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Bienzymatic Electrode for the Determination of Aspartame in Dietary Products

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A novel bienzymatic electrode for the determination of aspartame (*N*-L-α-aspartyl-L-phenylalanine 1-methyl ester) is constructed by chemical coimmobilization of carboxypeptidase A [EC 3.4.17.1] and L-aspartase [EC 4.3.1.1] on an ammonia gas sensing probe. The electrode response is linear in the concentration range 4.25×10^{-4} to 8.10×10^{-3} M with a slope of −45 mV/decade. The electrode is stable for more than 25 days and was successfully used for assay of aspartame in several dietary products.

Aspartame (*N*-L-α-aspartyl-L-phenylalanine 1-methyl ester) is a low-calorie nutritive sweetener composed from two amino acids, L-aspartic acid and L-phenylalanine. It is a white, odorless, crystalline powder with a sugarlike taste and a sweetness potency 180–200 times that of sucrose (*1–3*). Since its introduction under the trade name NutraSweet it received regulatory clearance from the Food and Drug Administration for use in carbonated beverages and several foods.

Aspartame was determined in various dietetic food products by chromatographic techniques (*4–8*). However, these methods are time-consuming and laborious.

A microbial amperometric sensor using immobilized *Bacillus subtilis* cells was developed for the determination of aspartame, but glucose and aspartame amino acid constituents caused some interference (*9*).

Recently, an enzyme electrode using L-aspartase was constructed by our group (*10*). Its response was in the concentration range of 1×10^{-3} to 1×10^{-2} M with a slope of −30 mV/decade and a lifetime of only 8 days.

In this paper, a new bienzymatic electrode is described for the determination of aspartame in several dietary products. This probe is based on the chemical coimmobilization of carboxypeptidase A [EC 3.4.17.1] and L-aspartase [EC 4.3.1.1] on an ammonia gas sensing electrode. The enzyme carboxypeptidase A specifically cleaves aspartame to α-ketoglutarate and aspartic acid (eq 1). The aspartic acid formed is then deaminated by L-aspartase with liberation of fumarate and ammonium ion (eq 2).

$$\text{aspartame} \xrightarrow{\text{carboxypeptidase A}} \alpha\text{-ketoglutarate} + \text{L-aspartic acid} \quad (1)$$

$$\text{L-aspartic acid} \xrightarrow{\text{L-aspartase}} \text{fumarate} + NH_4^+ \quad (2)$$

The ammonium ion generated is sensed by the ammonia electrode, the steady-state potential of which is proportional to the activity of NH_4^+ ions, i.e., the concentration of aspartame in the solution.

EXPERIMENTAL SECTION

Apparatus. The ammonia electrode (type 4000-4, Universal Sensors, P.O. Box 736, New Orleans, LA) was used as the base sensor. The enzymes were coimmobilized on the electrode's gas membrane and covered with a dialysis cellulose membrane (type Spectra/por 2 with a molecular weight cutoff of 12000–14000, Spectrum Medical Industries, Inc., Los Angeles, CA).

Potentiometric measurements were made with a Beckman Model 71 digital pH meter and recorded with a Radiometer Model REC 61 Servograph recorder.

All measurements were carried out at room temperature (25 ± 2 °C) and at a controlled magnetic stirring rate.

Reagents. Carboxypeptidase A (peptidyl-L-amino-acid hydrolase; type CO 261; EC 3.4.17.1); L-aspartase (L-aspartate ammonia-lyase; type A8147; EC 4.3.1.1), glutaraldehyde (GA), bovine serum albumin (BSA), aspartame, and Tris (tris(hydroxymethyl)aminomethane) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade and were used without further purification.

Tris buffer (0.1 M, pH 8.3) containing 3.0 mM $MgCl_2$ was prepared weekly in doubly distilled water and stored under refrigeration at 5 °C. Other buffer solutions of different pH were prepared in the same fashion.

Solutions of aspartame were always prepared fresh in Tris–HCl buffer, pH 8.3, containing 3.0 mM $MgCl_2$.

Equal powder and Equal tablets (NutraSweet Consumer Products, Inc., Chicago, IL), vanilla pudding (The Estee Corp., Parsippany, NJ), Diet Apple Slice (MSB Manufacturing Co., Memphis, TN), diet root beer, Diet Crush, and Diet Coke (Louisiana Coca-Cola Bottling Co., Gretna, LA) were purchased from a local food store.

Electrode Preparation. The gas membrane was attached to the bottom of the plastic jacket of the ammonia electrode with an O-ring. Approximately 2.0 mg (2.6 units) of L-aspartase was placed on the membrane and dissolved with 20 μL of phosphate buffer, pH 6.86. Thirty microliters of an aqueous toluene suspension of carboxypeptidase A (27 units) and 3 μL of 2.5% BSA were added and mixed carefully. Five microliters of 2.5% glutaraldehyde was added and gently mixed for a few seconds. The membrane was allowed to dry in a desiccator for 3–4 h. It was then rinsed with distilled water and soaked first in 0.1 M glycine phosphate buffer solution for 10 min and then in phosphate buffer, in order to elute or neutralize the excess of bifunctional agent. A cellulose dialysis membrane was mounted over the enzyme layer as the outer membrane.

The electrode jacket was assembled according to the manufacturer's instructions. An internal filling solution of 0.1 M NH_4Cl containing saturated $AgNO_3$ was used.

The compensation electrode for measurements of background in Diet Coke solutions was constructed in the same fashion, but L-aspartase was replaced by the same amount of BSA solution.

The electrodes were equilibrated in 0.1 M Tris–HCl buffer, pH 8.3, with 3.0 mM $MgCl_2$ for 4–5 h at room temperature before measurements and were stored at 5 °C in the buffer when not in use. They are now commercially available (Universal Sensors, New Orleans, LA 70148).

Procedure. The electrode was immersed in a 10-mL beaker containing 3 mL of Tris–HCl buffer stirred at a moderate controlled speed with a magnetic stirrer. When the electrode potential attained a stable value, 1.0 mL of aspartame standard solution was added, and the response was determined after a steady-state value was reached. Between measurements, the electrode was rinsed with distilled water.

To measure the aspartame content in a solid sample, a known amount was weighed and dissolved in 10 mL of Tris–HCl buffer. For a liquid sample, 5.0 mL was diluted to 10 mL with the same

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Table I. Determination of Aspartame in Dietary Products Using the Aspartame Electrode

sample	% of aspartame reported	mg/L of aspartame reported	% of aspartame found[a]	mg/L of aspartame found[a]	rel error	coeff of variation
equal (powder)	3.5		3.44 ± 0.06		−1.71	1.13
equal (tablets)	20.27		20.09 ± 0.22		−0.88	1.06
pudding	1.23		1.27 ± 0.06		+3.25	4.09
diet apple juice		490		469 ± 9	−4.28	1.78
diet root beer		490		501 ± 20	+1.90	3.81
Diet Coke		518		489 ± 32	−5.60	5.72
Diet Crush		490		472 ± 11	−3.67	2.33

[a] Assay values represent the average of six (n = 6) determinations per sample with a confidence level of 95%.

buffer. CO_2 was removed from the sample by bubbling of nitrogen for 15 min. Then, 1.0 mL of these solutions was used in the same fashion described above for the standard solution and the aspartame concentration determined from a calibration plot.

For Diet Coke samples, the background potential determined by the compensation electrode was converted to an aspartame concentration, which was then subtracted from the working electrode response to determine the true response for aspartame.

RESULTS AND DISCUSSION

In order to evaluate the performance of the aspartame electrode, several parameters, including amount of each enzyme, response time, recovery time, slope of calibration curve, selectivity, limit of detection, and suitability for practical applications, were investigated.

Effect of Amount of Enzyme. The effect of different immobilized amounts of L-aspartase on the response of the aspartate electrode was initially investigated. It was determined that the response of the electrode increases with the increase in the amount used up to 2.6 units and then levels off between 2.6 and 6.5 units.

The amount of L-aspartase was fixed at 2.6 units (2.0 mg), and amount of carboxypeptidase A was varied from 5 to 35 units. The optimum amount of the carboxypeptidase A was 27 units, with an immobilizing matrix of 3.0 μL of 2.5% BSA and 5.0 μL of 2.5% glutaraldehyde. The response time in the linear concentration range was 4–8 min, with complete base-line recovery in about 15 min. This response time is comparable to that reported by Kobos (*11*) and shorter than the one reported by Guilbault (*10*). Also, it was found that the response and recovery times toward aspartate and aspartame solutions were identical, indicating that coimmobilizing of the two enzymes did not cause any major drawbacks.

Effect of pH. Carboxypeptidase A is stable in the pH range of 7.0–10.0 at 25 °C with V_{max} optimal at pH 7.5 (*12*). L-Aspartase is optimally stable at pH 7.5, and the V_{max} optimum is at pH 8.5 (*10*).

Figure 1 shows the effect of pH on the response of the electrode with both enzymes to a 5×10^{-4} M aspartame solution containing 3.0 mM $MgCl_2$. The pH optimum is 8.2, midway between the pH maximum of the two enzymes.

Maximum activity of L-aspartase was obtained at a magnesium concentration of 3.0 mM. Since the response of the ammonia gas probe is higher at alkaline pH and the response time is shorter, pH 8.3 was selected for this study as a good compromise.

Analytical Characteristics of Electrode. A typical calibration curve for the aspartame enzyme electrode is shown in Figure 2. The electrode response is linear in the concentration range of 4.25×10^{-4} to 8.10×10^{-3} M with a sub-Nernstian response of −45 mV/decade and correlation coefficient r equal to 0.9998. The stability of the electrode was determined by comparing its response to 5×10^{-4} M aspartame with its first-day response. Figure 3 shows the long-term stability and the operative lifetime of the electrode. It was concluded that stability depends on the operational and

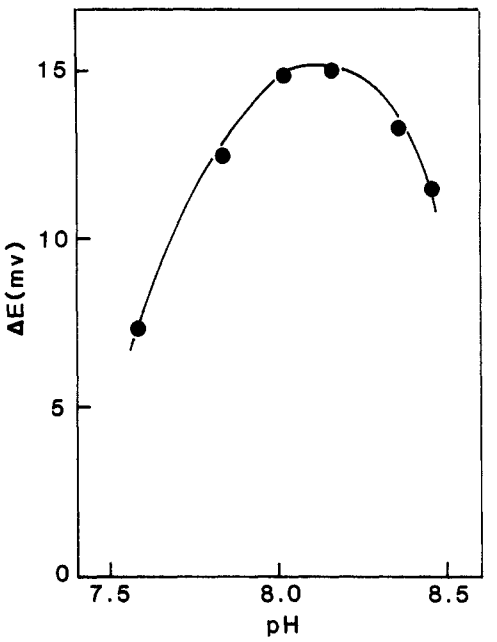

Figure 1. Effect of pH on the immobilized carboxypeptidase A–L-aspartase aspartame electrode at 25 °C in Tris–HCl buffer 0.1 M, Mg(II) 3.0 mM, and aspartame 5×10^{-4} M.

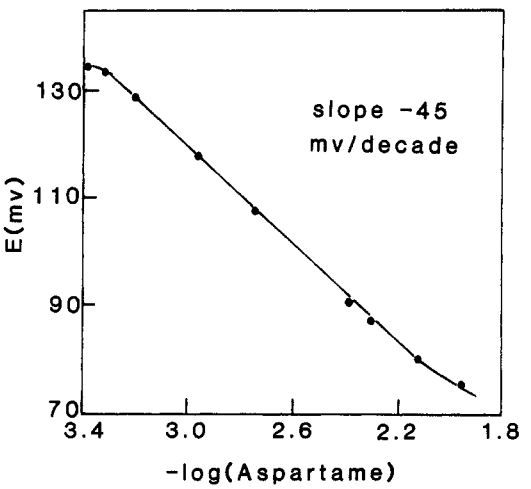

Figure 2. Typical calibration curve for the aspartame electrode at 25 °C in Tris–HCl buffer 0.1 M (pH 8.3) and Mg(II) 3.0 mM.

storage conditions. The enzyme electrode was stable for more than 25 days when it was stored at 5 °C in 0.1 M Tris–HCl buffer with 3.0 mM $MgCl_2$ at pH 8.3. Approximately 300 assays were performed with one electrode.

Many of the compounds normally found in dietary products (potassium benzoate, citric acid, caffeine, phenylalanine, thiamine, riboflavin, niacin, retinoic acid, and ascorbic acid) were tested as potential interferents. No interference from 10^{-2} M solutions of these compounds was observed. Never-

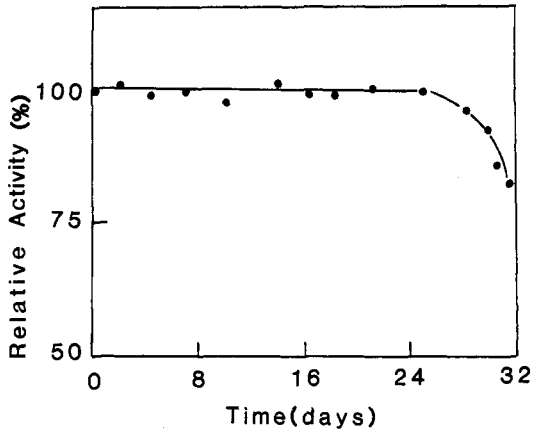

Figure 3. Long-term stability of aspartame electrode when stored in buffer solution at 5 °C.

theless, Diet Coke samples exhibited a very high background, and a compensating electrode was necessary. A positive Nessler test in conjunction with a negative headspace test for volatile amines suggests that the high background is probably due to the presence of some ammonium salts in the samples.

Table I presents a comparison between the reported and the experimentally determined percentage (or concentration in carbonated drinks) of aspartame in some dietary products. The results are in close agreement with those reported and within an acceptable range of error.

The aspartase electrode reported earlier (*10*) could only be used in a few dry powdered mixes. Its performance in real samples, especially in carbonated drinks, was not very successful. The coimmobilization of two enzymes improved the performance of the electrode with respect to selectivity, sensitivity, and response time and extended its applications to several aspartame products.

The proposed method is reliable, simple, and relatively fast, has minimum matrix effects (except in the case of Diet Coke), and does not require extensive preliminary sample treatment.

Registry No. EC 4.3.1.1, 9027-30-9; EC 3.4.17.1, 11075-17-5; aspartame, 22839-47-0.

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Hexadecylpyridinium–Phosphotungstate Ion Association in Construction of a Hexadecylpyridinium Cation Selective Electrode

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Phosphotungstic acid (PTA) forms an ion association with hexadecylpyridinium bromide (HDPBr) having a mole ratio of 1:3 (PT^{3-}:HDP^{+}). A poly(vinyl chloride) membrane selective electrode for HDP was constructed, based on incorporation of the $PT(HDP)_3$ ion association in the plastic film. Investigations of the effect of membrane composition and soaking on the electrode performance were conducted. The electrode showed long lifetime, Nernstian response within the concentration range of 6.3×10^{-6} to 3.1×10^{-3} M HDP at 25 °C over pH values from 2.0 to 8.5. The electrode was highly selective toward a large number of inorganic and organic cations, amino acids, sugars, and organic amines. HDP in aqueous solution was determined either by the standard addition method or by potentiometric titration using a standard solution of PTA as the titrant and the prepared electrode as the sensor.

Hexadecylpyridinium bromide (HDPBr) is one of the most important cationic surfactants, having very wide analytical and technological applications, among which are spectrophotometric determination of metal ions (*1–5*), inhibition of stainless steel corrosion (*6*), preparation of antimicrobial reagents (*7, 8*) improvement of foaming properties of detergents (*9*), preparation of cleaning solution (*10*), emulsification (*11*), solvent extraction of lanthanides and actinides (*12*), improvement of hair shampooing (*13*), initiation of polymerization (*14*), and water treatment (*15*).

HDP has been determined with various techniques, including thermogravimetry (*16*), potentiometry and biamperometry (*17*), spectrophotometry (*18, 19*) high-performance liquid chromatography (HPLC) (*20, 21*), gas chromatography (*22*), thin-layer chromatography (TLC) (*23*), and differential capacitance measurements (*24*). Nevertheless, most of these methods involve several manipulation steps before the final result of the analysis is obtained. This is in contrast to the potentiometric methods using ion-selective electrodes, which are simple, economical, and applicable to samples of different

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