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An Improved FIA Biosensor for the Determination of Aspartame in Dietary Food Products

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ABSTRACT

A flow injection analysis (FIA) biosensor system was developed for the determination of the artificial sweetener aspartame (L-aspartyl-L-phenylalanine methyl ester). The system consisted of an enzyme column of pronase immobilized on activated arylamine glass beads and a L-amino acid oxidase electrode connected in series. The dipeptide bond of aspartame was cleaved by immobilized pronase to release phenylalanine, which was in turn monitored by the enzyme electrode that used L-amino acid oxidase immobilized on a preactivated nylon membrane in combination with an amperometric electrode (platinum vs silver/silver chloride, 700 mV).

The response of the FIA biosensor was linear up to 1 m*M* aspartame with a lower detection limit of 25 μM and had good reproducibility (rsd 0.3%). The FIA biosensor was stable for at least 30 h of continuous use at T_r. Each assay takes 4 min giving a sample throughput of 15 h^{-1}. When applied to aspartame in dietary food products the results obtained agreed well with those reported by the product manufacturers.

Index Entries: Aspartame; biosensor; amperometry; immobilized L-amino acid oxidase; immobilized pronase; arylamine glass beads.

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INTRODUCTION

Aspartame (L-aspartyl-L-phenylalanine methyl ester) has been fast replacing saccharin as a low calorie sweetener in dietary products since its regulatory clearance in the early 1980s by the Food and Drug Administration. Owing to its increased application, there is now a demand to develop fast and accurate methods for aspartame detection. Aspartame has been determined by chromatographic procedures, such as high performance liquid chromatography and thin layer chromatography (*1–6*). However, these complicated and time-consuming techniques require considerable capital investment as well as trained personnel. The microbial biosensor using *Bacillus subtilis* cells with an oxygen electrode was nonspecific since it responded to glucose and other amino acids (*7*). The enzyme-based biosensor using immobilized carboxypeptidase A and aspartase in conjunction with an ammonia electrode had problems owing to interfering amines present in the food samples (*8*).

A FIA biosensor based on immobilized peptidase, aspartate-aminotransferase, and glutamate oxidase was developed for aspartame detection (*9*). The first enzyme acted on aspartame to release aspartate, which was then transaminated in the presence of α-ketoglutarate to form glutamic acid by aspartate-aminotransferase. The enzymatic oxidation of glutamic acid was then detected amperometrically using immobilized glutamate oxidase together with a hydrogen peroxide electrode. The biosensor technique was very successful in measuring aspartame in a variety of dietary products. Unfortunately, the major drawback to the possible commercialization of such a system was the necessity to purify the peptidase as well as the extremely high cost of the three enzymes involved.

The main purpose of this study was to develop an improved and simpler FIA biosensor for the determination of aspartame. Pronase was immobilized on arylamine glass beads to form an enzyme column and this enzyme was more efficient than peptidase in the cleavage of aspartame to aspartate and phenylalanine/phenylalanine methyl ester. L-amino acid oxidase was immobilized on a preactivated nylon membrane and attached to the tip of the hydrogen peroxide electrode. The sensitivity, reliability, accuracy, stability, and reusability of the FIA biosensor for aspartame determination in a variety of dietary food and beverage products were presented and discussed.

MATERIALS AND METHODS

Materials

L-amino acid oxidase Type IV (E.C. 1.4.3.2), NADH peroxidase, malic dehydrogenase, glutamic-oxalacetic transaminase (aspartate aminotransferase), lyophilized peptidase (30% protein), glutaraldehyde, aspartame,

phenylalanine, aminopropyl (pore 70 nm, 80–120 mesh), and arylamine (pore 7.5–10 nm, 200–400 mesh) glass beads were purchased from Sigma (St. Louis, MO). Lyophilized pronase (from *Streptomyces griseus*, 30% protein) was obtained from Boehringer Mannheim (Germany), sodium nitrite from Aldrich (Milwaukee, WI) and all remaining chemicals were purchased from Anachemia (Champlain, NY).

Immobilization of Enzymes

L-Amino Acid Oxidase on Membrane

A 1.5×1.5-cm piece of prewetted Immunodyne™ membrane (Pall Biosupport Corporation, Glencove, NY) was stretched on the top of a 1-cm diameter hollow plastic cylinder and held in place by an O-ring. The enzyme solution was layered on the stretched membrane and allowed to dry at T_r (20–22°C) for 45 min. The membrane was then removed and washed with 30 m*M* borate, 100 m*M* NaCl, pH 9.0 buffer, and stored at 4°C in buffer. The immobilized membrane was tested with respect to optimal loading, buffer compatibility, pH optimum, effect of flow rate, amino acid selectivity, and phenylalanine calibration.

Pronase on Aminopropyl and Arylamine Glass Beads

Aminopropyl glass beads (250 mg) were washed extensively with phosphate buffer saline PBS (9 g liter^{-1} sodium chloride, 20 m*M* phosphate, pH 7.0) and then activated by contacting with 3 mL of 2.5% (w/v^{-1}) glutaraldehyde in PBS for 2–3 h at T_r. The resulting orange-pink beads were washed thoroughly to remove excess glutaraldehyde and used for enzyme immobilization. Pronase enzyme solution was mixed with activated glass beads in a capped tube and slowly rotated overnight at 4°C. The immobilized enzyme beads were washed with 100 m*M* maleate, 100 m*M* NaCl buffer, pH 6.7 to remove unbound materials and stored at 4°C in buffer. The beads were packed into a 2.54 mm id tygon tubing, furnished with glass wool at the ends to retain the beads, to form a column reactor. Two hundred fifty milligrams of beads packed into approx a 12-cm column length.

Five hundred milligrams of arylamine glass beads were also washed with PBS buffer followed by a 1*M* HCl solution. The beads in 20 mL of 1*M* HCl were activated to the diazo form by the slow addition of 20 mL of 0.5*M* sodium nitrite solution at 0°C for 45 min. The beads were rinsed with cold 0.1*M* HCl followed by 0.2*M* sodium bicarbonate pH 8.0. The resulting reddish-brown beads were mixed with the pronase solution overnight at 4°C. After rinsing with maleate buffer the beads were packed into a column as described above. Five hundred milligrams of beads packed approx 2 6-cm column reactors. The immobilized enzyme reactor was tested with respect to optimal enzyme loading, buffer compatibility, pH optimum, effect of flow rate, column reactor length, and aspartame calibration.

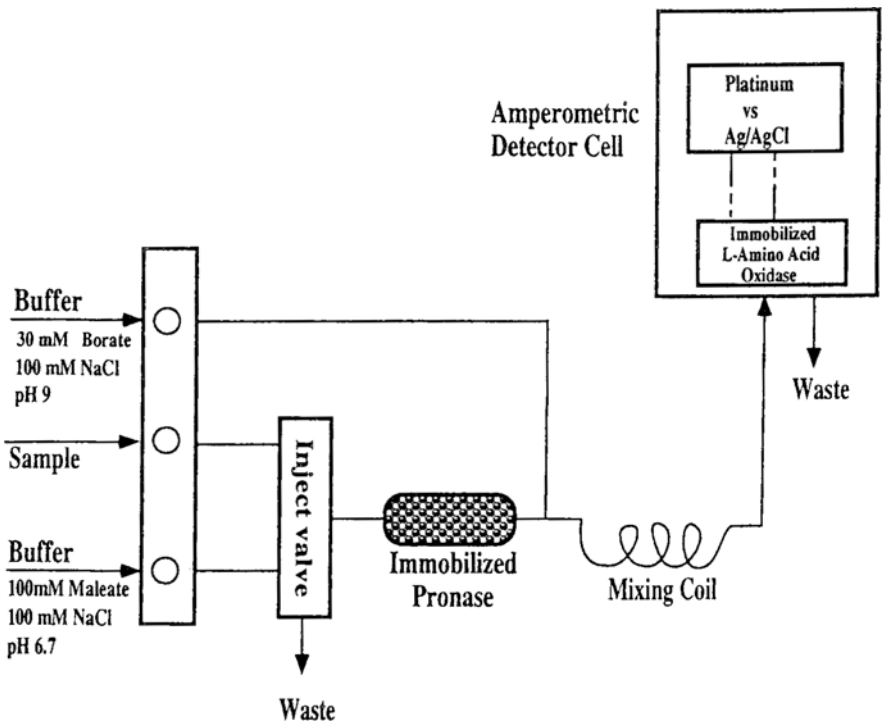

Fig. 1. Schematic diagram of the FIA biosensor for aspartame.

Measurement of Enzyme Activity

Amino acid oxidase activity was determined from the rate of NADH oxidation using a coupled enzyme assay containing 0.15 m*M* NADH, 0.48 U NADH peroxidase in 100 m*M* phospate buffer. The pH profile of the enzyme was determined using 0.5 m*M* phenylalanine. The aspartame peptide bond cleaving activity of the pronase was determined using a stopped time assay. The aspartame (1 m*M*) cleavage was performed in 100 m*M* maleate buffer. After 5 min a 100-μL aliquot was tranferred to a cuvet containing 10 m*M* α-ketoglutarate, 0.15 m*M* NADH, 80 U glutamic-oxalacetic transaminase in 150 m*M* phosphate buffer, pH 7.8. The metabolite assay for aspartate was initiated with the addition of 50 U of malic dehydrogenase. A coupling assay could not be performed owing to the potent inhibitory effect of maleate on the transaminase. For both assays the absorbance change for NADH at 340 nm was monitored spectrophotometrically (DU 7, Beckman Instruments Inc., Fullerton, CA).

Apparatus

The FIA biosensor system (Fig. 1) consisted of a peristaltic pump (FIA Pump 1000, Eppendorf North America Inc., Madison, WI) that delivered the sample and different buffers at a preset flow rate. A 75-μL sample was

injected into the maleate buffer stream (100 m*M*, 100 m*M* NaCl, pH 6.7) by a motorized injection valve (Eva injector, Eppendorf North America Inc.). The sample flowed through the immobilized pronase column before merging with the buffer stream containing 100 m*M* NaCl, 30 m*M* borate, pH 9.0. After flowing through a short mixing coil (the pH holds at 8.9) the phenylalanine produced was converted by the amino acid oxidase on the Immunodyne™ membrane and H_2O_2 was determined by the FIA detector system (Eva-zyme 500, Eppendorf North America Inc.). The detector consisted of a flow cell and an amperometric electrode (platinum vs silver/silver chloride, poised at +700 mV) covered by the membrane, which was held in place by an O-ring. The sampling and injection into the FIA were controlled by a master module (Eppendorf North America Inc.) and the output was recorded on a strip chart recorder. The response in peak height mode was expressed as relative units (RU), in which 1 RU was equal to 2.86 μV at the detection output.

Measurement of Aspartame Concentration in Dietary Products

Soft drink samples were diluted four-fold in the maleate buffer and were then ready for analysis. The acidity of these samples drops the pH from 6.7 to 6.4 (still within the optimal pH range for pronase) but when mixed with the borate buffer the resulting pH is still above 8.7 (the optimum for amino acid oxidase). The solid samples were extracted with 10% trichloroacetic acid followed by dilution (4–20-fold) in maleate buffer and pH adjustment to 6.7 with NaOH. The FIA biosensor was calibrated using aspartame solutions and the values obtained for the dietary products were compared to the manufacturer values. The aspartame concentrations were also determined by measurement with a Beckman 6300 high-performance analyzer.

RESULTS AND DISCUSSION

Optimization of Immobilized Enzyme Systems

Amino Acid Oxidase

Optimal enzyme-loading conditions were determined for amino acid oxidase by vortexing membranes in the presence of 0.5 m*M* phenylalanine for 30 s and then monitoring the H_2O_2 released by the NADH peroxidase coupling assay. The activity increased with increasing enzyme load (Fig. 2), with a maximum load achieved at about 1.9 U or 60 μL of stock enzyme. Consequently 1.9 U of amino acid oxidase were loaded onto membranes for all subsequent experiments.

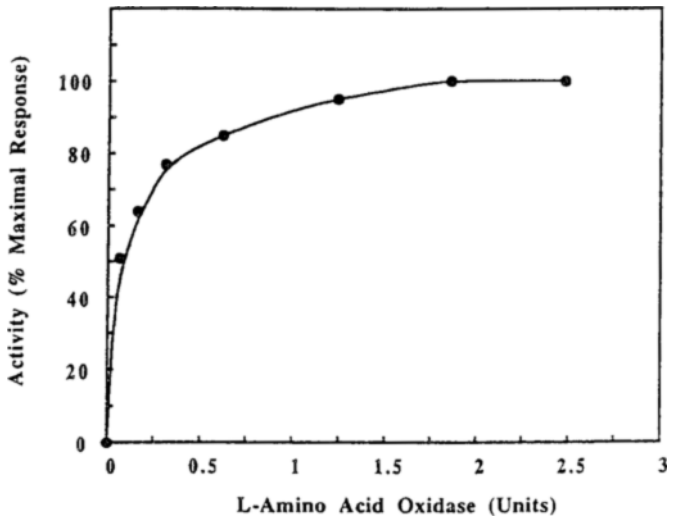

Fig. 2. Relationship between the amount of amino acid oxidase (SA 4.3 U mg^{-1}) loaded and the activity of immobilized enzyme.

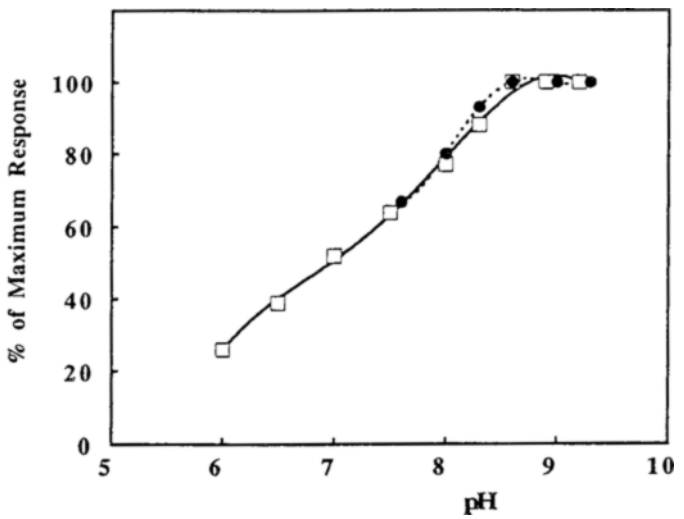

Fig. 3. Activity vs pH profile of immobilized amino acid oxidase: (□) phosphate; (●) borate.

Optimal operating conditions were determined for the immobilized amino acid oxidase utilizing the FIA system shown in Fig. 1 without the pronase enzyme reactor in place. The system was also operated without merging buffer streams to monitor the effect of pH in the presence of phosphate and borate buffer. As shown in Fig. 3, the pH optimum for the amino acid oxidase was reached at pHs above 8.5. Borate buffer did not reduce the sensitivity of the system at concentrations below 25 m*M*, however at 50 m*M* only 75% of the maximal response was retained. A borate

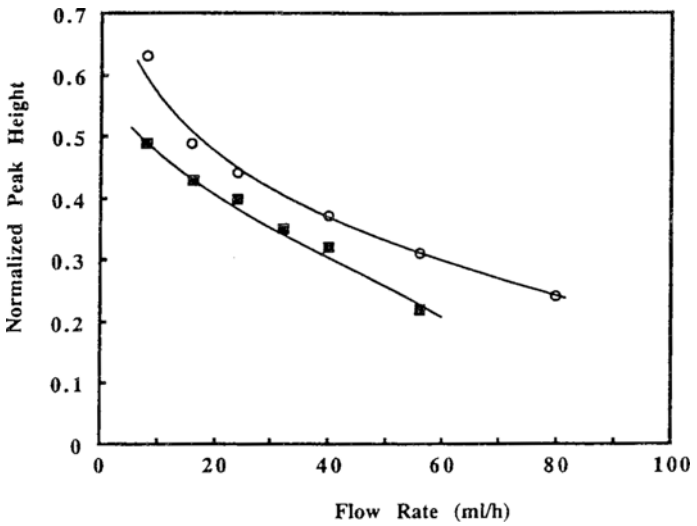

Fig. 4. Effect of flow rate at the detector on the FIA response with 75-μL injection volume: (○) phenylalanine; (■) aspartame. Peak height was normalized with respect to steady state responses for a flow rate of 20 mL h^{-1} (40 mL h^{-1} at the detector).

buffer concentration of 15 m*M* at the detector (30 m*M* in the merging system of Fig. 1) was more than sufficient to hold the pH above 8.5 when mixed with the maleate buffer stream. It should be noted that to achieve a similar effect with phosphate a concentration of over 1*M* would be required. As a result 30 m*M* borate buffer, pH 9.0, was chosen as a buffer. Sodium chloride was added to the buffer at 100 m*M* to help prevent fouling of the membrane and/or electrode and was shown to have no inhibitory effect on the amino acid oxidase at this concentration. The pH profile did not change from that of Fig. 3 when the system was operated with both maleate and borate indicating that maleate exhibited no detrimental effect on the amino acid oxidase. The pH profile of the soluble amino acid oxidase was very similar to that of the immobilized enzyme (figure not shown).

Fig. 4 illustrated the normalized peak height FIA response for 1 m*M* phenylalanine as a function of the flow rate utilizing the FIA system shown in Fig. 1. The flow rate at the detector will be double the input pump flow rate owing to the merging streams. As expected in accordance with the theoretical prediction for FIA systems with negligible mass transfer resistance in the bulk solution (*10*) and experimental observations (*9,11*) the response increased with a decrease in the sample flow rate. As a compromise between the sensitivity of analysis and sample throughput (assays/h) a flow rate of 20 mL h^{-1} (40 mL h^{-1} at the detector) was selected for all subsequent studies. The system was linear to concentrations of phenylalanine up to 1 m*M*. The linearity at such a high flow rate was likely because of the low K_m of amino acid oxidase with respect to phenylalanine

Table 1
Selectivity of Immobilized Amino Acid Oxidase to Analytes

Amino acid	Response relative to phenylalanine (%)
Phenylalanine	100
Tyrosine	183
Methionine	137
Tryptophan	115
Leucine	82
Isoleucine	45
Arginine	32
Histidine	23
Lysine	10
Cystine	8
Glutamine, valine, alanine, asparagine	< 5
Aspartate, glutamate, glycine, serine, proline, threonine,	0
Aspartame, L-phenylalanine methyl ester	0

(ca.100 μM). The system was operated continuously with injections of 1 mM phenylalanine for at least 30 h (450 injections) without significant loss of sensitivity (< 15%). The loss of sensitivity was attributed to the fouling of the electrode rather than enzyme stability, since cleaning the electrode after the experiment increased the response back to the original value. Electrode sensitivity could be easily restored by soaking it in hypochlorite solution followed by cleaning the surface of the electrode. The immobilized amino acid oxidase membrane was stable for at least 2 mo when stored in borate/salt buffer pH 9.0 at 4°C. Enzyme membranes prepared by crosslinking the enzyme with glutaraldehyde and bovine serum albumin did not possess greater stability and in fact had sensitivities of only about 50% that of the membranes where the enzyme was simply deposited.

The response of the amino acid oxidase was not sensitive to just phenylalanine, but also to many other amino acids, including tyrosine, methionine, tryptophan, leucine, isoleucine, arginine, and histidine (Table 1). This could pose a problem for reliable aspartame detection in samples containing such amino acids. However, the amino acid oxidase electrode was not sensitive to aspartame or L-phenylalanine methyl ester.

Some Characteristics of Immobilized Pronase

Commercially available pronase is ten-fold less expensive than peptidase. In addition, the activity of soluble enzyme (0.5 mg/mL lyophilized powder) for aspartame was four-fold higher than that of soluble peptidase (0.5 mg/mL lyophilized powder) using the stopped time assay as described

in the previous section. In view of this, pronase was used together with L-amino acid oxidase to develop a biosensor system for aspartame.

As reported by Male et al. (*9*), the crude preparation of peptidase was purified 19-fold before immobilization and about 2.4 mg of purified enzyme was immobilized onto 250 mg aminopropyl glass beads. Based on this protocol, about 1.2 mg crude pronase was immobilized onto 250 mg aminopropyl glass beads via its amino groups. A lower binding for pronase may imply that this enzyme possesses fewer amino groups in comparison to peptidase. In addition, this preparation undoubtedly contains other proteins that may compete with pronase to be covalently immobilized onto aminopropyl glass beads. A 10-cm column of these beads resulted in a signal for 1 m*M* aspartame of only 40% when compared to 1 m*M* phenylalanine, indicating that the complete conversion of the aspartame was not achieved. Similar behavior was also observed for crude peptidase (data not shown). As a result of this finding, rather than considering purification of the pronase a second immobilization procedure was attempted using arylamine glass beads. This coupling method involved diazotization of arylamine glass beads followed by reaction with the enzyme at slightly alkaline pH. The azo linkage was formed between arylamine glass beads and tyrosine groups of the enzyme. To 500 mg of arylamine beads 11.4 mg of protein bound, or more than 4 times that of aminopropyl glass beads. A 6-cm column of these beads resulted in identical responses for 1 m*M* solutions of phenylalanine or aspartame, indicating a complete conversion of the aspartame. As a result the arylamine beads were used for all further optimization studies. This constituted a significant cost reduction in comparison to using the expensive peptidase enzyme and the time-consuming purification protocol of the peptidase enzyme could now be avoided. It is of interest to note that the peptidase enzyme also bound to the arylamine beads (6 mg/500 mg beads) but unfortunately very little response was detected from the column (data not shown). Again, the azo linkage was expected to form between arylamine glass beads and tyrosine groups of this enzyme. This could be problematical if the tyrosine groups were either active sites or close to the binding sites of this enzyme for aspartame. Further investigation is required to elucidate the binding sites as well as the active sites of peptidase for aspartame. It should be noted that immobilized peptidase on aminopropyl glass beads via its amino groups possessed satisfactory activity (*9*).

Fig. 5 illustrates the effect of pH on the response of the pronase enzyme reactor (6 cm length) to 1 m*M* aspartame at a speed of 20 mL h^{-1} using maleate buffer. The pH optimum ranged between 6.5–7.5 and the soluble enzyme behaved similar to the immobilized enzyme. As reported (*9,12*), aspartame was quite unstable at pHs above 7.0, so as a result pH 6.7 was chosen for the maleate buffer. Maleate buffer was selected owing to its good buffering capacity and exhibited no effect on the response up to 200 m*M*. The concentration of 100 m*M* was considered sufficient to

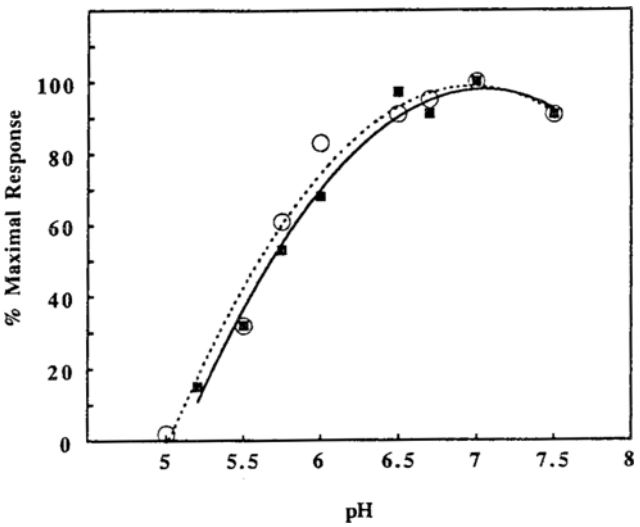

Fig. 5. Activity vs pH profile of pronase: (○) immobilized; (■) soluble.

hold the sample pH as well as not significantly reduce the pH after the merging point with the borate buffer. Phospate buffer was not utilized because it inhibited the pronase activity at the concentration necessary to buffer the samples. NaCl had no inhibitory effect on pronase up to 100 m*M* and this concentration was added to the buffer to prevent fouling of the reactor column.

Fig. 4 illustrates the normalized peak height FIA response for 1 m*M* aspartame as a function of flow rate. The flow rate of 20 mL h^{-1} (corresponding to 40 mL h^{-1} at the detector) was a good compromise between sensitivity and the throughput and was selected for further experiments. It should be noted that without the immobilized pronase column in place, the response of the FIA system to phenylalanine was somewhat higher. Obviously, the introduction of the immobilized enzyme column induced more convection/diffusion dispersion in a flowing stream than the empty tubing, thereby lowering the peak height response.

At the flow rate of 20 mL h^{-1}, a pronase column length of 4 cm gave a maximal response for 1 m*M* aspartame (Fig. 6) and as a result a 6-cm length was chosen for aspartame analysis. By lowering the flow rate to 12 mL h^{-1} the maximum response was obtained for a column of 2 cm but although the column length could be shortened and the response could be increased this condition would result in a lower throughput. These studies were performed in a peak area mode instead of peak height to account for the difference in peak heights caused by changing dispersions owing to varying column lengths.

Response of the Biosensor to Aspartame

In peak height mode there was an excellent linear response with the FIA biosensor to aspartame up to 1 m*M* (correlation coefficient to 1.0).

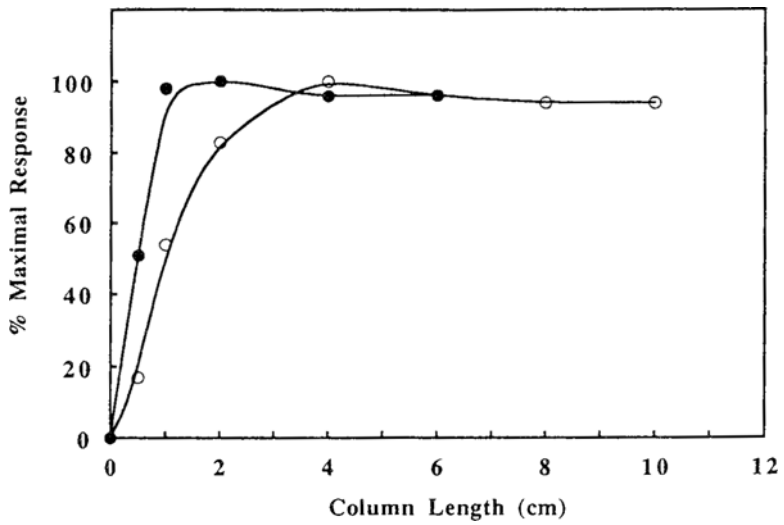

Fig. 6. Effect of column length on the FIA response to 1 m*M* aspartame: (●) 12 mL h^{-1}; (○) 20 mL h^{-1} (40 mL h^{-1} at the detector).

The sensitivity of the biosensor was determined to be 82.3 ± 0.26 RU μM^{-1} (95% confidence interval, $n = 8$) with a minimum detection level of 25 μM. Good reproducibility was obtained as reflected by the average response for 20 repeated analyses of 1.0 m*M* aspartame (81,700 ± 252 RU, at 95% confidence interval). In all cases, the background current was very stable and never exceeded 2% of the average response (81,700 ± 252 RU). Therefore, the auto-zero function of the FIA detector system was used to suppress the background current during each cycle of measurement. Each assay, including washing, was performed in 4 min giving a throughput of 15 h^{-1}. The system was run continuously with injections of 1.0 m*M* aspartame for at least 30 h (450 injections) without significant loss of sensitivity (< 15%). Again this loss of sensitivity could be owing to electrode fouling or slow decomposition of the aspartame solution over the long period of time. Replacement of the aspartame solution at the end of the experiment with a freshly prepared one partially restored the signal response. Immobilized pronase was observed to be stable for at least 2 mo when stored at 4°C in maleate buffer, pH 6.7.

Measurement of Aspartame in Dietary Food and Beverage Samples

The FIA biosensor system was applied to determine aspartame in various dietary food products. Aspartame concentrations determined by measuring hydrogen peroxide production by the FIA biosensor were in good agreement with those reported by the product manufacturers as well as those of the high performance analyzer (Table 2). The biosensor values plotted against those of the manufacturers and high performance analyzer resulted in straight lines with slopes of 0.998 and 1.05 respectively, as

Table 2
Aspartame Concentrations in Dietary Food Products Determined by Biosensor

Products	Aspartame Concentration[a]		
	Biosensor[b]	Reported by manufacturer	High performance analyzer
Diet Minute Maid	0.055	0.060	0.052
Diet Coke	0.048	0.052	0.049
Diet Pepsi	0.058	0.055	0.053
Diet Sprite	0.049	0.049	0.049
Diet 7-Up	0.050	0.049	0.050
Diet Fresca	0.039	0.049	0.039
Diet Ginger Ale	0.038	0.044	0.040
Equal	4.1	3.9	3.9
Jello (Raspberry)	3.5	3.8	4.3
Jelly (Strawberry)	2.9	2.9	2.9
Vanilla Pudding	1.5	1.5	1.6
Lemon Tea	1.3	1.2	1.4
Hot Chocolate	0.85	0.62	0.67

[a] mg aspartame/100 mg product
[b] Average of duplicate determinations

well as correlation coefficients of 1.0 ($n = 13$) for both (figure not shown). If only the beverages are plotted, the slopes of the lines were 1.06 and 0.980 respectively, with correlation coefficients of 1.0 ($n = 7$) for both. This excellent agreement thus indicated that the amino acid oxidase, although nonspecific, was sufficient to convert phenylalanine without any problems arising from interfering amino acids. This was especially encouraging with respect to the solid products and constitutes a significant cost reduction in comparison to the previous more specific biosensor utilizing aspartate amino transferase (glutamic-oxalacetic transaminase) and glutamate oxidase (*9*). A sample of Diet Pepsi™ was run continuously for 20 h (300 injections) and resulted in only a slight drop in sensitivity (< 10%), indicating that the real samples had no long term detrimental effect on the sensitivity of the FIA system.

In brief, an improved FIA biosensor for aspartame has been designed by replacing the costly peptidase enzyme ($280/column reactor), which also needed to be purified, with a crude pronase enzyme ($1/column reactor). In addition, the costly transaminase ($70/column reactor) and glutamate oxidase ($20/membrane) were replaced by amino acid oxidase ($10/membrane). The biosensor was successfully applied to the measurement of aspartame in a variety of dietary food products.

REFERENCES

1. Argoudelis, C. J. (1984), *J. Chromatogr.* **303,** 256–262.
2. Daniels, D. H., Joe, F. L., Warner, C. R., and Fazio, T. (1983), *J. Assoc. Off. Anal. Chem.* **67,** 513–515.
3. Hussein, M. M., D'Amelia, R. P., Manz, A. L., Jacin, H., and Chen, W-T. C. (1984), *J. Food Sci.* **49,** 520–524.
4. Issaq, H. J., Weiss, D., Ridlon, C., Fox, S. D., and Muschik, G. M. (1986), *J. Liq. Chromatogr.* **9(8),** 1791–1802.
5. Tsang, W-S., Clarke, M. A., and Parrish, F. W. (1985), *J. Agric. Food Chem.* **33,** 734–738.
6. Webb, N. G. and Beckman, D. D. (1984), *J. Assoc. Off. Anal. Chem.* **67,** 510–514.
7. Renneberg, R., Riedel, K., and Scheller, F. (1985), *Appl. Microbiol. Biotechnol.* **21,** 180–181.
8. Fatibello-Filho, O., Suleiman, A. A., Guilbault, G. G., and Lubrano, G. J. (1988), *Anal. Chem.* **60,** 2397–2399.
9. Male, K. B., Luong, J. H. T., and Mulchandani, A. (1991), *Biosensors and Bioelectronics* **6,** 117–123.
10. Olsson, B., Lunbäck, H., Johansson, G., Scheller, F., and Nentwig, L. (1986), *Anal. Chem.* **58,** 1046–1052.
11. Heider, G. H., Sasso, S. V., Huang, K., Yacynych, A. M., and Wieck, H. J. (1990), *Anal. Chem.* **62,** 1106–1110.
12. Homler, B. E. (1984), *Food Technol* **38(7),** 501–505.