[Chem. Pharm. Bull. 31(10)3609—3616(1983)]

L-Glutamate Oxidase from *Streptomyces violascens*. II. Properties

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(Received February 23, 1983)

The properties of L-glutamate oxidase, a novel L-glutamic acid oxidizing enzyme from *Streptomyces violascens* H82-N-SY7 were examined. The enzyme showed absorption maxima at 280, 390 and 470 nm and a marked shoulder at 490 nm, and contained 1 mol of flavin-adenine dinucleotide (FAD) per mol of enzyme. Enzyme activity was inhibited by Ag^+, Hg^{2+}, *p*-chloromercuribenzoate (PCMB) and *N*-bromosuccinimide. The enzyme catalyzed the oxidation of L-glutamic acid using molecular oxygen as a primary electron acceptor and produced α-ketoglutaric acid, NH_3 and hydrogen peroxide according to the following schema: L-glutamic acid $+ H_2O + O_2 \rightarrow$ α-ketoglutaric acid $+ NH_3 + H_2O_2$. L-Glutamate oxidase was used in a specific and sensitive determination procedure for L-glutamic acid.

Keywords——L-glutamate oxidase; *Streptomyces violascens*; flavoprotein; enzymatic determination of L-glutamic acid

We have found a new L-glutamic acid oxidizing enzyme in the culture filtrate of *Streptomyces violascens* H82-N-SY7 and tentatively named the enzyme "L-glutamate oxidase."[1)]

In our previous report,[1)] it was shown that L-glutamate oxidase produced by *Streptomyces violascens* is a new enzyme distinct from L-glutamate oxidases from *Azotobacter vinelandii*[2)] and spinach leaves.[3)] Also, we have reported the purification of L-glutamate oxidase from the culture filtrate 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 present paper describes the characteristics of the enzyme as a flavoprotein, substrate specificity, identification of the reaction product and the stoichiometry of the enzyme reaction. Also, suitable conditions for the assay of L-glutamic acid in biological materials were examined using L-glutamate oxidase.

Materials and Methods

Materials——L-Glutamate oxidase was prepared from the culture filtrate of *Streptomyces violascens* H82-N-SY7, as described previously.[1)] Horseradish peroxidase (90 units/mg), flavin-adenine dinucleotide, flavin mononucleotide, catalase (beef liver), and D-amino acid oxidase (porcine kidney, crystalline) were purchased from Sigma Chemical Co. α-Ketoglutaric acid, riboflavin, and *N,N*-dimethylaniline were purchased from Wako Pure Chemical Co. All other reagents were purchased from commercial sources and were of analytical grade.

Determination of Enzyme Activity——Assay Method I: In the determination of the enzyme unit and the investigation of substrate specificity at pH 6.8, enzyme activity was measured as described previously.[1)]

Assay Method II: In assay method I,[1)] produced color was unstable below pH 6.5. Thus, we employed another determination system for hydrogen peroxide.[4)] The enzyme preparation (0.05 ml) was added to 2 ml of 0.05 M citrate buffer (pH 5.0), containing 100 μg/ml 4-aminoantipyrine, 0.3 μl *N,N*-dimethylaniline and 2 units/ml peroxidase. This

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 at 37 °C for 30 min. 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 550 nm against the blank.

Assay Method III: In the investigation of enzyme properties, another method was employed to avoid the effect of peroxidase. Enzyme assay was based on the measurement of NH_3 generated during the oxidation of L-glutamic acid. Ammonia was measured by the method of Miller and Rice.[5] The reaction mixture consisted of 0.05 ml of enzyme solution, 0.5 ml of 35 mM L-glutamic acid and 2 ml of 0.05 M citrate buffer (pH 5.0). The reaction was carried out at 37 °C for 30 min and terminated by heating in a boiling water bath for 3 min after adding 0.2 ml of Dowex 50W-X16 (Na). Adsorbed ammonia on the resin was eluted with 4 M NaCl and determined by means of the indophenol color reaction.[5]

Spectrometry——The spectrophotometric determination was carried out using a Hitachi 200-10 spectrophotometer.

Flavin Determination——The purified enzyme (1.48 mg), dissolved in 1 ml of 0.1 M citrate buffer (pH 4.0), was kept in boiling water for 5 min, and then centrifuged to remove denatured protein. Thin layer chromatography of flavins was carried out with the use of cellulose powder thin layer chromatography (Merck, Darmstadt). *n*-Butanol–acetic acid–water (4:2:4) and *tert*-amyl alcohol–formic acid–water (3:1:1)[6] were used as solvent systems. All chromatographic experiments were performed in the dark. The flavin spots were detected by their fluorescence in ultraviolet (UV) light.

D-Amino acid oxidase apoenzyme for the identification of flavin adenine dinucleotide (FAD) was prepared according to the method of Burton.[7] D-Amino acid oxidase apoprotein activity was measured in the presence or absence of flavins, as described previously.[1]

Determination of Enzyme Products——Oxygen consumption was measured according to the method of Miller.[8] L-Glutamic acid was determined by the method of Yemm *et al.*[9] Ammonia was determined according to the method of Miller and Rice.[5] α-Ketoglutaric acid was determined by the method of Suda *et al.*[10]

Results

Prosthetic Groups of L-Glutamate Oxidase

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. 1). Addition of L-glutamic acid to the enzyme solution under anaerobic conditions resulted in the disappearance of the peaks at 390 and 470 nm (Fig. 1). The ratio of E_{280}/E_{470} was 15. The absorption spectra indicated that flavin nucleotide is the prosthetic group. The enzyme was kept in a boiling water bath for 5 min and then centrifuged to remove denatured protein. The yellow supernatant solution exhibited absorption maxima at 370 and 450 nm, identical with those of FAD. The extinction of extracted flavin was almost identical with that of the native

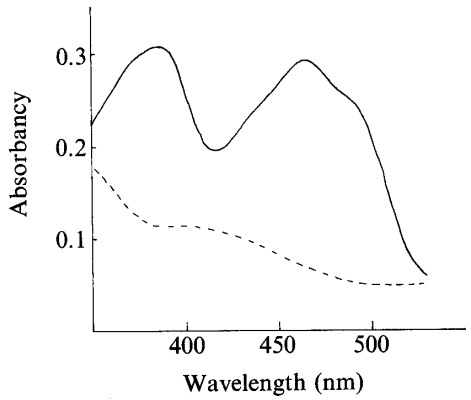

Fig. 1. Absorption Spectra of Native and Reduced L-Glutamate Oxidase

The solid line represents the native enzyme in 0.01 M citrate buffer (pH 5.0). The broken line represents the reduced enzyme after the addition of 1.75 μmol of L-glutamic acid under anaerobic conditions.

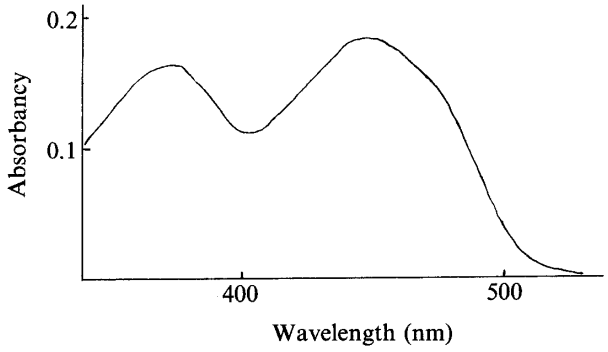

Fig. 2. Absorption Spectrum of Extracted Flavin Moiety

The purified enzyme (1.48 mg) was heated at 100 °C for 5 min, and centrifuged to remove denatured protein. The absorption spectrum of the supernatant solution was recorded.

enzyme. The flavin solution was subjected to cellulose powder thin layer chromatography. The chromatogram showed a single fluorescent spot with *Rf* values nearly identical with those of FAD. In addition, the supernatant solution activated apo-D-amino acid oxidase. Thus, the flavin moiety was identified as FAD.

FAD Content

When 1.48 mg of enzyme (2.39×10^{-8} mol on the basis of the molecular weight of 62000[1]) was heated as described above, the amount of FAD dissociated was estimated to be 1.73×10^{-8} mol as judged from the absorption at 450 nm ($\varepsilon = 1.13 \times 10^4$).[11] The result indicates that the enzyme contains 1 mol of FAD per mol of protein.

Effect of pH

The pH dependence of the enzyme activity was determined with L-glutamic acid as a substrate in various buffers (Fig. 3). The enzyme showed maximum reactivity at pH 5.0. The reaction rate declined markedly above pH 7.0 and below pH 4.0. The enzyme activity was not significantly influenced by the kind of buffer used. L-Glutamate oxidase was stable in the pH range from 3.0 to 7.0 at 37 °C for 1 h (Fig. 4).

TABLE I. Identification of the Flavin Moiety of L-Glutamate Oxidase by Cellulose Powder Thin-Layer Chromatography

Compound	*Rf*	
	Solvent I	Solvent II
Riboflavin	0.60	0.39
Flavin adenine dinucleotide	0.34	0.06
Flavin mononucleotide	0.52	0.26
Extracted flavin	0.33	0.04

The flavin spots were detected by their fluorescence under UV light. All chromatographic experiments were performed in the dark.
Solvent I: *n*-butanol–acetic acid–water (4:2:4).
Solvent II: *tert*-amyl alcohol–formic acid–water (3:1:1).

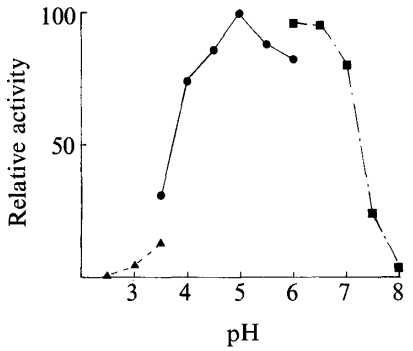

Fig. 3. Effect of pH on L-Glutamate Oxidase Activity

The reaction mixture consisted of 50 μl of enzyme solution (0.6 units/ml), 0.5 ml of 35 μmol/ml of L-glutamic acid and 2 ml of various buffers described below. Mixtures were incubated at 37 °C for 30 min. The produced ammonia was measured by the Miller–Rice method.[5]
pH 2.5—3.5, 0.05 M glycine–HCl buffer (▲), pH 3.5—6.0, 0.05 M citrate buffer (●), pH 6.0—8.0, 0.05 M phosphate buffer (■).

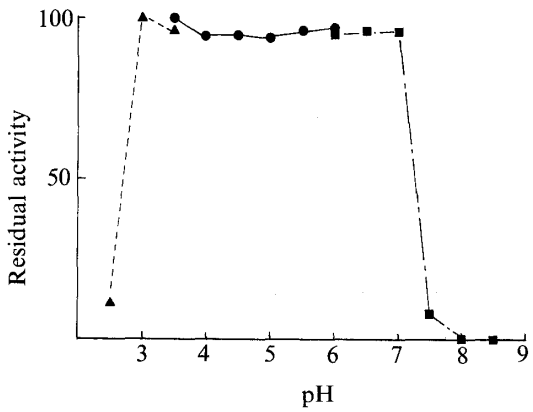

Fig. 4. pH Stability Curve of L-Glutamate Oxidase

The residual activities of L-glutamate oxidase were measured after standing at 37 °C for 1 h at the indicated pH. The buffers used were those described in Fig. 3.

Table II. Relative Oxidation Rates of Various Substrates by L-Glutamate Oxidase

Substrate	Relative rate	
	pH 5.0	pH 6.8
L-Glutamic acid	100	100
D-Glutamic acid	0	0
L-Glutamine	2.5	32.1
D-Glutamine	0	0
L-Aspartic acid	0	0
D-Aspartic acid	0	0
L-Asparagine	0	0
D-Asparagine	0	0
L-Tyrosine	2.7	0
L-Histidine	2.4	13.1
L-Phenylalanine	0	0
D-Phenylalanine	0	0
L-Tryptophan	0	0
L-Leucine	0	0
L-Isoleucine	0	0
L-Valine	0	0
L-Alanine	0	0
L-Glycine	0	0
L-Methionine	0	0
D-Methionine	0	0
L-Cysteine	0	0
L-Serine	0	0
L-Threonine	0	0
L-Proline	0	0
L-Lysine	0	0
L-Arginine	0	0
L-Ornitine	0	0
α-L-Glutamyl-L-alanine	0	0
Glutathione	0	0
L-Pyroglutamic acid	0	0
γ-Methyl-L-glutamic acid	0.6	0
L-γ-Glutamyl-*p*-nitroanilide	0	0

pH 6.8: 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 L-glutamate oxidase in a total volume of 2.55 ml. The reaction was carried out at 37 °C for 30 min, and the produced color was measured at 500 nm.

pH 5.0: The reaction mixture consisted of 17.5 μmol of substrate, 0.492 μmol of 4-aminoantipyrine, 2.37 μmol of *N*, *N*-dimethylaniline, 4 units of peroxidase and L-glutamate oxidase in a total volume of 2.55 ml. The reaction was carried out at 37 °C for 30 min, and the produced color was measured at 550 nm.

Substrate Specificity

The ability of the enzyme to catalyze oxidation of various L-amino acids, in particular L-glutamic acid derivatives, was investigated (Table II). L-Glutamate oxidase oxidized L-glutamic acid effectively in the pH range from 5.0 to 6.5 (Fig. 3). The enzyme oxidized L-glutamine and L-histidine at pH 6.8. However, the oxidation rates of L-glutamine and L-histidine were relatively low compared with that of L-glutamic acid. At pH 5.0, L-glutamine, L-tyrosine, and L-histidine were scarcely oxidized. γ-Substituted L-glutamic acid derivatives, glutathione and L-γ-glutamyl-*p*-nitroanilide, were not oxidized by L-glutamate oxidase. Further, the enzyme did not oxidize α-L-glutamyl-L-alanine.

Michaelis Constants

The Michaelis constants for the oxidation of L-glutamic acid, L-glutamine and L-histidine were determined by the method of Lineweaver and Burk.[12] K_m values for L-glutamic acid, L-glutamine, and L-histidine at pH 6.8 were 3.3×10^{-3} M, 6.7×10^{-3} M, and 5.0×10^{-3} M, respectively. K_m values for L-glutamic acid and L-glutamine at pH 5.0 were 1.1×10^{-3} M and 1.0×10^{-2} M, respectively. V_{max} values for L-glutamic acid at pH 5.0 and 6.8 were 92 μmol·min^{-1}·mg protein^{-1} and 128 μmol·min^{-1}·mg protein^{-1}, respectively.

Identification of Reaction Products

The reaction product of L-glutamic acid was identified as α-ketoglutaric acid by cellulose thin layer chromatography.[1] The reaction product formed from L-glutamic acid was also examined. After aerobic incubation of L-glutamic acid with L-glutamate oxidase in the presence or absence of catalase in 0.05 M citrate buffer (pH 5.0) at 37 °C for 18 h, reaction product(s) were purified by Bio-Rad AG-1 × 2 chromatography. The reaction product in the presence of catalase was identified as α-ketoglutaric acid by mixed melting point determination and comparison of the infrared (IR) spectrum with that of an authentic specimen. The reaction products in the absence of catalase were identified as succinic acid (main product) and α-ketoglutaric acid (minor product) by the same procedures as mentioned above.

Stoichiometry of the Reaction

The stoichiometry of the enzymatic oxidation of L-glutamic acid was investigated. As shown in Table III, the ratio of the amount of L-glutamic acid consumed, O_2 consumed, α-ketoglutaric acid formed and NH_3 formed was almost 2 : 1 : 2 : 2 in the presence of catalase. In the absence of catalase, succinic acid and α-ketoglutaric acid were detected in the reaction mixture by thin layer chromatography. Hydrogen peroxide was also detected in the reaction mixture without catalase. The amount of hydrogen peroxide, which was coupled with 4-aminoantipyrine and *N*,*N*-dimethylaniline in the presence of peroxidase, was almost equal to that of NH_3. Thus, hydrogen peroxide was thought to be an initial product in this oxidation. The oxidation of L-glutamic acid by L-glutamate oxidase seems to proceed as shown in Chart 1.

$$\text{L-glutamic acid} + H_2O + O_2 \xrightarrow{\text{L-glutamate oxidase}} \text{α-ketoglutaric acid} + NH_3 + H_2O_2$$

$$H_2O_2 \xrightarrow{\text{catalase}} 1/2\ O_2 + H_2O$$

$$\text{L-glutamic acid} + 1/2\ O_2 \longrightarrow \text{α-ketoglutaric acid} + NH_3$$

Chart 1

TABLE III. Stoichiometry of L-Glutamic Acid Oxidation Catalyzed by L-Glutamate Oxidase

Reaction time	Consumed or formed, μM			
	L-Glutamic acid consumed	O_2 consumed	α-Ketoglutaric acid formed	NH_3 formed
30 min	70.0	36.8	74.7	79.5
60 min	111	50.0	107	105

The enzyme reaction was performed in 0.05 M citrate buffer (pH 5.0). The reaction mixture consisted of 200 μM L-glutamic acid, 0.01 unit/ml purified L-glutamate oxidase, and 3.9 units/ml catalase.

Table IV. Effect of Inhibitors on L-Glutamate Oxidase Activity

Compound (1 mM)	Residual activity (%)
None	100
$AgNO_3$	0
$HgCl_2$	0
$CoCl_2$	96.1
$CuCl_2$	77.6
NaN_3	95.1
KCN	103
Diisopropylfluorophosphate	103
N-Ethylmaleimide	100
p-Chloromerculibenzoate	57.2
N-Bromosuccinimide	22.4
EDTA	100
α, α-Dipyridyl	106
o-Phenanthroline	102

The residual activity was measured by Assay Method III with L-glutamic acid as the substrate. The enzyme was preincubated with inhibitors for 30 min before the addition of substrate.

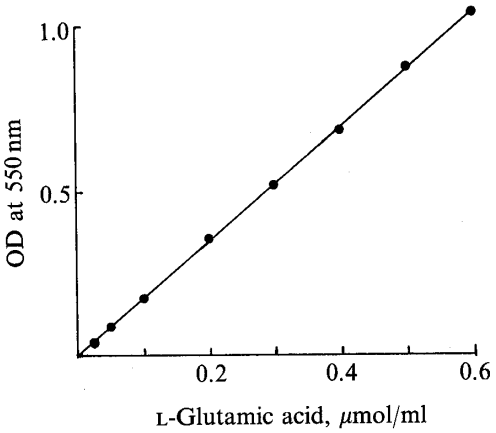

Fig. 5. Proportionality of Absorbance to L-Glutamic Acid Concentration

Half ml of L-glutamic acid solution was incubated with 2 ml of 0.05 M citrate buffer (pH 5.0), containing 100 μg of 4-aminoantipyrine, 0.3 μl of *N,N*-dimethylaniline, 2 units of horseradish peroxidase, and 0.25 units of L-glutamate oxidase for 30 min at 37 °C. The produced color was measured *vs.* a reagent blank at 550 nm.

Effect of Inhibitors on L-Glutamate Oxidase Activity

As shown in Table IV, L-glutamate oxidase activity was inhibited by Ag^+ and Hg^{2+} ions. *p*-Chloromercuribenzoate (PCMB) also inhibited the enzyme activity, but another SH reagent, *N*-ethylmaleimide, did not. *N*-Bromosuccinimide inhibited the enzyme activity. Metal chelating agents such as ethylenediaminetetraacetic acid (EDTA), α,α-dipyridyl and *o*-phenanthroline had no effect on the enzyme activity.

Specificity for Electron Acceptors

Nicotinamide-adenine dinucleotide (NAD^+), nicotinamide-adenine dinucleotide phosphate ($NADP^+$), methylene blue, thionine, and ferricyanide could not serve as electron acceptors. Because the purified enzyme did not exhibit dehydrogenase activities so far as studied, L-glutamate oxidase can be classified as an oxidase, not a kind of dehydrogenase. Addition of FAD and flavin mononucleotide (FMN) did not affect the oxidation rate of L-glutamic acid.

Application of L-Glutamate Oxidase to the Determination of L-Glutamic Acid

We next tried to determine L-glutamic acid, using L-glutamate oxidase. The amount of L-glutamic acid was determined by measuring the hydrogen peroxide generated during the oxidation of L-glutamic acid. The hydrogen peroxide was coupled with 4-aminoantipyrine and *N,N*-dimethylaniline in the presence of peroxidase to form a chromophore according to

TABLE V. Effect of Various Amino Acids and Reducing Agent on L-Glutamic Acid Determination

Compound	Concn. (μmol/ml)	Observed value (μmol/ml)	Difference (%)
None		0.500	
L-Glutamine	0.1	0.486	−2.8
	0.5	0.488	−2.4
	1.0	0.508	+1.6
L-Histidine	0.1	0.508	+1.6
	0.5	0.490	−2.0
	1.0	0.485	−3.0
L-Tyrosine	0.1	0.509	+1.8
	0.5	0.515	+3.0
	1.0	0.505	+1.0
L-Aspartic acid	0.1	0.513	+2.6
	0.5	0.503	+0.6
	1.0	0.510	+2.0
D-Glutamic acid	0.1	0.504	+0.8
	0.5	0.506	+1.2
	1.0	0.500	±0
2-Mercaptoethanol	0.1	0.444	−11.2
	0.5	0.209	−58.2
	1.0	0.082	−83.6

the method of Hirano *et al.*[4] Optimum conditions for the determination of L-glutamic acid were as follows. Half ml of L-glutamic acid solution was incubated with 2 ml of 0.05 M citrate buffer (pH 5.0), containing 100 μg of 4-aminoantipyrine, 0.3 μl of *N,N*-dimethylaniline, 2 units of horseradish peroxidase and 0.25 units of L-glutamate oxidase for 30 min at 37 °C. The produced color was measured *vs.* a reagent blank at 550 nm. The reaction was accomplished within 30 min at 37 °C, and the produced color was stable for 60 min at room temperature. Under the assay conditions, L-glutamic acid concentration was a linear function of absorbance at 550 nm at least up to an absorbance of 1.0 (Fig. 5). L-Glutamic acid was completely oxidized to α-ketoglutaric acid. The identity of the enzymatic reaction product was confirmed by thin layer chromatography. As shown in Table V, L-glutamine, L-histidine, L-tyrosine, L-aspartic acid, and D-glutamic acid did not affect the determination of L-glutamic acid. However, a high concentration of reducing agent inhibited the colorization in the L-glutamic acid assay system.

Discussion

L-Glutamate oxidase, a novel L-glutamic acid oxidizing enzyme was found in *Streptomyces violascens* H82-N-SY7. We have reported[1] that L-glutamate oxidase produced by *Streptomyces violascens* is distinct from L-amino acid oxidases including L-glutamate oxidases from *Azotobacter vinelandii*[2] and spinach leaves.[3]

Spectrophotometric studies with the purified enzyme indicated that L-glutamate oxidase is a flavoprotein. The flavin moiety dissociated from the enzyme protein was identified as flavin adenine dinucleotide. One mol of FAD per mol of enzyme protein was found to be present. The participation of FAD in the oxidation process of L-glutamic acid was confirmed by the spectral changes produced by adding substrate. Snake venom L-amino acid oxidase contains 2 mol of FAD/mol of enzyme.[13] L-Amino acid oxidase from rat kidney is a tetramer with Mr = 200000, and contains as a cofactor 0.5 mol[14] or 1 mol[15] of FMN/subunit. The

properties of these enzymes differ from those of L-glutamate oxidase from *St. violascens*. None of the metal chelating agents showed significant inhibition. It is considered that FAD is the only oxidation–reduction active group of this enzyme.

L-Glutamate oxidase oxidized L-glutamic acid effectively in the pH range from 5.0 to 6.5. The enzyme oxidized L-glutamine and L-histidine at pH 6.8. However, the oxidation rates of L-glutamine and L-histidine were relatively low compared to that with L-glutamic acid. At pH 5.0, L-glutamine and L-histidine were scarcely oxidized. γ-Substituted L-glutamic acid derivatives were not oxidized by the enzyme. Inhibition studies showed that L-glutamate oxidase was inhibited by SH reagents, such as Ag^+, Hg^{2+}, and PCMB. Thus, SH groups may be involved in the catalytic activity of the enzyme.

The enzyme reaction did not proceed without O_2. The reaction product of L-glutamic acid was identified as α-ketoglutaric acid in the presence of catalase. In the absence of catalase, the reaction products of L-glutamic acid were succinic acid and α-ketoglutaric acid. Hydrogen peroxide, another product of the reaction, may degrade α-ketoglutaric acid to succinic acid nonenzymatically.[16] The stoichiometry of the enzyme reaction indicated that L-glutamate oxidase might belong to the group of oxidases which catalyze two-electron reductions of oxygen. One mol of hydrogen peroxide was formed from one mol of oxygen.

L-Glutamate oxidase was also used in a specific and sensitive determination procedure for L-glutamic acid.

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