by symbols + and –, and the molecular sizes of the insert in these plasmids are given in kb. The minimal DNA size required for expression of *gox* gene is also indicated by an arrow. Abbreviations: Bg, *Bgl*II; Sp, *Sph*I; Sc, *Sca*I; B, *Bam*HI, Sm, *Sma*I; E, *Eco*RI; S, *Sal*I. (B) Strategy for sequencing the 2.6-kb DNA fragment of *gox* gene. The restriction sites used for subcloning DNA fragment into the pUC19 vectors are indicated. The thin arrows represent the directions of DNA sequencing.



Gox assay and isolation of the enzyme

Gox activity was measured spectrophotometrically by the 4-aminoantipyrine/phenol method (Allain et al. 1974). One unit of activity was defined as the formation of 1 μmol of hydrogen peroxide per min at 25°C under the standard conditions previously described. The enzyme was purified from the culture broth of *S. platensis* NTU3304 and *S. lividans* 66 transformed with pWLC09 (Fig. 2). Briefly, an 8-day-old culture grown in production medium was centrifuged to remove mycelia. The supernatant was filtrated with an UF membrane YM10 (Millipore, U.S.A.) to remove those proteins with a molecular mass below 10 kDa. Then, solid ammonium sulfate was added to give 50% saturation for precipitating Gox. The precipitate collected by centrifugation at 8000 × *g* for 30 min was dissolved in and dialyzed against 10 mM Tris–HCl (pH 8.0). The dialysate was applied to a Fractogel TSK DEAE-650M anion-exchange chromatography column equilibrated with 10 mM Tris–HCl (pH 8.0), and eluted with a linear gradient of NaCl (0.0–0.8 M) in 10 mM Tris–HCl (pH 8.0). The active fractions of Gox were collected and further purified by a Phenyl-Toyopearl 650 M hydrophobic interaction chromatography column which was equilibrated with 10 mM Tris–HCl (pH 8.0) containing 0.6 M $(NH_4)_2SO_4$, and eluted with a linear gradient of 1.0–0.0 M $(NH_4)_2SO_4$ in the same buffer. The active fraction from the second column was applied to

Fig. 3. Nucleotide sequence of the *S. platensis* NTU3304 *Bam*HI-*Sal*I fragment in plasmid pWLC09. The complete nt sequence for the 78-kDa peptide is shown with the (SD) core sequence boxed. The putative signal peptide is underlined and the processing site is indicated by a vertical arrow. Asterisks indicate the stop codon. Invert arrows indicate the transcriptional terminator. The N-terminal sequences for the 39-kDa (α), 19-kDa (β), and 16-kDa (γ) peptide subunits are boxed. In the upstream from the regulatory element of *gox* gene, the directed repeat sequences are indicated by horizontal arrows.

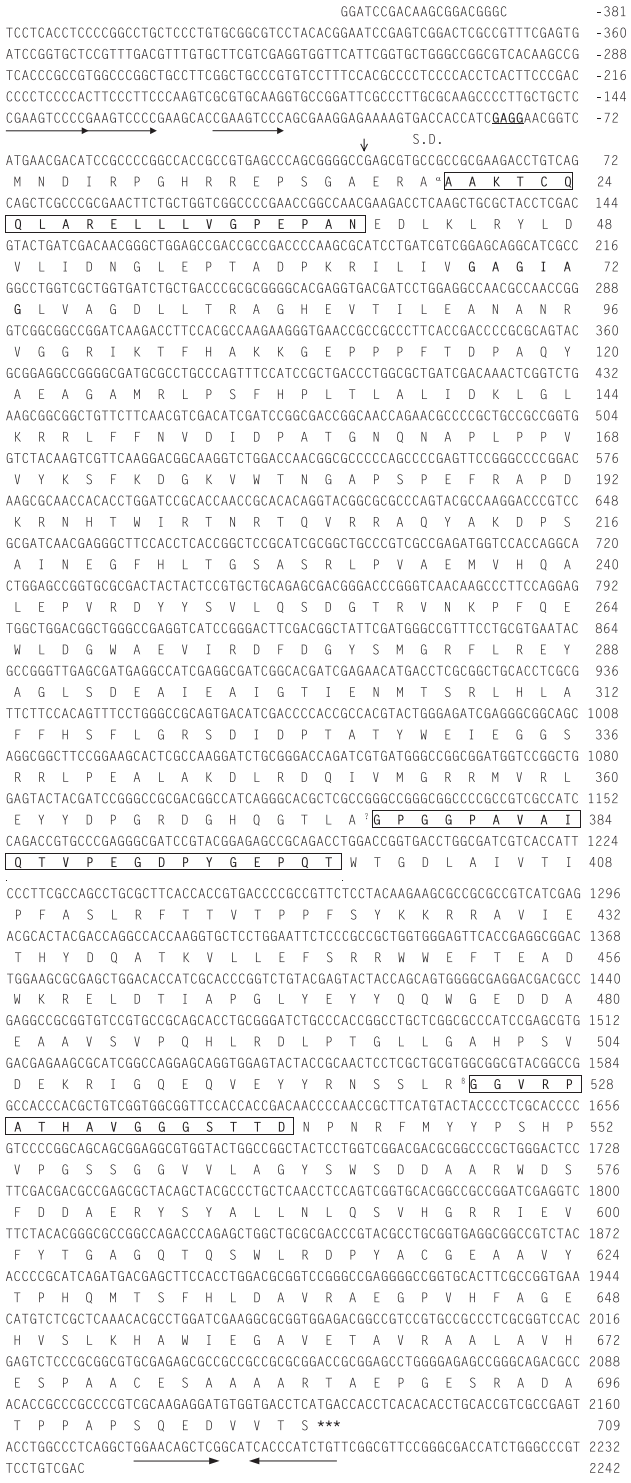


Table 1. Enzyme activity of L-glutamate oxidase produced by *S. platensis* NTU3304 and *S. lividans* 66 containing different recombinant plasmid.[a]

Strain	Total activity of L-glutamate oxidase (U)[b]	
	Extracellular	Intracellular
Streptomyces platensis NTU 3304	107±11	50±1
S. lividans 66 (pIJ702)[c]	ND[d]	ND
S. lividans 66 (pLCL05)	557±23	205±15
S. lividans 66 (pLCL08)	563±20	215±12
S. lividans 66 (pWLC09)	795±26	425±25

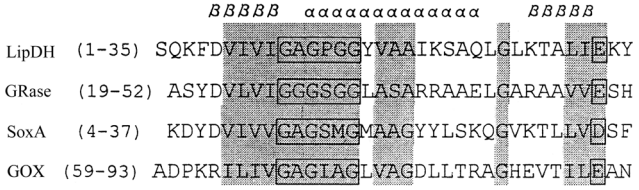
[a]Cells were cultivated for 5 days in 100 mL of production medium on a shaking incubator at 28°C. After centrifugation of the culture broth, total Gox activities of the supernatant and the packed cell were assayed, respectively. The Gox activities of the packed cell measured by using the cell extracts in 50 mM Tris–HCl buffer (pH 8.0) obtained by ultrasonication represent intracellular Gox and those Gox activities of supernatant represent extracellular Gox.

[b]Each value is the mean ± standard deviation calculated from the data of three experiments.

[c]The strain was transformed with the recombinant plasmid indicated in parentheses.

[d]ND, not detected.

Fig. 4. Comparison of amino acid sequences involved in FAD-binding motifs of L-glutamate oxidase and other flavoenzymes. Conserved amino acid residues are indicated by shadow. Residues (G-X-G-X-X-G motif) important for interacting with dinucleotide structure of FAD are boxed. LAOBc: L-amino acid oxidase from *Bacillus cereus*; LAONc: L-amino acid oxidase from *Neurospora crassa*; SoxA: sarcosine oxidase from *Arthrobacter* sp. TE1826.



a Sephadex G-150 gel filtration chromatography column and eluted with 10 mM Tris–HCl (pH 8.0). The purity of Gox was judged by polyacrylamide gel electrophoresis. The Gox activity of the packed cell was assayed by using the cell extracts in 50 mM Tris–HCl buffer (pH 8.0) obtained by sonication.

N-terminal amino acid sequence analysis

Amino acid sequence analysis was performed by Edman degradation method (Matsudaira 1987). Briefly, the three subunits of the purified Gox separated on the SDS-PAGE gel were electroblotted onto a polyvinylidenedifluoride membrane. After being visualized with Amido black stain, the membrane was cut into three pieces containing the respective subunit protein. The membrane pieces were directly applied to a protein sequencer (model 477A: Applied Biosystems, Forster City, Calif.) for N-terminal sequence analysis.

DNA isolation and manipulation

Plasmid DNA was prepared according to the method of Kieser et al. (1984). Isolation of chromosomal DNA from *S. platensis* NTU3304 and transformation of *S. lividans* 66 with the recombinant plasmid DNA were performed by the methods of Hopwood et al. (1985), while the transformation of *E. coli* was performed by the method of Maniatis et al. (1989). Restriction endonuclease di-

Table 2. Purification of L-glutamate oxidase of *S. lividans* 66 (pWLC09).

Step	Volume (mL)	Total activity (U)	Protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (*n*-fold)
Filtrate	1800	12871	4347.2	2.96	100	1
Ammonium sulfate	200	8652	477.59	18.12	67.22	6.12
DEAE-Fractogel	178	8104	257.92	31.42	62.96	10.61
Phenyl-Toyopearl	120	7721	123.35	62.59	59.99	21.15
Sephadex G-150	100	7119	109.21	65.18	55.31	22.02

gestion, agarose gel electrophoresis, and isolation of DNA fragment from the low melting agarose (Sigma) were done according to Maniatis et al. (1989).

Cloning of *gox* gene

Chromosomal DNA prepared from *S. platensis* NTU3304 was partially digested with *Sau*3A. The chromosomal DNA fragments with a molecular size ranging from 9–15 kb isolated from the low melting agarose gel by using Wizard DNA clean-up Kit (Promega) were ligated to *Bgl*II-cleaved pIJ702. The ligated mixture was used to transform the protoplast of *S. lividans* 66, and the transformants regenerated on R2YE agar plate were selected by overlaying a soft agar containing thiostrepton (Hopwood et al. 1985; Katz et al. 1983).

To detect the Gox producing colonies, a Gox assay based on a colorimetric reaction of L-glutamic acid with Gox and peroxidase (Allain et al. 1974) was performed. The enzyme assay mixture included 0.82 mM 4-aminoantipyrine, 14 mM phenol, 1% L-glutamic acid, 2 U/mL horseradish peroxidase, and 1% agar in 0.1 M sodium phosphate buffer (pH 7.0). In the detection experiment, the enzyme assay mixture was overlaid onto colonies grown on agar plate. The Gox activity of the test colonies were observed by the development of red color due to the formation of quinoneimine.

DNA sequencing and nucleotide sequence accession number

The strategy for the DNA sequencing of *gox* gene is illustrated in Fig. 2B. Briefly, the *Bam*HI-*Sal*I DNA fragment of pWLC09 was digested with the restriction enzymes indicated in Fig. 2B, and the resultant fragments were respectively subcloned into pUC19. Nucleotide sequences of plasmid DNA were determined on both strands by primer walking, using the dideoxy nucleotide chain-termination procedure (Sanger et al. 1977). The nucleotide sequence in Fig. 3 has been submitted to the GenBank Nucleotide Sequence Data Library under accession No. AF239797.

Western immunoblot analysis

The cells of *S. lividans* 66 (pIJ702) and *S. lividans* 66 (pWLC09) grown in the production medium were suspended in 1 to 2 volumes of 0.05 M Tris–HCl (pH 7.5). They were then sonicated in a sonicator (Soniprep 150, MSE, Leicestershire LE11 OTR); sonication was applied at an amplitude of 18–22 microns 6 times with 5-min cooling intervals every 3 min. Sonic extracts were centrifuged at 45 000 × *g* for 30 min. The supernatant was mixed with an equal volume of sample buffer (containing 0.1% β-mercaptoethanol, 12% glycerol, 50 mM Tris–HCl, pH 6.8), separated by SDS-PAGE, and transferred to a nitrocellulose membrane (Hybond-C Super, Amersham). After blocking with 5% skim milk in PBS, the membrane was reacted with rabbit polyclonal anti-Gox subunit peptide antibodies, which were prepared by intramuscular injection of the respective purified subunit peptide of Gox into rabbit three times with 1-week intervals. The blots were then treated with horseradish peroxidase-conjugated goat anti-rabbit antibody (Amersham), and developed with an ECL system (Amersham).

Table 3. Effect of carbon source on the production of L-glutamate oxidase by *S. lividans* 66/pLCL05.[a]

Carbon source	Relative activity (%)	PCV (mL/10 mL)[b]	Final pH
Glucose	19.06	1.3	7.48
Fructose	86.35	1	7.94
Galactose	49.42	0.9	7.01
Xylose	16	0.7	6.76
Sucrose	36.24	1	8.6
Maltose	48.94	1.2	7.15
Lactose	65.41	1	8.39
Potato starch	100	1.2	8.34
Cellulose	35.76	0.6	8.82

[a]Cultivations were carried out in 300 mL triple-baffled flasks containing 50 mL basal production medium with 2% (w/v) carbon source at 27°C on a shaking incubator for 8 days.

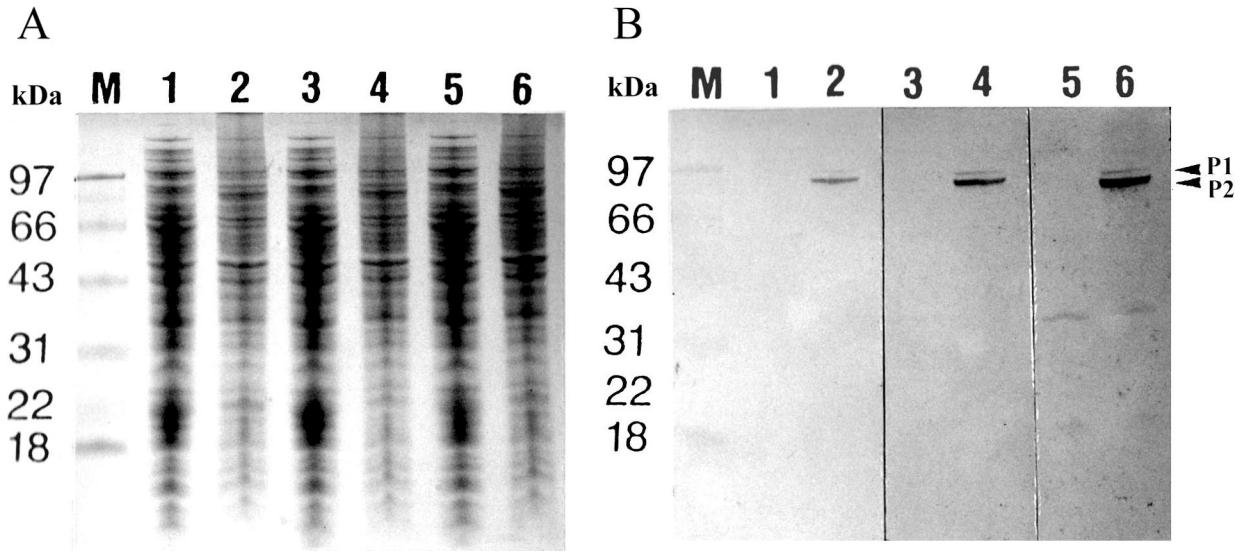
[b]10 mL of the 8-day-old fermentation broth sample was centrifuged at 3000 × *g* for 10 min and the volume of the packed cell was measured immediately after the centrifugation.

Results and discussion

Construction and screening of a *Streptomyces platensis* NTU3304 chromosomal library

Fragments of 9–15 kb were isolated from partially digested *S. platensis* NTU3304 chromosomal DNA, and ligated into the *Bgl*II site of pIJ702. *Streptomyces lividans* 66 cells transformed with the recombinant plasmids were screened by a Gox assay based on a colorimetric reaction of L-glutamic acid with Gox and peroxidase (Allain et al. 1974). Of 5700 colonies containing a plasmid gene bank of *S. platensis* NTU3304 DNA, one *S. lividans* 66 colony capable of growing in the presence of thiostrepton became red in color after the colorimetric reaction test. The recombinant plasmid, designated as pLCL05, isolated from the cells of this colony was employed to retransform *S. lividans* 66. All transformants gave a positive reaction in the colorimetric reaction test. This result suggests the color reaction phenotype is plasmid encoded, since *S. lividans* 66 alone does not have such a phenotype (Table 1). The pLCL05 physical map was determined by digesting the plasmid with a variety of restriction enzymes. It contained an insert of 11.7 kb (Fig. 2), and the DNA fragment in the insert encoding Gox was designated as the *gox* gene. SDS-PAGE analyses of the active enzyme produced by the cells of *gox* gene clone showed that the purified protein was composed of three subunits, and the size of these subunits were consistent with those produced by *S. platensis* NTU3304 (Fig. 1). The Gox activity

Fig. 5. Western immunoblot analysis of intracellular Gox. (A) SDS-PAGE of the cell extracts from *S. lividans* 66/pIJ702 (lanes 1, 3, 5) and *S. lividans* 66/pWLC09 (2, 4, 6). (B) Immunoblotting analysis corresponding to panel A. In panel B, lanes 1 and 2 were immunoblotted with anti-α peptide antibody; lanes 3 and 4 were immunoblotted with anti-β peptide antibody; and lanes 5 and 6 were immunoblotted with anti-γ peptide antibodies. The intact precursor peptide (P1) and the precursor peptide without signal peptide (P2) of Gox in lanes 2, 4, and 6 are indicated by arrows on the right.



produced by *S. lividans* 66 containing pLCL05 was 5-fold higher than that produced by *S. platensis* NTU3304 cells, and no appreciable Gox activity was detected in the *S. lividans* 66 cells containing pIJ702 without an insert (Table 1).

Subcloning of *gox* gene

As shown in Fig. 2, the *gox* gene was located on a 2.6-kb *Bam*HI-*Sal*I fragment after restriction mapping and subcloning of the fragment. The Gox activity produced by *S. lividans* 66 containing pWLC09 (Fig. 2) was eight-fold higher than that of *S. platensis* NTU3304, and had two-fold higher intracellular Gox activity than strains containing pLCL05 and pLCL08 (Table 1). This may be attributed to the higher copy number of recombinant plasmid pWLC09 in the host cell.

SDS-PAGE analysis and amino terminal sequence of Gox

The enzymes produced by *S. lividans* 66 containing pWLC09 and *S. platensis* NTU3304 were purified by sequential ion exchange, hydrophobic interaction, and gel filtration chromatographies. Finally, the purified enzyme with 22-fold purification and 55% recovery yield was obtained (Table 2). SDS-PAGE analysis revealed that the purified active enzyme consisted of three subunit peptides designated as α, β, and γ with a molecular masses of 39, 19, and 16 kDa, respectively (Fig. 1). The SDS-PAGE data (Fig. 1) show four main bands in the purified enzyme preparation from both *S. platensis* and *S. lividans* 66 harboring pWLC09. Besides the α-, β-, γ- subunit protein bands, the fourth protein band of around 34 kDa may be the uncleaved γ-β dimer. To identify the product of the *gox* gene and the cleavage site of the subunits in the precursor polypeptide that gives rise to the mature enzyme, we determined the N-terminal sequence of the purified subunit peptide of Gox. The N-terminal sequence of the respective subunit determined by the Edman degradation method is shown in Fig. 3.

Nucleotide sequence analysis

Analysis of the sequence revealed one open reading frame (ORF) beginning with an ATG codon 381 nucleotides from the *Bam*HI site and ending with a TGA codon 112 nucleotides from *Sal*I site of the *Bam*HI-*Sal*I fragment for a protein sequence of 709 amino acid residues and a putative molecular weight of 78 kDa (Fig. 3). A potential ribosome-binding site for Gox can be located six nucleotides upstream from the initial ATG codon (position 1, Fig. 3). The overall G+C content of *gox* gene is 69%, and is consistent with those G+C contents (62% to 74%) of *Streptomyces* sp. (Hsieh and Jones 1995; Bruhlmann and Keen 1997; Bey et al. 2000; Mendes et al. 2000). The N-terminal amino acid sequence of the α, β, and γ shown in Table 2 are located at position 19–39, 524–540, and 376–398, respectively. The order of the subunit in the precursor polypeptide encoded by the ORF from N-terminus to C-terminus were identified as α–γ–β (Fig. 3) with the predicted molecular masses of 38.94, 16.17, and 19.42 kDa, respectively. These values agree very well with results determined by SDS-PAGE analysis of the active Gox produced by *S. platensis* NTU3304 and the *gox* gene clones (Fig. 1). Since Gox is a secreted enzyme, a precursor to the enzyme should possess a signal that permits its transport to the outside of the cell. Indeed, preceding the N-terminal end of the α subunit, a putative signal peptide can be found (Fig. 3). This proposed sequence meets most of the criteria for a signal peptide characterized by Perlman and Halvorson (1983). For example, the proposed Gox signal peptide has one arginine residue at position 5 and two alanine residues at positions 15 and 18 near the signal cleavage site (Fig. 3). This Ala-X-X-Ala sequence resembles the most frequent protease (signal) cleavage sequence of Ala-X-Ala. These collective observations support the hypothesis that a signal peptide is present for transporting the intact Gox precursor across the cytoplasmic membrane. As compared with other flavoenzymes, the region of the ADP-binding fold of

FAD is well conserved in the α subunit (residues 59–93) of Gox (Fig. 4). The hydrophobic N-terminus of other reported flavoenzymes usually is composed of a common flavin adenine dinucleotide (FAD)-binding domain (Rounchi et al. 1982). As shown in Fig. 4, the sequence of Gly-X-Gly-X-X-Gly (residues 68–73) located in the region of α subunit of the mature Gox is identical with those flavin adenine dinucleotide-binding fold of other flavoenzymes. This strongly suggests that Gox is a member of the flavoenzyme family.

Regulating elements of the nucleotide sequence

A ribosomal binding site (GAGG), which is complementary to the 3′-terminal region of 16S rRNA of *S. lividans* (3′-UCUUUCCUCC-5′) (Suzuki and Yamada 1988), is located 8 bp upstream from the initiation codon (Fig. 3). Although we are unable to identify the promoter sequence upstream from this ribosome-binding site, three direct repeat sequences are found upstream from the coding region. As found in the *gox* gene, other different direct repeat sequences are also found upstream of the galactokinase (*gal*) gene of *S. lividans* (Sibylle et al. 1993) and the chitinase (*chi*) gene of *S. plicatus* (Delic et al. 1992 ; Miyashita et al. 1993; Miyashita et al. 1997). The direct repeat sequences upstream of the *chi* structural gene are implicated in the regulation of two chitinase promoters, which are subject to carbon catabolite control in *Streptomyces* (Delic et al. 1992). Interestingly, another experiment studying the effect of carbon source on the biosynthesis of Gox revealed that *S. platensis* NTU3304 could grow well, but its biosynthesis of Gox was reduced five-fold using glucose instead of potato starch as the carbon source in the basal culture medium (Table 3). This result suggests that the biosynthesis of Gox is subject to carbon catabolite control. Thus, it is possible that the function of the directed repeat sequences located upstream of the *gox* structural gene may be related to the mechanism of carbon catabolite control. An inverted repeat sequence which might serve as transcription terminator is found downstream from the coding region of Gox.

Expression

Streptomyces lividans 66 cells transformed with plasmid pLCL05, pLCL08, pWLC09 (Fig. 2A), and pIJ702 were cultivated at 28°C for 5 days in the production medium. The enzyme activity obtained from the supernatant represents extracellular enzyme. On the other hand, the enzyme activity of the sonicated cell extract represents the intracellular enzyme. Results in Table 1 show that *S. lividans* 66 (pLCL05) and *S. lividans* 66 (pWLC09) produced five- and eight-fold higher levels of both intracellular and extracellular Gox activity, respectively, than those produced by *S. platensis* NTU3304, whereas *S. lividans* carrying pIJ702 does not produce any detectable enzyme activity, demonstrating the expression of *gox* gene in *S. lividans* 66 cells. To investigate the post-translational processing of Gox, we isolated the intracellular gene product from the intracellular preparation, and analyzed the precursor of Gox by immunoblot with polyclonal antibody against subunits α, β, and γ, respectively. As shown in Fig. 5, a major protein (76 kDa) and a minor protein (78 kDa), whose molecular masses are in agreement with those predicted for the gene product of *gox* gene (Fig. 3), were recognized by all three antibodies, suggesting that both proteins are the precursors of Gox. The smaller precursor (76 kDa) in the major band may be produced by the cleavage of signal peptide from the intact precursor (78 kDa).

Acknowledgements

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