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# L-Glutamate Oxidase from *Streptomyces violascens*. I. Production, Isolation and Some Properties

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L-Glutamate oxidase, a novel L-glutamic acid oxidizing enzyme, has been found in the culture broth of *Streptomyces violascens*. The enzyme has been purified 914-fold by precipitation with ammonium sulfate, affinity chromatography on L-glutamic acid-Sepharose 6B and L-glutamine-Sepharose 6B, hydroxyapatite chromatography and gel filtration on Sephadex G-100. The purified enzyme showed a single band on polyacrylamide disc gel electrophoresis and SDS–polyacrylamide gel electrophoresis. The molecular weight of the enzyme was estimated to be about 60000. The purified enzyme exhibited a characteristic flavoprotein spectrum. The enzyme catalyzed the oxidation of L-glutamic acid and L-glutamine without a requirement for any exogenous cofactor.

**Keywords**——L-glutamate oxidase; L-glutamic acid oxidation; *Streptomyces violascens*; affinity chromatography; flavoprotein

L-Amino acids are oxidized nonspecifically by L-amino acid oxidase from snake venoms and animal tissues.[1] L-Glutamic acid metabolism is dependent on an electron transport system in *Azotobacter vinelandii*.[2] In plants, Tsukamoto[3] has reported that L-glutamic acid was metabolized in the presence of an oxidation–reduction dye such as methylene blue or thionine. It has also been reported that D-glutamic acid was metabolized by *Aerobacter*[4,5] or *Aspergillus ustus*.[6–9]

However, there is no report of L-glutamic acid metabolism without a requirement for any exogenous cofactor.

We have screened many microorganisms metabolizing L-glutamic acid and found that *Streptomyces violascens* produces a L-glutamic acid oxidizing enzyme. The present paper describes an effective purification method, using L-glutamic acid-Sepharose 6B and L-glutamine–Sepharose 6B affinity chromatography, and presents properties of the L-glutamic acid oxidizing enzyme. The L-glutamic acid oxidizing enzyme from *Streptomyces violascens* is apparently different from the other enzymes described above in reaction mode, substrate specificity and subcellular distribution, and we have tentatively named the enzyme L-glutamate oxidase (L-glutamic acid: oxygen oxidoreductase). This is the first report of L-glutamate oxidase.

## Materials and Methods

**Materials**——Horseradish peroxidase (90 units/mg), D-amino acid oxidase (porcine kidney, crystalline), catalase (beef liver), and γ-methyl-L-glutamate were purchased from Sigma Chemical Co. L-Amino acid oxidase (snake venom, *Crotalus*) was purchased from Boehringer Mannheim GmbH. Other materials were purchased from the following sources: 1,4-butanediol diglycidyl ether (Aldrich Chemical Co.), Sephadex G-100 and Sepharose 6B (Pharmacia Fine Chemicals), hydroxyapatite (Seikagaku Kogyo Co.). All other reagents were purchased from commercial sources and were of analytical grade.

**Determination of Enzyme Activity**——Enzyme assay was based on the measurement of hydrogen peroxide generated during the oxidation of L-glutamic acid. The hydrogen peroxide was coupled with 4-aminoantipyrine and phenol in the presence of peroxidase to form a quinoneimine dye, according to the method of

Allain *et al.*[10] The amount of quinoneimine dye was measured spectrophotometrically at 500 nm. The enzyme preparation (0.05 ml) was added to 2 ml of 0.05 M phosphate buffer (pH 6.8) containing 2 μmol of 4-aminoantipyrine, 35 μmol of phenol and 4.75 units of peroxidase. The solution was mixed well and allowed to stand for 3 min at 37°C, then 0.5 ml of 35 μmol/ml of L-glutamic acid was added, and the whole was incubated for 30 min at 37°C. The enzyme reaction was terminated by heating the mixture in a boiling water bath for 3 min. The mixture was cooled to room temperature, and the absorbance was read at 500 nm against the blank. One unit of L-glutamate oxidase activity was defined as the amount forming 1 μmol of hydrogen peroxide per min at 37°C.

**Screening**——Microorganisms were inoculated into 100 ml of the screening medium, which consisted of 1.5% soluble starch, 1% glucose, 1.5% soybean protein, 0.5% dried yeast (Ebios), 0.25% NaCl, 0.3% $CaCO_3$ and 0.1% $K_2HPO_4$ (pH 7.6) in 500 ml Sakaguchi flasks. The cultivations were done at 27°C for 4 d on a reciprocal shaker. The L-glutamate oxidase activity in the culture filtrate was determined by the assay method described above.

**Production Medium**——The culture medium consisted of 1.5% soluble starch, 1% polypeptone, 0.1% $K_2HPO_4$, 0.05% KCl, and 0.05% $MgSO_4 \cdot 7H_2O$ (pH 7.6). The inoculation medium consisted of 1% maltose, and 0.2% yeast extract (pH 7.0).

**Improvement of Productivtiy of L-Glutamate Oxidase by Mutation**——*Streptomyces violascens* H82-N-SY7 was mutated according to the method of Oki *et al.*[11] The cells were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine at a concentration of 1 mg/ml in 0.2 M Tris–HCl buffer (pH 8.5) with shaking at 28°C for 60 min (killing rate 99.95%), and plated on YS agar (0.3% yeast extract, 1% soluble starch, and 1.5% agar, pH 7.2). About 300 colonies were picked out, cultured on YS agar, and tested for ability to produce L-glutamate oxidase.

**Preparation of Affinity Gel**——Epoxy-activated Sepharose 6B was prepared according to the method of Matsumoto.[12] Sepharose 6B was washed with a sufficient volume of water, suction-dried under a vacuum on a glass filter funnel for 5 min and weighed. Washed and suction-dried Sepharose 6B (100 g) was suspended in 100 ml of 0.6 N NaOH containing 200 mg of $NaBH_4$, and mixed with 100 ml of 1,4-butanediol diglycidyl ether. The suspension was incubated at 25°C for 8 h with shaking. It was then transferred to a glass filter funnel and the gel was washed with a sufficient volume of water.

Coupling of L-glutamine to epoxy-activated Sepharose 6B was performed as follows. Suction-dried and epoxy-activated Sepharose 6B (100 g) was suspended in 400 ml of 0.1 N NaOH containing 130 mmol of L-glutamine. The suspension was incubated at 40°C for 24 h with shaking and was then washed with a sufficient volume of water.

Coupling of L-glutamic acid to Sepharose 6B was performed as follows. γ-Methyl-L-glutamate (100 mmol) was esterified with *N*-hydroxy-succinimide (200 mmol) in the presence of dicyclohexylcarbodiimide (200 mmol) in purified dimethylformamide (300 ml) at 4°C for 16 h. Suction-dried and epoxy-activated Sepharose 6B (200 g) was suspended in 300 ml of ammonium hydroxide (28%). The suspension was incubated at 40°C for 1.5 h with shaking, washed with water and then washed with anhydrous dioxane. The resulting amino Sepharose 6B (167 g) was mixed with the activated γ-methyl-L-glutamate (100 mmol) obtained above and incubated at 25°C for 22 h. The gel suspension was washed with a mixture of dimethylformamide and dioxane (1: 2 v/v), followed by 1 M NaCl, and then water. The γ-methylester group of γ-methyl-L-glutamyl Sepharose 6B was hydrolyzed in 0.075 N NaOH at room temperature for 2 h. The resulting gel was washed with a sufficient volume of water.

**Determination of Protein**——Protein concentration of an enzyme preparation was measured by the method of Lowry *et al.*[13]

**Electrophoresis**——Polyacrylamide disc gel electrophoresis was carried out at pH 4 by the method of Reisfeld *et al*,[14] and SDS–polyacrylamide gel electrophoresis was performed by the method of Weber *et al.*[15]

**Determination of Molecular Weight**——The molecular weight of the purified enzyme was estimated by SDS–polyacrylamide gel electrophoresis and Sephadex G-100 gel filtration. The marker proteins used were cytochrome C (Mr=11700), trypsin (Mr=23300), alcohol dehydrogenase (Mr=41000), and bovine serum albumin (Mr=68000).

## Results

### Selection of Microorganisms

Two strains (31-SY4 and H82-N-SY7) out of 500 *Actinomycetes* strains were found to produce L-glutamate oxidase. H82-N-SY7 produced more enzyme than 31-SY4. H82-N-SY7 was a stock culture of our laboratory and had been identified previously as *Streptomyces violascens* H82-N-SY7.[16] However, the enzyme productivity of *St. violascens* was still low. Thus, we obtained a more productive organism by monospore selection and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutation. One of these strains, H82-N-SY7-M270, was found to produce a large amount of L-glutamate oxidase.

### Time Course of Enzyme Production

The relationship between the enzyme production and medium pH change was investigated. As shown in Fig. 1, L-glutamate oxidase activity was detected in the medium as early as 3 d, and reached a maximum at 5 d. The enzyme activity decreased on further cultivation. When the medium pH had fallen to 6.2, the cultivation could be terminated.

### Purification of L-Glutamate Oxidase

All procedures were carried out at 4°C unless otherwise stated.

**Step 1**——After cultivation for 5 d, mycelia were removed by filtration. Solid ammonium sulfate was added to the filtrate to give 40% satuation. After standing overnight, the precipitate was collected by centrifugation and was dissolved in 0.01 M citrate buffer (pH 4.0). This solution was dialyzed against the same buffer for 24 h.

**Step 2**——The dialyzed solution (192 ml, 1860 units and 2.08 g as protein) was subjected to L-glutamic acid–Sepharose 6B chromatography on a column (2.5×25 cm) pre-equilibrated with 0.01 M citrate buffer (pH 4.0). The charged column was washed with a sufficient volume of the same buffer and eluted with a linear concentration gradient of NaCl from 0 to 0.4 M in the same buffer. The elution profile is shown in Fig. 2.

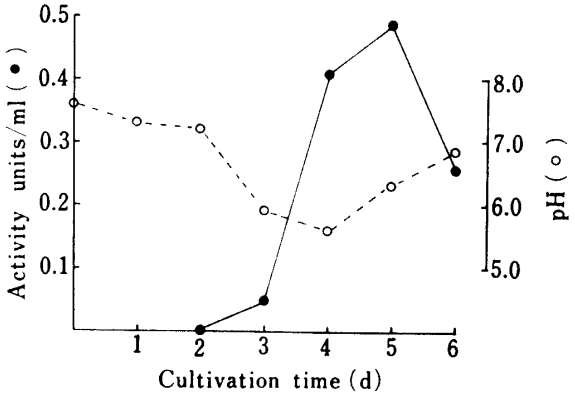


Fig. 1. Time Course of Formation of L-Glutamate Oxidase by *Streptomyces violascens* H82-N-SY7-M270

The cultivation medium consisted of 1.5 % soluble starch, 1 % polypeptone, 0.1 % $K_2HPO_4$, 0.05 % KCl, and 0.05 % $MgSO_4 \cdot 7H_2O$ (pH 7.6).

**Step 3**——The active fractions were combined, concentrated by ultrafiltration, and dialyzed against 0.01 M citrate buffer (pH 4.0). The dialyzed solution (59 ml, 743 units and 50.8 mg as protein) was subjected to L-glutamine–Sepharose 6B chromatography on a column (2.5×25 cm) pre-equilibrated with 0.01 M citrate buffer (pH 4.0). The charged column was washed and eluted under the same conditions as described for L-glutamic acid–Sepharose 6B column chromatography (Fig. 3).

**Step 4**——The active fractions were combined, concentrated by ultrafiltration, and dialyzed against 0.1 M phosphate buffer (pH 6.0). The dialyzed solution (70 ml, 678 units and 23.5 mg as protein) was subjected to hydroxyapatite chromatography on a column (1.5×25 cm) pre-equilibrated with 0.1 M phosphate buffer (pH 6.0). The charged column was washed with a sufficient volume of the same buffer and eluted with a linear concentration gradient of NaCl from 0 to 0.9 M in the same buffer.

**Step 5**——The active fractions were combined, concentrated by ultrafiltration, and dialyzed against 0.1 M citrate buffer (pH 4.0). The dialyzed solution (8.6 ml, 548 units and 9.4 mg as protein) was subjected to Sephadex G-100 chromatography on a column (3×70 cm) pre-equilibrated with 0.1 M citrate buffer (pH 4.0), and eluted with the same buffer (Fig. 5). The active fractions (404 units and 6.7 mg as protein) were combined and concentrated by ultrafiltration. This fraction was used for the characterization of the enzyme.

A summary of the purification procedures is shown in Table I. The enzyme preparation was purified to 914-fold in specific activity with a recovery of 27.5% of the original activity.

### Homogeneity

The purified enzyme gave a single band on polyacrylamide disc gel electrophoresis carried out at pH 4.0 and on SDS-polyacrylamide gel electrophoresis (Fig. 6).

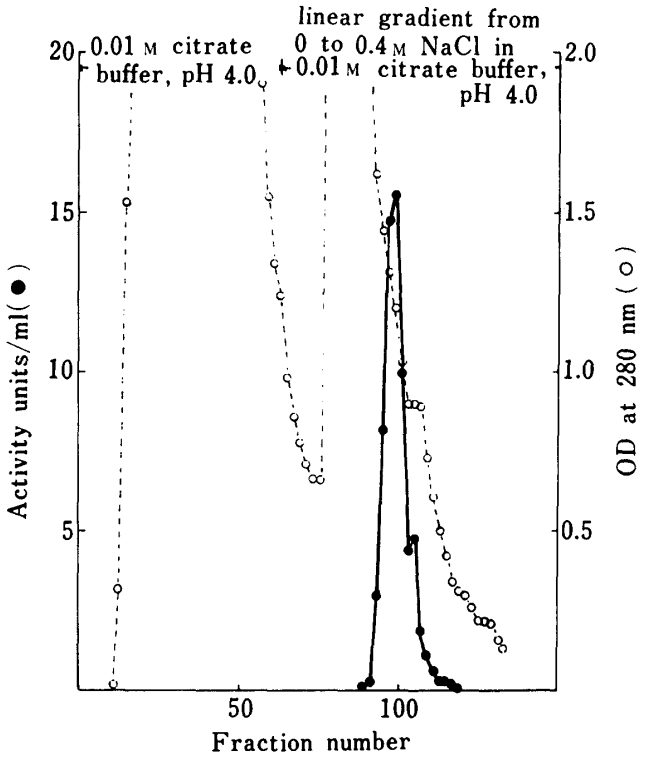


Fig. 2. Chromatography of L-Glutamate Oxidase on a L-Glutamic Acid–Sepharose 6B Column

The ammonium sulfate-precipitated and dialyzed fraction (1860 units, 2.08 g as protein) was subjected to affinity chromatography on L-glutamic acid–Sepharose 6B (2.5×25 cm). The column was washed with 0.01 M citrate buffer (pH 4.0) and eluted with a linear gradient concentration of NaCl from 0 to 0.4 M in the same buffer, at a flow rate of 60 ml/h; 5 ml fractions were collected.

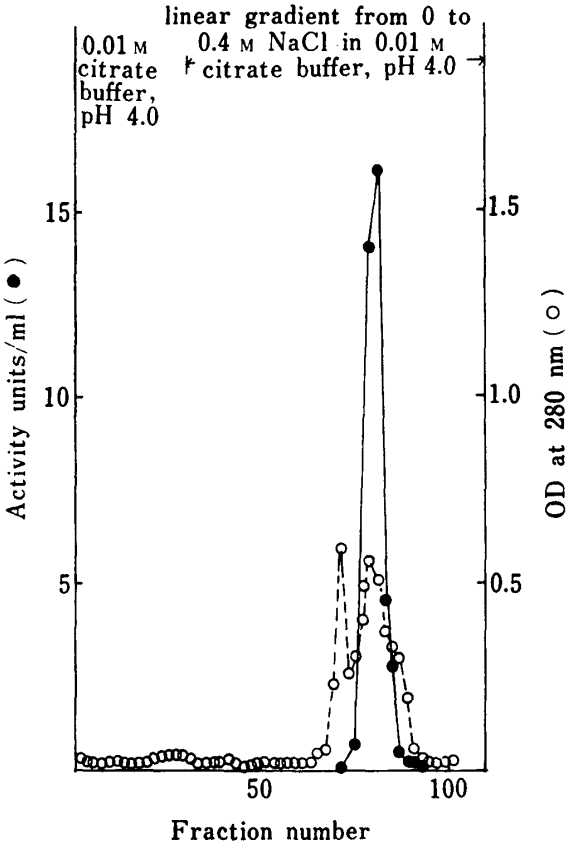


Fig. 3. Chromatography of L-Glutamate Oxidase on a L-Glutamine–Sepharose 6B Column

After chromatography on L-glutamic acid–Sepharose 6B, the enzyme preparation (743 units, 50.8 mg as protein) was subjected to affinity chromatography on L-glutamine–Sepharose 6B (2.5×25 cm). The charged column was washed and eluted under the same conditions as described in Fig. 2.

## Molecular Weight

In SDS–polyacrylamide gel electrophoresis, the molecular weight of the enzyme was estimated to be about 60000. By Sephadex G-100 column chromatography, the molecular weight of the enzyme was estimated to be about 62000.

## Absorption Spectrum

The purified enzyme showed an absorption spectrum characteristic of a flavoprotein with absorption maxima at 280, 390 and 470 nm and a marked shoulder at 490 nm (Fig. 7).

## Substrate Specificity

The substrate specificity of L-glutamate oxidase from *St. violascens* was investigated with various amino acids which were oxidized by L-glutamic acid oxidizing enzyme from Azotobacter[2] or spinach leaves.[3] The relative oxidation rates of amino acids by L-glutamate oxidase were also compared with L-amino acid oxidase and D-amino acid oxidase. The results are shown in Table II. L-Glutamate oxidase oxidized L-glutamic acid and L-glutamine, but did not oxidize D-glutamic acid, L-aspartic acid, D-asparagine, D-methionine or D-phenylalanine. L-Amino acid oxidase and D-amino acid oxidase could not oxidize L-glutamic acid. L-Amino acid oxidase oxidized L-glutamine, L-methionine, L-phenylalanine and L-leucine. D-Amino acid oxidase oxidized D-methionine, D-phenylalanine and D,L-alanine.

The substrate specificity of L-glutamate oxidase was considerably different from those of

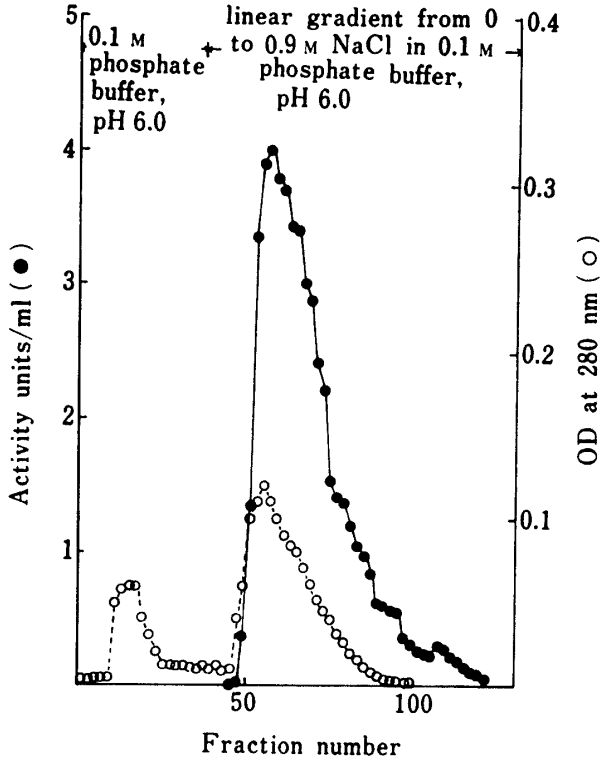


Fig. 4. Chromatography of L-Glutamate Oxidase on a Hydroxyapatite Column

After chromatography on L-glutamine-Sepharose 6B, the enzyme preparation (678 units, 23.5 mg as protein) was subjected to hydroxyapatite column chromatography (1.5×25 cm). The column was washed with 0.1 M phosphate buffer (pH 6.0) and eluted with a linear gradient concentration of NaCl from 0 to 0.9 M in the same buffer, at a flow rate of 180 ml/h; 5 ml fractions were collected.

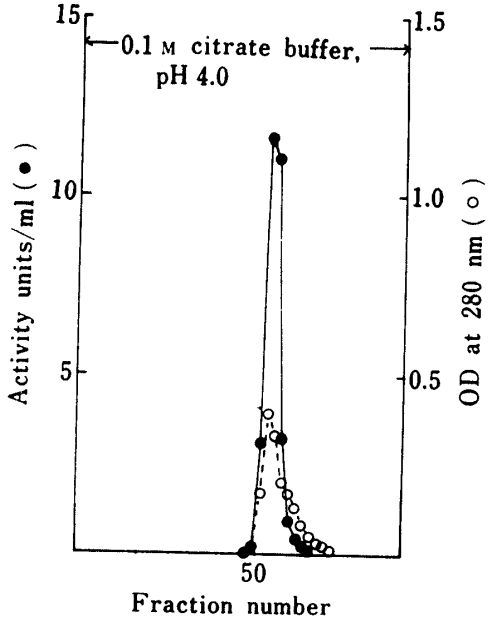


Fig. 5. Gel Filtration of L-Glutamate Oxidase on Sephadex G-100

After chromatography on hydroxyapatite, the enzyme preparation (548 units, 9.4 mg as protein) was applied to a Sephadex G-100 column (3×70 cm). The column was eluted with 0.1 M citrate buffer, pH 4.0, at a flow rate of 12 ml/h; 3 ml fractions were collected.

TABLE I. Summary of the Purification of L-Glutamate Oxidase

| Fraction | Total units | Total protein (mg) | Specific activity (units/mg) | Recovery (%) |
|---|---|---|---|---|
| Broth filtrate | 1470 | 22300 | 0.066 | 100 |
| $(NH_4)_2SO_4$ ppt. and Dialyzate | 1860 | 2080 | 0.894 | 127 |
| L-Glutamic acid-Sepharose 6B column | 743 | 50.8 | 14.6 | 50.5 |
| L-Glutamine-Sepharose 6B column | 678 | 23.5 | 28.9 | 46.1 |
| Hydroxyapatite column | 548 | 9.4 | 58.3 | 37.3 |
| Sephadex G-100 gel filtration | 404 | 6.7 | 60.3 | 27.5 |

L-amino acid oxidase and D-amino acid oxidase.

**Identification of Reaction Product**

The reaction product from L-glutamic acid was identified by cellulose powder thin layer chromatography. The enzyme reaction was performed in 0.05 M phosphate buffer (pH 6.8) with or without catalase. After incubation at 37°C for 15 h, the reaction mixture was subjected to thin layer chromatography. Table III shows that the *Rf* values of the reaction product in the presence of catalase were identical with those of authentic α-ketoglutaric acid, and the *Rf* values of the product in the absence of catalase were identical with those of succinic acid. Another product of L-glutamate oxidase, hydrogen peroxide, may degrade α-ketoglutaric acid to succinic acid in the absence of catalase.[7]

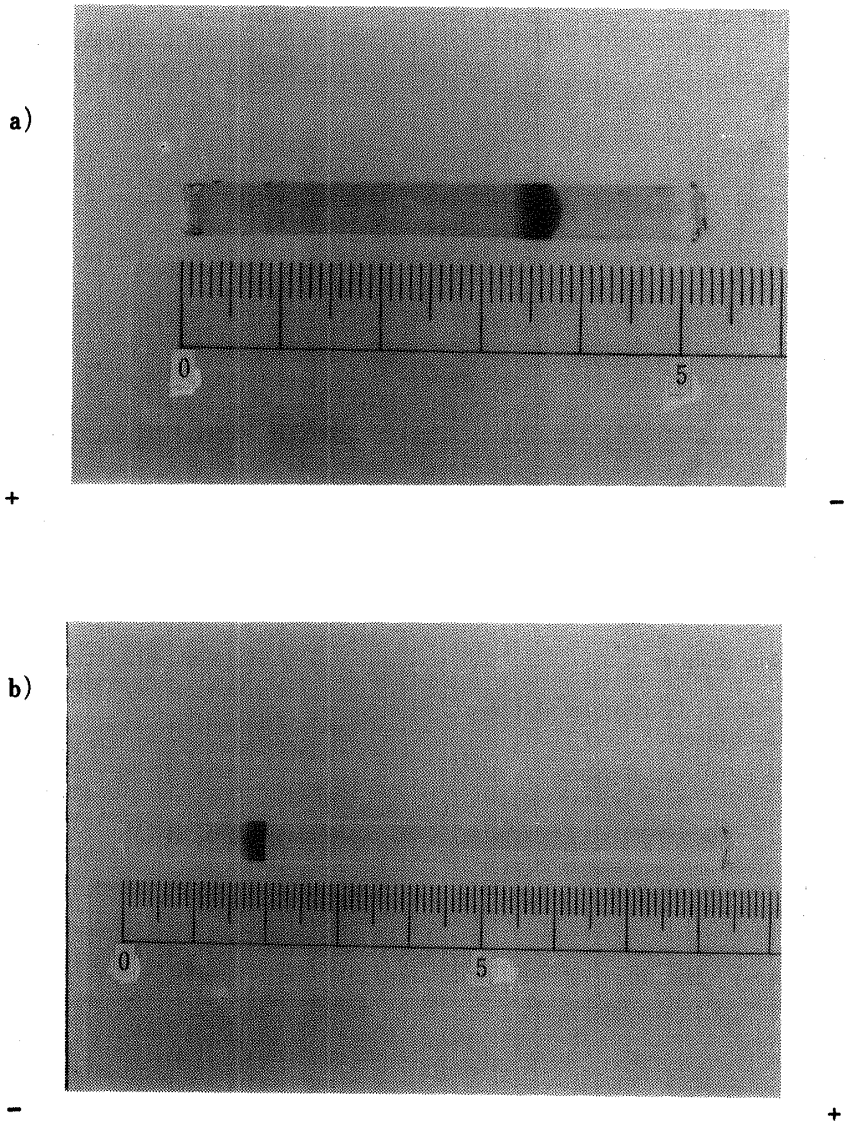


Fig. 6. Electrophoretic Profiles of Purified L-Glutamate Oxidase

a) Polyacrylamide disc gel electrophoresis.
b) SDS-polyacrylamide gel electrophoresis.

## Discussion

The present studies have shown that L-glutamate oxidase produced by *Streptomyces violascens* is a new enzyme. The enzyme reaction proceeded in the presence of oxygen without any requirement for exogenous cofactors.

L-Glutamic acid oxidation in *Azotobacter vinelandii* occurs in the membrane and is integrated into the *Azotobacter* electron transport system.[2] *Azotobacter* L-glutamic acid oxidizing enzyme can oxidize D-asparagine, D-methionine and D-phenylalanine.[2] L-Glutamic acid oxidation in the leaves of spinach occurs in cytoplasmic juice and is dependent on an oxidation–reduction dye such as methylene blue or thionine.[3] Plant L-glutamic acid oxidizing enzyme can oxidize L-aspartic acid.[3] However, L-glutamate oxidase produced by *Streptomyces violascens* is an extracellular enzyme, and can oxidize L-glutamic acid and L-glutamine

without any requirement for exogenous cofactors. Oxidation–reduction dyes (methylene blue and thionine) were not capable of serving as electron acceptors (data not shown). L-Glutamate oxidase did not oxidize L-aspartic acid, D-asparagine, D-methionine or D-phenylalanine. Further, the substrate specificity of L-glutamate oxidase was very different from those of L-amino acid oxidase and D-amino acid oxidase. From these results, we conclude that L-glutamate oxidase is a new enzyme, on the basis of its substrate specificity, subcellular distribution and reaction mechanism.

From the spectral data, the present

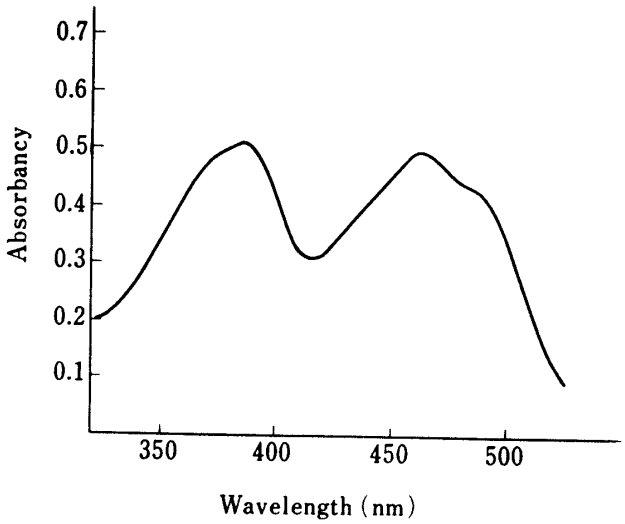


Fig. 7. Absorption Spectrum of Purified L-Glutamate Oxidase in 0.1 M Citrate Buffer, pH 4.0

TABLE II. Relative Oxidation Rates of Various Amino Acids by L-Glutamate Oxidase, L-Amino Acid Oxidase and D-Amino Acid Oxidase

| Substrate | Relative rate | | |
|---|---|---|---|
| | L-Glutamate oxidase (pH 6.8) | L-Amino acid oxidase (pH 7.2) | D-Amino acid oxidase (pH 8.3) |
| L-Glutamic acid | 100 | <0.5 | <0.5 |
| D-Glutamic acid | <0.5 | <0.5 | <0.5 |
| L-Aspartic acid | <0.5 | <0.5 | <0.5 |
| D-Aspartic acid | <0.5 | <0.5 | <0.5 |
| L-Glutamine | 32.1 | 10.1 | <0.5 |
| D-Glutamine | <0.5 | <0.5 | <0.5 |
| L-Asparagine | <0.5 | 4.2 | <0.5 |
| D-Asparagine | <0.5 | <0.5 | <0.5 |
| L-Methionine | <0.5 | 106 | <0.5 |
| D-Methionine | <0.5 | <0.5 | 126 |
| L-Phenylalanine | <0.5 | 68.4 | <0.5 |
| D-Phenylalanine | <0.5 | <0.5 | 80.3 |
| L-Leucine | <0.5 | 100 | <0.5 |
| D, L-Alanine | <0.5 | 4.7 | 100 |

The reaction mixture consisted of 17.5 μmol of substrate, 2 μmol of 4-aminoantipyrine, 35 μmol of phenol, 4.75 units of peroxidase and the test enzyme in total volume of 2.55 ml. The reaction was carried out at 37 °C for 30 min, and the color produced was measured at 500 nm. Relative activity was calculated as follows: in the case of L-glutamate oxidase, L-glutamic acid was used as the standard; in the case of L-amino acid oxidase, L-leucine was used as the standard; and in the case of D-amino acid oxidase, D,L-alanine was used as the standard.

TABLE III. Identification of Reaction Product of L-Glutamate Oxidase by Cellulose Powder Thin Layer Chromatography

| Sample | *Rf* | |
|---|---|---|
| | Solvent I | Solvent II |
| Reaction product (+catalase) | 0.54 | 0.51 |
| Reaction product (−catalase) | 0.74 | 0.71 |
| α-Ketoglutaric acid | 0.54 | 0.51 |
| Succinic acid | 0.74 | 0.71 |

The chromatograms were sprayed with 0.1% 2, 6-dichlorophenol-indophenol (EtOH sol.)
Solvent I: BuOH-$H_2O$-HCOOH (10:5:1), upper layer.
Solvent II: BuOH-EtOH-$H_2O$-HCOOH (10:2:2:2).

enzyme is considered to be a flavoprotein. L-Glutamic acid oxidizing enzyme from spinach leaves,[3] D-glutamate oxidase from *Aspergillus ustus*,[8] D-amino acid oxidase from kidney,[17] and L-amino acid from snake venom[1] are also considered to be flavoproteins.

L-Glutamate oxidase was purified to an electrophoretically homogeneous state. In the purification step of salting out and dialysis, recovery of the enzyme reached 127%, it may be considered that some enzyme inhibitor was removed by salting out and dialysis. It was difficult to purify L-glutamate oxidase by conventional methods. Thus, we developed new affinity adsorbents, L-glutamic acid–Sepharose 6B and L-glutamine–Sepharose 6B. The enzyme could be effectively purified by affinity chromatography using L-glutamic acid–Sepharose 6B and L-glutamine–Sepharose 6B.

The results of a detailed investigation on the characteristics of L-glutamate oxidase will be described in a subsequent report.

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