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Immobilization of L-Glutamate Oxidase and Peroxidase for Glutamate Determination in Flow Injection Analysis System

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ABSTRACT

Streptomyces SP.N 14, isolated from soil samples, produced extracellular L-glutamate oxidase (GOD) in liquid culture. After a two-step ammonium sulfate purification and dextran G-150 chromatography, the specific activity was reached at 28.2 U/mg. The partial purified enzyme and horseradish peroxidase (HRP) were covalently coupled to alkylamine controlled pore glass (CPG) by means of glutaraldehyde. About 200–300 U/g of immobilized GOD and 300–400 U/g of immobilized HRP were obtained. The immobilized enzymes were packed into a teflon tube and used in flow injection analysis (FIA) for glutamate in broth. A good linear range was observed for this immobilized enzyme system at 0.1–2.0 m*M*, and the precision was 2.8% (n = 25). More than 80 samples were measured within an hour. One enzyme column with about 4 U of immobilized GOD and 5 U of immobilized HRP, applied for 50 assays/d, has been used for more than 50 d. The concentration of L-glutamate remaining lower than 2.0 m*M*, the determination of glutamate in this system was not affected by pH and temperature within the range of 6.0–7.0 and 25–35°C, respectively. The system was applied to determine L-glutamate in broth samples during L-glutamate fermentation, and good correlation was achieved between results obtained with the system and with the Warburg's method.

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Index Entries: L-glutamate oxidase; immobilized enzyme; L-glutamate; flow injection analysis.

INTRODUCTION

L-glutamate is an economically important biotechnological product. It is contained in many kinds of foods and contributes to their enhanced flavor. The monitoring of L-glutamate is essential for food processing and fermentation control. In addition, glutamate determination can be used to estimate glutamate levels, glutamate oxaloactate transaminase, and glutamate pyruvate transaminase activities in serum, which are important analytes in clinical diagnosis. The determination of L-glutamate has been based mainly on chromatographic techniques (*1*). However, these methods involve complicated and time-consuming procedures. Enzymatic methods recently have been developed for the more rapid and selective determination of L-glutamate. Nikolelis (*2*) used a combination of soluble glutamate dehydrogenase and an ammonium gas-sensing electrode for the determination of this compound in foods. Microbial sensors based on glutamate decarboxylase-containing microorganisms in conjunction with a carbon dioxide gas-sensing electrode have been fabricated by Hikuma et al. (*3*). Riedl and Scheller (*4*) proposed an amperometric microbial sensor for L-glutamate. However, the stability of these sensors was less than 3 wk and response times were long. More recently, a new enzyme, oxidizing L-glutamate as substrate and producing hydrogen peroxide, was isolated from different sources (Table 1). Even if the biological importance of the more specific L-amino acid oxidases is still unknown, they provide a new method for determination of L-glutamate and related substances. Biosensors with immobilized glutamate oxidase were also reported (*5,6*). However, the capabilities of these microorganisms for producing L-glutamate oxidase are poor (Table 1), and commercially available L-glutamate oxidase is very expensive.

In this paper, we describe a strain of Streptomyces SP.N14, isolated from soil that produces extracellular L-glutamate oxidase in submerged culture. After 40–48 h of submerged fermentation at 28°C, 14 U/mL broth and specific activity of 2.5 U/mg were obtained. After a two-step ammonium sulfate purification, specific activity was increased to 14.8 U/mg. Further purification with dextran G-150 chromatography produced a specific activity of 28.2 U/mg. The partially purified enzyme and HRP were successfully immobilized on CPG by cross-linking with glutaraldehyde. The immobilized enzymes were packed in a teflon tube and applied in flow injection analysis for glutamate in broth. The method described here is simple, rapid, cheap, and particularly effective. The sample containing glutamate is injected into and mixed with carrier solution that is allowed to flow through the enzyme reactor. In the course of the enzymatic reactions, the oxygen receptor was oxidized to a colored product. The

Table 1
Various L-glutamate Oxidase from Different Sources

Strains	Mode of culture	Founder	Date	Activity, U mL^{-1}	Reference
sp. viola	liquid (extracell.)	Matsuzaki, A.	82.3	0.066	(*10*)
sp. A7700	liquid (intracell.)	Ishikawa, H.	82.3		(*11*)
sp. A8063	liquid (intracell.)	Ishikawa, H.	82.3		(*11*)
sp. X-119	solid (extracell.)	Kusakabe, H.	82.6	0.056	(*12*)
sp. endus	liquid (extracell.)	Passarge, M.	86.11	0.024	(*13*)
sp. plate	liquid(extracell.)	Chen, C. Y.	90.8		(*14*)

change in absorbance at 550 nm is measured, and the concentration of L-glutamate is calculated.

MATERIALS AND METHODS

Reagents

1. GOD: crude solution 28.2 U/mg, was harvested from SP.N14 in submerged fermentation and partially purified.
2. HRP: Horseradish peroxidase (Institute of Biochemistry, Academia Sinica, Shanghai, China) was used.
3. CPG: controlled pore glass (120–200 mesh, mean pore diameter 750 A) was obtained from the Institute of Silicate Chemistry, Academia, Shanghai, China.
4. Glutaraldehyde (25% aqueous solution) and (3-Aminopropyl) triethoxysilane were purchased from Fluka.
5. Buffer solution: phosphate buffer, 0.1*M*, pH 5.5–7.5.
6. Carrier solution: 0.05*M* phosphate buffer, pH 6.5, which contained phenol (0.2 g/L), 4-aminoantipyrine (50 mg/L), and sodium azide (0.2 g/L), was used as carrier solution in flow injection analysis for glutamate determination.
7. Glutamate standard: L-glutamate (AR) was dried in an oven at 80°C for 3 h, and 5 m*M* glutamate solution containing 0.14% (w/v) benzoic acid was made as stock solution. A series of standard working solutions with glutamate concentration in the range of 0–2.0 m*M* were prepared by diluting the stock solution with 0.14% benzoic acid solution.

Apparatus

A flow injection analysis system (LZ-1000, Shenyang, Liao Ning, China) with 721 spectrophotometer was used for determination of glutamate (Fig. 1). The recorder (XWC-100, Shanghai) was used to record the

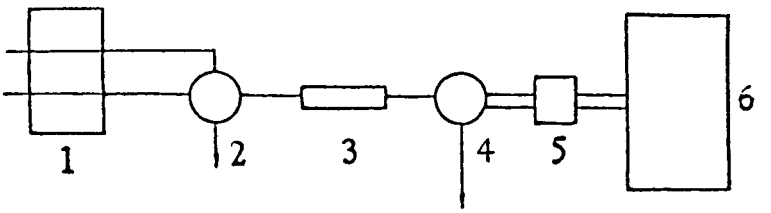

Fig. 1. Diagram of FIA for L-glutamate determination using GOD and HRP reactor. 1, pump; 2, sample valve; 3, immobilized enzyme reactor; 4, detector; 5, logarithmic transformer; 6, recorder.

output signal of 721 spectrophotometer after logarithmic transformation. All connecting tubes were made from teflon and had an internal diameter of 0.5 mm.

Determination of the Activity of Native and Immobilized GOD Oxidase

The activity of GOD was determined using a spectrophotometric method based on the production of H_2O_2. A reaction mixture containing 1.4 mL of N,N-diethyl-m-toluidine (0.26 μ 1/mL, pH 6.5), 1.0 mL of 4-aminoantipyrine (121.5 μ g/mL, pH 6.5), 0.5 mL of L-glutamate (11 mg/mL, pH 6.5), and 0.1 mL of a HRP (60 U/mL) was incubated at 37°C in a water bath for 2 min. Then 0.1 mL of L-glutamate oxidase solution was added and the mixture was incubated at 37°C for another 10 min. The concentration of hydrogen peroxide was determined by measuring the absorbance at 550 nm. Enzyme activity was determined by comparison with a calibration curve for hydrogen peroxide. One unit of activity is determined as the amount of the enzyme which can catalyze L-glutamate oxidation reaction to produce 1 μM hydrogen peroxide per min under the conditions described. Determination of the activity of immobilized L-glutamate oxidase was similar to the assay for the native enzyme.

Determination of Protein

The protein was determined according to the method described by Lowry et al. (*7*). Immobilized protein was calculated from the amount of protein added for immobilization minus the amount remaining after immobilization.

Preparation of Immobilized Enzyme Reactor

1. Silanization of CPG: 1 g of CPG was immersed in 10 mL of 10% γ-aminopropyltriethoxysilane in 95% ethanol. The mixture was degassed by applying suction, and was shaken for 2 min. The solid was isolated by filtration, washed with 95% ethanol, and then cured by heating to 110°C for 10 min.

2. Immobilization of GOD: to 0.2 g of alkylamine glass, 2 mL of 2.5% (w/v) solution of glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.0) was added. The solution-solid mixture was degassed by applying suction and was shaken in a shaker for 60 min. The solid was washed with distilled water to remove the unreacted glutaraldehyde. To the glass, 0.5 mL of GOD solution (18 mg/mL) in 0.1 mol/L phosphate buffer (pH 6.5) was added. The reaction took place in a vacuum dissector for 10 min and then at 4°C overnight. The immobilized enzyme was washed with distilled water to remove the free enzyme and stored at 4°C in phosphate buffer (0.1*M*, pH 6.5).
3. Immobilization of HRP: the same as the method of immobilization GOD except that pH 7.5 of phosphate buffer was used instead of pH 6.5 of phosphate buffer.
4. Construction of the immobilized enzyme reactor: the immobilized GOD and HRP were packed into a teflon tube (2 mm ID, length 30 mm) and both ends were sealed with nylon net. The packed-bed reactor contained about 4–5 U GOD and HRP. The immobilized enzyme reactor was applied in a flow injection analysis (FIA) system.

RESULTS AND DISCUSSION

Immobilization of Enzyme

Silanization of CPG

The procedure of Silanization of CPG varied with the solvent used for dissolving the silane reagent: (1) distilled water: the pH of the reaction must be adjusted to 3.5 precisely in the Silanization procedure (*8*), (2) Toluene: the reaction is carried out under reflux at 110°C in a liquid paraffin bath (*9*). The silanized materials produced are stable, but the procedure is tedious; (3) ethanol: the procedure is comparatively simple and results are almost the same as that of toluene in our experiments.

The activity of immobilized enzyme was affected by the purity of the enzyme added (Table 2). The activity of immobilized enzyme can be improved by using high purified enzyme. It is supposed that some of the impurities preferentially bind to the glass.

Effect of pH and Temperature

The effect of pH on the determination of glutamate by using the system shown in Fig. 1 was studied by determination of standard glutamate solutions in phosphate buffer (0.1*M*) at various pH values. The results showed that pH had no effect on the determination of glutamate within the range of pH 6.0–7.0, if the concentration of glutamate remained below 2.0 m*M*.

Table 2
Effect of GOD Purity on the Immobilization of GOD on CPG

Native GOD purity, U/mg	2.4	7.6	4.8	28.2
GOD yield, U/g	28	102	200	320
Protein yield, mg/g	70	57	50	28.3
Immobilized GOD purity, U/mg	0.4	1.8	4.0	11.3
GOD purity yield, %	16.7	23.7	27	40

Enzyme purity yield = immobilized enzyme purity/native enzyme purity.

Table 3
The Specificity of the FIA System

Substrate, 200 mg L^{-1}	Relative response, %
L-Glutamate	100
D-Glutamate, L-Glutamine, L-Leu, L-Ile, L-Ser, L-Arg, L-Pro, L-Lys, L-His, L-Thr, L-Met, L-Cys, α-Ketoglutaric acid, Butyric acid	0

The effect of temperature on the determination of glutamate in the system shown in Fig. 1 was investigated by determination of standard glutamate solution with phosphate buffer (pH 6.5, 0.1*M*) at various temperatures. Within the range of 25–35°C, temperature had no significant effect on the determination of glutamate when the concentration of glutamate remained lower than 2.0 m*M*. The determination of glutamate in this system was not affected by pH and temperature in the range of pH 6.0–7.0 and 25–35°C, respectively; it's suggested that the immobilized enzyme reactor has enough activity to completely convert glutamate to hydrogen peroxide under such conditions. It is helpful for practical use of this system.

Specificity

The relative responses of this system to some other amino acids are shown in Table 3. There was no response to other amino acids examined. The specificity of the novel L-glutamate oxidase was superior to that of a commercially available L-glutamate oxidase.

Calibration

The assay procedure was carried out by the method described in the Materials and Method section. The calibration curve was obtained under the optimum conditions described above was shown in Fig. 2. A typical chart recorder output is shown in Fig. 3. The linear range of the calibration curve was 0.1–2.0 m*M* L-glutamate and the determination rate reached 80 samples per hour.

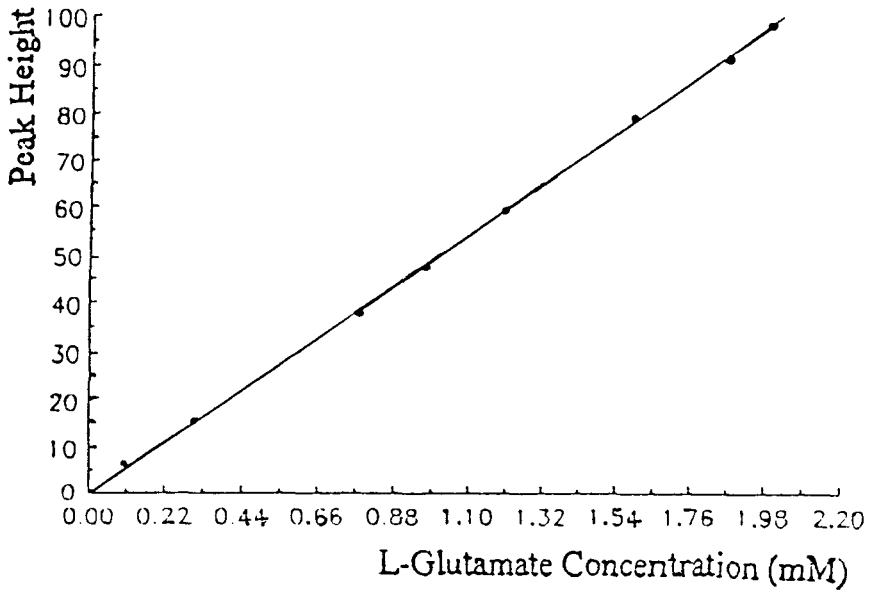

Fig. 2. Calibration curve for L-glutamate using FIA system.

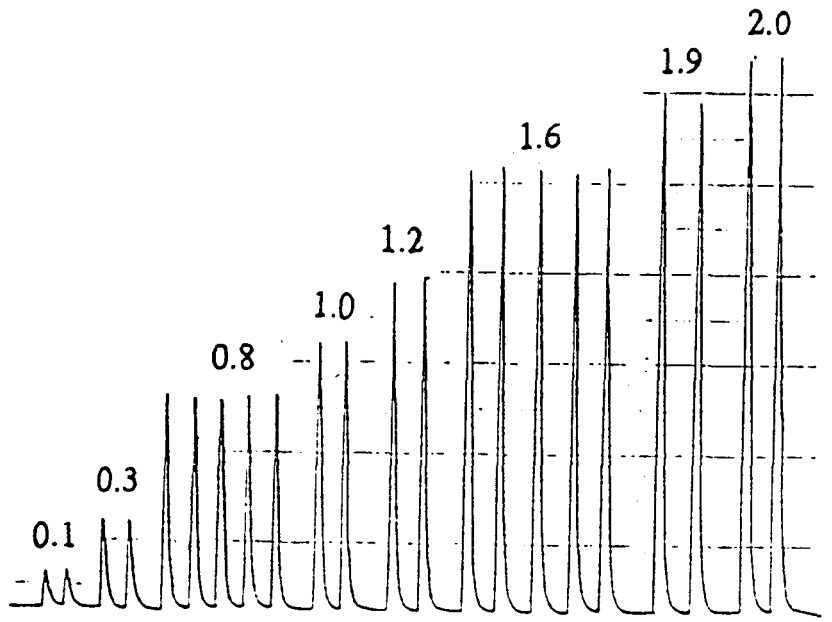

Fig. 3. Typical signals obtained in FIA system for L-glutamate determination using the immobilized enzymes.

Stability

The stability of immobilized GOD and HRP reactor was tested in the FIA for glutamate determination over a period of 2 mo, during which 50 assays were performed daily, and the enzyme reactor was stored at 4°C in phosphate buffer when not in use. The immobilized GOD and HRP reactor can be used 50 d. The reproductivity of this system for 2.0 m*M* glutamate standard solution determination is good (cv 2.8%, n = 25).

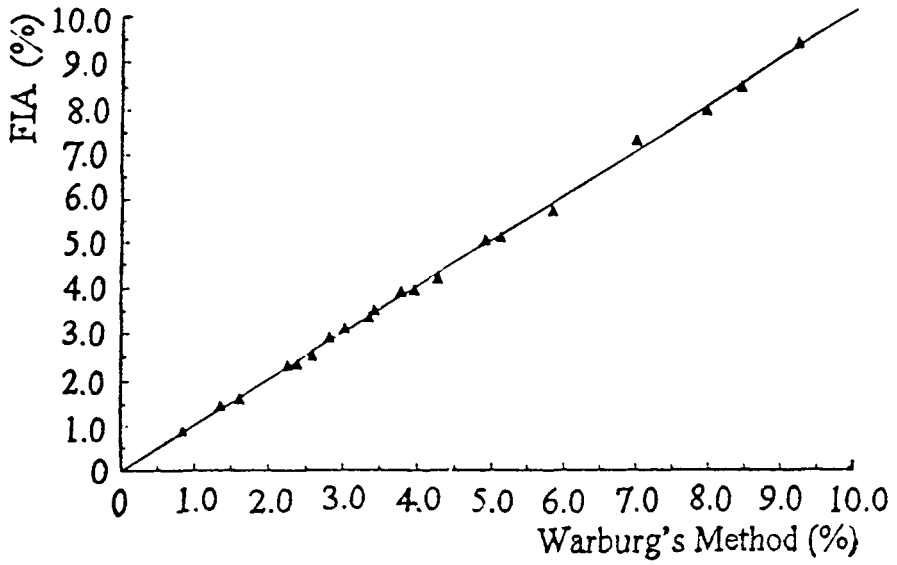

Fig. 4. Correlation and regression line for broth glutamate determined by immobilized enzyme method against that determined by Warburg's method.

Determination of Glutamate in Broth

Different samples were taken at different stages during the whole L-glutamate fermentation process and the L-glutamate concentrations were determined by Warburg's respiration apparatus and the immobilized GOD reactor described in this paper. The results are shown in Fig. 4. A high correlation was obtained between the two methods; the correlation coefficient was 0.990 and the regressing equation for the data was $Y = 0.985X + 0.058$ (Y, enzyme reactor; X, Warburg's method).

CONCLUSION

The novel L-glutamate oxidase appears to be a good tool enzyme because of its superiority in substrate specificity and simplicity in large production. The immobilized GOD and HRP reactor used in FIA for determination of glutamate in broth is very simple, cheap, and suitable for monitoring L-glutamate concentration during the fermentation process.

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