Bienzyme Carbon Paste Electrodes for L-Glutamate Determination

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Amperometric bienzyme electrodes were constructed for the direct assay of L-glutamate in a flow injection system (F1) working at a potential of -50 mV vs. Ag/AgCl. Electrode characteristics were obtained and compared for carbon paste electrodes based on different peroxidases [horseradish peroxidase (HRP) or fungal peroxidase (ARP)], co-immobilized with L-glutamate oxidase (GlOx) using different immobilization techniques. Addition of polyethylenimine (PEI) into the sensing chemistry was found beneficial, enhancing the glutamate sensitivity of the sensor by 40%. The G1Ox-HRP-PEI bienzyme electrodes displayed superior biosensor characteristics than those based on ARP. Linear calibration curves between 8 and 800 µM, both for L-glutamate and hydrogen peroxide, were registered with a sensitivity for glutamate of 6 µA/mM and a detection limit of 5 µM (calculated as twice signal-to-noise ratio). The optimized glutamate biosensor was further used as a detection unit in a column liquid chromatographic system for the analysis of some blood plasma and urine samples.

L-glutamate is a significant analyte playing an important role in various areas, such as amino acid biosynthesis, bioprocess monitoring, the food industry, and in medicine. It is widely used as a flavor enhancer in various foodstuffs and seasonings (1), as well as being connected to the Chinese restaurant syndrome (2). It has been associated with several diseases; hence, it is used as a clinical marker for general and protein metabolism (3), and for nutritional status (4). Glutamate is a principal neurotransmitter in the mammalian brain (5,6) playing a vital role in understanding the dynamics of the energy balance of the brain and evaluation of selective neuronal injury following various damages to the nervous system (7,8). Its quantification was found to be useful for the diagnosis of some myocardial and hepatic diseases (9), for the pathology of neurological (10) and psychiatric disorders (11). It is also often used in clinical laboratories for the determination of aminotransferase activities (12).

Many different methods have already been proposed for L-glutamate determination, mainly: chromatographic techniques (13), kinetic potentiometry (14), or capillary electrophoresis with electrochemical detection (15). Since these methods involve complicated and time-consuming procedures, they are inadequate for rapid analysis of a large number of samples or for timely monitoring. In the past 10 years, attention was mainly focused on the use of amperometric enzyme electrodes, taking advantage of the inherent high substrate selectivity of the enzymes. Most often NAD^+ dependent glutamate dehydrogenase (16-24) and GlOx (6,12,25-52) have been employed for the determination of L-glutamate in different systems. However, glutamate dehydrogenase based systems require the addition of soluble NAD^+, complicating thus the sensor construction. This drawback can be eliminated by the use of GlOx.

Most of the GlOx based systems detect glutamate by direct electrochemical reduction/oxidation of the co-substrate (O_2) or the formed H_2O_2, both reactions requiring high overpotentials, thus, resulting in repressed electrochemical selectivity. Therefore, either a gas permeable or the combination of several sizes and charges excluding membranes is required, complicating the sensor design. Attempts to decrease the working potential for H_2O_2 detection were made modifying the electrode surface (12,25,33,44,45), incorporating various mediators into the sensor (32,34,40,41), or by using coupled GlOx-HRP systems (39,52).

In this work, amperometric glutamate biosensors are described, based on coupled glutamate oxidase and horseradish or fungal (*Arthromyces ramosus*) peroxidase, co-immobilized into carbon paste. The electrodes were operated at -50 mV *vs.* Ag/AgCl, the "optimal" working potential for a biosensor. At this potential, interfering signals are minimal from very few electrochemically-oxidizable compounds (often present in the complex samples: serum, blood, microdialysate, fermentation broths), and the system is characterized by low levels of noise and background currents. One should, however, keep in mind that chemical (*vs.* electrochemical) interferences remain a possibility since H_2O_2 can be reacted with many reducing agents. Sensors were prepared by different immobilization techniques, and were stabilized with polyethylenimine. Biosensor characteristics are compared for the various electrode configurations. The selectivity of the optimized biosensor was determined towards a broad range of various amino acids, and the biosensor was coupled to a liquid chromatographic system and used as an electrochemical detector for L-glutamate determination in urine and plasma.

Experimental

Chemicals

L-glutamate oxidase (**GlOx**, EC 1.4.3.11, lyophilized powder, from *Streptomyces* sp., activity 8 U mg^{-1}), kindly donated by Yamasa Shoyu Co., Chiba-ken, Japan (cat. no. 7804), horseradish peroxidase (**HRP**, EC 1.11.1.7, lyophilized powder, 288 U mg^{-1}), from Sigma Chem. Co., MO, USA (cat. no. P 8375), and fungal peroxidase (**ARP**, EC 1.11.1.7, lyophilized powder, from *Arthromyces ramosus*, activity 250 U mg^{-1}), a gift from Suntory Ltd., Osaka, Japan (lot no. 900511), were used as received. Glutaraldehyde (**GA**, cat. no. G 5882, 25% aqueous solution), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (**CDI**, cat. no. E-7750), and polyethylenimine (**PEI**, cat. no. P 3143, 50% aqueous solution) were provided from Sigma. The aqueous GA solution was purified in order to remove polymeric material by shaking with active carbon followed by centrifugation. The untreated GA was kept below -18° C before use. All amino acids were from Sigma; L-alanine (cat. no. A 7627), L-arginine (cat. no. A 5006), L-aspargine (cat. no. A 9256), L-glycine (cat. no. G 7126), L-histidine (cat. no. H 8000), L-isoleucine (cat. no. I 2752), L-leucine (cat. no. L 8000), L-lysine (cat. no. L 5501), L-methionine (cat. no. M 9625), L-phenylalanine (cat. no. P 2126), L-proline (cat. no. P 0380), L-serine (cat. no. S 4500), L-threonine (cat. no. T 8625), L-tryptophan (cat. no. T 0254), L-valine (cat. no. V 0500), L-glutamine (cat. no. G 3126), L-glutamic acid (cat. no. G 1626), and L-aspartic acid (cat. no. A 9256). Hydrogen peroxide (cat. no. 7209), and sodium dihydrogen phosphate (cat. no. 7294) were obtained from Merck, Darmstadt, Germany. In all cases, solutions were prepared using water produced in a Milli-Q system from Millipore, Bedford, MA, USA.

Instrumentation

The carbon paste working electrode was inserted in a conventional, three electrode flow-through amperometric cell (53), containing a platinum wire auxiliary and a Ag/AgCl (0.1 M KCI) reference electrode. The cell was connected to a potentiostat (Zäta Electronics, Lund, Sweden) and the response was read on a strip chart recorder (Kipp and Zonen, The Netherlands, model BD 111). Phosphate buffer (0.1 M, pH 7.0) was delivered with a flow rate of 0.7 ml $min.^{-1}$, using one peristaltic pump (Minipuls 2, Gilson, Villiers-le-Bel, France), while samples were delivered with another one (Model 2150, LKB, Bromma, Sweden). 50 µl of sample were injected using a pneumatically operated injection valve (Cheminert type SVA, Cotati, CA, USA). All connections were made with Teflon tubings and commercially available screw couplings.

When the electrode was coupled to a liquid chromatographic (LC) system, a column was chosen which would operate without organic solvents in the mobile phase. A column containing a reversed phase polymer material (PLRP-S, 5 µm, 100Å, 150 x 0.4 mm I.D., from Polymer Laboratories, Church Stretton, Shropshire, UK) was used for glutamate separations with phosphate buffer at pH 6.0 (25 mM) as the mobile phase delivered by a liquid chromatographic pump (model 2150, LKB, Bromma, Sweden). The mobile phase was passed through a 0.45 mm filter (Waters Chromatography Division, Millipore, Bedford, MA, USA), and degassed with helium prior to use. Connections between the various parts of the LC systems were made with PTFE tubings, 0.25 mm i.d. 5 µl samples were injected with an injector (model 7000) from Rheodyne, Cotati, CA, USA.

Samples for chromatographic analysis were handled as follows. The urine samples from a healthy pregnant woman were diluted with HPLC-grade water and thereafter directly injected into the LC system. Blood samples were taken to 1.0 ml plastic test tubes and allowed to clot for 10 min. We added 100 µl of perchloric acid (Merck) to 1.0 ml of serum, and the sample was centrifuged for 15 min. at 4000 g. The supernatant was decanted and stored at -20° C until use. Afterwards, the samples were directly injected into the chromatographic system.

Construction of the enzyme electrode

Plain graphite-paraffin oil carbon pastes, prepared by thoroughly mixing 40 µl of paraffin oil (Fluka, Buchs, Switzerland, cat. no. 76235) with 100 mg of graphite powder

T1

L-glutamate oxidase based amperometric biosensors.

Enzyme	Transducer	Detection Principle	Immobilization	Linear Response µM	LDL µM	S / µA/mM	Response Time	Sample	Ref.
GlOx SE ampl.	Pd/Au sputtered carbon	H_2O_2	gelatine entrapment	0.5-1000	1	-	<1 min.	buffer	25
GlOx SE	Pt	FI, H_2O_2, +600 mV	gelatine entrapment	1-1000	-	43 nA/mM*	2 min.	buffer	26
GlOx SE	Pt	O_2, -600 mV or H_2O_2, +600 mV	gelatine entrapment	10-1000	1	15.3 µA/mMcm^2	<1 min.	buffer, protein hydrolyzate	27
GlOx ampl.	Pt	H_2O_2, +500 mV	+ BSA covered by a nylon net	0.002-10	0.3 nM	-	-	buffer	28
GlOx ampl.	Pt	H_2O_2, +500 mV	+ BSA, GA	0.002-10	0.2 nM	50*	-	buffer	30
GlOx	silanized Pt sheet	FI, H_2O_2, +500 mV	+ BSA, GA	5-1000	4	0.75*	1 min.	buffer, seasoning	29
GlOx SP	cellulose triacetate membrane	O_2	GA + spacers	120-840	120	-	<3 min.	buffer, fermentation broth	35
GlOx	PCP	H_2O_2, E>+400 mV	CDI coupling	0-25	1	67*	5 s	buffer	33
GlOx	PCP	H_2O_2, +320 mV	ads	2-2000	2	67 µA/mMcm^2	1 min.	serum	12
GlOx	graphite pellets	H_2O_2, +220 mV, DMF mediated	+ BSA, GA	0.2-2000	-	28 nA/mM*	1 min.	foodstuff	32
GlOx	spectrograph, graphite	H_2O_2, +400 mV, mediated	redox hydrogel FMSEP	<200	10	10 nA/mM	1 min.	buffer	34
GlOx ampl.	Pt	FI, H_2O_2 or O_2, + 650 or -650 mV	Immobilon-AV membrane	0.05-500	35 nM	0.5*	<2 min.	buffer, spices, serum	31, 37, 38
GlOx	Pt	H_2O_2	Immobilon-AV membrane	1-1000	-	-	<1 min.	human sera	36
GlOx	graphite	H_2O_2, mediated with NMP + TCNQ	CDI coupling stabilized with DE and LA	-	-	-	-	buffer	40
GlOx + HRP	CP	FI, H_2O_2, 0 mV	+ PEI, ads	10-1000	-	-	-	buffer	39
GlOx	CP	H_2O_2, +150 mV + TTF mediator	BSA + GA, membrane covered	2.6-800	2.6	1.5*	2 min.	buffer and food	41
GlOx	PCFD, 7 µm ID	H_2O_2, +600 mV, pulsed amperometry	+ BSA in PVA-SbQ	50-800	-	5 nA/mM*	12 s	buffer	43
GlOx	Pt	amperometric detection	+ BSA in gelatine	0-100	0.5	-	1 min.	ACSF, and µdialysate	42
GlOx	rhodiumized carbon rods	FI, H_2O_2, +400 mV	BSA, and GA	100-1500	100	-		buffer and cell culture	45
GlOx	chip/silicon wafers, Pt	O_2	BSA + GA in fluorinated ethylene-propylene membrane	5-500	5	-	<3 min.	buffer	46
GlOx	Pt/thin film	FI, H_2O_2	polyimine gel	up to 5mM	-	2.4* nA/mM	<1 min.	buffer	47
GlOx	PCP or Pt	Flow cell, H_2O_2, +600 mV	CDI coupling or gel trapped + BSA, GA	100- mM range, 2-100	100 2	0.7 µA/mM 1.8 µA/mM	<1 min. 1.5 min.	ACSF, µdialysate	44
GlOx	screen printed/thick film, silanized Pt or C	H_2O_2, +600 mV	BSA and GA	10-50	10	5	-	soups, sauces	49
GlOx S	Teflon coated Pt-Ir wire	H_2O_2, +600 mV	+ BSA, GA	2-60	0.2	0.1	<2 s	buffer, rat brain	48
GlOx	Pt microelectrode (10 and 25 µm ID)	H_2O_2, +850 mV	PFDA	100-15 mM	100	13 pA/mM (10 µm)	-	buffer	6
GlOx	Pt microelectrode (10 and 25 µm ID)	H_2O_2, +850 mV	PFDA and PPy	100-15 mM	100	120 pA/mM PFDA 180 pA/mM PPy (both 25 µm)	-	buffer	50
GlOx + HRP	SnO_2	H_2O_2, +250 mV	encapsulation in PPy or + GA on silanized el. + mediator	0.1-100 PPy	0.1	-	1 min.	buffer	52
GlOx	chip/thin film, Pt	FI, H_2O_2, +500 mV	pHEMA membrane	100-5 mM	100	-	<15 s	buffer	51

Where: potential values are given *vs.* Ag/AgCl
sensitivity values are labelled with * were estimated from calibration curves
ACSF: artificial cerebrospinal fluid
ampl.: amplified systems using coupled GlOx and GlDH (glutamate dehydrogenase)
BSA: bovine serum albumin
CDI: various carbodiimide derivatives
CP: carbon paste
DEAE: diethyl-aminoethyl-dextrane
DMF: 1,1' dimethylferrocene
FI: flow injection
FMSEP: ferrocene-modified siloxane-ethylene polymer
GA: glutaraldehyde
GlOx: *Streptomyces* sp. X119-6 from Yamasa Shoyu Co.
LA: lactitol
NMP + TCNQ: N-methylphenazinium tetracyanoquinodimethane
PCFD: platinized carbon fiber disc
PCP: platinized carbon paper
PFDA: poly(ortho-phenylenediamine)
pHEMA: poly(2-hydroxyethlmethacrylate)
PVA-SbQ: polyvinylalcohol-stilbazole quaternized
PPy: polypyrrole
S: from Sigma
SP: *Streptomyces platensis*
SE: *Streptomyces endus*
TTF: tetrathiafulvalene
µdialysate: system was coupled to microdialysis sampling

(Fluka, cat. no. 50870), were filled into 1.0 ml plastic syringes (Pharma-Plast A/S, Rødby, Denmark, with a tip of 7.0 mm o.d. and 1.8 mm i.d.), leaving about 3-4 mm empty in the top of the tip to be filled with the enzyme-modified paste. A silver thread inserted into the paste provided the electrical contact. Enzyme modified paste (see below) was filled into the top of the syringes, and the top (surface) was gently rubbed on a smooth glass surface, to produce a flat shining electrode surface with a geometric area of about 0.024 cm^2. The final electrode was inserted as the working electrode into the cell connected to the flow injection system described above.

Chemically modified carbon pastes were prepared as follows (**A-C**): The graphite powder was pretreated by heating at 700° C for 15 s in a muffle furnace and cooled to ambient temperature in a desiccator (54). The modified carbon pastes were prepared by different co-immobilization techniques. When simply adsorbed (**A**), 200 µl of 0.1 M phosphate buffer (pH 7.0) containing 1.0 mg of HRP (288 U/mg) or ARP (250 U/mg), and 1.0 mg of GlOx (8 U/mg), were added to the pretreated graphite powder. The graphite-enzyme mixture was allowed to react at 4° C for 2 h followed by vacuum drying for 3 h and then finally mixing with 40 µl of paraffin oil to produce the modified paste. When the two enzymes were cross-linked (**B**), GA (0.12% final concentration) was mixed with the graphite powder together with the enzyme solution. The covalently immobilized pastes (**C**) were prepared using CDI as the coupling reagent (following a previously published protocol (55)), when the pretreated graphite was first activated with CDI prior to the addition of the enzyme solution mentioned under A. The polycationic additive containing pastes were prepared adding 200 µl of PEI (0.32% in 0.1 M phosphate buffer pH 6.0) together with the enzyme solution to the above mentioned pastes (A-C). The modified pastes were kept in a dessicator at 4° C between measurements.

Results and Discussion

GlOx was mostly reported from various *Streptomyces* species (***T1***). The most stable enzyme, and the one displaying the highest activity and selectivity towards L-glutamate is produced by the Yamasa Shoyu company (X-119-6, purified from wheat bran culture) (1). Polycationic additives (40) were reported to further increase its stability. The enzyme stated is almost exclusively selective towards L-glutamate in aqueous solution, displaying only <1% activity towards aspartate (1). However, it was recently found when immobilized onto controlled pore glass to be active for another L-amino acid (ß-ODAP; ß-N-oxalyl-L-α,ß-N-diaminopropionic acid), found in grass pea (*Lathyrus sativus*). For man ß-ODAP is a potent neurotoxin. It is thus of great importance for countries where grass pea is largely cultivated to develop sensitive and selective methods for its determination (56-58). GlOx contains 2 FAD moieties per enzyme molecule and displays a Michaelis-Menten coefficient (K_m) for L-glutamate of 0.21 mM (solution). It is stable between pH 5.5-10 with a rather broad pH optimum around 6.5-8.5 (1,59).

GlOx catalyzes the following:

$$\text{L-glutamate} + O_2 + H_2O \xrightarrow{\text{GlOx}} \alpha\text{-ketoglutarate} + NH_3 + H_2O_2$$

T1 presents various heretofore developed electrode configurations for the determination of L-glutamate. Most of them make use of GlOx and as can be seen, the obtained biosensor characteristics [lower detection limit (LDL), dynamic linear range, apparent K_m (K_m^{app}), sensitivity, stability, interference-eliminating ability], were highly dependent of the electrode material and sensing chemistry. Generally, the use of various protecting membranes, needed especially for interference elimination, decreased the sensitivity for glutamate, increased the detection limit and response time, and also caused a shift in the linear dynamic range. Similarly, the obtained K_m^{app} greatly varied (28 µM (33), 0.2 mM (34), 2.9 mM (12), 5.7 mM (41), 9.1 mM (6)), demonstrating the decisive influence of the diffusion properties of the matrix used for electrode construction. However, no substantial differences could be observed between the optimal pH values of the various presented electrodes, they were found to be between pH 7.0-7.4, except for the GlOx-HRP bienzyme electrode with an optimum pH of 6.4 (52).

Carbon paste electrodes are cheap, easy to handle and display the inherent advantage of bulk modification (60). The use of coupled oxidase-peroxidase design was attractive because of the possibility of constructing reagentless electrodes and the convenient optimal working potential (-50 mV *vs.* Ag/AgCl). The bienzyme electrodes described here were optimized with regard to influence of the used peroxidase, of the co-immobilization method, effect of a polycationic additive, and pH.

Flow Injection Experiments

Effect of co-immobilization

T2 summarizes the results obtained for the different co-immobilization techniques. The effect of the various reagents was evaluated comparing the obtained sensitivities for the various configurations with those measured for the bienzyme configurations together with the two enzymes co-immobilized only by adsorption. The influence of using either HRP or ARP was evaluated by comparing similar co-immobilization methods. Generally, the response to the substrates, L-

glutamate and H_2O_2, was higher for electrodes using HRP; 3-4 times higher for adsorption, 6 times for adsorption and addition of PEI, 12 times for crosslinking with GA, 7 times when PEI was added together with GA, and 10 times for CDI immobilization and addition of PEI. However, no significant difference could be observed when the enzymes were only covalently immobilized.

The crosslinking and coupling reagents influenced the two enzymes differently of the systems revealed by different responses for the two substrates. Crosslinking with GA caused no changes in H_2O_2 sensitivity when using HRP, while the signal decreased 5 times when using ARP. The glutamate signal decrease was also much more pronounced (3 times more) for the ARP based configuration. Covalent immobilization with CDI drastically decreased the signal for the HRP bienzyme electrodes both for H_2O_2 (4 times) and glutamate (10 times), while only a slight decrease could be observed for the GlOx-ARP electrodes. Thus, it seems that GA and CDI mainly decrease the activity of GlOx and not that of the peroxidases.

Addition of PEI was beneficial for the GlOx-HRP electrode. The glutamate sensitivity increased for all immobilization procedures with the degree of increase being different; 0.4 times for adsorption, 2 times for crosslinking with GA, and 5-6 times for CDI coupling. The H_2O_2 signal decreased 2 times upon crosslinking, while it was not significantly influenced for adsorption and covalent coupling. However, PEI always had a negative effect on the GlOx-ARP system for all electrode configuration and for both substrates. It was thus assumed, that PEI (similar to dextrane and lactitol (40)) stabilized GlOx, but it had a less decisive role than the nature of the co-immobilized peroxidase.

Calibration curves are depicted in ***F1A*** and ***F1B***, for GlOx-HRP-PEI and GlOx-ARP-PEI electrodes, respectively. The former one represents the optimal configuration, being characterized by an apparent Michaelis-Menten coefficient (K_m^{app}) of 0.78 mM/Eadie-Hofstee plots, i_{max} of 5.1 µA glutamate, a sensitivity of 6.5 µA/mM (current density of 0.27 mA mM^{-1} cm^{-2}), and a dynamic linear range between

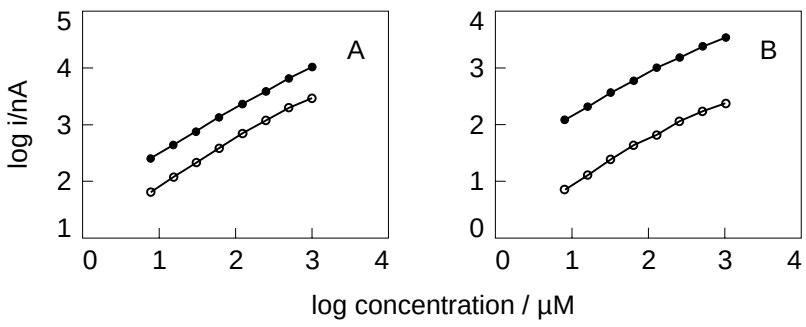
T2

FI responses to 0.1 mM H_2O_2 and 0.5 mM L-glutamate for co-immobilized GlOx and HRP/ARP electrodes using different co-immobilization reagents.

Co-immobilization technique (paste type)	i(H2O2)/µA	i(glu)/µA	i(H2O2)/µA	i(glu)/µA
	GlOx/HRP		GlOx/ARP	
paste A adsorption	1.800 1.440 2.100	2.300 1.480 2.040	0.448 0.776 0.464	0.392 0.776 0.424
mean	1.78±0.33	1.94±0.42	0.562±0.19	0.531±0.21
adsorption + PEI	2.280 2.220 1.880 1.700 1.730	2.840 2.680 2.210 2.750 2.210	0.200 0.224 0.312 0.232 0.216	0.424 0.488 0.576 0.448 0.408
mean	1.96±0.27	2.68±0.30	0.236±0.044	0.53±0.21
paste B GA	2.100 1.740 1.760	0.500 0.290 0.250	0.334 0.420 0.446	0.025 0.030 0.028
mean	1.87±0.2	0.35±0.13	0.40±0.06	0.028±0.003
GA + PEI	0.940 0.890 0.740	0.810 0.800 0.540	0.304 0.236 0.276	0.116 0.088 0.092
mean	0.86±0.1	0.72±0.15	0.27±0.03	0.099±0.02
paste C CDI	0.400 0.500 0.500	0.130 0.230 0.220	0.472 0.448 0.396	0.252 0.240 0.204
mean	0.43±0.12	0.19±0.06	0.44±0.04	0.23±0.03
CDI + PEI	0.670 O.590 -	1.220 0.920 -	0.216 0.212 0.224	0.164 0.148 0.148
mean	0.63	1.07	0.22±0.006	0.15±0.009

F1

Calibration curves obtained for (A) GlOx-HRP-PEI and (B) ARP-GlOx-PEI electrodes. Signs represent (❍) H_2O_2, and (●) L-glutamate signals. Applied potential: -0.05 V vs. Ag/AgCl, flow rate: 0.7 ml $min.^{-1}$, injection volume: 50 µl, carrier: 0.1M phosphate buffer at pH 7.0

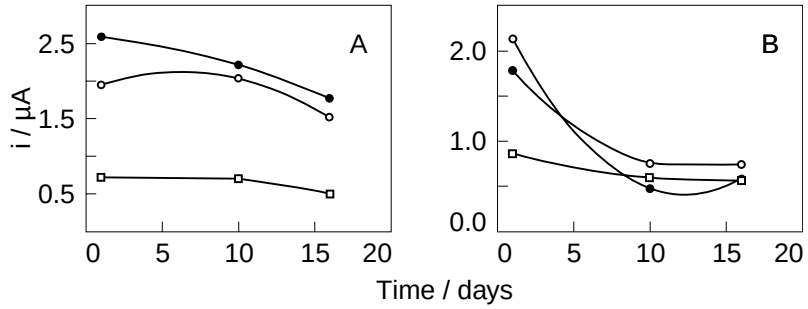


8-800 µM. The detection limit calculated as a 2:1 signal-to-noise ratio was 5 µM. The glutamate conversion efficiency, calculated as the ratio between the glutamate and H_2O_2 sensitivity, was about 30%. This result was the highest obtained so far, with various coupled oxidase-peroxidase configurations, in our laboratory (39,54,55,61,62,64).

The storage stability of three different bienzyme electrodes was tested for 0.1 mM H_2O_2 and 0.5 mM L-glutamate; only adsorbed, adsorbed with PEI addition, and crosslinked with GA and PEI addition (***F2A*** and ***F2B***). Between experiments, the electrodes were stored dry at 4° C. Both the simply adsorbed and GA crosslinked electrodes displayed a stable glutamate signal for approximately 10 days, thereafter (day 16) the decrease was of 21% and 28% for the adsorbed and crosslinked-PEI electrodes, respectively. The "stabilizing" effect of PEI was not confirmed in these experiments. The glutamate response decreased by 14% in 10 days and an additional 30% in 16 days. The H_2O_2 signal decreased for all three electrode-types in the first 10 days and was almost stable during the remainder of the tests. The storage stability of the paste was not studied in this experiment, but it is expected to be superior to that of the electrodes in use as previously observed for other configurations (61). However, coating the electrodes with an additional membrane and/or exchanging the presently used polycation might improve the stability of the electrodes, as previously noticed (55, 61).

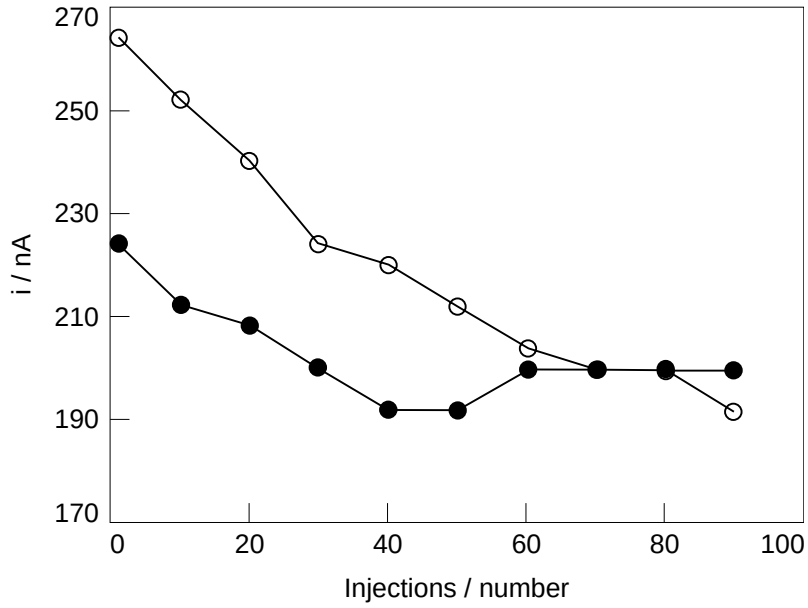
F2

Storage stability of bienzyme electrodes (A) HRP-GIOx and (B) ARP-GIOx, enzymes co-immobilized (❍) by adsorption, (●) by adsorption and addition of PEI, and (❑) by crosslinking with GA and addition of PEI. Other experimental conditions as in F1.



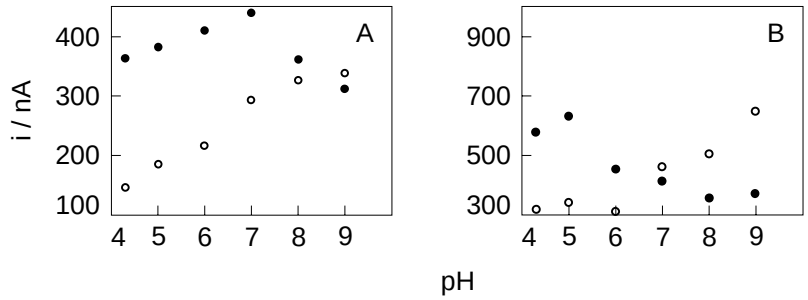
F3

Operational stability of HRP-GIOx-PEI electrodes. Sample throughput 120 injections h^{-1}. (❍) H_2O_2 concentration: 0.1 mM and (●) L-glutamate concentration: 0.5 mM. Other conditions as in F1.



F4

pH profile of the bienzyme electrodes. (A) represents glutamate signal for (●) HRP-GlOx-PEI, and for (❍) ARP-GIOx-PEI electrode, (B) illustrates the corresponding H_2O_2 response for (❍) HRP-GIOx-PEI and (●) for ARP-GIOx-PEI electrodes.



The operational stability of G1Ox-HRP-PEI electrodes was studied by continuously injecting 0.1 mM H_2O_2 and 0.5 mM L-glutamate at a sample throughput of 120 injections h^{-1} (***F3***). Electrodes were monitored during 1.5 h and during this period a sensitivity decrease of 27% and 12% for H_20_2 and glutamate respectively was observed. Efforts to improve the stability of this bienzyme electrode are currently in progress.

The pH profile of the two different bienzyme electrodes (G1Ox-HRP-PEI or G1Ox-ARP-PEI) radically differed (***F4A*** and ***F4B***). The HRP based electrode displayed a dependence of the response to glutamate with pH having an optimum around pH 7.0 as in previously published works (***T1***). However, the optimal range is restricted for this electrode compared with that encountered for the free enzyme (6.5-8.5). For the ARP based electrode, the glutamate response increased with increasing pH, with a tendency to level off around pH 8.0. A similar variation was previously found when co-immobilizing HRP or ARP with D-amino acid oxidase (62). The H_2O_2 signal dependency of pH confirmed previous results obtained for carbon fiber electrodes (CF) (63). HRP, at a maximum pH

of 5.0, decreased the signal with increasing pH values, and ARP based electrodes increased the signal with increasing pH values. However, for CF electrodes, a leveling off tendency was observed for ARP with little difference in sensitivity between pH 6.0-8.0. This change in the pH profile of the ARP based CP electrodes may be attributed to the carbon paste matrix and the presence of the PEI.

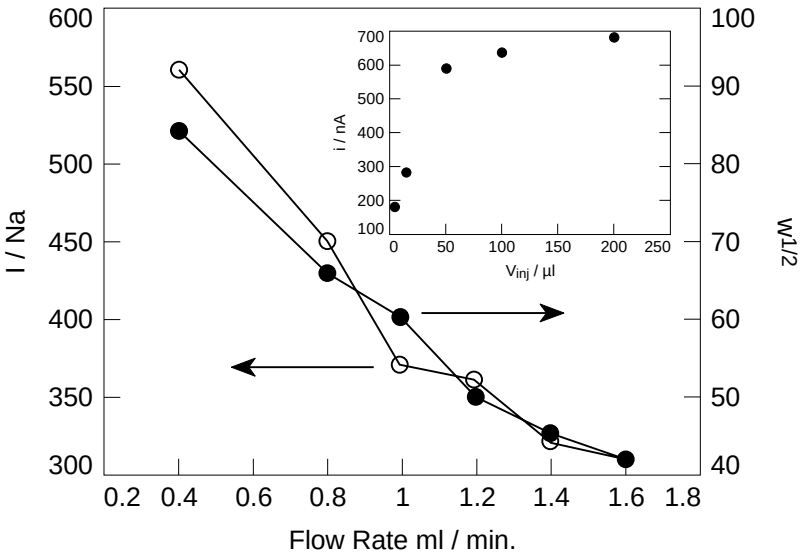
The selectivity of the optimized electrode was tested for a broad range of amino acids. The results are shown in ***T3***. Previously published work showed no (52) or very low (<2%) (48) interfering signals for the most common L-amino acids in biological systems. However, previous work done in our laboratory with co-immobilized H_2O_2 producing oxidase-peroxidase bienzyme electrodes showed that the selectivity of the oxidase was changed when immobilized into carbon paste compared to its aqueous soluble form (alcohol oxidase (55), L-amino acid oxidase (64), and D-amino acid oxidase (62)). As seen from ***T3***, the G1Ox-HRP-PEI electrode responded for 13 amino acids out of 18 tested. The highest sensitivities were found for L-glutamate and L-aspartic acid as expected from the literature (1).

T3

Relative responses to 17 L-amino acids obtained with the GlOx-HRP-PEI bienzyme electrode.

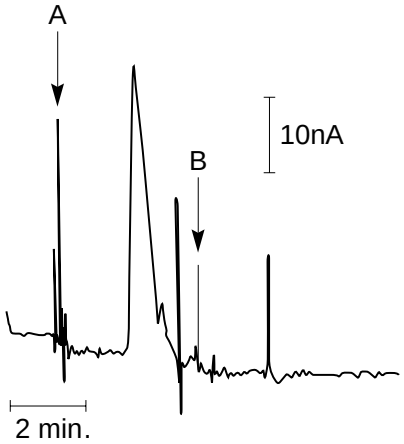
amino acid	relative response/%	amino acid	relative response/%
L-glutamate	100.0	L-leucine	7.8
L-aspartic acid	18.2	L-methionine	7.8
L-serine	9.9	L-histidine	7.8
L-aspargine	8.8	L-phenylalanine	7.8
L-valine	8.8	L-alanine	0
L-glutamine	8.3	L-arginine	0
L-proline	8.3	L-lysine	0
L-threonine	8.3	L-tryptophan	0
glycine	7.8		

F5



HRP-GlOx-PEI bienzyme electrode used as detector in the liquid chromatographic system. (❍) L-glutamate sensitivity, and (●) peak width at half height ($w^{1/2}$) as a function of the flow rate of the carrier. Injection volume: 5 µl, carrier: 25 mM phosphate buffer at pH 6.0. Insert: L-glutamate sensitivity as a function of the sample injection volume of the chromatographic system.

F6



Chromatogram obtained for (A) plasma spiked with 0.5 mM L-glutamate and (B) blank plasma. Flow rate of mobile phase: 0.8 ml min^{-1}. Other conditions as in F5.

Liquid Chromatography Experiments

The optimized biosensor (G1Ox-HRP-PEI) was coupled as the detection unit to a liquid chromatographic (LC) system in some preliminary applications. Based on results found in previous experiments where coupled enzyme and electrochemical reactions were studied (55,62,64), it can be assumed that the first enzyme reaction (G1Ox) is the rate limiting step. The flow rate dependence and its impact on the sensitivity, demonstrated that lower flow rates (0.4 ml $min.^{-1}$) yielded higher sensitivities. Doubling the flow rate caused a signal decrease by a factor or two. Interestingly, an increase of the flow rate of the mobile phase by a factor of 4, only decreased the signal response to less than half (***F5***). This could be of practice for clinical or medical applications where the number of processed samples are of vital importance and, therefore, a high flow rate might be worthwhile. Since an injection volume of only 5 µl was used, this can be increased if sensitivity would be a problem. Peak broadening is also strongly dependent on the flow rate, which can be expected. But since several reactions occur sequentially,

the peak width at half height ($w^{1/2}$) will even be more related to the chosen flow rate.

As seen in ***F5***, $w^{1/2}$ varied in a fashion similar to that of the peak currents. However, these tabulated values do not show that the peak tailing increased strongly at lower flow rates, resulting in more extended run times. By using a flow rate of 0.4 ml/min. a total analysis time of 15 min. was needed, while at 1.6 ml/min., a new sample could be run every 4 minutes.

The simple chromatographic setup was applied to the analysis of biological fluids, such as urine from a pregnant woman and blood plasma from a healthy volunteer. Traditional sample handling steps were used prior to injection into the analytical system (see *Experimental*). In order to maintain operational stability, the injection volume was minimized so that the system was not exposed to excessive amounts of matrix compounds present in the samples.

Typical chromatograms are shown in ***F6*** where plasma and the same plasma spiked with 0.5 mM glutamate are shown. As illustrated, the selectivity of the system is very good. Only a minor void volume response (appearing after 1 min. at a flow rate of 0.8 mL $min.^{-1}$) can be seen, followed by the glutamate peak. The LC column is primarily used to delay the glutamate from the void volume response. Blank plasma (***F6B***) showed a sharp peak appearing after 2.4 minutes, which does not correspond to that of glutamate. This peak was repeatedly found to appear in these plasma samples, and it has not been identified yet. The separation of the plasma spiked with 0.5 mM glutamate, illustrated in ***F6A***, is equivalent to a total amount of 2.8 nmol.

A decline in operational stability of the biosensor was noticed when continuously used for more than 10 injections of plasma samples. This was more pronounced at flow rates higher than 1 mL $min.^{-1}$. As the enzymes in the paste were not covalently bound, a slow diffusion of the aqueous soluble components is expected with a decrease in sensitivity as a result. Previous experience in covering the electrode surface with various membranes (55) showed that the operational stability could be significantly increased without affecting the sensitivity very much.

Conclusions

An amperometric bienzyme electrode is described for the determination of L-glutamate. The carbon paste material and the oxidase-peroxidase bienzyme system required a low operating potential, thus, increasing the electrochemical selectivity of the biosensor. HRP containing electrodes exhibited superior characteristics to those based on ARP. The selectivity pattern of the G1Ox was changed somewhat when immobilized in the carbon paste matrix as compared with published data for the enzyme in aqueous solution. The optimized electrode could, when coupled to a liquid chromatographic separation, be used as an electrochemical detector for the determination of L-glutamate in biological samples.

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