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O. Fatibello-filho[a]; A. Suleiman[bc]; G. G. Guilbault[bc]
[a] Departamento de Quimica, Universidade Federal de Sao Carlos, Sao Carlos, S.P., Brazil [b] Department of Chemistry, University of New Orleans, New Orleans, Louisiana [c] Universal Sensors New Orleans, Louisiana

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POTENTIOMETRIC DETERMINATION OF L-ASPARAGINE WITH AN ENZYMATIC ELECTRODE

O. FATIBELLO-FILHO,† A. SULEIMAN, and G. G. GUILBAULT*

Department of Chemistry
University of New Orleans
New Orleans, Louisiana 70148

Universal Sensors
New Orleans, Louisiana 70148

ABSTRACT

A new enzyme electrode for the determination of *L*-asparagine is constructed by chemical immobilization of *L*-aspartase [E.C. 4.3.1.1] on an ammonia-gas-sensing probe. *L*-Asparagine was determined over a concentration range of 1.6×10^{-5} to 1.5×10^{-3} *M* with a subnerstian response of -51 mV/decade. The electrode was stable for more than 30 days. The effect of pH, substrate and metal activator concentrations, amount of immobilized enzyme, and interferents on the electrode response are reported.

INTRODUCTION

The ammonia-gas sensor has been widely used for the determination of *L*-asparagine [1-8]. Various biocatalysts (e.g., bacterial cells, enzymes, and tissue slices) were directly immobilized on the surface of the gas sensor's membrane [1, 3, 5-8], used in soluble form, or immobilized in a tubular re-

†On leave from Universidade Federal de Sao Carlos, Departamento de Quimica, Caixa Postal 676, 13560 Sao Carlos, S.P., Brazil.

actor [2, 4]. The above-mentioned methods are based on the conversion of *L*-asparagine to *L*-aspartate and ammonia by *L*-asparaginase [E.C. 3.5.1.1], and the subsequent determination of NH_3 by an ammonia gas electrode. Other nonenzymatic potentiometric methods described include the use of a copper tubular electrode which responds to the anionic form of the amino acid [9] and the use of a fluoride electrode utilizing the reaction of *L*-asparagine with 2,4-dinitrofluorobenzene [10]. However, these methods offered low sensitivity and poor selectivity.

In this communication a new enzyme electrode for potentiometric determination of *L*-asparagine is proposed. The electrode was constructed by chemically immobilizing *L*-aspartase [E.C. 4.3.1.1] on a Celgard membrane. The enzyme catalyzes the deamination of asparagine, and the ammonia generated is sensed by the electrode described.

EXPERIMENTAL

Apparatus

The ammonia electrode (Type 4000.4, Universal Sensors, P.O. Box 736, New Orleans, Louisiana 70148) was used as the base sensor. The enzyme was immobilized on Celgard microporous film (Type 2402, Celanese Corporation, P.O. Box 32414, Charlotte, North Carolina 28232) which was placed at the bottom of the electrode and covered with a dialysis cellulose membrane (Type spectra/por 2 with MW cutoff 12 000 to 14 000, Spectrum Medical Industries, Inc., Los Angeles, California 90054).

Potentiometric measurements were made with a Beckman Model ϕ71 digital pH meter and were recorded with a radiometer, Model REC 61 Servograph recorder.

All measurements were carried out at room temperature (25 ± 2°C) in 10 mL beakers with controlled magnetic stirring.

Reagents

Analytical grade reagents were used to prepare buffers and standard asparagine solutions.

Tris buffer was prepared as described before [11]. Solutions of *L*-asparagine and other amino acids (Sigma Chemical Co.) were always prepared fresh, a few minutes before determinations in 0.1 *M* Tris-HCl buffer, pH 8.5, containing 2.5 m*M* $MgCl_2$.

The enzyme used was *L*-aspartase [E.C. 4.3.1.1] (Type A8147, Sigma

Chemical Co.) from Hafnia Alvei (*Bacterium Cadaveris*) with 1.3 units/mg protein.

Electrode Preparation

A piece of Celgard membrane was placed at the bottom of the plastic jacket of the ammonia sensor and held in place by an O-ring. Approximately 2.0 mg (2.6 units) of lyophilized enzyme was placed on the flat membrane and dissolved with 20 μL of phosphate buffer pH 6.86. Two μL of 2.5% glutaraldehyde was added and gently mixed with the enzyme solution for a few seconds, then allowed to dry in a desiccator for 4-5 h. The electrode was rinsed with distilled water and soaked first in 0.1 *M* glycine phosphate buffer solution for 15 min and then in phosphate buffer in order to elute or neutralize the excess of bifunctional agent. No BSA was used due to the inherent high protein content of *L*-aspartase. A cellulose dialysis membrane was mounted to further fix the membrane.

The rest of the electrode was assembled according to the manufacturer's instructions. The internal filling solution used was 0.1 *M* NH_4Cl, which was reported in our previous work [11] to improve the performance of the electrode.

The electrode was equilibrated before measurements in 0.1 *M* HCl-Tris buffer, pH 8.5, containing 2.5 m*M* $MgCl_2$ for at least 4 h at room temperature. When not in use, the electrode was stored at 5°C in buffer solution or in 1 m*M* dithiothreitol solution containing 2.5 m*M* $MgCl_2$.

PROCEDURES

The gas electrode equipped with the enzymatic membrane was dipped in a 10-mL beaker containing 3 mL of Tris-HCl buffer stirred at moderate speed with a magnetic stirrer. When the electrode potential attained a stable value, 1.0 mL of asparagine standard solution was added and the response was determined after reaching a steady-state value. Between measurements the electrode was rinsed with distilled water and kept in 0.1 *M* Tris-HCl buffer solution.

RESULTS AND DISCUSSION

The effect of aspartase on asparagine has not been discussed in the literature. However, the results of this study suggest that asparagine is deaminated by the enzyme. Further research is needed to unveil the nature of this reaction.

In order to evaluate the performance of the asparagine electrode in terms of response time, recovery time, slope of calibration curve, selectivity, and limit of detection, several parameters were studied.

Effect of pH and Metal Activator

The effect of pH, buffer system used, magnesium concentration, and the performance of the base NH_3 electrode on the response of an enzymatic aspartase electrode were reported in our previous paper [11]. It was determined that maximum activity of the enzyme was obtained at pH 8.4-8.5 with magnesium concentrations of 2.5 m*M*. In the present work, all studies were carried out in 0.1 *M* HCl-Tris buffer, pH 8.5 with 2.5 m*M* $MgCl_2$.

Effect of the Amount of Enzyme

The effect of different immobilized amounts of *L*-aspartase on the response of electrode and the response time are shown in Fig. 1. The response of the electrode increases with increasing amount of enzyme up to 2.5 units, then levels off at 2.6-6.5 units. On the other hand, the response time increases linearly with the amount of enzyme used. The results are in agreement with the Ross et al. study of gas-sensing membrane probes [12], indicating an increase in response time as the thickness of the enzyme film increases. Further working electrodes were constructed using 2.5 units of *L*-aspartase. The response time of the indicated electrode was 5 min for 125 μM *L*-asparagine solution, with a complete baseline recovery of 10 min.

Analytical Characteristics of the Electrode

A typical calibration curve for the asparagine enzyme electrode is shown in Fig. 2. The electrode response plot is linear in the concentration range of 1.6×10^{-5} to 1.5×10^{-3} *M* with a subnerstian response of -51 mV/decade and a correlation coefficient $r = 0.998$. Figure 3 shows the long-term stability and the operative lifetime of the electrode. These parameters depend on the operational and storage conditions. The enzyme electrode was stable for more than 23 days when it was stored at 5°C in 0.1 *M* Tris-HCl buffer solution and for at least 30 days without significant loss of enzyme activity when stored in 1 m*M* dithiothreitol solution containing 2.5 m*M* $MgCl_2$. Dithiothreitol has proven to be a good stabilizer of the sulfhydryl active groups of the enzyme [13]. Approximately 400 assays were performed with one electrode.

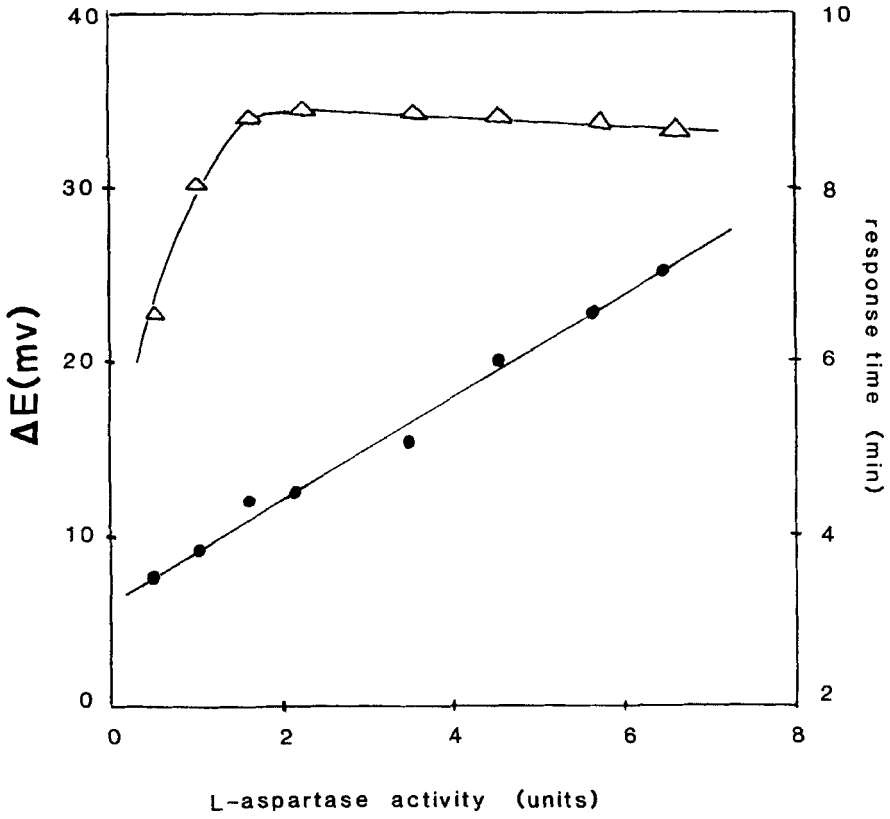

FIG. 1. Effect of *L*-aspartase on the potential response (△) and response time (●) for 125 μM *L*-asparagine solution.

Selectivity studies were performed by testing the response of the enzyme electrode to several other *L*-amino acids as well as *D*-asparagine and *DL*-asparagine. It is evident (Table 1) that the electrode is not totally specific to *L*-asparagine, contrary to the common belief that *L*-aspartase is one of the most selective enzymes [14-17].

This study and our recent work [11] show interferences from *L*-histidine and *L*-glutamine, although most of the response from the latter is due to decomposition by base. The interference from *L*-histidine can be eliminated by

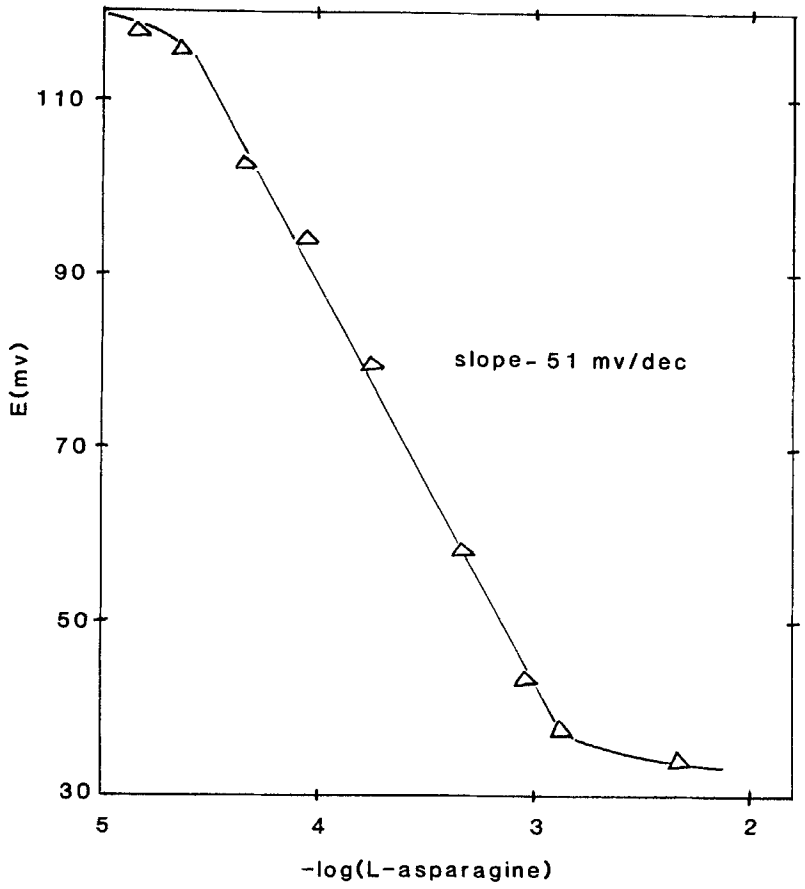

FIG. 2. Typical calibration curve for an *L*-asparagine electrode at 25°C in Tris-HCl 0.1 *M* buffer (pH = 8.5). *L*-Asparagine concentration in moles/liter.

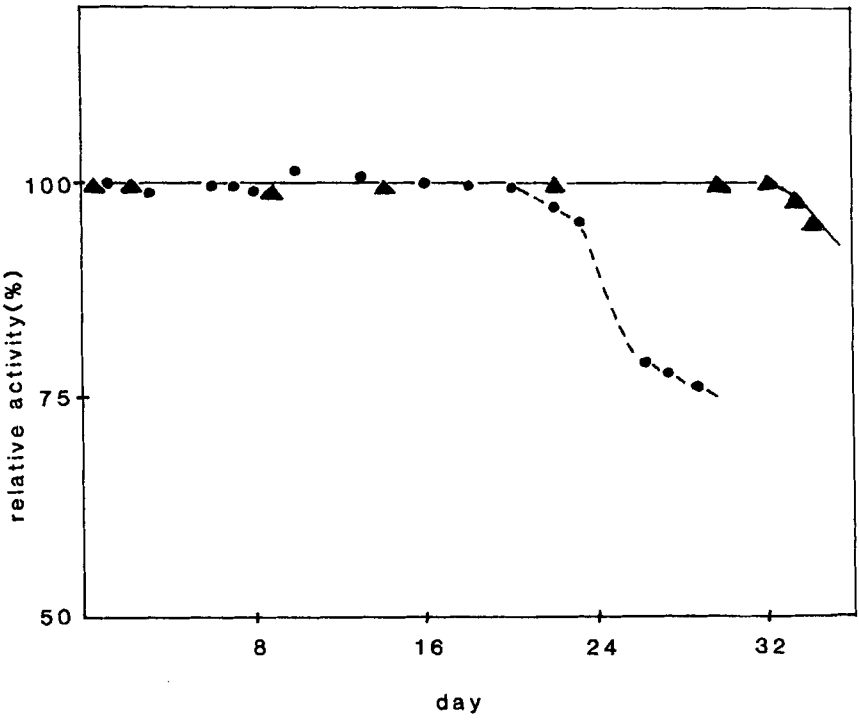

FIG. 3. Long-term stability of an *L*-aspartase electrode when stored in buffer solution (●) and in dithiothreitol (▲) at 5°C.

the selective extraction of the protonated histidine from acid solution with a solution of dibenzo-18-crown-6 in dichloromethane [18]. It is also interesting to note that the electrode is more sensitive to *L*-asparagine than to *L*-aspartate.

The proposed method can be used for the determination of *L*-asparagine with a limit of detection of 2.1 μg/mL. This method is more sensitive than the previously reported *L*-asparaginase electrodes [1, 3, 5-8] and enzyme reactors [2, 4] as well as the nonenzymatic potentiometric methods [9, 10].

The stability, response range, and slope are also improved in comparison to other reported electrodes [1, 3, 5-8].

TABLE 1. Relative Activity of the Immobilized *L*-Aspartase Electrode Toward Various Amino Acids

Substance	Relative activity[a]	Substance	Relative activity
L-Asparagine	100		
D-Asparagine	47	Hydroxy *L*-proline	0
D,L-Asparagine	86	*L*-Isoleucine	6
L-Alanine	0	*L*-Leucine	3
L-Arginine	4	*L*-Lysine	0
L-Aspartate	32	*L*-Methionine	3
L-Cysteine	7	*L*-Phenylalamine	0
L-Cystine	0	*L*-Serine	0
L-Glutamic acid	0	*L*-Threonine	0
L-Glutamine	63[b]	*L*-Thyptophan	2
Glycine	0	*L*-Tyrosine	0
L-Histidine	51	Valine	0

[a]All measurements were made in 5×10^{-3} *M* amino acid solution, except *L*-tryptophan (10^{-5} *M*) and *L*-tyrosine (10^{-4} *M*).

[b]Fast nonenzymatic and enzymatic decomposition of this pH (see Ref. 11).

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