Analyst, May 1997, Vol. 122 (487–490) 487

Amperometric Bienzymic Sensor for Aspartame

D. Compagnone[a], D. O'Sullivan[b] and G. G. Guilbault[b]

[a] *Dipartimento di Scienze e Tecnologie Chimiche, Università di Roma 'Tor Vergata,' via della Ricerca Scientifica, 00133 Rome, Italy*

[b] *Chemistry Department, University College Cork, Cork, Ireland*

An amperometric enzyme electrode for the determination of aspartame was developed by covalent immobilization of alcohol oxidase and α-chymotrypsin. A platinum based hydrogen peroxide electrode was used as the detector. Excellent sensitivity was obtained using batch, flow-through and flow injection methods with detection limits of 2×10^{-7}, 4×10^{-7} and 10^{-6} mol l^{-1}, respectively. Different strategies for eliminating interfering compounds, including the introduction of an additional alcohol oxidase–catalase membrane and signal subtraction using an alcohol electrode, were employed. A recovery study on seven food samples was carried out and the results were satisfactory.

Keywords: *Aspartame; food analysis; amperometric bienzymic sensor*

Aspartame (l-aspartyl-l-phenylalanine methyl ester) is a low calorie artificial sweetener widely used in foodstuffs and soft drinks. Although many different chromatographic procedures[1–4] for the determination of aspartame have been optimized, there is still a demand for an inexpensive, rapid and automated method. Since enzyme electrodes coupled with flow injection (FI) meet these requirements, considerable efforts towards the development of an aspartame electrode have recently been made. l-Aspartase[5,6] or carboxypeptidase A and aspartate ammonia lyase[7] immobilized on an ammonia-sensitive electrode and also a three enzyme system including aspartate aminotransferase, glutamate oxidase and aspartame hydrolysing enzyme[8] coupled with an H_2O_2 probe have been reported. The major drawback of these biosensors is interference from l-aspartate, which is usually present in samples. Another bienzymic system for the determination of aspartame using immobilized alcohol oxidase on an O_2 electrode and α-chymotrypsin in solution was reported by Smith *et al.*[9] One of the products of the cleavage of aspartame by α-chymotrypsin is methanol, which can be oxidized by alcohol oxidase to formaldehyde and H_2O_2 with concomitant consumption of O_2. A biosensor with both enzymes immobilized has been developed more recently[10] and was shown to have improved stability.[11]

In this paper, we describe the development of an aspartame electrode using alcohol oxidase and α-chymotrypsin in contact with an H_2O_2 electrode. The biosensor has been used for the measurement of aspartame with batch, flow-through and FI methods. The biosensor has much improved sensitivity for aspartame and, using different strategies, electrochemical interferences have been eliminated. The biosensor has been tested for the measurement of aspartame in soft drinks and tablets with satisfactory recoveries.

Experimental

Reagents and Apparatus

α-Chymotrypsin (EC 3.4.21.1; bovine pancreas), alcohol oxidase (EC 1.1.3.13; *Hansenula* species), catalase (1.11.1.6; bovine liver), aspartame, dithiothreitol, BSA, α-chymotrypsin inhibitors, phenylalanine and glutaraldehyde were purchased from Sigma (St. Louis, MO, USA). All other reagents were of analytical-reagent grade from BDH (Poole, UK). Cellulose acetate membranes with a 100 Da molecular mass cut-off (MMCO) were prepared following a published procedure.[12] Polycarbonate membranes of porosity 0.03 μm, were purchased from Nuclepore (Pleasanton, CA, USA).

Amperometric measurements were performed using an H_2O_2 electrode consisting of a platinum working electrode polarized at +650 mV *versus* an Ag/AgCl reference electrode connected to an amperometric biosensor detector (ABD), both from Universal Sensors (Metairie, LA, USA). A Model 4500 Microscribe *x–t* recorder from The Recorder Company (San Marcos, TX, USA) was used to monitor changes in current.

Flow-through and FI measurements were carried out with the biosensor inserted in a wall-jet flow cell (Universal Sensors; volume 40 μl) and connected to a Minipuls 3 peristaltic pump (Gilson, Villiers-le-Bel, France). The injection valve used in FI was a Model 5020 from Rheodyne (Cotati, CA, USA) with a 500 μl Teflon loop.

Procedures

Enzyme immobilization was carried out using equivalent activities of each enzyme. Amounts of 0.2 mg of α-chymotrypsin and 0.5 mg of alcohol oxidase (5 U each) were dissolved in 20 μl of 0.1 m phosphate buffer (pH 7.5) and mixed. Glutaraldehyde (final concentration 0.05%) was added and the solution was immediately applied to the surface of a 1 cm^2 polycarbonate membrane and allowed to react for 1 h. The resulting enzymic membrane was sandwiched between the polycarbonate and the cellulose acetate membrane, placed on the electrode jacket (via an O-ring) and mounted on the working electrode with the cellulose acetate in close contact with the platinum surface. Unreacted glutaraldehyde was then blocked by soaking the electrode in 0.1 m glycine for 10 min.

Measurements in batch were carried out by injections of aliquots of standard aspartame solutions (or sample) after equilibration of the electrode in 5 ml of 0.1 m phosphate buffer (pH 7.5). For flow-through analysis, 10 ml of standard aspartame solutions (or sample) in working buffer were used. In both procedures steady-state currents were related to the concentration of aspartame in solution for the construction of calibration curves. Peak height current values (after subtraction of the background) were used to construct calibration curves in FI.

Stock solutions of liquid samples were prepared by 1 + 1 dilution with the working buffer; a 10% concentration in the same buffer was used for solid samples. Stock solutions were stored at 4 °C (maximum 3 d) until processed for analysis. All experiments were carried out at room temperature.

Results and Discussion

Aspartame can be cleaved by the enzymic action of α-chymotrypsin[13] to give methanol, a product that is detectable using alcohol oxidase. The overall reaction is as follows:

$$H_2NCH(CH_2CO_2H)CONHCH(CH_2C_6H_5)CO_2CH_3 +$$
$$\alpha\text{-chymotrypsin}$$
$$H_2O \rightarrow CH_3OH +$$
$$H_2NCH(CH_2CO_2H)CONHCH(CH_2C_6H_5)CO_2H$$
$$\text{alcohol}$$
$$CH_3OH + O_2 \rightarrow HCHO + H_2O_2$$
$$\text{oxidase}$$

An aspartame biosensor with long term stability based on the immobilization of α-chymotrypsin (bovine pancreas) and alcohol oxidase (*Hansenula* sp.) was developed by Chou *et al.*[10,11] The enzyme electrode studied in this work consisted of α-chymotrypsin and alcohol oxidase (activity ratio 1:1) immobilized *via* glutaraldehyde and retained at the surface of an H_2O_2 electrode (working Pt polarized at +650 mV *versus* Ag/AgCl). Two additional membranes were used in the electrode assembly: a 100 Da MMCO cellulose acetate membrane in contact with the platinum surface and a 0.03 μm porosity polycarbonate membrane externally. Calibration curves for aspartame using batch, flow-through and FI methods carried out in 0.1 M phosphate buffer (pH 7.5) are shown in Fig. 1. A linear response for batch and flow-through analysis was obtained in the range 1×10^{-6}–7.5×10^{-4} mol l^{-1} with detection limits (calculated as three times the noise of the baseline) of 2×10^{-7} and 4×10^{-7} mol l^{-1}, respectively. Linearity up to 10^{-3} mol l^{-1} and a detection limit of 10^{-6} mol l^{-1} were found when the electrode was used in FI. The best flow rates were attained at 0.6 ml min^{-1} for flow-through and 0.8 ml min^{-1} for FIA. The repeatability was tested using 10 injections of 10^{-4} mmol l^{-1} aspartame; the RSDs were 4.0, 3.6 and 1.8% for batch, flow-through and FI, respectively. The performance of this electrode, in terms of sensitivity, is superior to that of any aspartame biosensor developed to date. This can be attributed to a high rate of conversion of aspartame into a detectable species by α-chymotrypsin and to the sensitivity of the H_2O_2 electrode. It should also be noted that alcohol oxidase has been reported to have the ability to oxidize formaldehyde at a lower rate than methanol,[14–16] thus yielding 2 equiv. of H_2O_2 per molecule of methanol.

Interferences

Two different kinds of interfering compounds need to be considered for enzyme electrodes, electrochemical and enzymic. Protection from electrochemical interferents in the biosensor developed was obtained by means of a 100 Da MMCO cellulose acetate membrane. This membrane has already been used in clinical and food analysis[17–20] and its behaviour toward many potentially interfering compounds is well known. However, in order to test the 'electrochemical selectivity' of our probe, compounds that are normally present in beverages were added to the working solution at a concentration of 1 mmol l^{-1}. Lactose, sucrose, citric acid, ascorbic acid, sodium benzoate and phenylalanine, one of the amino acid breakdown products of aspartame, did not give any detectable signal.

Enzymic interferences have represented a major drawback for the aspartame enzyme electrodes developed to date.[5–11] Using the biosensor developed in this work, problems can arise from molecules having alcoholic functions because of the broad substrate specificity of alcohol oxidase.[17–19] Apart from short chain alcohols, an interfering signal has been also reported[11] using TRIS buffer with the O_2 based biosensor (owing to the propane-1,3-diol part of the molecule). When the H_2O_2 based biosensor was tested for alcohol interference by injections of 10^{-4} mmol l^{-1} of methanol, ethanol and propanol, the resulting changes in current (at the steady state) were 170, 105 and 62%, respectively, relative to the same concentration of aspartame (Fig. 2).

The first attempt to reduce interferences was made by application of an external additional enzymic membrane. This membrane consisted of alcohol oxidase (5 units) and catalase cross-linked *via* glutaraldehyde. The catalytic action of catalase can be described by the reaction

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

Using the proposed sensor configuration, alcohols present in the sample are eliminated by the external alcohol oxidase–catalase membrane with no net production of H_2O_2. Aspartame, on the other hand, can diffuse freely and reach the internal α-chymotrypsin–alcohol oxidase membrane producing the electroactive species. Fig. 2 shows the selectivity of such a sensor to the three short chain alcohols. A significant decrease in current was obtained for methanol, ethanol and propanol whereas the response for aspartame was still linear over the range 5×10^{-6}–2×10^{-3} mol l^{-1} with a detection limit of 2×10^{-6} mol l^{-1} (Fig. 3). When more than 2 mg of catalase were used, no further improvement in selectivity was observed. The

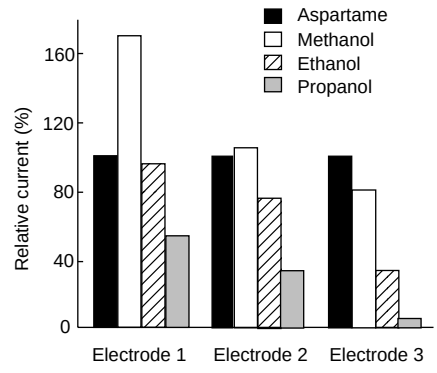

Fig. 2 Response to alcohols (batch mode) of the single bienzymic electrode (1) and of the double bienzymic electrode containing 1 mg (electrode 2) and 2 mg (electrode 3) of catalase.

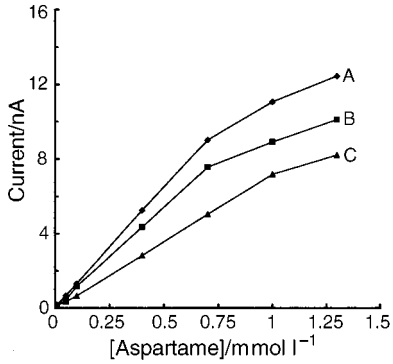

Fig. 1 Calibration curves for aspartame in A, batch; B, flow-through; and C, flow injection modes.

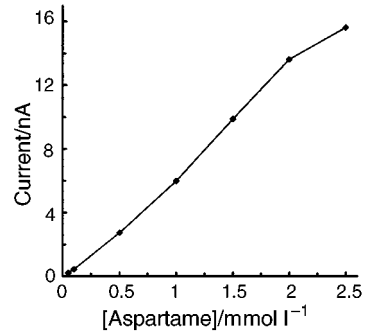

Fig. 3 Calibration curve for aspartame using the double bienzymic electrode in batch analysis.

residual current generated by the short chain alcohols can be explained by taking into account the activity of alcohol oxidase towards the product of the enzymic reaction (as mentioned in the previous section). It needs also to be considered that both formaldehyde and acetaldehyde are electroactive under our experimental conditions (Pt working electrode polarized at +650 mV *versus* Ag/AgCl). Thus, in the case of non-quantitative conversion by alcohol oxidase, they can contribute to the residual current undergoing oxidation at the electrode surface. From the data obtained it can be concluded that the double bienzymic membrane configuration can be used for samples not containing methanol and/or ethanol (the response is still 80 and 33%, respectively). However, alcohols of higher molecular mass do not interfere because their corresponding aldehydes are oxidized at a lower rate by alcohol oxidase or at the electrode surface.

A different strategy was attempted using α-chymotrypsin inhibitors and the single bienzymic membrane electrode. The biosensor was allowed to equilibrate in a mixture containing 10^{-4} mmol l^{-1} aspartame and methanol and then different amounts of chicken egg or soybean α-chymotrypsin inhibitors were injected into the solution. Only the chicken egg inhibitor was able to inhibit completely the immobilized α-chymotrypsin at a concentration of 8 mg ml^{-1}, reducing the steady-state current to the value expected for a 10^{-4} mmol l^{-1} solution of methanol. Unfortunately, the aspartame electrode did not recover its initial activity, even after extensive washing in buffer (60% after 2 h).

Another possible method of eliminating enzymic interferences is obtained by separation of the signal of alcohol oxidase from the signal of α-chymotrypsin–alcohol oxidase. A possibility is to use immobilized alcohol oxidase and α-chymotrypsin in solution as proposed by Smith *et al.*[9] The signal coming from any interfering compound is then used as a baseline and the change in current after addition of α-chymotrypsin is related to the aspartame content. This approach was tested by replacing α-chymotripsin with BSA in the aspartame electrode described previously and challenging it with the 10^{-4} mol l^{-1} aspartame–methanol mixture. After stabilization of the steady-state signal due to methanol, 1 U of α-chymotrypsin was injected in solution. Complete consumption of aspartame was obtained in 10 min. However, subsequent measurements on the aspartame–methanol mixture gave increased current values for the steady state, indicating absorption of the soluble enzyme on the external polycarbonate membrane. Thus, taking the long response time and consumption of α-chymotrypsin into consideration, this approach was not considered feasible.

Finally, it was decided to process each sample both with the alcohol electrode (no α-chymotrypsin) and with the aspartame electrode and to extrapolate the true value for aspartame by subtraction of the signals. Although the procedure may appear complex, it is the only one that ensures complete discrimination of interferences and works fairly well.

Food Samples

A recovery study on seven food samples was carried out to assess the matrix effect on the aspartame electrode. A 1 : 25 dilution of each sample stock solution (see Experimental) was tested using the alcohol electrode as described previously. No or very little current (5–10 pA) was obtained for each of the samples tested, indicating that there was no electrochemical and/or enzymic interfering compounds present in the samples. The recovery study was then carried out in batch analysis and in FI using the aspartame electrode only (Table 1). The results were satisfactory, the recoveries being between 94.7 and 105.9% for batch analysis and between 97.2 and 105.3% for FI.

In order to check the reliability of the measurement proposed in the previous section using the alcohol and the aspartame enzyme electrodes, four samples were spiked with methanol (5 mmol l^{-1}) and the aspartame recovery was calculated (Table 2). The values obtained were between 90.8 and 109.9% for batch analysis and between 97.9 and 104.1% for FI. The difference in the recovery ranges for the two systems can be explained by considering the RSDs of the batch (4%) and FI (1.8%) procedures. In fact, measurement with the alcohol electrode introduces an additional error to the determination of aspartame that can be minimized using the FI procedure.

The operational stability of the aspartame electrode using the FI procedure was also tested by consecutive injections of a Diet

Table 1 Recovery of aspartame using the developed enzyme electrode in batch analysis and FI modes

	Found/ mmol l^{-1}		Added/ mmol l^{-1}	Expected/ mmol l^{-1}		Measured/ mmol l^{-1}		Recovery (%)	
Sample	Batch	FI		Batch	FI	Batch	FI	Batch	FI
Canderel	35.64	33.62	5	40.64	38.62	41.20	40.65	101.6	103.5
Hermenesetas	6.85	6.59	5	11.85	11.59	11.55	11.74	97.5	101.6
Half spoon	5.91	6.19	5	10.91	11.19	11.31	10.96	103.7	97.9
Diet Coke	6.11	6.21	5	11.11	11.21	10.75	11.85	96.8	105.3
Diet Up	3.15	3.43	5	8.15	8.43	8.10	8.30	99.4	98.7
One Cal	11.09	11.16	5	16.09	16.16	16.45	16.24	102.2	100.4
Ribena	10.44	10.14	5	15.44	15.14	14.62	14.71	94.7	97.2

Table 2 Recovery of aspartame in samples spiked with 5 mmol l^{-1} of methanol and processed with the alcohol and aspartame electrodes

	Methanol found/ mmol l^{-1}		Aspartame found/ mmol l^{-1}		Aspartame expected/ mmol l^{-1}		Recovery (%)	
Sample	Batch	FI	Batch	FI	Batch	FI	Batch	FI
Canderel	4.85	4.91	33.21	32.92	35.64	33.62	93.2	97.9
Hermenesetas	5.12	4.92	7.53	6.86	6.85	6.59	109.9	101.1
Diet Coke	4.84	5.01	5.61	6.41	6.11	6.21	91.8	103.8
Diet Up	4.89	4.96	2.86	3.57	3.15	3.43	90.8	104.1

Coke sample during a day. After 200 analyses the electrode still retained 75% of the initial activity.

Conclusions

An aspartame electrode based on the covalent immobilization of alcohol oxidase and α-chymotrypsin on the surface of a hydrogen peroxide electrode has been developed. A significantly improved sensitivity in comparison with other aspartame electrodes constructed to date was obtained. Different procedures for the elimination of interferences have been tested and discussed. The electrode was used in batch and FI modes for aspartame in seven different food matrices, with good recoveries.

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Paper 6/07715C
Received November 13, 1996
Accepted January 30, 1997