J. Biochem. **134**, 805–812 (2003)
DOI: 10.1093/jb/mvg206

Recombinant Expression, Biochemical Characterization and Stabilization through Proteolysis of an L-Glutamate Oxidase from *Streptomyces* sp. X-119-6

Jiro Arima[1], Takashi Tamura[1], Hitoshi Kusakabe[2], Makoto Ashiuchi[3], Toshiharu Yagi[3], Hidehiko Tanaka[1] and Kenji Inagaki[*,1]

[1]Department of Bioresources Chemistry, Faculty of Agriculture, Okayama University, Okayama 700-8530; [2]Research Laboratories, Yamasa Shoyu Co., Ltd., Choshi, Chiba 288-0056; and [3]Department of Bioresources Science, Faculty of Agriculture, Kochi University, Nankoku, Kochi 783-8502

Received May 10, 2003; accepted August 27, 2003

L-Glutamate oxidase (LGOX) from *Streptomyces* sp. X-119-6 is a protein of 150 kDa that has hexamer structure $\alpha_2\beta_2\gamma_2$. The gene encoding LGOX was cloned and heterologously expressed in *Escherichia coli*. LGOX isolated from the *E. coli* transformant had the structure of a one chain polypeptide. Although the recombinant LGOX exhibited catalytic activity, it was inferior to the LGOX isolated from *Streptomyces* sp. X-119-6 in catalytic efficiency. The recombinant LGOX exhibited low thermostability compared to the LGOX isolated from *Streptomyces* sp. X-119-6 and was an aggregated form. Proteolysis of the recombinant LGOX with the metalloendopeptidase from *Streptomyces griseus* (Sgmp) improved its catalytic efficiency at various pH. Furthermore, the Sgmp-treated recombinant LGOX had a subunit structure of $\alpha_2\beta_2\gamma_2$ and nearly the same enzymological character as the LGOX isolated from *Streptomyces* sp. X-119–-6. A higher molecular species observed for the recombinant LGOX was not detected for the Sgmp-treated recombinant LGOX. These results prove that proteolysis by Sgmp is involved in the stabilization of the recombinant LGOX.

Key words: L-glutamate oxidase, precursor form, proteolysis, *Streptomyces* sp.

Abbreviations: LGOX, L-glutamate oxidase; IPTG, isopropyl 1-thio-β-D-galactopyranoside; MBTH, 3-methyl-2-benzothiazoline hydrazone.

L-Glutamate oxidase (LGOX) [EC 1.4.3.11] catalyzes the oxidative deamination of an L-glutamate to a 2-ketoglutarate along with the production of ammonia and hydrogen peroxide *via* an imino acid intermediate. LGOX is classified as an L-amino acid oxidase that has high substrate specificity. This enzyme is a useful analytical tool for the quantitative assaying of L-glutamate existing in food and in a fermentation process. It has also attracted considerable attention due to its potential application to the diagnosis of liver function, *i.e.* it could be used to measure the serum glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) levels in the clinical laboratory (*1*).

There have been some reports on LGOXs purified from genus *Streptomyces* (*2–5*). Common characteristics of these LGOXs are that they are exoenzymes that are stable as to pH and temperature, and are flavoproteins that contain non-covalently bound FAD as a cofactor. Recently, gene cloning and expression of LGOX that has hexamer structure $\alpha_2\beta_2\gamma_2$ from *Streptomyces platensis* was reported (*6*). The LGOX from *S. platensis* is expressed in *Streptomyces lividans* cells as a precursor that is a single polypeptide, and the mature enzyme modified by protease is observed in the extracellular fraction. However, despite these data, nothing is known about its physiological role or structure function relationship, or the processing of the precursor form of LGOX.

Streptomyces sp. X-119-6 produces the same type of enzyme as the LGOX from *Streptomyces platensis* that has hexamer structure $\alpha_2\beta_2\gamma_2$ (*5*). This enzyme was predicted to be expressed as a precursor form that has the structure of a one chain polypeptide. Although information concerning the gene encoding the LGOX is available, the properties of the single polypeptide enzyme, the precursor form of LGOX, remain unclear. In this paper, we report on recombinant expression in *E. coli*, characterization, and proteolysis of the recombinant LGOX that has the structure of a one chain polypeptide. Our studies also indicate that proteolysis is involved in the activation and stabilization of the structure under various conditions.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions—*Streptomyces* sp. X-119-6 (*5*) was used as the donor strain for the *Lgox* gene. Plasmid pUC19 and phage λZAP Express (*7*) containing the phagimid pBK-CMV region were used as the cloning vector. Plasmid pKK223-3 (*8*) was used as the expression vectors. *Escherichia coli* JM109 (*9*) and XL1-blue MRF′ were used as the host strains for general cloning procedures.

Streptomyces sp. X-119-6 was grown aerobically at 30°C in a YEME medium [0.3% yeast extract, 0.5% polypeptone, 0.3% malt extract, 34% sucrose, 1% glucose,

*To whom correspondence should be addressed. Tel: +81-86-251-8299, Fax: +81-86-251-8299, E-mail: kinagaki@cc.okayama-u.ac.jp

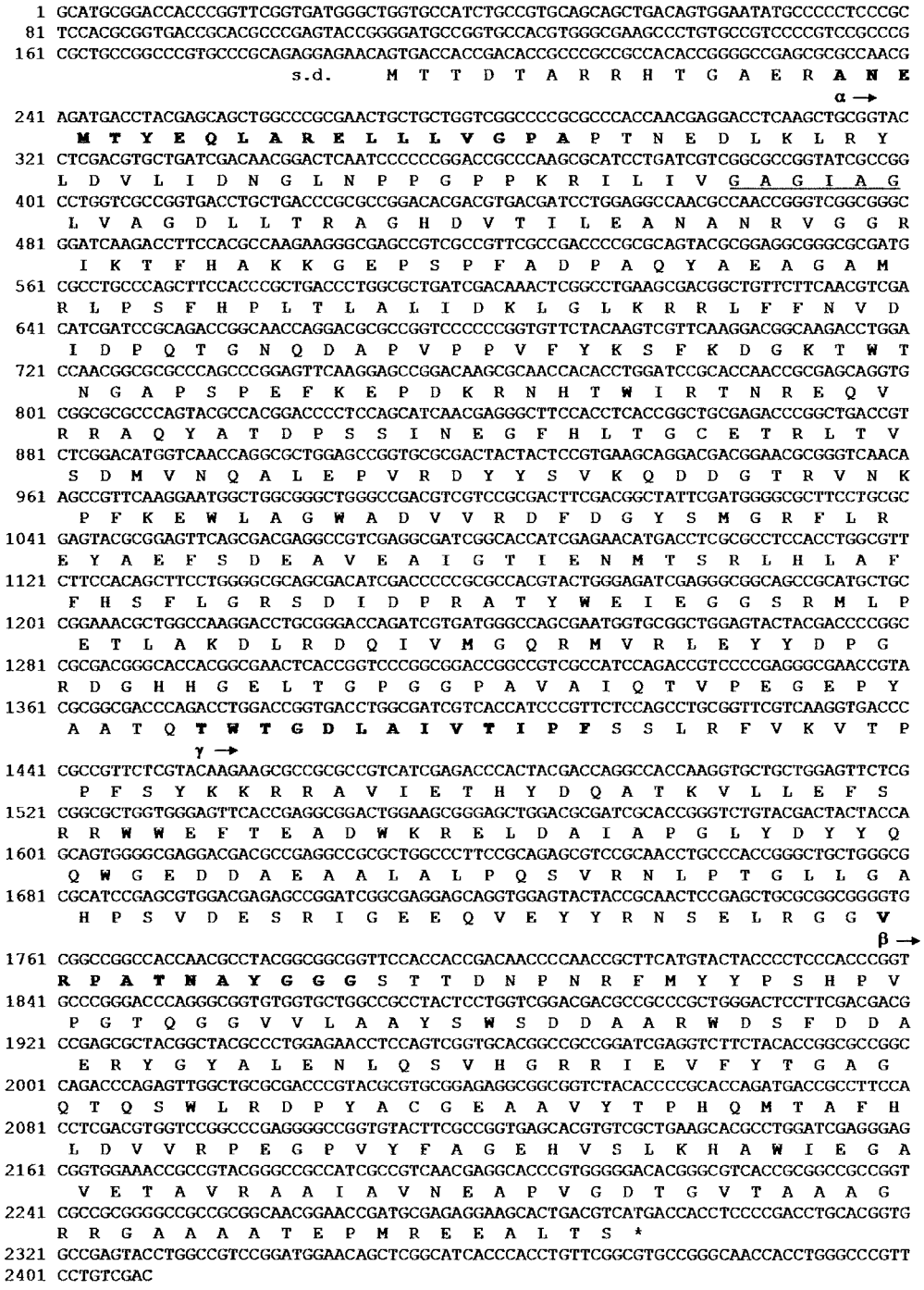

Fig. 1. **Nucleotide sequence of the L-glutamate oxidase gene and the deduced amino acid sequence.** The deduced amino acid sequence is given under the nucleotide sequence. The putative ribosome binding site is underlined in the nucleotide sequence. The stop codon is indicated by an asterisk. The N-terminal amino acid sequences of the subunits, α, β, and γ, are given in bold letters. Residues (G-X-G-X-X-G motif) important for interaction with the dinucleotide structure of FAD are underlined in the amino acid sequence. The conserved TKVLL sequence is boxed.

0.5% $MgCl_2$ $6H_2O$, and 0.5% glycine, (pH 7.3)] (*10*). The *E. coli* transformant was routinely grown aerobically at 37°C or 22°C in 2× TY medium (*11*) containing suitable concentrations of antibiotics.

General Methods—The techniques used for restriction enzyme digestion, ligation, transformation, and other standard molecular biology manipulations were based on methods described by Maniatis *et al.* (*11*). Chromosomal DNA of *Streptomyces* sp. X-119-6 was prepared by the method of Saito and Miura (*12*). Plasmid DNA was prepared by the alkaline extraction procedure (*13*). Hybridization of DNA on a membrane was performed as described by Southern (*14*). The nucleotide sequence was determined by the dideoxy chain termination method (*15*). A search for the amino acid sequence was performed with the BLAST program (*16*). Protein was analyzed by

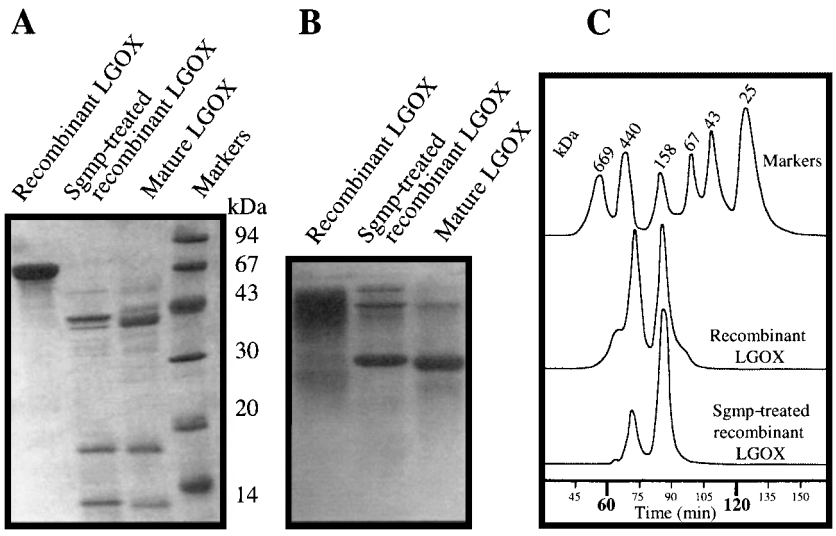

Fig. 2. **Electrophoretic and chromatographic analyse of the recombinant LGOX and Sgmp-treated recombinant LGOX.** (A) 15% SDS-PAGE of the recombinant LGOX, its Sgmp digestion products, and the mature LGOX from *Streptomyces* sp X-119-6. Molecular mass markers are shown on the right-hand side of the gel. (B) 15% native PAGE of the recombinant LGOX, its Sgmp digestion products, and the mature LGOX isolated from *Streptomyces* sp X-119-6. (C) FPLC gel-filtration profiles of the recombinant LGOX (2 mg/injection), and the Sgmp-treated recombinant LGOX (2 mg/injection).

SDS-PAGE under denaturing conditions (*17*). The gels were stained with Coomassie brilliant blue. N-Terminal amino acid sequencing was performed with a sequanator (model 477A) from Applied Biosystems (Warrington). The native molecular mass of the purified LGOX was determined by Superdex 200 gel filtration using a fast protein liquid chromatography system.

Cloning of the Lgox Gene—For the screening for genomic DNA fragments containing the *Lgox* gene, a complementary oligonucleotide [5′-AACGAGATGAC(CG)TACGAGCA-3′, 20 bp] was designed from the N-terminal amino acid sequence (-Asn-Glu-Met-Thr-Tyr-Glu-Gln-). The oligonucleotide was radiolabeled with T4 polynucleotide kinase and [γ-^{32}P]ATP, and then used as a hybridization probe. A genomic library was constructed with *Bam*HI restriction fragment 1–3 kb and *Bam*HI-digested pUC19. This library was screened by colony hybridization with a ^{32}P-end labeled oligonucleotide probe (*11*). One positive clone harboring the recombinant plasmid, pGB1, was obtained. A second genomic library was constructed with *Sac*I restriction fragment 4–6 kb and λZAP Express. This library was screened with the insert fragment of pGB1 labeled with [α-^{32}P]dCTP (220 TBq/mmol; Amersham International plc, Little Chalfont, England) (*18*) as a probe. The positive plaque was then subcloned into phagimed pBK-CMV by the single clone excision method (*12*, *19*)

Construction of an Expression Vector—To construct an expression plasmid for the *Lgox* gene product, the proposed translational start codon of *Lgox*, an *Eco*RI site and a *Pst*I site were introduced by PCR. The *Lgox* gene that lacked the putative signal peptide region was amplified by PCR using sense primer U1 (5′-CCACACCGGGGCCGAATTCATGAACGAGAT-3′) and antisense primer L1 (5′-AGGTACTCGGCCACCCTGCAGGTC-3′). The PCR product was subcloned into pKK223-3 (yielding pKK-LGOX). The final plasmid, pKK-LGOX, was confirmed by sequencing.

Enzyme Assays—Monitoring of the increase in hydrogen peroxide was performed by the 4-aminoantipyrine phenol method (*20*) In the assay, 0.2 ml of 0.5 M L-glutamate was added to 1.8 ml of a mixture comprising 70 mM potassium phosphate, pH 7.4, 0.5 mM 4-aminoantipyrine, 1.7 mM phenol, 10 U/ml of peroxidase and enzyme at 30°C, and then the increase in A_{505} per minute was monitored. One unit of LGOX activity was taken as the amount of enzyme that liberated 1 μmol of hydrogen peroxide per minute. Another LGOX activity assay involved the determination of 2-ketoglutarate formed with 3-methyl-2-benzothiazolone hydrazone hydrochloride as described previously (MBTH method) (*21*). One unit of LGOX activity was taken as the amount of the enzyme that liberated 1 μmol of 2-ketoglutarate per minute.

Purification of the Recombinant LGOX—E. coli JM109 (pKK-LGOX) cells were cultivated at 22°C for 24 h in 3-liters of 2× TY medium. Expression of the LGOX was induced by incubation at 22°C with 0.5 mM IPTG for 24 h. The harvested cells were suspended in 20 mM potassium phosphate buffer (KPB) (pH 7.4), and then disrupted by ultrasonication on ice. After removal of the cell debris, the supernatant was brought to 20% saturation with ammonium sulfate. The resultant supernatant was brought to 45% saturation with ammonium sulfate. The resultant precipitate was dissolved in 20 mM KPB (pH 7.4) containing 20% ammonium sulfate. This solution was loaded onto a Butyl-Toyopearl 650M (Tosoh, Tokyo) column (ϕ4.8 × 15 cm, 270 ml) equilibrated with 20 mM KPB (pH 7.4) containing 20% ammonium sulfate. The column was washed with the equilibration buffer and then eluted with a 1.2-liter linear gradient of 20 to 0% ammonium sulfate at the average flow rate of 4 ml/min. Fractions exhibiting high specific activity were pooled and dialyzed against 20 mM KPB (pH 7.4). The dialyzed solution was loaded onto a DEAE-Toyopearl 650M (Tosoh) column (ϕ4.8 × 10 cm, 180 ml) equilibrated with 20 mM KPB (pH 7.4). The column was washed with the same buffer containing 100 mM NaCl and then eluted with 200 mM NaCl at the average flow rate of 4 ml/min. The enzyme solution was then applied to a Superdex 200 column (ϕ1.6 × 90 cm, 180 ml) equilibrated with 20 mM KPB (pH 7.4) containing 200 mM NaCl. The proteins were eluted at the flow rate of 0.5 ml/min. Protein concentrations of fractions were determined by measuring the absorbance at 280 nm.

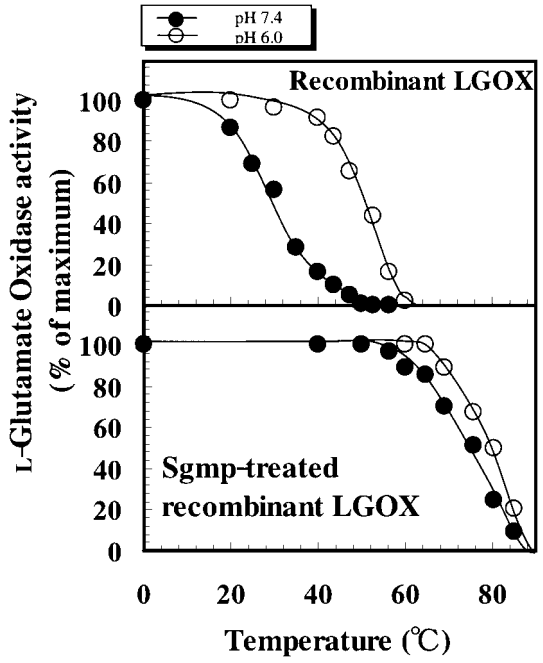

Fig. 3. **Effects of temperature on the stabilities of the recombinant LGOX and Sgmp-treated recombinant LGOX.** Each enzyme was used at 10 μg/ml. Values are expressed as percentages of the maxima (100% = 0.26 and 0.57 μM/min for the recombinant LGOX and Sgmp-treated recombinant LGOX, respectively). The enzyme activity was measured at pH 7.4 after incubation of each enzyme at the indicated temperatures for 30 min.

Proteolysis and Comparison of the Enzymological Characteristics of the Recombinant LGOX and Digested LGOX—Proteases (trypsin, chymotrypsin, and Sgmp) were purchased from Sigma Chemical Protease digestion was carried out by treating a 1 ml aliquot of purified recombinant LGOX (20 mg/ml in 20 mM KPB, pH 7.4) with a 20 μl aliquot of protease (1 mg/ml in 20 mM KPB, pH 7.4) for 4 h at 25°C. The digested sample was purified by DEAE-Toyopearl 650 M column chromatography.

Estimation of the activity at different pH values and temperature was performed using the purified enzymes. To determine the optimal pH, the following buffers (100 mM) were used: acetate buffer (pH 3.4 to 6.2), KPB (pH 6.2 to 8.2), and boric acid buffer (pH 8.2 to pH 10.2). The optimal temperature and thermostability were determined between 0 and 80°C in 20 mM KPB (pH 6.0 and pH 7.4). To determine the thermostability, each enzyme was preincubated for 30 min at the corresponding temperature, and later LGOX activity was measured by the 4-aminoantipyrine phenol method at 30°C. To determine the optimal temperature, each enzyme was preincubated for 5 min at the target temperature, and later LGOX activity was measured by the MBTH method at the same temperature as for the preincubation. Activation energy values were calculated according to Segel (*22*).

RESULTS

Cloning and Sequencing of the Lgox Gene and Expression in E. Coli—Using the methods and conditions described under "MATERIALS AND METHODS," a genomic library of *Streptomyces* sp. X-119-6 was constructed and subsequently screened with the ^{32}P-labeled nucleotide as a probe. Two rounds of screening resulted in several positive phages that contained the entire *Lgox* gene. By the single clone excision method, the inserts of positive phages were placed into phagimid pBK-CMV (pGS1).

The determined nucleotide sequence of the *Lgox* gene in pGS1 contained one open reading frame (Fig. 1). Amino acid sequences obtained by automated Edman degradation of the N-terminus of each subunit of the purified LGOX from *Streptomyces* sp. X-119-6 were identified in the deduced primary amino acid sequence (bold type in Fig. 1). Database searches revealed the identify of the LGOX from *Streptomyces* sp. X-119-6 with the enzyme from *S. platensis*, the sequence similarity being about 85%.

E. coli JM109 harboring pGS1 exhibited no expression of LGOX. To obtain overexpression of the LGOX in *E. coli* JM109, we constructed an expression plasmid (pKK-LGOX) containing the *Lgox* gene that lacked the signal peptide region in the same orientation as the tac promoter of pKK-LGOX, as described under "MATERIALS AND METHODS." The LGOX was expressed constitutively in *E. coli* JM109 in a soluble and active form up to a concentration equivalent to 20% of the total soluble cellular protein (data not shown)

Characterization of the Recombinant LGOX—LGOX expressed in the *E. coli* transformant had a precursor form that showed a molecular mass of 76 kDa on SDS-PAGE. The recombinant LGOX was isolated from *E. coli* JM109 harboring pKK-LGOX with a yield of 300 mg of pure protein, as judged on SDS-PAGE, from a 3-liter culture. The purified enzyme could be stored for several months in 20 mM KPB (pH 7.4) at 4 or –20°C without significant loss of activity. The molecular mass of the recombinant LGOX in natural conditions was estimated by gel filtration chromatography to be 150 kDa, 300 kDa, and higher (Fig. 2C). Comparison of this value with the molecular mass determined from the primary amino acid sequence suggests that the recombinant LGOX exists not only in a dimeric form but also as a higher molecular species. Native PAGE of the recombinant LGOX gave a broad smear that is not possible to detect as a band (Fig. 2B).

The thermostability was examined in the temperature range of 0–60°C at pH 6.0 and 7.4. It was found that the 50% thermal inactivation temperature of the recombinant LGOX was about 30°C at pH 7.4, and about 50°C at pH 6.0 (Fig. 3). The temperature dependence of the activity of the recombinant LGOX was examined in the temperature range of 5–78°C at pH 6.0 and 7.4. The optimum temperature at pH 6.0 was higher than that at pH 7.4. The LGOX precursor showed temperature optima of 35°C at pH 7.4 and 50°C at pH 6.0 (Fig. 4). Arrhenius diagrams of L-glutamate oxidation in the temperature range of 10–30°C allowed calculation of activation energies of 72 and 86 kJ/mol at pH 7.4 and pH 6.0, respectively (Table 1). The pH dependence of k_{cat} was assayed in the pH range of 3.2–10.6. The recombinant LGOX showed a narrow pH optimum of around pH 7.0 (Fig. 5). On measuring the initial reaction rates with different L-glutamate concentrations, a K_m of 5.0 mM and a k_{cat} of 33 s^{-1} were found at pH 7.4. However, a different initial reac-

Table 1. **Properties of the mature LGOX isolated from *Streptomyces* sp.** X-119-6, recombinant LGOX, and Sgmp-treated LGOX.

Properties	Mature LGOX from *Streptomyces* sp. X-119-6	Recombinant LGOX	Sgmp-treated LGOX
Composition[a,b]	$\alpha_2\beta_2\gamma_2$ (5)	Homodimer	$\alpha_2\beta_2\gamma_2$
Optimum temperature (°C) at pH 7.4[c]	–	35	58
Optimum temperature (°C) at pH 6.0[c]	–	50	58
Optimum pH[d]	6.0–8.5 (5)	7.0	6.0–8.5
50% thermal inactivation temperature (°C) at pH 7.4[d,f]	75 (5)	30	75
50% thermal inactivation temperature (°C) at pH 6.0[d,f]	80 (pH 5.5)(5)	50	80
V_{max} (mol/min/mg) at pH 7.4 (30°C)[d]	55.1 (5)	26	57
K_m (mM) at pH 7.4 (30°C)[d]	0.23 (5)	5.0	0.23
K_m (mM) at pH 6.0 (30°C)[d]	–	0.23	0.23
k_{cat} (s^{-1}) at pH 7.4 (30°C)[e]	72.5[g]	33	75
k_{cat} (s^{-1}) at pH 6.0 (30°C)[e]	–	27	49
k_{cat}/K_m (M^{-1} s^{-1}) at pH 7.4 (30°C)	3.2×10^5 [g]	6.6×10^3	3.3×10^5
k_{cat}/K_m (M^{-1} s^{-1}) at pH 6.0 (30°C)	–	1.2×10^5	2.1×10^5
E_a (kJ/mol) at pH 7.4	–	72	41
E_a (kJ/mol) at pH 6.0	–	86	51

[a]Determined by gel filtration. [b]Determined by SDS-PAGE. [c]Measured by MBTH method. [d]Measured by 4-amino antipyrine/phenol method. [e]Calculated from V_{max} values. [f]Incubated for 30 min. [g]Calculated from the value given in Ref. *5*.

tion rate was observed at pH 6.0, *i.e.* a K_m of 0.23 mM and a k_{cat} of 27 s^{-1}.

Proteolysis of the Recombinant LGOX—The recombinant LGOX has a precursor form that has the structure of a one chain polypeptide. Table 1 indicates that this enzyme was inferior to the LGOX isolated from *Streptomyces* sp. X-119-6 in catalytic efficiency and stability. Therefore, we investigated how the properties of the recombinant LGOX were affected by proteolysis. Trypsin and chymotrypsin digestion caused activation and improvement of the affinity to substrates of the recombinant LGOX. However, the thermostability, k_{cat} value and subunit structure (58 kDa and 18 kDa) differed from those of the LGOX isolated from *Streptomyces* sp. X-119-6 (data not shown). We tried digestion of the recombinant LGOX with the metalloendopeptidase from the same genus, *Streptomyces griseus* (Sgmp), which exhibits specificity for large aliphatic and aromatic amino acids. The recombinant LGOX was digested with Sgmp at the LGOX: Sgmp ratio of 1,000:1 (w/w), which allowed completion of the reaction within 4 h. The ability to catalyze deamination of L-glutamate was not lost at all in the recombinant LGOX on digestion with Sgmp. The Sgmp-treated enzyme gave major bands corresponding to approximately 44, 43, 17, and 10 kDa on 15% SDS-PAGE (Fig. 2A). A similar profile was obtained for mature LGOX from *Streptomyces* sp. X-119-6. These fragments were subjected to N-terminal sequence analysis. The sequences of the 44 and 43 kDa fragments were established to be M-N-E-M-T-Y-E, that of the 17 kDa one to be E-L-R-G-G-V-R-P, and that of the 10 kDa one to be Y-A-A-T-Q-T-W-T. This showed that the Sgmp attacks near the N-terminals of the β and γ-subunits. Native-PAGE of the Sgmp-treated recombinant LGOX gave a major band at a slight different position than with mature LGOX (Fig. 2B). Gel filtration analysis of the Sgmp-treated recombinant LGOX revealed molecular masses of 150 and 300 kDa. The ratio of the quantities of these materials differed remarkably with the recombinant LGOX (Fig. 2C). The 150 kDa peak is fairly larger than the 300 kDa one for the Sgmp-treated recombinant LGOX.

Characterization of Sgmp-Treated Recombinant LGOX—In comparison to the recombinant LGOX, the kinetic properties of the Sgmp-treated recombinant LGOX exhibit some differences. On measuring the initial reaction rate with different L-glutamate concentrations, a K_m of 0.23 mM and a k_{cat} of 75 s^{-1} at pH 7.4, and a k_{cat} of 49 at pH 6.0 were found. These values resemble those of mature LGOX. Table 1 summarizes the kinetic parameters of each enzyme form. These results showed that the Sgmp-digested enzyme exhibited improved affinity to substrates at pH 7.4 and activity.

The stability and optimum parameters of Sgmp-treated recombinant LGOX also differ from those of the recombinant enzyme. The stability of the Sgmp-treated recombinant LGOX was examined in the temperature range of 0–80°C. The 50% thermal inactivation temperature of the Sgmp-treated enzyme was about 75°C at pH 7.4, and about 80°C at pH 6.0 (Fig. 3). The activity of the Sgmp-treated recombinant LGOX was examined in the temperature range of 5–78°C at pH 6.0 and 7.4. The Sgmp-treated recombinant LGOX showed a bell-shaped curve as a function of temperature, with an optimum temperature of 58°C (Fig. 4). Arrhenius diagrams of L-glutamate oxidation in the temperature range of 10–30°C allowed calculation of activation energies of 41 and 51 kJ/mol at pH 7.4 and 7.0, respectively (Table 1). The activity of the Sgmp-treated recombinant LGOX was examined in the pH range of 3.2–10.6. The Sgmp-treated recombinant LGOX showed a bell shaped curve as a function of pH, with an optimum pH of 6.8–8.3 (Fig. 5). These results showed that the properties of the recombinant LGOX were improved by Sgmp treatment, resulting in similar ones to those of the mature LGOX isolated from *Streptomyces* sp. X-119-6.

DISCUSSION

In this paper, we have described the characterization of the recombinant LGOX produced from an *E. coli* transformant that has a precursor form, and Sgmp digestion analysis of this enzyme. The identified structure of the

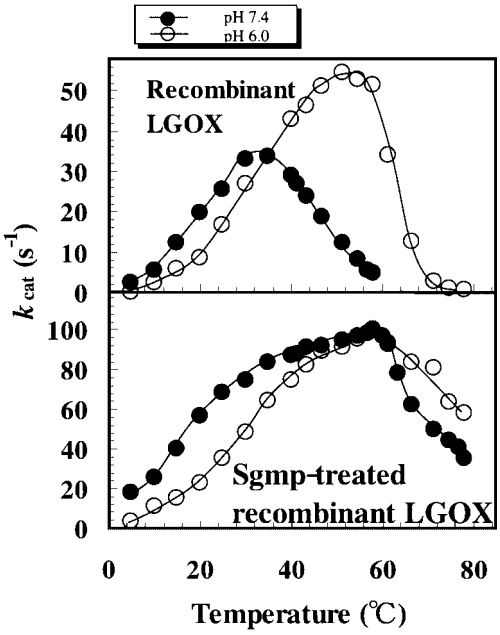

Fig. 4. **Temperature dependence of the oxygenation of 50 mM L-glutamate by 10 μg/ml recombinant LGOX and Sgmp-treated recombinant LGOX at pH 7.4 and 6.0.** Values are expressed as k_{cat}.

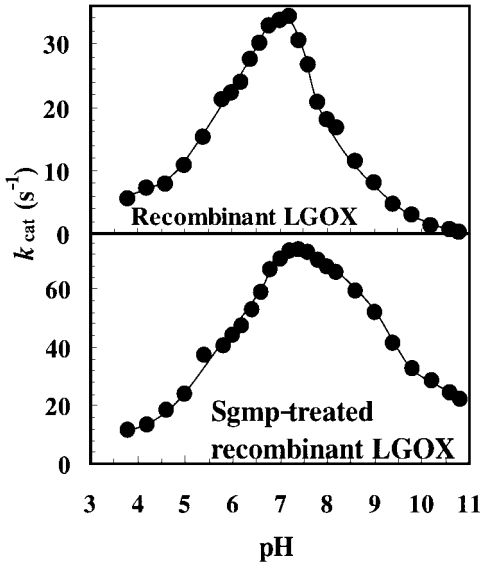

Fig. 5. **Effects of pH on the activities of the recombinant LGOX and Sgmp-treated recombinant LGOX.** Each enzyme was used at 10 μg/ml. Values are expressed as k_{cat}.

LGOX from *Streptomyces* sp. X-119-6 comprised from an $\alpha_2\beta_2\gamma_2$ subunit, originating from a single *Lgox* gene encoding an open reading frame for one polypeptide chain (Fig. 1). The primary amino acid sequence of this enzyme shows no apparent similarity with those of flavoenzymes except for the LGOX from *Streptomyces platensis*. Several related proteins, such as L-amino acid oxidase, tryptophan-2-monooxigenase and D-amino acid oxidase, exhibit partial sequence similarity with LGOX (10–25%). In particular, the N-terminal sequences of these enzymes were conserved so that the βαβ-fold, which binds to the ADP region of FAD (*23*), was present. Then, a conserved sequence, TKVLL, which is related to degradation of the imino intermediate, was observed in the γ-subunit (*24–26*) (boxed in Fig. 1).

We made use of recombinant expression to characterize the LGOX that had the structure of a one chain polypeptide. A major finding in this investigation is that the precursor-formed recombinant LGOX is an active enzyme, which exhibits inferior catalytic efficiency compared with mature LGOX isolated from *Streptomyces* sp. X-119-6. We expect that the LGOX activity that catalyzes the oxidation of L-glutamate along with the production of ammonia and hydrogen peroxide is toxic for or has a negative influence on the growth of cells. Consequently, this result predicts that the LGOX is present in cells as a precursor form that has low activity, and activation by proteolysis occurs after secretion. Properties, such as thermostability, optimum temperature, the pH dependence of k_{cat}, and the quaternary structure of the recombinant LGOX, differ greatly from those of mature LGOX. In particular, a higher molecular species was detected for the recombinant enzyme. We speculate that this higher molecular species might be an aggregated form of the recombinant enzyme. The thermostability of the recombinant LGOX was sensitive to pH, a lower pH stabilizing this enzyme as to temperature (pH 6.0 > pH 7.4). In addition, an excessive decrease in the K_m value of this enzyme was observed at a low pH (pH 6.0 < pH 7.4). In contrast to these results, the activation energy (E_a) of the reaction catalyzed by the recombinant LGOX at pH 6.0 was higher than that of the reaction catalyzed by this enzyme at pH 7.4. The k_{cat} value of the recombinant LGOX at pH 6.0 is also lower than that of this enzyme at pH 7.4 (Table 1). It was expected from these results that the structure of the recombinant LGOX that has a precursor form may be affected as to protonation, *i.e.* the precursor form enzyme may lose some of its structural flexibility, thereby causing loss of its polarity. If true, the enzyme being present as a precursor form is not a necessary condition. The nature of this enzyme differs greatly from that of the mature LGOX isolated from *Streptomyces* sp. X-119-6. We supposed that the difference in nature between the recombinant LGOX and the mature enzyme is caused by the subunit structure.

In this study we succeeded in apparent activation and stabilization of the recombinant LGOX by proteolysis using Sgmp. Native-PAGE of the Sgmp-treated recombinant LGOX gave a major band at a slightly different position than with the mature LGOX and without the smear in the case of the recombinant LGOX (Fig. 2B). Also, the Sgmp-treated recombinant LGOX did not produce a higher molecular species, as judged from the results of gel filtration (Fig. 2C). Judging from these results, digestion of the recombinant LGOX by Sgmp stabilized its quaternary structure without separation of the proteolytic fragment or a decrease in its activity. The Sgmp-treated recombinant LGOX showed approximately 20-fold lower K_m and 2-fold higher V_{max} (and k_{cat}) values at pH 7.4 compared to the recombinant LGOX, leading to 50-fold higher catalytic efficiency (k_{cat}/K_m) at this pH. The thermostability was also improved by the Sgmp digestion. We tried digestion of the recombinant with trypsin

and chymotrypsin instead of Sgmp previously. Such digestion improved its activity, but stabilization of the recombinant LGOX did not occur (K_m value of 0.23 mM for both enzymes at pH 7.4, and k_{cat} values of 44 and 59 s^{-1} for the trypsin-treated recombinant LGOX and chymotrypsin treated recombinant LGOX at pH 7.4, respectively). The digestion patterns of the recombinant LGOX with these proteases were also different from in the case of the mature LGOX isolated from *Streptomyces* sp. X-119-6 and the recombinant LGOX digested with Sgmp (58 kDa and 18 kDa). There is a strong possibility that the recombinant LGOX is activated by various proteases. However, it is not absolutely sure that stabilization occurs through digestion. Therefore, it seems that better proteases for stabilization of the recombinant LGOX that has a precursor form is Sgmp and similar proteases.

We suggest that the Sgmp-treated recombinant LGOX has a quaternary structure similar to the mature form. Improvement of the thermostability was observed when the Sgmp-treated recombinant LGOX was kept at pH 6.0 (Fig. 3). Similar data were obtained for mature LGOX from *Streptomyces* sp. X-119-6 (*5*). The activation energy (E_a) of the reaction catalyzed by the Sgmp-treated recombinant LGOX was lower than that of the reaction catalyzed by the recombinant LGOX with each pH. However, with decreasing pH the activation energy of the reaction catalyzed by the Sgmp-treated recombinant LGOX increased. These results showed that the Sgmp-treated recombinant LGOX also loses structural flexibility at pH 6.0, like the recombinant LGOX. Although similar data were obtained for the Sgmp-treated and non-treated recombinant LGOX as to pH dependent parameters, the Sgmp-treated recombinant LGOX did not show such extreme changes in properties at different pHs as the recombinant LGOX that has a precursor form. These observations for the Sgmp-treated recombinant LGOX revealed that this enzyme has a stable nature as compared to the recombinant precursor enzyme.

In conclusion, it is suggested that the enzyme being present as a precursor form is not a necessary condition, it having low catalytic activity and stability. Proteolysis of this one-chain enzyme by Sgmp generates an L-glutamate oxidase with improved activity and stability as to temperature. Furthermore, the LGOX digested with Sgmp was not present as a higher molecular species, as found on gel filtration. As to the maturation of this enzyme on proteolysis, we speculate there is one possibility for the subunit structure of $\alpha_2\beta_2\gamma_2$. First, LGOX is expressed as the precursor form of a dimer protein (α-β-γ_2) that may have an incompletely active form structure. Second, the LGOX precursor is digested by an appropriate endopeptidase, and has a completely active form of $\alpha_2\beta_2\gamma_2$ structure without separation of the large proteolytic fragment. We also speculate that the endopeptidase involved in the LGOX maturation is similar to Sgmp. The observations in this study indicate that proteolysis by Sgmp is not only related to activation, but also involved in stabilization of the structure for existence under various conditions.

Nucleotide sequence accession number. The nucleotide sequence has been assigned accession number AB085623 in the DDBJ database.

REFERENCES

1. Cooper, J.M., Calum, M.J., and Julia, A.S. (1991) Amperometric enzyme electrode for the determination of aspartate aminotransferase and alanine aminotransferase is serum. *Anal. Chim. Acta* **245**, 57–62
2. Kamei, T., Asano, K., Suzuki, H., Matsuzaki, M., and Nakamura, S. (1983) L-Glutamate oxidase from *Streptomyces violascens*. I. Production, isolation and some properties. *Chem. Pharm. Bull.* **31**, 1307–1314
3. Kamei, T., Asano, K., Kondo, H., Matsuzaki, K., and Nakamura, S. (1983) L-Glutamate oxidase from *Streptomyces violascens*. II. Properties. *Chem. Pharm. Bull.* **31**, 3609–3616
4. Bohmer, A., Muller, A., Passarge, M., Liebs, P., Honeck, H., and Muller, H.G. (1989) A novel L-glutamate oxidase from *Streptomyces endus*. Purification and properties. *Eur. J. Biochem.* **15**, 327–332
5. Kusakabe, H., Midorikawa, Y., Fujishima, T., Kuninaka, A., and Yoshino, H. (1983) Purification and properties of a new enzyme, L-glutamate oxidase, from *Streptomyces* sp. X-119–6 grown on wheat bran. *Agric. Biol. Chem.* **47**, 1323–1328
6. Chen, C.Y., Wu, W.T., Huang, C.J., Lin, M.H., Chang, C.K., Huang, H.J., Liao, J.M., Chen, L.Y., and Liu, Y.T. (2001) A common precursor for the three subunits of L-glutamate oxiase encoded by *gox* gene from *Streptomyces platensis* NTU3304. *Can. J. Microbiol.* **47**, 269–275
7. Short, J.M., Fernandez, J.M., Sorge, J.A., and Huse, W.D. (1988) Lambda ZAP: a bacteriophage lambda expression vector with in vivo excision properties. *Nucleic Acids Res.* **11**, 7583–600
8. Amann, E., Brosius, J., and Ptashne, M. (1983) Vectors bearing a hybrid trp-lac promoter useful for regulated expression of cloned genes in *Escherichia coli*. *Gene* **25**, 167–178
9. Yanisch, P.C., Vieira, J., and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119
10. Hopwood, D.A., Bibb, M.J., Chater, K.F., Kieser, T., Thompson, C.J., Kieser, H.M., Lydite, D.J., and Schrempf, H. (1985) *Genetic Manipulation of Streptomyces: a Laboratory Manual*, John Innes Foundation, Norwich, UK
11. Maniatis, T., Fritsch, F.F., and Sambrook, J. (1989) *Molecular cloning: a Laboratory Manual*, 2nd ed., Cold Spring Harbor Lab., Cold Spring Harbor, New York
12. Saito, H. and Miura, K. (1963) Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochim. Biophys. Acta* **72**, 619–629
13. Birnboim, H.C. and Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **24**, 1513–1523
14. Southern, E.M. (1975) Detection of specific sequence among DNA fragment separated by gel electrophoresis. *J. Mol. Biol.* **98**, 503–517
15. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl Acad. Sci. USA* **74**, 5463–5467
16. Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389–3402
17. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **15**, 680–685
18. Feinberg, A.P. and Vogelstein, B. (1983) A technique for radiolabelling DNA restriction endonuclease fragment to high specific activity. *Anal. Biochem.* **132**, 6–13
19. Short, J.M. and Sorge, J.A. (1992) *In vivo* excision properties of bacteriophage lambda ZAP expression vectors. *Methods Enzymol.* **216**, 495–508
20. Allain, C.C., Poon, L.S., Chan, C.S., Richmond, W., and Fu, P.C. (1974) Enzymatic determination of total serum cholesterol. *Clin. Chem.* **20**, 470–475

21. Soda, K. (1968) Microdetermination of D-amino acids and D-amino acid oxidase activity with 3-methyl–2-benzothiazolone hydrazone hydrochloride. *Anal. Biochem.* **24**, 228–235
22. Segel, I.H. (1976) *Biochemical Calculations*, pp. 277–281, John Wiley and Sons, New York
23. Wierenga, R.K., Terpstra, P., and Hol, W.G. (1986) Prediction of the occurrence of the ADP-binding βαβ-fold in proteins, using an amino acid sequence fingerprint. *J. Mol. Biol.* **187**, 101–107
24. Pawelek, P.D., Cheah, J., Coulombe, R., Macheroux, P., Ghisla, S., and Vrielink, A. (2000) The structure of L-amino acid oxidase reveals the substrate trajectory into an enantiomerically conserved active site. *EMBO J.* **19**, 4204–4215
25. MacHeroux, P., Seth O., Bollschweiler, C., Schwarz, M., Kurfurst, M., Au, L.C., and Ghisla, S. (2001) L–Amino acid oxidase from the Malayan pit viper *Calloselasma rhodostoma*. Comparative sequence analysis and characterization of active and inactive forms of the enzyme. *Eur. J. Biochem.* **268**, 1679–1686
26. Binda, C., Coda, A., Angelini, R., Federico, R., Ascenzi, P., and Mattevi, A. (1999) A 30 Å long U-shaped catalytic tunnel in the crystal structure of polyamine oxidase. *Structure* **7**, 265–276