JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Simple and Inexpensive Flow L-Glutamate Determination Using Pumpkin Tissue

NICÁCIO J. M. ARRUDA,† JOSÉ L. L. FILHO,† MARIA C. B. S. M. MONTENEGRO,‡ ALBERTO N. ARAÚJO,‡ AND VALDINETE L. SILVA*,§

Biochemistry Department, Laboratory of Immunopathology Keizo Asami (LIKA), UFPE-50670-901-Recife-PE, Brazil, Department Química Física/Faculty of Pharmacy, REQUIMTE, 4000-Porto, Portugal, and Chemical Engineering Department, Environmental and Quality Engineering Laboratory, UFPE-50740-521-Recife-PE, Brazil

This work refers to a very easy to implementate flow injection system with potentiometric detection for L-glutamate determination in food samples. The proposed procedure is based on measurement of carbon dioxide produced by decarboxylation of L-glutamate catalyzed by L-glutamate decarboxylase (E.C. 4.1.1.1.5) from *Cucurbita maxima* (pumpkin). The FI potentiometric system includes an enzymatic reactor with a length of 8 cm and thickness of 5 mm packed with 200 mg of a *C. maxima* outer layer cut in to small pieces. The proposed procedure allowed L-glutamate determinations in the concentration interval of 10−100 mmol L^{-1} for an injected sample volume of 50 μL. A phosphate buffer (0.1 mol L^{-1}, pH 5.5) solution flowing at 1.4 mL min^{-1} was used as the carrier solution in the system. The results obtained in the analysis of food samples revealed a relative error lower than 5% when compared with those provided by the spectrophometric reference procedure. The immobilized reactor retained its initial activity for 21 days. It was possible to measure 40 samples/h with the flow system proposed.

KEYWORDS: L-Glutamate; glutamate decarboxylase; potentiometry; flow analysis; pumpkin; *Cucurbita maxima*

INTRODUCTION

L-Glutamate is a biotechnological product present in different kinds of foods that contributes to their flavor. Thus, its monitoring is essential during food processing and fermentation control because ingestion of high concentrations of L-glutamate derived from food can induce the appearance of neurological diseases, mainly Parkinson's and Alzheimer's. Testing for glutamate in food is normally carried out using chromatographic (*1*), spectrophotometric (*2*, *3*), and fluorimetric methods (*4*). The proposal of automated procedures led to an additional reduction of time, cost of analysis, and simplified sample pretreatment. Therefore, some alternatives presented in the literature (*5*−*16*) resort to flow techniques with enzymatic reaction. Most of them use commercial glutamate oxidase enzymes (*7*, *8*, *10*, *13*, *14*), glutamate dehydrogenase (*9*, *11*, *12*, *15*), or glutamate decarboxylase (*15*, *16*) immobilized as columns (*5*, *7*, *10*−*12*, *16*) or at a surface of electrodes (*8*, *9*, *14*).

In a previous work (*16*), it was evidenced that the extraction of glutamate decarboxylase from natural products (fruits) provides a low cost approach for the development of enzymatic reactors. However, the preparation of enzymatic reactors based on enzyme extracts was revealed to be a cumbersome and laborious procedure. *Cucurbita maxima* (pumpkin) constitutes a natural source of L-glutamate decarboxylase (GAD), the enzyme that catalyses the reaction

$$\text{L-glutamate} \xleftrightarrow[\text{PLP}]{\text{GAD}} \gamma\text{-aminobutirate} + CO_2$$

and has the advantages of reduced cost and elevated quantity. These aspects are made evident in this work by describing the preparation of an enzymatic reactor with the enzyme naturally immobilized in pieces of the outer layer and its incorporation into a continuous flow system coupled to a potentiometric detector. The system implemented was used in the analysis of different commercial food products.

EXPERIMENTAL PROCEDURES

Reagents and Solutions. In the preparation of different solutions, distilled and deionized water were used together with analytical grade reagents without any additional purification. To prepare a 0.1 mol L^{-1} phosphate buffer solution, 20.6 g of sodium hydrogen phosphate was weighed and dissolved in 500 mL of water. To this, a 0.1 mol L^{-1} sodium dihydrogen phosphate solution was added until a pH of 5.5 at 25 °C was attained. Finally, pyridoxal phosphate (PLP) (Sigma, ref 9255) was added as a mediator of the enzymatic reaction to attain the final concentration of 10^{-4} mol L^{-1} in solution. The buffer solution described was used both in evaluation tests of enzymatic activity and

* To whom correspondence should be addressed. Tel: +055 081 32718711. Fax: + 055 081 32747278. E-mail: vlins@ufpe.br.
† Laboratory of Immunopathology Keizo Asami (LIKA).
‡ REQUIMTE.
§ Environmental and Quality Engineering Laboratory.

10.1021/jf0344791 CCC: $25.00
Published on Web 10/25/2003

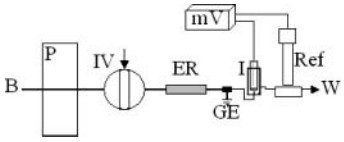

Figure 1. Schematic representation of the implemented flow injection system. B, carrier solution; P, peristaltic pump; IV, proportional injector; ER, enzymatic reactor; GE, grounding electrode; I, indicator electrode; Ref, reference electrode; W, waste; and mV, decimilivoltimeter.

as a carrier in the flow system. Pyridine, phosphate, and acetate were used as alternative buffers.

In the preparation of 0.10 mol L^{-1} sodium L-glutamate solution, 1.45 g (Sigma, ref G1626) of the product was weighed out and dissolved in 50 mL of buffer solution. The more diluted L-glutamate solutions were prepared daily from the stock solution by rigorous dilution with the buffer previously described.

The outer layer from *C. maxima* was cut in to 2 mm pieces and was used to prepare the enzymatic column (8 cm × 0.5 cm). The enzymatic column was stored in a refrigerator at 4 °C until it was used.

To carry out the determinations in food samples, 5.0 g of different products, namely, meat softener, meat soup, and spice, was dissolved at room temperatue (25 °C) in 50 mL of distilled water and injected directly without any further pretreatment.

Apparatus. The flow system (**Figure 1**) used an Ismatec peristaltic pump (model IPC-8) to impel the different solutions, equipped with Tygon (0.8 mm i.d.) propulsion tubes of the same brand, replaced weekly. A two way acrylic injector was also used in the manifold (*17*). The analytical signals were obtained by means of a Radelkis-OP-208/1 decimilivoltimeter equipped with a Radelkis OP-9363 carbon dioxide gas sensor and a Radelkis OP-0830P calomel type as a reference electrode. To register the analytical signals, a single pen Graphic 1000 (Lloyd Instruments) recorder was used. A Femto 435 spectrophotometer was employed to assess L-glutamate levels in the food samples by the conventional procedure.

Methods. *Preparation of the Enzymatic Reactor*. For the preparation of the enzymatic reactor, a polyethylene column with a length equal to 8.0 cm (0.5 cm i.d.) was filled with square pieces with 2 mm sides of the outer layer of *C. maxima*. For this, a piece of the fruit was first cut and abundantly rinsed with distilled water. Thereafter, the outer layer was sliced in square pieces with 2 mm sides and 1 mm thick and then washed with phosphate buffer solution. An amount of 200 mg of these pieces was used for reactor preparation. The enzymatic columns were stored at 4 °C in the refrigerator between utilizations.

Flow and Conventional Procedures. A solution of L-glutamate (10−100 mmol L^{-1}) was injected into the system (**Figure 1**) and carried by a phosphate buffer stream (pH 5.5, 0.1 mol L^{-1}) at the flow rate of 1.4 mL min^{-1} through the column packed with the naturally immobilized enzyme. On these conditions, L-glutamate was converted into carbon dioxide and γ-aminobutyric acid. A gas electrode enabled us to detect the concentration of carbon dioxide formed; it was the response proportional to the logarithm of L-glutamate concentration in the sample. The flow system implemented and optimized was applied to the analysis of food products, and the results obtained were compared with those provided by the application of the conventional method to the same samples. This method consisted of the spectrophotometric determination of L-glutamate at the wavelength of 494 nm, based on the use of immobilized L-glutamate dehydrogenase from a commercial kit for food analysis (*19*).

RESULTS AND DISCUSSION

Flow System Optimization. First, the effect of different carrier buffers, namely, pyridine, phosphate, and acetate, on the activity of the immobilized column was carried out by injecting 60 μL of a 50 mmol L^{-1} glutamate solution into the system at a fixed flow rate of 2 mL min^{-1}. The best performance for the enzyme was obtained by using phosphate buffer (0.1 mol L^{-1}, pH 5.5) for which a maximum relative response (100%) was obtained when compared with those given by the other buffers solutions. The rate of enzymatic reaction was lower in acetate (40%) than in pyridine buffer solution (90%). In fact, after some trials were performed with crude enzyme preparations, it was shown (*18*) that the inhibitory effect of acetate arises from competitive inhibition between L-glutamate and acetate by the active site of the enzyme. Different pH values were also tested for the above-mentioned buffer solutions. The maximum response was obtained at pH 5.5, where most of the γ-carboxyl is present as carboxylate ion. This result confirmed previous observations (*19*) in which amounts of 36 and 96% of carboxylate ion were found at pH values of 4.0 and 5.6, respectively. Using the same flow conditions, the response of the system incorporating different enzymatic reactors was evaluated. The results obtained show that an increase of the column length up to 8 cm corresponded to an increase of the analytical signals obtained as a consequence of the increase of the contact time between the substrate and the immobilized enzyme. Nevertheless, the use of reactors higher than 8 cm determined the decrease in intensity of the analytical signal due to the overlapping effect of sample dilution in the interior of the reactor. Similar results were obtained by some authors (*20*) when a 10 cm length glass column with L-glutamate oxidase immobilized in cellulose was used.

The effect of injection volume on the system response was assessed by varying it in the range of 20−100 μL. After the column length settled at 8 cm and the flow rate settled at 1.8 mL min^{-1}, several calibrating solutions contained L-glutamic acid concentrations up to 60.0 mmol L^{-1}; it was observed that although there was an increase in substraction amount available for reaction, the intercalation of volumes greater than 50 μL determined an insufficient dispersion of the carrier in the sample segment and consequently a deviation from the optimum relative to conditions of pH and enzymatic mediator available.

The effect of the flow rate was also evaluated in the interval between 0.6 and 2.5 mL min^{-1}. The obtained results show that the increase of this parameter favors the sampling rate. However, a significant increase of sample dispersion was observed for values higher than 1.5 mL min^{-1}. Hence, a flow rate of 1.4 mL min^{-1} was chosen as a compromise between sensitivity and sampling rate. The optimized system enabled the attainment of a linear response of potential as a function of logarithm of L-glutamate concentration in the interval of 10−100 mmol L^{-1}, corresponding to the calibration line with the equation of signal (mV) = 104.4 (±0.5) + 44.8 (±0.3) × log[L-glutamate (mol L^{-1})] (r = 0.998). The analytical frequency of 40 samples h^{-1} was also obtained.

Kinetic Enzyme Characterization. Using the optimized flow system, a double reciprocal plot of activity vs substract concentration was carried out to determine the apparent Michaelis Menten constant (K_M) and the maximum enzymatic rate (V_{max}) (**Figure 2**). The values obtained for these two parameters were 1.83 × 10^4 μmol min^{-1} mg^{-1} of protein and 6.25 mmol L^{-1}, respectively. When compared with other published K_m values (*21*−*26*) and not considering that this was evaluated under flow conditions, in this work, it could be concluded that only Osuji (*24*) reported a higher value (7.7 mmol L^{-1}) for the enzyme extracted from *Arachis hypogaea* (peanuts). This result indicates lower affinity of the enzyme for the considered substrate. Relative to the V_{max} found, it could be concluded that it is 100 times higher than that reported for glutamate decarboxylase extracted from *Escherichia coli* (*21*).

Application to Analysis of Real Samples. The developed flow procedure was applied to the analysis of food products, and the results obtained were compared with those furnished

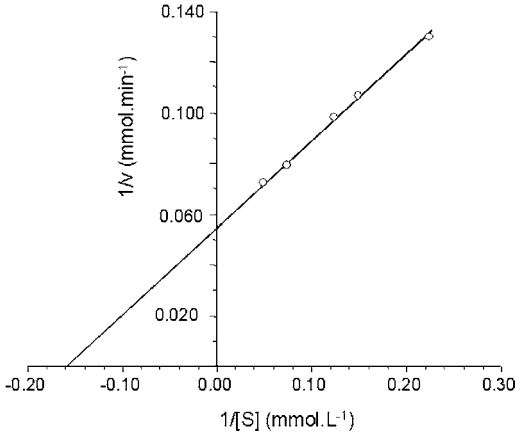

Figure 2. Lineweaver–Burk plot obtained for the reactor containing L-glutamate decarboxylase from *Cucurbita maxima* outer layer. Sample injection volume, 50 μL; carrier solution, phosphate buffer solution (pH 5.5, 0.1 mol L^{-1}); and flow rate, 1.40 mL min^{-1}.

Table 1. Results Obtained after Triplicate Analysis of Food Samples by the Conventional Procedure and by the Proposed Procedure

	results (mg g^{-1})		
sample	spectrophotometry	proposed procedure	relative error (%)
meat softener	4.95 ± 0.32	4.71 ± 0.23	4.85
meat soup	34.3 ± 0.59	32.76 ± 0.60	4.49
spices	90.0 ± 0.44	87.22 ± 0.62	3.09

from the application of the conventional method to the same samples. As can be observed (**Table 1**), deviations of results lower than 5% were verified. Moreover, the evaluation of the precision of results obtained showed that the implemented procedure has a precision comparable to that of the conventional procedure. Besides the good results obtained comparatively to the conventional method usually used, it was verified that it was possible to use the same enzymatic column for 21 days or to perform about 200 determinations without significant change of determination sensitivity (**Figure 2**). To evaluate the selectivity of the proposed procedure, recovery trials were performed using 50 mmol L^{-1} of L-glutamate solutions containing potential interfering species in the same concentration. It was verified that the presence of ascorbic acid, asparagine, aspartic acid, citric acid, glycine, and lysine did not interfere in the determination of L-glutamate once 100 ± 1% recovery values were obtained. However, the presence of oxalic acid produced an increase of the analytical signals obtained of about 26%.

CONCLUSIONS

The results obtained in this work show that the automated procedure implemented using a naturally immobilized L-glutamate decarboxilase from *C. maxima* could be considered advantageous for simple and low cost determinations of L-glutamate in food products. The implemented procedure uses a natural enzyme as a process of selective enzymatic determination of L-glutamate avoiding the use of tedious sample pretreatments. When compared with other proposed enzymatic procedures using chemical immobilization, this method presents the advantage of using a natural product with comparable enzymatic activity.

LITERATURE CITED

(1) Anderson, L. W.; Zaharevitz, D. W.; Strong, J. M. Glutamine and glutamate: automated quantification and isotopic enrichments by gas chromatography/mass spectrometry. *Anal. Biochem.* **1987**, *163* (2), 358−368.

(2) Lee, K. H.; Ishikawa, T.; McNiven, S. J.; Nomura, Y.; Hiratsuka, A.; Sasaki, S.; Arikawa, Y.; Karube, I. Evaluation of chemical oxygen demand (COD) based on coulometric determination of electrochemical oxygen demand (EOD) using a surface oxidized copper electrode. *Anal. Chim. Acta* **1999**, *398*, 161−171.

(3) Fonda, M. L. Glutamate decarboxylase from bacteria. *Methods Enzymol.* **1985**, *113*, 11−16.

(4) Chapman, J.; Zhou, M. Microplate-based fluorometric methods for the enzymatic determination of L-glutamate: application in measuring L-glutamate in food sample. *Anal. Chim. Acta* **1999**, *402*, 47−52.

(5) Puchades, R.; Lemieux, L.; Simard, R. E. Sensitive rapid and precise determination of L-glutamic acid in cheese using a flow-injection system with immobilized enzyme column. *J. Food Sci.* **1987**, *54*, 423−427.

(6) Kusakabe, Y. M.; Fujishima, T. Methods for determining L-glutamate in soy sauce with L-glutamate oxidase. *Agric. Biol. Chem.* **1984**, *48*, 181−184.

(7) Yao, T.; Kobayashi, N.; Wasa, T. Flow-injection analysis for L-glutamate using immobilized L-glutamate oxidase: comparison of an enzyme reactor and enzyme electrode. *Anal. Chim. Acta* **1990**, *231*, 251−121.

(8) Mizutani, F.; Sato, Y.; Hirata, Y.; Yabuki, S. High-throughput flow-injection analysis of glucose and glutamate in food and biological samples by using enzyme/polyion complex-bilayer membrane-based electrodes as detectors. *Biosens. Bioelectron.* **1998**, *13*, 809−815.

(9) Villarta, R. V.; Cunningham, D. D.; Guilbault, G. G. Amperometric enzyme electrodes for the determination of L-glutamate. *Talanta* **1991**, *38* (1), 49−55.

(10) Matsumoto, K.; Asada, W.; Murai, R. Simultaneous biosensing of inosine monophosphate and glutamate by use of immobilized enzyme reactors. *Anal. Chim. Acta* **1998**, *358*, 127−136.

(11) Stalikas, C. D.; Karayannis, M. I.; Tzouwara-Karayanni, S. Immobilization of glutamate dehydrogenase on glass derivatives. A method for the assay of glutamates in real samples with simplex optimized automated FIA-system. *Talanta* **1994**, *41*, 1561−1567.

(12) Matsumoto, K.; Sakoda, K.; Osajima, Y. Determination of L-glutamate by amperometric flow-injection analysis using immobilized glutamate oxidase: manifold for simultaneous detection of component signal and blank signal. *Anal. Chim. Acta* **1992**, *261* (1−2), 155−159.

(13) Dremel, B. A. A.; Schmid, R. D.; Wolfbeis, O. S. Comparison of two fibre-optic L-glutamate biosensors based on the detection of oxygen or carbon dioxide, and their application in combination with flow-injection analysis to determination of glutamate. *Anal. Chim. Acta* **1991**, *248*, 351−359.

(14) Male, F. B.; Luong, J. H. T.; Mulchandani, A. Determination of aspartame in dietary food products by a FIA biosensor. *Biosens. Bioelectron.* **1991**, *6*, 117−123.

(15) Shi, R.; Stein, K. Flow injection methods for determination of L-glutamate using glutamate decarboxylase and glutamate dehydrogenase reactors with spectrophotometric detection. *Analyst* **1996**, *121*, 1305−1309.

(16) Oliveira, M. I. P.; Pimentel, M. C.; Montenegro, M. C. B. S. M.; Araújo, A. N.; Pimentel, M. F.; Silva, V. L. L-Glutamate determination in food samples by flow-injection analysis. *Anal. Chim. Acta* **2001**, *448*, 207−213.

(17) Bergamin, F. H.; Reis, B. F.; Jacintho, A. O.; Zagatto, E. A. G. Ion exchange in flow injection analysis: Determination of Ammonium Ions at the μg l^{-1} level in Natural Waters with Pulsed Nessler Reagent. *Anal. Chim. Acta* **1980**, *117*, 81−89.

(18) Shukuya, R.; Schwert, G. W. *J. Biol. Chem.* **1960**, *235*, 1658−1661.

(19) Najjarin, V. Determination of Amino Acids by Specific Bacterial Decarboxylases in the Waburg Apparatus. In *Methods in Enzymology (III)*; Colowick, S., Kaplan, N., Eds; Academic Press: NY, 1957; p 4621.

(20) Matsumoto, K.; Ritchie, L.; Chen, C. Sequential enzymatic monitoring of glucose, ethanol and glutamate in bioreactor fermentation broth containing a high salt concentration by a multichannel flow-injection analysis method. *Anal. Chim. Acta* **1992**, *308*, 145−151.

(21) Chu, W. C.; Metzler, D. E. Enzymatically active truncated cat brain glutamate-decarboxylase-expression, purification and absorption-spectrum. *Arch. Biochem. Biophys.* **1994**, *313*, 287−295.

(22) Satyanarayan, V. S.; Madhusudanan, N. Purification and characterization of glutamate decarboxylase from *Solanum tuberosum*. *Eur. J. Biochem*. **1985**, *150*, 53−60.

(23) Nomura, M.; Nakajima, I.; Fujita, Y.; Kobayashi, M.; Kimoto, H.; Sukuki, I.; Aso, H. *Lactococcus latis* contains only one glutamate decarboxylase gene. *Microbiology* **1999**, *145*, 1375−1380.

(24) Osuj, I. G. Peanut glutamate dehydrogenase: a target site of herbicide action (reprinted from plant nutrition for sustainable food production and environment). *Soil Sci. Plant Nutr*. **1997**, *43*, 1159−1164.

(25) Haor, R.; Schmit, J. C. Purification and characterization of glutamate decarboxylase from *Neurospora-crassa conidia*. *J. Biol. Chem.* **1991**, *266*, 5135−5139.

(26) Michalik, M.; Nelson, J.; Erecinska, M. BABA production in rat islets of langerhans. *Diabetes* **1993**, *42*, 1506−1513.

Received for review May 8, 2003. Revised manuscript received September 11, 2003. Accepted September 22, 2003. We thank CAPES/GRICES Proc. 4.1.3, CNPq, and FACEPE for their financial support.

JF0344791