An Enzymatic Method for the Kinetic Measurement of L-Asparaginase Activity and L-Asparagine with an Ammonia Gas-Sensing Electrode

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A simple kinetic method to assay L-asparaginase and L-asparagine with an ammonia gas-sensing electrode is described. The method is based upon the de-amination of L-asparagine by L-asparaginase from *Escherichia coli*, resulting in the production of ammonia. The initial rate (mV/min) of ammonia release is proportional to the activity of L-asparaginase and also to the concentration of L-asparagine in the presence of a large amount of the enzyme. Optimal temperature, buffer composition and pH for the assays are specified. L-Asparaginase was determined in the range of 0.4—1.6 U in a 0.1 ml sample; the recovery was 98.1—103.8% for 16 determinations and σ_n was 1.59. L-Asparagine was determined in the concentration range of 1×10^{-4}—1×10^{-3} M with σ_{n-1} 1.92. The method was applied to the determination of 1—5×10^{-4} M asparagine added to human serum with σ_{n-1} 1.96 for 5 determinations.

Keywords L-asparagine; L-asparaginase; ammonia gas-sensing electrode; kinetic method; hydrolysis; gas-permeable membrane; initial rate; potentiometric method; assay

L-Asparagine has recently been shown to be an essential amino acid in certain tumors, and asparaginase has a striking antineoplastic effect in the treatment of malignant disease .[1] Therefore, there is much interest in asparaginase and asparagine in the medical and pharmaceutical fields.

Various methods have been developed for the determination of asparagine and the measurement of asparaginase activity including paper or column chromatography,[2] enzymatic spectrophotometric methods[3] and an enzyme electrode.[4] These methods tend to be time-consuming and to be subject to interference by organic impurities. The enzyme electrode method is a new assay method for asparagine, but the response (Δ mV) is directly proportional to the logarithmic concentration of asparagine. A simple, specific, interference-free method is still required.

The aim of this study was to establish an analytical method to determine asparagine or asparaginase activity simply by an initial-rate kinetic approach. The initial rate of ammonia production is selectively measured with an ammonia gas-sensing electrode and is directly proportional to the enzyme activity or asparagine concentration.

Experimental

Apparatus The ammonia gas-sensing electrode used was a Horiba model 52002A-06T, consisting of an ammonia gas-permeable membrane, pH glass-electrode, and silver–silver chloride reference electrode with 0.1 M ammonium chloride filling solution as the internal electrolyte. The potential measurement system consisted of a pH/mV meter (Hitachi-Horiba F-7ss) and a recorder (Toa Electronic EPR-221E polyrecorder). All measurements were carried out in a 10-ml cell and consisted of dipping a sensor into a sample solution. The cell was kept at a constant temperature by circulating water with a Sharp TE-104 constant-temperature circulater. Initial rate readings were recorded at a chart speed of 20 mm/min and a range of $\pm$50 mV.

Reagents and Solutions Reagent-grade chemicals were used without further purification, and solutions were prepared with distilled water. Asparaginase (EC 3.5.1.1) from *Escherichia coli* with an activity of 100 units/0.5 mg (one unit liberates 1.0 μmol of ammonia nitrogen from L-asparagine per min at pH 8.6 at 37 °C) was obtained from Sigma, and asparagine from Kanto Chemical Co. Human serum type AB (Product No. 14—490A) was obtained from Whittaker Bioproducts, Inc. A 0.01 M stock substrate solution was prepared by dissolving 0.15014 g of L-asparagine in 100 ml of distilled water. An enzyme stock solution (100 units/0.5 mg) was prepared by dissolving the enzyme in 5 ml of 50% (v/v) glycerin. The solution was stored in a refrigerator. Working solutions of substrate or enzyme were prepared by appropriate dilutions with water (substrate) or 50% glycerin (enzyme), just before measurements. The 0.2 M Tris–HCl buffer solution was prepared by dissolving tris-hydroxymethylaminomethane (6.0578 g) in *ca.* 200 ml of distilled water and adjusting the solution to the optimal pH with 1 N HCl to give a final volume of 250 ml.

Determination of L-Asparaginase Activity Initially, 4 ml of 0.2 M Tris–HCl buffer solution (pH 9.0) and 1 ml of 0.05 M L-asparagine were pipetted into a 10-ml reaction cell thermostatically controlled at 30 $\pm$ 0.1 °C unless otherwise stated. The cell contained a small Teflon-coated stirring bar. The electrode was washed with water and immersed in the solution. After a stable potential reading had been obtained for the stirred solution, 0.1 ml of enzyme solution was added. The rate curve was recorded for 5 min and the initial rate of potential change (mV/min) was computed by dividing the Δ mV value at 5 min by 5. The initial rate was plotted as a function of the enzyme activity to yield a standard curve for determining the activity of other L-asparaginase samples.

Determination of L-Asparagine in Aqueous Solution or Serum Four milliliters of 0.2 M Tris–HCl buffer (pH 8.5) and 1 ml of L-asparagine standard or sample were added to the reaction cell. After being washed, the ammonia electrode was immersed in the solution and the assay procedure was started by injecting 0.1 ml of enzyme solution (0.4 U) into the cell to initiate the reaction. The L-asparagine concentration was determined from a calibration graph of the initial reaction rate *vs.* the standard L-asparagine concentration.

Results and Discussion

The ammonia gas-sensing electrode is based on the de-amination of L-asparagine in the presence of L-asparaginase.

$$\text{L-asparagine} \xrightarrow{\text{L-asparaginase}} \text{L-aspartate} + NH_3$$

Asparaginase activity or asparagine is measured in terms of the initial rate of ammonia production monitored by the ammonia gas-sensing electrode.

Effects of Buffer Composition and pH When the electrode is immersed in a buffered solution, the initial rate is directly proportional to the asparaginase activity or asparagine concentration. When the solution is not buffered, the measured electrode response using an ion-selective electrode, enzyme electrode or pH-sensing gas-permeable membrane electrode follows the Nernstian equation and is directly proportional to the logarithm of the concentration.[5] On the other hand, when the solution is buffered, the response is directly proportional to the concentration.[6] Such a linear response can be explained in terms of the buffer capacity as suggested by Adams and Carr[7] and also Papariello and co-workers.[8] Therefore, the effects of buffer composition and pH were studied to find conditions that

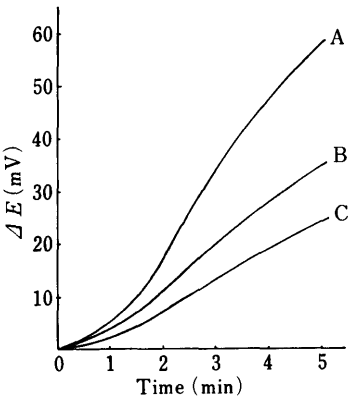

Fig. 1. Recorded Curves of Potential *vs.* Time for the Asparaginase-Catalyzed Hydrolysis of Asparagine at pH 9

(A) Tris–HCl buffer; (B) sodium diethylbarbituric acid–HCl buffer; (C) glycine–NaOH buffer.

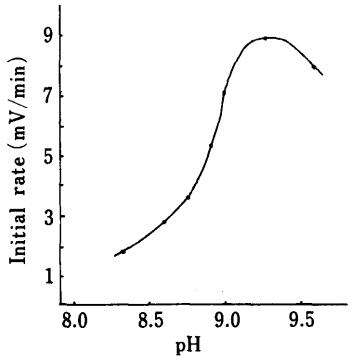

Fig. 2. Effect of pH on Hydrolysis of Asparagine

provide good sensitivity. The initial rates of ammonia production in three different buffers at pH 9 were monitored at 30 °C, using 1.6 U/mg asparaginase and 0.05 M asparagine, as shown in Fig. 1. The reactivity increased in the following order: glycine–NaOH buffer < sodium diethylbarbituric acid–HCl buffer < Tris–HCl buffer.

On the basis of the data, subsequent experiments were carried out in the Tris–HCl buffer solution for pH control. The initial rates of ammonia production at different pH values were monitored at 30 °C, using 1.6 U/mg L-asparaginase and 0.05 M substrate. The optimal pH for L-asparaginase was reported to be pH 6.9—8.7,[9] but the pH profile shown in Fig. 2 indicated a pH optimum of 9.25. The pH optimum was influenced by both the enzyme activity and the pH for optimum electrode operation (response of the ammonia gas-sensing electrode increased with increasing pH value), consequently shifting the pH to the alkaline region.

Effect of Temperature A study of the effect of temperature in the range of 20—42 °C showed that the initial rate of potential change increased with increasing temperature, but the change in the potential of the electrode became unstable at temperatures over 40 °C. Therefore, the reaction rates were measured at 30 °C to ensure stable potential response.

Determination of L-Asparaginase The effects of pH on the calibration plot in 0.2 M Tris–HCl buffer solution using 0.05 M asparagine at 30 °C were studied, as shown in Fig. 3. At pH 8.75 and 9.00, a linear calibration plot was obtained at 0.4—1.6 U/mg enzyme. At pH 9.25, the slope of the

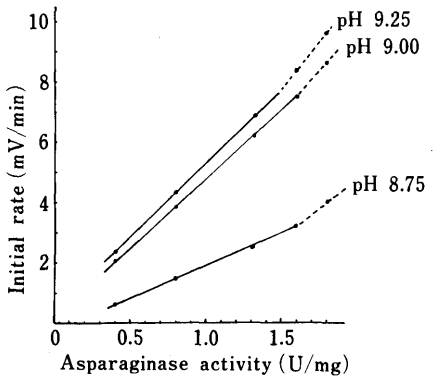

Fig. 3. Effect of pH on the Calibration Plot in 0.2 M Tris–HCl Buffer for Asparaginase

TABLE I. Determination of Asparaginase in Aqueous Solution

Taken (U/mg)	Number	Found (U/mg)	Recovery (%)
0.40	1	0.397	99.2
	2	0.399	102.5
	3	0.402	99.5
	4	0.410	101.8
			Mean 100.8
0.80	1	0.796	99.5
	2	0.783	98.2
	3	0.800	100.0
	4	0.774	97.7
			Mean 98.9
1.33	1	1.305	98.1
	2	1.343	100.3
	3	1.333	100.5
	4	1.337	101.7
			Mean 100.2
1.60	1	1.661	103.8
	2	1.617	100.3
	3	1.587	99.7
	4	1.566	99.4
			Mean 100.8

Average mean 100.2%. σ_n 1.59.

calibration plot increased, but the range of linearity decreased. On the basis of the above data, the most suitable experimental conditions for initial rate measurements were concluded to be pH 9 in 0.2 M Tris–HCl buffer solution at 30 °C. Under the optimum conditions described, L-asparaginase could be determined in the range of 0.4—1.6 U/mg of sample. The average of the means of 16 determination (Table I) was calculated to be 100.2% and σ_n was 1.59. Another method[10] for the estimation of L-asparaginase activity requires 0.68—3.38 U/mg. The limit of detection is lower than that in the other method, while the maximum detection is less.

Determination of L-Asparagine The determination of asparagine is similar to that of asparaginase activity. The principal advantage of this method is that the initial rate (mV/min) of ammonia evolution is proportional to the asparagine concentration. The most suitable conditions of buffer composition, pH and temperature have already been described. Therefore, under those optimal conditions, the effect of asparagine concentration on the initial rate was studied. The initial rates of ammonia evolution were directly proportional to the asparagine concentration, using 4 U/mg L-asparaginase in 0.2 M Tris–HCl buffer solution at

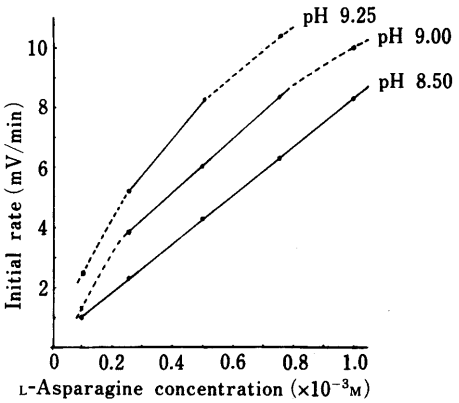

Fig. 4. Effect of pH on the Calibration Plot in 0.2 M Tris–HCl Buffer for Asparagine

TABLE II. Determination of Asparagine in Aqueous Solution

Asparagine ($\times 10^{-4}$ M)	Taken	1.00	2.50	5.00	7.50	10.0
Asparagine ($\times 10^{-4}$ M)	Found	0.98	2.56	5.10	7.43	9.94
Relative error (%)		−2.0	+2.4	+2.0	−0.9	−0.6

Average mean 100.2%. σ_{n-1} 1.92.

TABLE III. Determination of Asparagine Added to Serum

Asparagine ($\times 10^{-4}$ M)	Added	1.00	2.00	3.00	4.00	5.00
Asparagine ($\times 10^{-4}$ M)	Found	1.02	2.06	2.99	3.92	5.05
Relative error (%)		+1.8	+3.0	−0.3	−2.0	+1.0

Average mean 100.7%. σ_{n-1} 1.96.

30 °C. The effect of pH on a calibration plot of the initial rate *vs.* the asparagine concentration was examined, as shown in Fig. 4. At pH 8.5, a linear calibration plot was obtained at 10^{-4}—10^{-3} M L-asparagine. At pH 9.0, a linear calibration plot was obtained at $2.25 \times 10^{-4} - 7.5 \times 10^{-4}$ M L-asparagine. At pH 9.25, the slope of the calibration plot increased, but the range of linearity decreased. Under the optimum conditions at pH 8.5, L-asparagine could be determined in the concentration range of $1 \times 10^{-4} - 1 \times 10^{-3}$ M, and σ_{n-1} was 1.92 (Table II).

In order to study the applicability of the method to biological fluids, experiments were performed using commercially available human serum. The method was used to determine the L-asparagine concentration arising from the addition of known quantities of asparagine solution to serum. The recovery was then computed on the basis of the amount added (Table III). The electrode method was able to determine the asparagine concentration with σ_{n-1} 1.96, indicating the absence of any significant interference.

Stability of Electrode Potential Variations in the electrical source voltage and electrical or electromagnetic induction effects[11] result in variations in electrode potential. In this case, the electrode potentials result in about 2—4 mV variations from a straight line (Fig. 3). Therefore, experiments have to be performed in an electrically stable state.

Conclusion

The electrode method offers a simple kinetic means for specific measurements of L-asparaginase and L-asparagine, and is advantageous because of its simplicity, accuracy, and lower cost compared with conventional methods. Further, the electrode method does not suffer from turbidity problems, such as can be encountered with fecal samples. Protein degradation products which interfere with the absorption method would not affect the electrode method. The drawback of this method is that variations in the electrical environment described above result in variations in electrode potential.

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