Anal. Chem. **1997,** *69,* 3471−3475

LC−Biosensor System for the Determination of the Neurotoxin *β*-*N*-Oxalyl-L-α,*β*-diaminopropionic Acid

Abebaw Belay,[†] Tautgirdas Ruzgas,[‡] Elisabeth Csöregi,[§] Ghirma Moges,[†] Merid Tessema,[†] Theodros Solomon,[†] and Lo Gorton*,[§]

Department of Chemistry, Addis Ababa University, P.O. Box 1176, Addis Ababa, Ethiopia, Institute of Biochemistry, Mokslininku 12, 2600 Vilnius, Lithuania, and Department of Analytical Chemistry, University of Lund, P.O. Box 124, S-221 00, Lund, Sweden

An analysis system is described for the determination of the neurotoxin *β*-*N*-oxalyl-L-α,*β*-diaminopropionic acid (*β*-ODAP). The system is based on liquid chromatographic separation of *β*-ODAP from interfering amino acids on an anion exchange column coupled with an amperometric enzyme electrode for the registration of *β*-ODAP. The electrode is based on a graphite rod modified with an $Os^{2+/3+}$ redox polymer cross-linked with L-glutamate oxidase and horseradish peroxidase. This LC−bienzyme electrode analytical system enabled monitoring of as low as 4 μM *β*-ODAP (injection volume 100 μL). The *β*-ODAP content in real grass pea samples was measured to range between 3.3 and 5.2 g kg^{-1} in dry grass pea.

Lathyrus sativus (LS; grass pea, also called *guaya* in Amharic and *khesari* in India and Bangladesh) is a popular drought- and flood-tolerant crop and foodstuff in drought-prone areas of Africa and Asia. Seeds of LS provide a nourishing diet of high-quality protein and carbohydrates and may be the only food available during periods of famine. The protein content of this edible seed is about 26−30%. However, consumption of seeds of LS for more than three months as a staple diet can cause a lower motor neuron degenerative disease known as lathyrism or neurolathyrism (irreversible paralysis of the lower limbs).[1] The disease reaches epidemic proportions sometimes following drought and flood-triggered famine in those areas.[2] The toxin responsible for human lathyrism, identified 30 years ago, is the nonessential amino acid *β*-*N*-oxalyl-L-α,*β*-diaminopropionic acid (*β*-ODAP).[3,4] The level of this compound in the dry seed varies widely depending on genetic factors and environmental conditions. Analysis of a large number of samples of LS collected from an endemic area of India showed that the *β*-ODAP content in the dry seeds ranged from 1 to 25 g kg^{-1}.[5] A similar study in northwest Ethiopia showed that the toxin levels of whole seed samples collected from the study areas ranged from 2 to 8 g kg^{-1}.[6]

Intensive research for developing low or zero toxin varieties of LS seeds is going on in several institutes focusing on agricultural reserves. The most obvious option to achieve this goal entails plant breeding and postharvest processing. This involves processing of a large number of samples, requiring a fast and selective method for monitoring the neurotoxin. Different nonenzymatic methods have been reported, but all of them were found to be experimentally inconvenient and nonselective between the nontoxic α-ODAP and its toxic isomer, *β*-ODAP.[7−10] Naturally in the seed the nontoxic α-ODAP coexists with the toxic isomer to the extent of ∼5%. *β*-ODAP, however, undergoes a slow transformation to the α-isomer in water at room temperature. The interconversion is facilitated at elevated temperatures. The reported equilibrium concentrations of the α- and the *β*-isomers are between 60−65 and 40−35%, respectively.[11−14] Thus, there is clearly an evident need of a *β*-selective method of assay.

Recently, a selective enzymatic method for the determination of *β*-ODAP using an immobilized L-glutamate oxidase (GLOx, from *Streptomyces* sp.) reactor was reported.[13] The described flow injection-GLOx reactor analysis system is based on the monitoring of a colorimetric reaction where the product of the enzymatic oxidation of *β*-ODAP, hydrogen peroxide, is reduced in a second enzyme step using immobilized horseradish peroxidase (HRP) and a color-forming reagent. The GLOx reactor, however, displays some limitations when real samples of grass pea are analyzed. These include the following: a decrease in the response of the analytical reactor system to ∼30% after ∼50 injections of crude extracts of LS[15] and the GLOx reactor is also prone to interference from L-glutamate, the major substrate of GLOx. Previously it was shown that ∼34% of the free amino acids in grass pea seed meals of LS might consist of L-glutamate and L-aspartate and their content can be increased up to 72% during seed meal fermentation.[16] It was demonstrated that a small GLOx prereactor could be used to oxidize L-glutamate before entering a second GLOx reactor where the toxin is oxidized for its analysis.[13]

[†] Addis Ababa University.
[‡] Institute of Biochemistry.
[§] University of Lund.

(1) Roa, S. N. L.; Malathi, K.; Sarma, P. S. In *World Review of Nutrition and Dietetics*; Bourne, G. H., Ed.; Karger AG: Basel, 1969; Vol. 10, p 214.
(2) Haimanot, R. T.; Kidane, Y.; Wuhib, E.; Kalissa, A.; Alenu, T.; Zein, Z. A.; Spencer, P. S. *Int. J. Epidemiol.* **1990**, *19*, 664.
(3) Murti, V. V. S.; Seshadri, T. R.; Venkitasubramanian, T. A. *Phytochemistry* **1964**, *3*, 73.
(4) Rao, S. L. N.; Adiga, P. R.; Sarma, P. S. *Biochemistry* **1964**, *3*, 432.
(5) Roy, D. N.; Narasinga, B. S. *Curr. Sci.* **1968**, *37*, 395.
(6) Haimanot, R. T.; Abegaz, B. M.; Wuhib, E.; Kassina, A.; Kidane, Y.; Kebede, N.; Alemu, T.; Spencer, P. S. *Nutr. Res.* **1993**, *13*, 1113.
(7) Rao, S. L. N. *Anal. Biochem.* **1978**, *14*, 387.
(8) Kisby, G. E.; Roy, D. N.; Spencer, P. S. *J. Neursci. Methods* **1988**, *26*, 45.
(9) Khan, J. K.; Kebede, N.; Kuo, Y. H.; Lambein, F.; De Bruyn, A. *Anal. Biochem.* **1993**, *208*, 237.
(10) Geda, A.; Briggs, C. J.; Venkataram, S. *J. Chromatogr.* **1993**, *635*, 338.
(11) Bell, E. A.; O'Donovan, J. P. *Phytochemistry* **1966**, *15*, 1211.
(12) Abegaz, B. M.; Nunn, P. B.; De Bruyn, A.; Lambein, F. *Phytochemistry* **1993**, *33*, 112.
(13) Moges, G.; Johansson, G. *Anal. Chem.* **1994**, *66*, 3834.
(14) Belay, A.; Moges, G.; Solomon, T.; Johansson, G. *Anal. Lett.*, in press.
(15) Belay, A. M.Sc. Thesis, Addis Ababa University, Addis Ababa, Ethiopia, 1995.
(16) Kuo, Y.-H.; Bau, H.-M.; Quemener, B.; Khan, J. K.; Lambein, F. *J. Sci. Food Agric.* **1995**, *69*, 81.

S0003-2700(96)01098-0 CCC: $14.00

Combination of these two reactors actually requires additional calibration-like procedures to prove required conversions of L-glutamate and β-ODAP, resulting in a complicated analysis procedure.

In the present work, an application of a bienzyme-modified electrode for the selective determination of the neurotoxic β-ODAP is demonstrated for the first time. The solid graphite electrode was modified with a redox hydrogel layer containing immobilized GLOx and HRP. This electrode was used as a postcolumn amperometric detector. The major interfering amino acids (L-glutamate and L-aspartate) were fully baseline separated from β-ODAP using an anion exchange column. By the bienzyme electrode design, combining a hydrogen peroxide-producing oxidase and peroxidase, the detection of the analyte could be done at a working potential of −50 mV vs Ag/AgCl, thus circumventing the often reported problems, e.g., unstable response and biases from easily oxidizable species, associated with direct electrochemical oxidation of hydrogen peroxide at a Pt electrode necessitating much higher potentials (>+600 mV vs Ag/AgCl). This fact has also recently been recognized by specialist manufacturers by marketing the first redox hydrogel-based hydrogen peroxide-based electrode for use in HPLC and flow injection applications.[17]

EXPERIMENTAL SECTION

Chemicals and Reagents. L-Glutamate oxidase from *Streptomyces* sp. (EC 1.4.3.11, 6.8 units mg^{-1}, Yamasa Corp., Tokyo, Japan, Catalog No. 7804) and horseradish peroxidase (EC 1.11.1.7, 1000 units mg^{-1}, Boehringer-Mannheim, Mannheim, Germany, Catalog No. 814407) were purchased as lyophilized powders. Poly(1-vinylimidazole){osmium (4,4′-dimethylbpy)$_2$Cl}$^{2+/+}$ redox polymer was synthesized according to a previously published protocol.[18] Elemental analysis exhibited the presence of one Os redox center at every seventh vinylimidazole unit in the redox polymer denoted as PVI$_7$-dme-Os. Poly(ethylene glycol) (400) diglycidyl ether (PEGDGE; Polysciences, Warrington, PA, Catalog No. 08210) was used to cross-link the enzymes with the redox polymer. L-Glutamic acid (Catalog No. G-6904), β-ODAP monohydrate (Catalog No. O-5382), L-aspartic acid (Catalog No. A-9256), and bovine serum albumin (BSA, Catalog No. A-6003), all from Sigma (St. Louis, MO), were used as received. The solutions were prepared using HPLC-grade water produced in a Milli-Q system (Millipore, Bedford, MA). All experiments were carried out at room temperature.

Instrumentation. The LC system used in this work consisted of an LC pump (Model 600, Waters, Millipore, Milford, MA), an automated switching valve (Model P/N 60057, Waters), and a Carbopac anion exchange column (PA1, 4 × 250 mm i.d., Model P/N 35391, Dionex, Sunnyvale, CA). The effluent of the column was passed through either a refractive index detector (Model 2142, LKB, Bromma, Sweden) or a flow-through amperometric cell of the well-jet type[19] containing a Pt wire, Ag/AgCl (0.1 M KCl), and a graphite rod modified with enzyme-redox hydrogel layer as the counter, the reference, and the working electrodes, respectively. The three-electrode cell was connected to a potentiostat (Zäta-Elektronik, Lund, Sweden) and operated at a potential of −50 mV vs Ag/AgCl. The output of the potentiostat was recorded on an *X*−*Y* recorder (W+W Electronics, Lausanne, Switzerland, Model 1107). The mobile phase (0.1 M phosphate buffer, pH 7.0) was filtered through a 0.45 μm filter (Millipore, S. A., Molshem, France, type HA) and degassed with helium prior to use.

Preparation of the Bienzyme-Modified Electrode. A graphite rod (type RW 001, Ringdorff-Werke, GmbH, Bonn, Germany, o.d. 3.05 mm) was polished on wet fine emery paper (Tufback, Durite, P1200) using an in-house-made polishing machine and then washed thoroughly with deionized water. After drying at room temperature, a premixed solution consisting of 1 μL of HRP (10 mg mL^{-1} in 0.1 M phosphate buffer, pH 7.0), 0.8 μL of PVI$_7$-dme-Os (10 mg mL^{-1} in water), 0.4 μL of a freshly prepared PEGDGE solution (5 mg mL^{-1}), and 2 μL of GLOx (10 mg mL^{-1} in 0.1 M phosphate buffer, pH 7.0) was placed on the electrode surface. To accelerate the cross-linking reaction, the electrode was exposed under thermostated conditions at 50 °C for 10 min. Before the bienzyme electrode was inserted into the electrochemical cell, it was kept in a desiccator at room temperature. The reported results are the mean of three equally prepared electrodes.

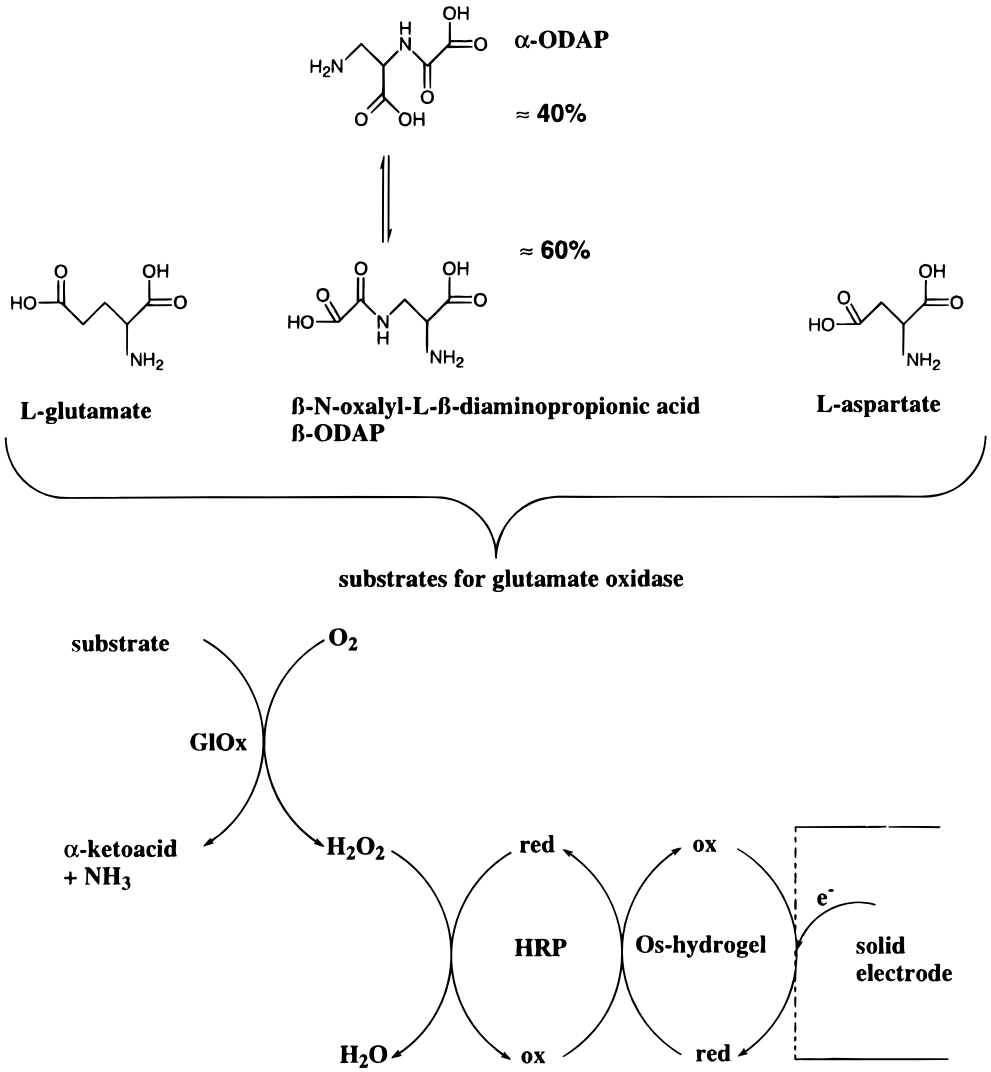
Extraction of β-ODAP from Real Samples. β-ODAP was extracted from 100 mg of grass pea powder in 10 mL of 0.1 M phosphate buffer (pH 7.0) at room temperature. The extraction was effected by rotating the sample containing test tubes at 20 rpm in a rotating oven for 2 h.[13] Particles were removed by centrifugation and filtration through a 0.45 μm filter (Millipore). Proteins were separated from the sample by an ultrafiltration membrane (Amicon, Beverly, MA, molecular cutoff 10 000) using centrifugation at 4000 rpm.

RESULTS AND DISCUSSION

β-ODAP is oxidized by GLOx in the presence of molecular oxygen. The enzymatic oxidation of the neurotoxin was suggested to be a typical amino acid oxidase-catalyzed reaction resulting in hydrogen peroxide, ammonia, and an α-keto acid.[13] Either of the first two products could be subsequently used for a qualitative and quantitative assay of the toxin. Efficient detection of H_2O_2 is possible using electrodes modified with horseradish peroxidase through a mediatorless or mediated reaction.[20] A redox hydrogel-based electrode was, however, considered as an interesting design since it allows immobilization of a high amount of enzyme within the sensing layer and through the action of the redox mediator speeds up the electron transfer between the electrode and the oxidized forms of peroxidase. Both features are of special importance for the design of a biosensor for β-ODAP since the activity of GLOx for this substrate was previously reported to be very low, being equal to only 0.8% compared with that of its main substrate, L-glutamate.[21] The reaction sequence at the bienzyme electrode for the determination of β-ODAP together with the chemical structures of the main substrates of GLOx is presented in Scheme 1. The composition of the sensing layer (ratio of GLOx/HRP/Os-polymer/PEGDGE) for optimal sensing of L-glutamate was the subject of an independent study.[22] Because of the low activity of GLOx for β-ODAP, the main priority was here ascribed to the immobilization of a high amount of this enzyme without severely reducing the response of the sensor for H_2O_2

(17) Peroxidase Electrode Kit. Bioanalytical Systems, Inc., West Lafayette, IN (BAS, Catalog No. MF-9079).
(18) Ohara, T. J.; Rajagopalan, R.; Heller, A. *Anal. Chem.* **1994**, *66*, 2451.
(19) Appelqvist, R.; Marko-Varga, G.; Gorton, L.; Torstensson, A.; Johansson, G. *Anal. Chim. Acta* **1985**, *169*, 237.
(20) Ruzgas, T.; Csöregi, E.; Emnéus, J.; Gorton, L.; Marko-Varga, G. *Anal. Chim. Acta* **1996**, *330*, 123.
(21) Moges, G.; Solomon, T.; Johansson, G. *Anal. Lett.* **1994**, *27*, 2207.
(22) Belay, A.; Csöregi, E.; Ruzgas, T.; Kissinger, P.; Gorton, L., manuscript in preparation.

Scheme 1. Structures of the Substrates for L-Glutamate Oxidase and Reaction Sequence at the L-Glutamate Oxidase/Horseradish Peroxidase/Redox Hydrogel-Modified Electrode



and decreasing the stability of the hydrogel layer. The following amounts, 20, 10, 8, and 2 μg of GLOx, HRP, PVI$_7$-dme-Os, and PEGDGE, respectively, were placed and cured on the top of the solid graphite rod. Investigations of such electrodes showed that continuous measurement of L-glutamate in a flow injection system under the following conditions, sample concentration 50 μM, injection volume 50 μL, injection frequency 30 h^{-1}, and flow rate of the carrier 0.4 mL min^{-1}, only revealed less than 4% decrease in sensitivity after 8 h.[22] Calibration of this bienzyme electrode at two different flow rates and the dependence of the response on the flow rate in the flow injection mode (omitting the separation column from the system) are presented in Figure 1. It can be seen that the response increased with a decrease in the rate of the flow carrier. This was anticipated based on the enzyme kinetics limiting the response behavior of the electrodes, as a result of the low activity of GLOx for β-ODAP. Unfortunately, the calibrations graphs for β-ODAP are nonlinear at both low and high flow rates. The sensor responded, though, linearly for L-glutamate in the concentration range between 1 and 200 μM. Presentation of the calibration plots for β-ODAP in Lineweaver−Burk and Eadie−Hofstee coordinates resulted also in nonlinear dependencies pointing to more complex limiting factors for the sensor response than simple Michaelis−Menten enzyme kinetics. To improve the linearity of the response, further optimization of the composition of the sensing layer is needed. Despite the inconvenient nonlinearity of the calibration curve, a lower limit of detection of 4 μM β-ODAP was possible with this bienzyme electrode using the present flow analysis setup. However, the current detection limit obtained in the present work is more than twice an improvement when compared with 10 μM β-ODAP for the previously reported analytical system based on the GLOx reactor.[13,15]

The selectivity of the GLOx/HRP/redox hydrogel electrode was tested by comparing the sensor response for 20 μM L-glutamate, β-ODAP, and L-aspartate at a flow rate of 0.4 mL min^{-1}. Peak currents of 104, 28, and 0 nA were recorded, respectively. There was no response for L-aspartate even at a concentration of 50 μM. The response for 100 μM L-aspartate was only 40% of that for 10 μM β-ODAP. This might indicate that the binding of β-ODAP to the enzyme is favored or the bimolecular constant for β-ODAP oxidation is higher compared with similar characteristics for L-aspartate, and hence the activity of GLOx for L-aspartate is 2.6 times lower than that for β-ODAP (0.3 and 0.8% of the activity for L-glutamate, respectively).[23] Since the electrode responded to three analytes, a selective detection of β-ODAP was thus impossible without using additional selectivity-enhancing tools.

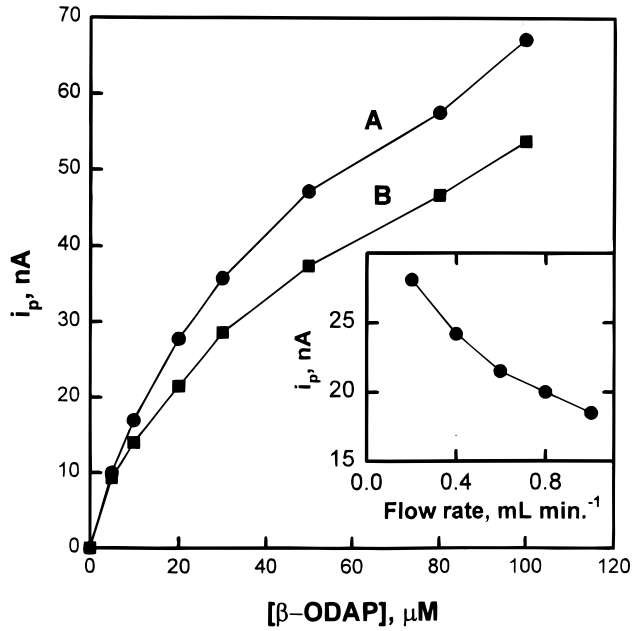


Figure 1. Dependence of the peak current on the concentration of β-ODAP. Graphite electrodes were modified with $Os^{3+/2+}$ redox hydrogel containing cross-linked L-glutamate oxidase and horseradish peroxidase. Conditions: applied potential −50 mV vs Ag/AgCl; injection volume 50 μL; flow carrier 0.1 M phosphate buffer (pH 7); flow rate (A) 0.2 and (B) 0.8 mL min^{-1}. Inset shows the response to 20 μM β-ODAP as a function of flow rate.

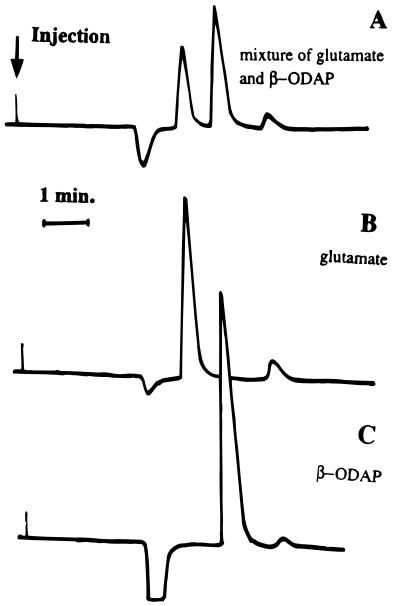


Figure 2. Chromatograms revealing separation of L-glutamate and β-ODAP using a Carbopac anion exchange column and refractive index detection. Conditions: perfusion rate 0.4 mL min^{-1}; injection volume 100 μL; mobile phase 0.1 M phosphate buffer (pH 7). Peaks correspond to (A) a mixture of 5 μM L-glutamate and 5 μM β-ODAP, (B) 10 μM L-glutamate, and (C) 10 μM β-ODAP.

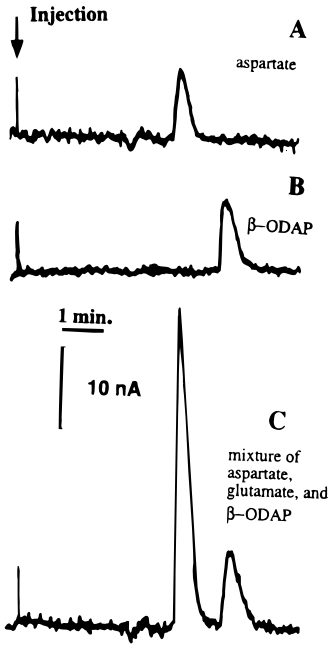


Figure 3. Chromatograms of L-aspartate, L-glutamate, and β-ODAP using a Carbopac anion exchange column and bienzyme electrode detection. Conditions: applied potential −50 mV vs Ag/AgCl; perfusion rate 0.4 mL min^{-1}; injection volume 100 μL; mobile phase 0.1 M phosphate buffer (pH 7). Peaks correspond to (A) 100 μM L-aspartate, (B) 20 μM β-ODAP, and (C) a mixture containing 5 μM L-glutamate, 100 μM L-aspartate, and 20 μM β-ODAP.

This was ensured by separating β-ODAP from L-glutamate and L-aspartate in a liquid chromatographic step and using the bienzyme electrode as a postcolumn amperometric detector.

The main criterion for selecting the chromatographic separation system was to obtain baseline separation of β-ODAP from L-glutamate and L-aspartate isocratically using only phosphate buffer (pH 7.0) as mobile phase since this electrolyte was compatible with the bienzyme electrode and used in the previously reported extraction method of β-ODAP from real samples.[21] An anion exchange column for the separation of the three amino acids was therefore chosen. Chromatograms resulting from separate injections of 10 μM L-glutamate, 10 μM β-ODAP, and a mixture containing of 5 μM of each are presented in Figure 2. For these experiments a flow rate of 0.4 mL min^{-1} and a refractive index detector were used. It can be seen that L-glutamate and β-ODAP could be well separated isocratically, L-glutamate being eluted first. In separate experiments, it was noted that L-aspartate coeluted with L-glutamate (not shown), resulting in elution of β-ODAP as a separate chromatographic peak free from interfering amino acids.

In the experiments described below, the detection unit in the LC system was replaced by the electrochemical flow-through cell containing the bienzyme electrode. A calibration graph for β-ODAP was recorded. Due to peak broadening caused by the LC column, the peak current decreased to ~30% of that obtained without the column; however, other features of the calibration curve were retained (i.e., nonlinear peak current−concentration dependence). This calibration curve (not shown) was used for the evaluation of the content of β-ODAP in test solutions and real samples. The present old-fashioned graphical interpolation used in this work for quantitation of β-ODAP is expected to be replaced by a linear regression method when using an improved bienzyme electrode configuration in which the turnover rate of GLOx for β-ODAP is increased (subject of a forthcoming publication). Different concentrations of L-aspartate, L-glutamate, and β-ODAP separately as well as mixtures thereof were injected into the analytical system. Examples of chromatograms obtained are presented in Figure 3. As stated above, L-aspartate was found to

(23) Moges, G. Ph.D. Thesis, Addis Ababa University, Addis Ababa, Ethiopia, 1994.

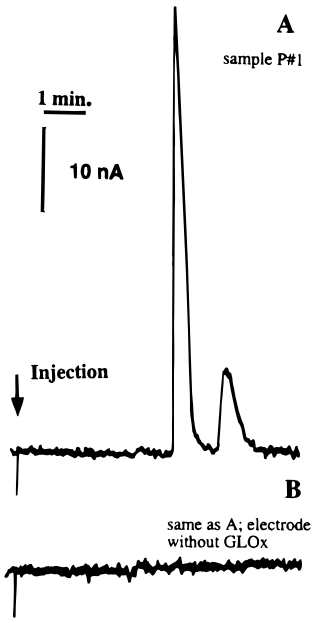


Figure 4. Chromatograms obtained by analyzing the extract of sample P#1. (A) Other conditions as in Figure 3. (B) Chromatogram for the same sample and analysis conditions except that L-glutamate oxidase is exchanged for BSA when the enzyme electrode is prepared.

coelute with L-glutamate, however, both being well baseline separated from β-ODAP. The analytical system showed a relative standard deviation of 8% ($n = 5$) for injections of the same 20 μM β-ODAP concentration.

Three real samples of grass pea obtained as a powder from the University of Addis Ababa (P#1, P#2, P#3) were processed to extract β-ODAP according to the method described in the Experimental Section. The final solutions were diluted 10 times and injected into the LC system. A chromatogram for one sample (P#1) is presented in Figure 4 including a record where the bienzyme electrode was replaced with one modified with BSA instead of GLOx. It can be seen that a sensor response was recorded only when GLOx was present in the redox hydrogel. The results of the measurements using real samples are summarized in Table 1. It can be noted that the analytical system exhibited the same response due to L-glutamate and L-aspartate present in all three samples. The content of β-ODAP, however, was different. Additionally, sample P#3 was further spiked with 30 μM β-ODAP and results showed a recovery of 98%. It can be mentioned that the content of β-ODAP calculated per kilogram of the samples is in the range of that previously reported when other methods of analysis were used.[23] Control injections of standard solutions of β-ODAP and L-glutamate after running real samples showed no statistical proofs for any changes in the response characteristics of the bienzyme electrode or separation performance. Single-sample analysis including chromatographic separation and postcolumn amperometric detection could be completed within 7 min.

Table 1. Content of β-ODAP Determined with the LC−Enzyme Electrode Analytical System[a]

sample	L-glutamate and L-aspartate, μM	β-ODAP, μM	β-ODAP, g/kg of dry seeds
P#1	10 ± 0.0	28.6 ± 0.0	4.9
P#2	10.4 ± 0.2	18.9 ± 0.0	3.3
P#3	9.9 ± 0.2	29.7 ± 0.4	5.2
spiked P#3	9.9 ± 0.2	59.0 ± 0.3	

[a] Number of repeated injections, 5. Other conditions for the analysis as stated in Figure 3.

CONCLUSION

An amperometric electrode containing a redox hydrogel layer with immobilized GLOx and HRP is proposed for the selective detection of low concentrations of the neurotoxin β-ODAP. Calibration curves for this analyte were, however, nonlinear. A detection limit of 4 μM β-ODAP was possible (calculated as three times the signal-to-noise ratio). The combination of liquid chromatography with enzyme electrochemical detection provides a selective detection of β-ODAP in real samples. Linearization of the calibration curves and validation of the method are the objectives for further development and characterization of the analytical LC−enzyme electrode setup.

ACKNOWLEDGMENT

This work was financially supported by the Swedish Natural Science Research Council (NFR), the Swedish Council for the Engineering Science (TFR), the Swedish Board for Industrial and Technical Development (NUTEK), and the Swedish International Development Cooperation Agency (SIDA). Prof. Jan Åke Jönsson and Mr. Nelson Torto, Dept. of Analytical Chemistry, Lund University, are appreciated for their valuable advice regarding the LC separation of β-ODAP from other amino acids and Prof. A. Heller, Dept. of Chemical Engineering, The University of Texas at Austin, TX, is appreciated for the valuable discussions regarding the redox hydrogel. The Swedish Institute and Prof. Peter Kissinger (Bioanalytical Systems, Inc., West Lafayette, IN) are acknowledged for financial support by A.B. and T.R., respectively.

Received for review October 28, 1996. Accepted June 16, 1997.[⊗]

AC961098W

[⊗] Abstract published in *Advance ACS Abstracts*, August 1, 1997.