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

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L-Glutamate oxidase (LGOX) from *Streptomyces* sp. X-119-6 is a protein of 150 kDa that has hexamer structure \(a_{6}b_{2}g_{2}\). 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 \(a_{6}b_{2}g_{2}\) 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-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 food and in a fermentation process. It has also attracted considerable attention due to its potential application to food and in a fermentation process. It has also attracted considerable attention due to its potential application to food and in a fermentation process. It has also attracted considerable attention due to its potential application to food and in a fermentation process. It has also attracted considerable attention due to its potential application to food and in a fermentation process.

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 \(a_{6}b_{2}g_{2}\) 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 \(a_{6}b_{2}g_{2}\) (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 single 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,
0.5% MgCl₂ 6H₂O, 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. 1. Nucleotide sequence of the L-glutamate oxidase gene and the deduced amino acid sequence.](image)

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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/G/C/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-32P]ATP, and then used as a hybridization probe. A genomic library was constructed with BamHI restriction fragment 1–3 kb and BamHI-digested pUC19. This library was screened by colony hybridization with a 32P-end labeled oligonucleotide probe (11). One positive clone harboring the recombinant plasmid, pGB1, was obtained. A second genomic library was constructed with SacI restriction fragment 4–6 kb and BamHI-digested pUC19. This library was screened with the insert fragment of pGB1 labeled with [α-32P]dCTP (220 TBq/mmol; Amersham International plc, Little Chalfont, England) ([18] as a probe. The positive plaque was then subcloned into phagedin 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 EcoRI site and a PstI site were introduced by PCR. The Lgox gene that lacked the putative signal peptide region was amplified by PCR using sense primer U1 (5'-CCACACCGGGGCGC-GAAATCTAGAAGCAGAT-3') and antisense primer L1 (5'-AGGTACTCGGCCACCTGCGAGTCT-3'). The PCR product was subcloned into pKK235-3 (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-aminopyridine 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-aminopyridine, 1.7 mM phenol, 10 U/ml of peroxidase and enzyme at 30°C, and then the increase in A505 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-benzothiazoline hydrazide 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 2x 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. The column was washed with the equilibration buffer and then eluted with a 1.2-liter linear gradient of 20 to 0% 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 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. 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.
activity was measured by the MBTH method at the same for 5 min at the target temperature, and later LGOX
the optimal temperature, each enzyme was preincubated
the indicated temperatures for 30 min. The enzyme activity was measured at pH 7.4 after incubation of each
LGOX and Sgmp-treated recombinant LGOX, respectively). The
of the maxima (100% = 0.26 and 0.57
was preincubated for 30 min at the corresponding tem-
pH 7.4). To determine the thermostability, each enzyme
temperature was performed using the purified enzymes.
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
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 deter-
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Amino acid sequences obtained by automated Edman
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Characterization of the Recombinant LGOX—
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
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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 pos-
itive 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
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about 85%.

*E. coli* JM109 harboring pGS1 exhibited no expression
of LGOX. To obtain overexpression of the LGOX in *E. coli*
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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 diges-
tion 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.

Proteolytic digestion was used to create specific cleavage
sites that could be used to release functional subunits
of the LGOX. The purified LGOX from *E. coli*—
S. platensis sp. X-119-6 was constructed and
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Fig. 3. Effects of temperature on the stabilities of the recom-
binant 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
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Characterization of the Recombinant 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
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ture. The purified enzyme could be stored for several
months in 20 mM KPB (pH 7.4) at 4 or –20°C without signif-
nicant loss of activity. The molecular mass of the recom-
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Mature LGOX from X-119-6, recombinant LGOX, and Sgmp-treated LGOX.

<table>
<thead>
<tr>
<th>Properties</th>
<th>Mature LGOX from X-119-6</th>
<th>Recombinant LGOX</th>
<th>Sgmp-treated LGOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimum pH</td>
<td>6.0–8.5 (5)</td>
<td>7.0</td>
<td>6.0–8.5</td>
</tr>
<tr>
<td>Optimum temperature (°C) at pH 7.4</td>
<td>75 (5)</td>
<td>30</td>
<td>75</td>
</tr>
<tr>
<td>Optimum temperature (°C) at pH 7.4</td>
<td>80 (pH 5.5) (5)</td>
<td>50</td>
<td>80</td>
</tr>
<tr>
<td>50% thermal inactivation temperature (°C) at pH 7.4</td>
<td>75 (5)</td>
<td>30</td>
<td>75</td>
</tr>
<tr>
<td>50% thermal inactivation temperature (°C) at pH 6.0</td>
<td>80 (pH 5.5) (5)</td>
<td>50</td>
<td>80</td>
</tr>
<tr>
<td>Vmax (mol/min/mg) at pH 7.4 (30°C)</td>
<td>55.1 (5)</td>
<td>26</td>
<td>57</td>
</tr>
<tr>
<td>Km (mM) at pH 7.4 (30°C)</td>
<td>0.23 (5)</td>
<td>5.0</td>
<td>0.23</td>
</tr>
<tr>
<td>hcat (s⁻¹) at pH 7.4 (30°C)</td>
<td>72.5e</td>
<td>33</td>
<td>75</td>
</tr>
<tr>
<td>hcat (s⁻¹) at pH 6.0 (30°C)</td>
<td>–</td>
<td>27</td>
<td>49</td>
</tr>
<tr>
<td>hcat / Km (M⁻¹ s⁻¹) at pH 7.4 (30°C)</td>
<td>3.2 × 10⁵ e</td>
<td>6.6 × 10³</td>
<td>3.3 × 10⁵</td>
</tr>
<tr>
<td>hcat / Km (M⁻¹ s⁻¹) at pH 6.0 (30°C)</td>
<td>–</td>
<td>1.2 × 10⁵</td>
<td>2.1 × 10⁵</td>
</tr>
<tr>
<td>Ecat (kJ/mol) at pH 7.4</td>
<td>–</td>
<td>72</td>
<td>41</td>
</tr>
<tr>
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<td>–</td>
<td>56</td>
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*Calculated by gel filtration. **Determined by SDS-PAGE. ***Measured by MBTH method. ****Measured by 4-amino antipyrine/phenol method.

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

**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 Km of 0.23 mM and a kcat of 75 s⁻¹ at pH 7.4, and a kcat 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
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-monoxygenase 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 \( \beta\beta\)A-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 \( \gamma \)-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 \( k_{\text{cat}} \), 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 \( k_{\text{cat}} \) 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 \( V_{\text{max}} \) (and \( k_{\text{cat}} \)) values at pH 7.4 compared to the recombinant LGOX, leading to 50-fold higher catalytic efficiency (\( k_{\text{cat}}/K_m \)) 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 ($\alpha-\beta-\gamma$) 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.

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