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CHEMICAL & BIOSENSORS

Effect of L-Aspartate Concentration on the Response of the Amperometric L-Glutamate Sensor for the Measurement of L-Glutamate and Aspartate Aminotransferase Activity in Serum

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Abstract: L-glutamate oxidase was immobilized in a photo-cross-linkable polymer membrane on a palladium strip electrode for the amperometric measurement of aspartate aminotransferas eactivity. The sample, serum for example, was injected into a buffered L-aspartate and α-ketoglutarate solution. L-aspartate is the essential substrate and can transfer to L-glutamate via the aspartate aminotransferase catalyzing reaction. Aspartate aminotransferase activity can be measured by determining the increasing rate of L-glutamate. Under the optimal condition, the current increasing rate was proportional to the aspartate aminotransferase activity of the sample in the range of 8–200 U/L. The data are in good correlation ($R^2 = 0.998$) with data from

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a commercial aspartate aminotransferase assay kit. Good reproducibility (relative standard deviation = 3.03%, $n = 8$) was obtained from a sample with 50 U/L aspartate aminotransferase activity. The sensor is expectable to be applied in a clinical point-of-care diagnosis.

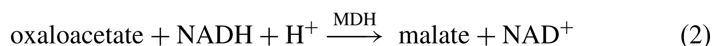
Keywords: Biosensor, aspartate aminotransferase, L-glutamate oxidase, polyvinyl alcohol bearing a styrylpyridinium residue, amperometric sensor

INTRODUCTION

Elevated serum levels of aspartate aminotransferase (AST) indicate liver or heart function disorder (Rietz and Guilbault 1975a). When the liver is damaged, the enzyme will be released into the bloodstream, where it is measured to obtain clinical diagnostic information.

Most of the clinical laboratories employ spectrophotometric or fluorimetric methods (Karmen 1955; Reitman and Frankel 1957; Rietz and Guilbault 1975b; Clark 1987) for determining AST activity. These methods are based on the measurement of absorbance decrease of NADH, which is the coenzyme involved in the reaction of malate dehydrogenase (MDH). The MDH-catalyzing reaction is summarized as follows (Clark 1987).

$$\text{L-aspartate} + \alpha - \text{ketoglutarate AST} \quad \text{oxaloacetate} + \text{L-glutamate} \qquad (1)$$



$$\text{oxaloacetate} + \text{NADH} + H^{+} \xrightarrow{\text{MDH}} \text{malate} + \text{NAD}^{+} \qquad (2)$$

However, these methods rely on complicated and expensive instruments and thus are not suitable for point-of-care applications. Alternatively, amperometric sensors appear very attractive because they can provide specific and repetitive assays by miniaturizable low-cost devices. A carbon strip electrode, prepared with a screen-printing technique, was often employed for constructing an amperometric biosensor. The following reaction, using L-glutamate oxidase (L-GLOx) is an alternative to the amperometric sensor to measure AST activity.

$$\text{L-glutamate} + O_2 \xrightarrow{\text{L-GLOx}} \alpha\text{-ketoglutarate} + NH_3 + H_2O_2 \qquad (3)$$

The reference range for AST activity in normal adults is between 30 and 40 U/L only. The determination of transaminase activity on an amperometric sensor usually requires a rather long time (Wollenberger et al. 1989; Compagnone et al. 1992; Cooper et al. 1991), owing to the low AST activity in the serum sample (Hart et al. 1999). For achieving a rapid and highly sensitive measurement, the following approachs are taken into consideration. First, hydrogen peroxide liberated from enzymatic reactions in the vicinity of the electrode surface must be oxidized immediately.

In one of our recent studies, palladium metal was deposited on the electrode (Chang et al. 2003). The deposition of palladium on the electrode surface provides a very porous structure consisting of aggregates of fine palladium particles. This results in a significant increase in the electrode surface area, which leads to an increase in surface activity for the measurement of the hydrogen peroxide liberated from enzymatic reactions (White et al. 1994; Newman et al. 1995; Chi and Dong 1993; Johnston et al. 1995). Second, the substrate must diffuse quickly through the thin layer to undergo the enzymatic reaction. Third, substrate and oxygen must diffuse quickly through the enzyme membrane layer to undergo the enzymatic reaction (Mizutani et al. 1998). The use of an electrochemical mediator is useful to shuttle electrons from the active site of the enzyme or biomolecule to the electrode surface. However, the electroactive species employed in such a system would be easily diffused away from the electrode surface and thus rendered unsuitable for continued use. A photo-cross-linkable polymer polyvinyl alcohol bearing a styrylpyridinium residue (PVA-SbQ) has also been reported to be suitable for fabrication of a thin enzyme membrane (about 1 μm thick) (Sohn et al. 1997). The enzyme can be immobilized in the matrix with high surface density (Jaffrezic-Renault et al. 1999). The authors presented a highly sensitive L-glutamate sensor for measuring the activity of alanine aminotransferase (ALT) in serum (Chang et al. 2003). The sensor is composed of immobilized L-GLOx in a photo-cross-linkable polymer, PVA-SbQ, membrane on a palladium-deposited screen-printed carbon electrode. PVA-SbQ is a polyvinyl alcohol bearing a styrylpyridinium residue as its photosensitive group. The cross-linking of the polymer is performed using florescent light or UV radiation to produce cyclodimerization of the styrylpyridinium groups (Ichimura and Watanabe 1980). PVA-SbQ has various advantages when applied as an immobilizing agent for proteins (Ichimura 1984; Newman et al. 1995; Rouillon et al. 1994; Perdomo et al. 1999). Its cross-linking reaction is induced in the light with a wavelength longer than 320 nm: 1) there occurs no photo-induced deactivation to an enzyme because such a light is not absorbed by proteins and 2) chemical denature is prevented because no covalent bonds form between the enzyme and the matrix (Ichimura 1984). The measurement of AST activities in the serum was more difficult than the measurement of ALT activities because the substrate for AST catalytic reaction, L-aspartate, may cause a cross-reaction with L-GLOx and generate a cathodic signal in the sensor, as has been reported (Kusakabe et al. 1983; Zilkha et al. 1995; Janasek and Spohn 1999). In preliminary tests, the L-glutamate sensor did not work when the reagent contained high concentrations of substrate. In this research, an optimized study was performed for the measurement of AST activity in serum. The response curves of low AST concentration and high AST concentration were compared at the same substrate dose of reagent. The detection time, reproducibility, and sensitivity of the sensor for the measurement of AST activity in serum were also determined.

EXPERIMENTAL SECTION

Reagents

Bovine serum albumin (BSA), monosodium L-glutamate (MSG), L-aspartate, α-ketoglutarate (α-KG), glutaraldehyde, and AST (EC 2.6.1.1, from porcine heart) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). PVA-SbQ (degree of polymerization 1700, degree of saponification 87%) was obtained from Toyo Gosei Kogyo Chemical (Tokyo, Japan). The enzyme L-GLOx (EC 1.4.3.11) was produced from *Streptomyces sp.* NTU 3304 in our laboratory (Chen and Su 1991). All other reagents were of analytical grade.

Electrode Modification

A commercial glucose test strip (Boehringer-Mannheim Roche Diagnostics Corp., Indianapolis, USA) consisting of two palladium strip electrodes was used. The palladium connecting strips of the commercial electrode were used as electrodes by cutting off the enzyme loaded pads. The prepared palladium strips were then modified and connected to a laboratory-built potentiostat as the work and counter electrodes.

Enzyme Immobilization

The enzyme was immobilized by a combination process of PVA-SbQ photo-cross-linking and glutaraldehyde exposure. The enzyme solution with activity of 70 U/mL was prepared by mixing 50 mg PVA-SbQ, 10 mg BSA, and 140 mg of L-GLOx with activity of 70 U/mL. Then 1 μL of the mixture was applied onto the active area of the electrode. The sensor was then placed in a dark sealed box with saturated glutaraldehyde vapor. The box was kept at 4°C for 12 hours to complete the cross-linking reaction among the protein molecules by glutaraldehyde. The sensor was then exposed to florescent light for 25 min to complete the photo-cross-linking reaction among PVA-SbQ molecules.

Apparatus and Procedure

Amperometric measurements were performed using a laboratory-built potentiostat. Input and output signals from the potentiostat were coupled to a PC (Pentium 166 MHz) using a peripheral interface card (AT-MIO-16E, National Instruments, Austin, TX, USA). The interface card consisted of a 16-channel analog-to-digital (A/D) converter (12 bit) and a 2-channel digital-to-analog (D/A) converter (12 bit). Voltage output, data display, and signal recording were programmed using the LabVIEW 5.1 software

package (National Instruments, Austin, TX, USA). All measurements were taken with a three-electrode system, using an Ag/AgCl electrode as the reference and the modified palladium strips as the working and the counter electrodes.

The amperometric measurement was performed at 0.4 V vs. Ag/AgCl in a thermostat water-jacketed cylindrical cell. Phosphate buffer (PBS, pH 7.0) was used as the working solution. All experiments were carried out at 35°C. For the amperometric measurement of MSG, 100 mM of MSG solution were injected into a 9.9-mL working solution, using a microsyringe, when a steady state of the operation system had been reached. The baseline current and the response current following the injection of MSG were displayed and simultaneously logged by the computer until a steady state was achieved. Magnetic stirring was used to ensure the homogeneity of the solution. The difference between the baseline and the steady-state current was used to calculate the concentration of MSG.

For the determination of AST activities in serum, a buffered substrate solution was prepared by dissolving 1 mM α-KG and 25 mM L-aspartate in 100 mM phosphate buffer (pH 7.0). The electrodes at working potential of 0.4 V vs. Ag/AgCl were settled in the reaction cell with a given volume of the buffered substrate solution. Samples were injected into the reaction cell when a baseline current had been reached. The response current was recorded continuously, and the current-to-time curve was recorded. The standard sample solutions for which the AST activity had been determined by the Sigma AST assay kit were used to establish the calibration graph.

RESULTS AND DISCUSSION

Fabrication of the L-Glutamate Sensor

The L-glutamate produced in the AST catalytic reaction is converted into α-KG and hydrogen peroxide by L-GLOx. The hydrogen peroxide was then oxidized on the palladium electrode by the polarizing potential applied to the electrode. The response current obtained from the oxidation of hydrogen peroxide corresponds to the concentration of hydrogen peroxide, L-glutamate, and the AST activity within the working capacity of L-GLOx.

The AST activity is as low as 30 U/L in human serum (Mizutani et al. 1998). A thin membrane of immobilized enzyme with satisfactory diffusion characteristics is desirable for achieving a rapid and highly sensitive measurement of AST activity. PVA-SbQ has been reported to be suitable for the fabrication of an immobilized enzyme membrane as thin as 1 μm with good diffusion characteristics (Ichimura 1984). It was chosen as the enzyme- immobilizing agent in this study and further combined with

glutaraldehyde to fabricate the immobilized enzyme membrane. The reaction between glutaraldehyde and a primary amino group by the formation of the Schiff-base bond has been investigated and applied in covalently binding amino groups on an enzyme molecule, L-GLOx for example (Chang et al. 2003; Chen 1987). Protein molecules with no catalytic activity, such as BSA, are usually co-cross-linked with the enzyme molecules to increase the size of cross-linked molecules. The authors' recent work showed that the optimal composition for the enzyme immobilization was 25% PVA-SbQ and 2.5% BSA, by weight (Chang et al. 2003). The immobilized enzyme membrane on the active area of the electrode was constructed according to the procedure previously described. The optimal period of exposure to glutaraldehyde vapor in the dark sealed box was 12 hours (Table 1). Another study was conducted to examine the effect of the applied volume of the enzyme solution on the response of the sensor. The results showed that a solution of 1 μL enzyme yielded maximal response and 0.5 μL resulted in a rapid response. The between-electrode reproducibility of the sensor, however, was poor when the applied volume was less than 1 μL because the enzyme membrane was unable to distribute evenly on the electrode. The applied volume of the enzyme solution was thus decided as 1 μL in the following experiments.

The stability and reproducibility of the response obtained with the same electrode was satisfactory. The within-electrode relative standard deviation was 2.6% for the response to 10 μM MSG over 100 tests. The sensor was stable for 5 mo in dry condition under room temperature. The immobilized enzymes can retain their functional characteristics to a large extent for several months upon repetition of wetting and drying (Chang et al. 2003). The satisfactory stability can be attributed to the method of combining photo-cross-linking of PVA-SbQ with a chemical-cross-linking agent (e.g., glutaraldehyde), to immobilize the enzyme effectively, and to the presence of a great number of −OH groups in the PVA-SbQ matrix that offer a hydrophilic environment around the enzyme molecules.

Table 1. Effect of the exposure time in saturated glutaraldehyde vapor on the response characteristics of the sensor

Exposure time (h)	Response[a] (nA)	SD (nA)	RSD (%)
0	1215	246	20.24
6	1113	182	16.32
12	1088	92	8.46

[a]Each item of the data is the mean value of three measurements. Measurements were carried out with 100 μM MSG as the substrate in 100 mM sodium phosphate buffer (pH 7.0). The polarizing potential applied to the sensor was 0.4 V vs. Ag/AgCl.

Effect of pH and Temperature on the Response of the Sensor

As described in our previous paper (Chang et al. 2003), the L-GLOx electrode exhibited an anodic response to hydrogen peroxide starting at +0.2 V, reaching its diffusion current limit at about +0.4 V. This result is in agreement with the findings (Chi and Dong 1993: Newman et al. 1995) in similar experiments. In order to minimize the interference from electroactive species (e.g., ascorbic acid and uric acid), an operation potential of +0.4 V was selected for the following tests.

The effect of pH on the response of the sensor was examined in the pH range from 5.0 to 9.5. The maximal sensitivity was observed in buffer solutions with pH 7.0. The fact that optimal pH for immobilized L-GLOx was close to that of the soluble L-GLOx revealed that the immobilizing process did not change the catalytic behavior of L-GLOx due to the pH variation (Chen 1987). The positively charged PVA-SbQ matrices were reported to change the optimal pH value for some immobilized enzymes (Ichimura and Watanabe 1980). However, it had only a negligible influence on the optimal pH value of L-GLOx in the present work.

The effect of temperature on the sensitivity of the sensor was also studied in the range from 15 to 80°C (Fig. 1). The maximum sensitivity was observed at 65°C. It is 15°C higher than the optimal temperature for soluble L-GLOx, indicating that the immobilization process improved the thermal stability of the enzyme (Chen 1987). The decrease in response when the temperature was higher than 65°C is considered as thermal inactivation of L-GLOx. Although the response of the sensor was enhanced at higher temperatures, the response of the sensor became unstable at a temperature higher than

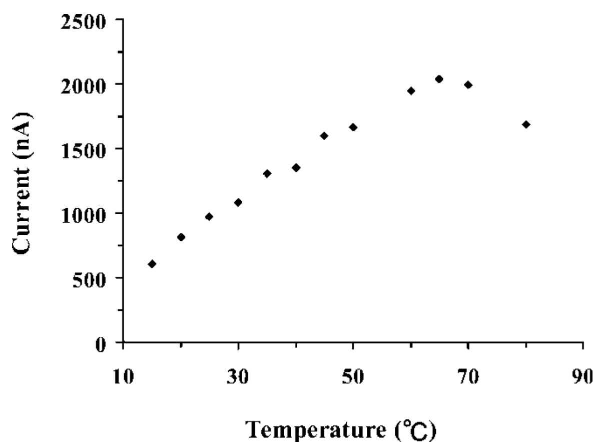


Figure 1. Effect of temperature on the response of the sensor. Measurements were carried out with 100 μM MSG as the substrate in 100 mM sodium phosphate buffer (pH 7.0). The polarizing potential applied to the sensor was 0.4 V vs. Ag/AgCl.

50°C (data not shown). The temperature of 37°C was chosen for the present study under the consideration of human body temperature.

Determination of MSG Concentration

Two calibration curves for the determination of MSG were established by the steady-state method and the kinetic method, respectively. The sensor exhibited a linear calibration range, from 50 nM to 100 μM, with a slope of 12.18 nA/μM and a correlation coefficient of 0.997 ($n = 10$) based on the steady-state method. The detection limit of MSG was 50 nM (S/N = 3). The calibration curve obtained from the kinetic method showed dynamic linear ranges from 50 nM to 75 μM, with a correlation coefficient of 0.994.

Optimization of Substrate Composition

α-KG and L-aspartate are necessary substrates for the measurement of AST activity. Responses of the enzyme-catalyzed reactions have been reported to be proportional to the substrate concentration (Mizutani et al. 1998). However, as shown in Fig. 2, the sensitivity of the L-glutamate sensor was severely inhibited by α-KG and L-aspartate.

In order to achieve a better sensitivity for the measurement of AST activity using our L-glutamate sensor, the optimal concentration of α-KG and L-asparatate was investigated. Table 2 shows the effect of L-aspartate and α-KG concentration on the response of the sensor in the measurement of AST activity. A standard AST solution with 30 U/L activity (the normal range in human

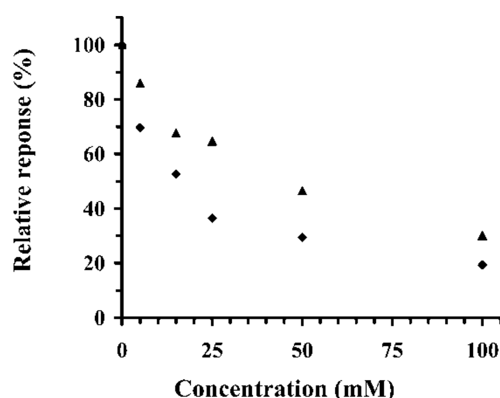


Figure 2. Effect of (▲) L-aspartate and (◆) α-ketoglutatate concentrations on the response of the sensor to 100 μM MSG. The sensor was operated in 100 mM sodium phosphate buffer (pH 7.0) at 35°C. The polarizing potential applied to the sensor was 0.4 V vs. Ag/AgCl.

Table 2. Effect of L-aspartate and α-ketoglutarate concentration on the response of the sensor for 30 U/L AST

	Current increase rate(nA/min) α-ketoglutarate concentration (mM)					
L-aspartate concentration (mM)		0	0.5	1	2	4
15		0	5.74	3.46	—[a]	nd[b]
25	0	6.97	8.80	—[a]	nd[b]	
50	0	3.10	3.89	5.27	—[a]	

[a]Signal could not be detected because of noise distortion.
[b]Measurement was not performed.

blood is 25–30 U/L) was injected into the working solution. The concentrations of α- KG and L-aspartate in the working solution were varied in the range from 0–4 mM and 15–50 mM, respectively. When the concentration of α-KG was more than 2 mM, the response current of the sensor became very unstable and the increase rate of the response current could not be determined. The optimal substrate composition for the measurement of AST activity was decided as 1 mM α-KG and 25 mM L-aspartatem as shown in Table 2.

Measurements of AST Activity

Comparison of the response curves obtained from previous study for the determination of ALT activity (Chang et al. 2003) with the response curves for the determination of AST activity (Fig. 3) revealed that the latter obtained a higher background current. L-Aspartate causes a cross-reaction to L-GLOx, yielding a cathodic signal to the sensor (Kusakabe et al. 1983; Zilkha et al. 1995; Janasek and Spohn 1999), which produces high background current. As shown in Fig, 3 line B, steady-state background current could be obtained when the sensor was settled in the reaction cell filled with working solution. The current profiles for standard solution of 50 U/L ALT and AST were as shown in Fig, 3. L-Glutamate is a product of the AST catalytic reaction. The activity of AST can be determined from the increase of the response current of the L-glutamate sensor. Electrochemically active substances in serum, such as ascorbic acid and uric acid, may cause severe interference on an electrochemical sensor system. In this study, the responses from electrochemically active species caused an elevation of the baseline. They created no interference on a dynamical AST determination when the dynamic data are taken after the steady state of an elevated baseline has been reached. As shown in Fig. 3, the current increased with extension of reaction time after standard ALT was injected, indicating that the hydrogen peroxide released in the reaction was produced by the L-GLOx immobilized

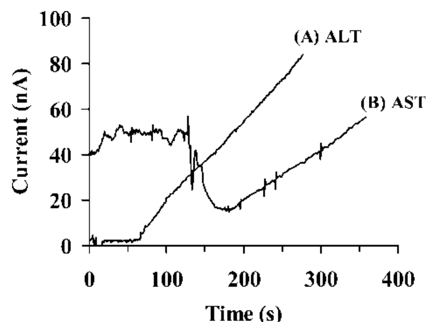


Figure 3. Response curves of the sensor for (A) ALT and (B) AST standard solution with 50 U/L concentrations, which were added to the working solution at the time point indicated. The sensor was operated in 100 mM sodium phosphate buffer (pH 7.0) containing 1 mM α-ketoglutarate, 25 mM L-aspartate (for AST) and/or 100 mM L-alanine (for ALT) at 35°C. The polarizing potential applied to the sensor was 0.4 V vs. Ag/AgCl.

in the PVA-SbQ membrane. Interestingly, the response current decreased immediately after the sample solution was injected. The response curve was different from the response curves obtained for the determination of 50 U/L ALT activity. The decrease of response current is caused by the dilution of L-aspartate and the consequent reduction of the background current when the sample solution was added. Two minutes elapsed before the response

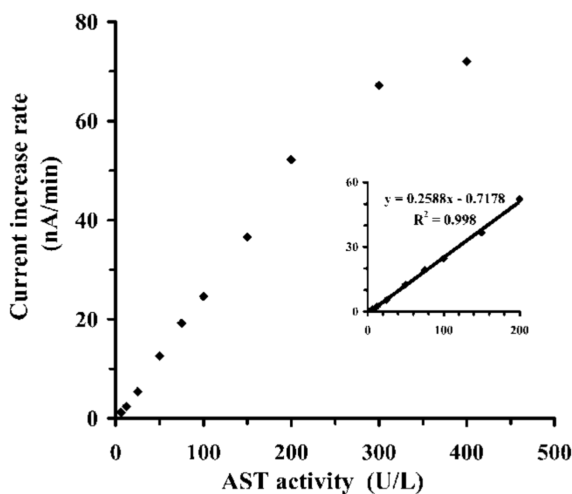


Figure 4. Calibration graphs of the sensor for the determination of AST activity. The sensor was operated in 100 mM sodium phosphate buffer (pH 7.0) containing 1 mM α-ketoglutarate and 25 mM L-aspartate at 35°C. The polarizing potential applied to the sensor was 0.4 V vs. Ag/AgCl.

Table 3. Response of the sensor during the test of within electrode reproducibility of 50 U/L AST

AST response	(nA/min)
Mean[a]	12.58
SD	0.385
RSD (%)	3.03

[a]Data are the mean values of eight measurements. Measurements were carried out with 1 mM α-ketoglutarate and 25 mM L-aspartate as the substrate in 0.1 M sodium phosphate buffer (pH 7.0). The polarizing potential applied to the sensor was 0.4 V vs. Ag/AgCl.

current began to increase linearly with the extension of reaction time. The increasing rate of the response current was related to AST activity. The increasing rate of the response current was accordingly measured from 2 min after the injection of sample solution. The slopes (nA/min) obtained from various AST standard solutions were plotted against the AST activity, determined by the AST assay kits over the range 8–200 U/L ($y = 0.2588 \times -0.7178$, $R^2 = 0.998$, $n = 10$), to yield a calibration graph (Fig. 4). The within-electrode reproducibility of the sensor showed a relative standard deviation of 3.03% (Table 3) for AST with 50 U/L activity in eight measurements. However, the reproducibility of the sensor showed a relative standard deviation of more than 10% for AST with 8 U/L activity. This result demonstrates that the present method was poor in reproducibility for the measurement of low ALT concentration. Since the L-GLOx in this experiment could catalyze the L-aspartate, there was high background current to interfere with the amperometric reaction. The sensitivity and reproducibility of the L-GLOx biosensor can be enhanced by modifying the characteristic of L-GLOx to reduce the background reaction between L-GLOx and L-aspartate.

CONCLUSION

A simple and convenient AST measuring device for diagnostic and point-of-care purposes is required. A L-glutamate sensor, based on a palladium-deposited screen-printed carbon electrode and an immobilized L-GLOx membrane, was developed for the determination of AST activity in serum. The time required for measurement of AST activity was 4 min. The linear range of the sensor was 8–200 U/L AST activity ($R^2 = 0.998$). The reproducibility of the sensor was relative standard deviation of 3.03% ($n = 8$). The present L-glutamate sensor is simpler and less expensive than commercial devices for measuring AST. However, a cross- reaction between the L-GLOx and L-asparate, and thus a high background current, was observed.

The substrate composition in the buffered working solution has been optimized to maximize the sensitivity of the sensor. Further study concerning a device for sequentially or simultaneously measuring AST and ALT activities is currently being undertaken in our laboratory.

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