New Reagentless Glutamate Biosensors Based on Mesophilic and Thermophilic Glutamate Dehydrogenases

Valeri Pavlov,[1] Serve Kengen,[2] Ioanis Katakis[*1]*

Bioanalysis and Bioelectrochemistry Group, Chemical Engineering, Universitat Rovira i Virgili, E-43006, Tarragona (Catalonia), Spain, and Laboratory of Microbiology, Department of Biochemical Science, Wageningen Agricultural University, Hesselink van Suchtelenweg 4, Wageningen NL-6703 CT, Netherlands.

RECEIVED DATE

[*] To whom the correspondence should be addressed. E-mail: ioanis.katakis@urv.net**,** Tel: +34 977 55 9655, Fax: +34 977 55 9667.

[1]Universitat Rovira i Virgili.

[2]Wageningen Agricultural University.

ABSTRACT. Two novel methods for NAD^+ and dehydrogenase immobilization on the graphite electrode surfaces have been developed and applied to the construction of reagentless biosensors for L-glutamate based on mesophilic glutamate dehydrogenase (GLDH) from bovine liver and thermophilic glutamate dehydrogenase from *Pyrococcus furiosus.* The methods rely on modification of graphite electrodes with new NADH oxidizing polymer [Os(1,10-phenanthroline-5,6-phendione)$_2$(PVP)$_4$Cl]Cl followed by physical adsorption of dehydrogenase and alginic acid modified with NAD^+ (NAD^+-alginate) on the surface of electrodes or on entrapment of NAD^+ and dehydrogenase in the hydrogel formed *in situ* by crosslinking of PVP bearing amino groups ("binder" polymer) with polyethyleneglycol diglycedyl ester (PEGDGE) on electrodes. Biosensors constructed with the use of bovine GLDH and NAD^+-alginate showed detection limit equal to 0.5 mM and linear range 1.852-6.0 mM. The immobilization of bovine GLDH in the hydrogel gave sensors with the detection limit equal to 0.33 mM and the linear range 1.4-4.3 mM. The response time for both configurations was 100 s. When GLDH from *Pyrococcus furiosus* was adsorbed together with NAD^+-alginate on the electrode surface the resulting sensors showed the detection limit equal to 4 mM and linear range 13-38.5 mM. Crosslinking of this thermophilic enzyme together with "binder" polymer yielded biosensors demonstrating detection limit equal to 6 mM and the linear range 6.6-34 mM. The response time of the last two configurations was 70 s. The 90 ^oC. The sensors constructed with the use of bovine GLDH demonstrated rapid loss of response at this elevated temperature. In addition, disposable glutamate sensors based on thermophilic and mesophilic glutamate glutamate biosensors based on the thermophilic enzyme showed ability to operate at elevated temperature equal to dehydrogenases mixed with stabilizers have been constructed and their shelf life time was studied to demonstrate that the use of thermophilic GLDH allows to extend the shelf stability of glutamate biosensors by 10 times.

KEYWORDS. glutamate analysis, thermophilic, glutamate dehydrogenase, reagentless biosensors, NAD^+.

Glutamate determination is very important in the food analysis, pharmacology, and medicine because L-glutamic acid is an important neurotransmitter[1] implicated in the development of neurological diseases such as amnesia, depression, and schizophrenia.[2,3] It also is a marker in the diagnosis of miocardic diseases and hepatitis.[4] Different analytical methods have been developed for the detection of glutamate: gas chromatography,[5] kinetic potentiometry based on the reaction of glutamate with dinitroflourobenzene,[6] high performance liquid chromatography,[7] and capillary electrophoresis.[8]

In order to reduce analysis time bioanalytical methods based on glutamate decarboxylase, glutamate oxidase, and glutamate dehydrogenase (GLDH) coupled with optical transduction[9-11] have been developed. Amperometric transduction was employed in biosensors based on glutamate oxidase.[12-14] The biosensors based on glutamate oxidase are dependent on oxygen. The dependence of biosensensor response on oxygen can be eliminated by using $NAD(P)^+$ dependent GLDH as a biorecognition element. Glutamate biosensors constructed by the immobilization of GLDH on carbon fiber microelectrodes were based on direct NADH oxidation on the carbon surface[15] requiring high applied overpotential (>0.55 V *vs*. SCE). The overpotential can be lowered by employing enzymatic NADH oxidation via diaphorase and NADH oxidase.[16-17] Another alternative is the use of mediators catalyzing the electrochemical oxidation of NAD(P)H.[18]

The above mentioned glutamate sensors require NAD^+ in a sample solution for their operation. Reagentless biosensors based on dehydrogenases, in general, can be produced by immobilization of enzymes, NAD^+ and mediator for the coenzyme reoxidation on the surface of an electrode. Five strategies for the coenzyme immobilization have been demonstrated in the literature: entrapment in hydrogels formed *in situ* by polymeric macromolecules,[19,20] adsorption onto pre-prepared polymeric membranes,[21] entrapment in electropolymerized films,[22] entrapment in carbon paste,[24-26] and attachment

to self-assembled monolayers.[27] Biosensors in which NAD^+ was entrapped in hydrogels formed *in situ* have good sensitivities because of very fast transport of analyte to the enzyme, but suffer from low stability in the reagentless mode of operation due to easy desorption of the coenzyme and the enzyme. When NAD^+ was entrapped in electropolymerized films and pre-prepared membranes the sensitivity was low due to the slow transport of analyte. The most stable reagentless biosensors were produced on the basis of carbon paste in which a "reserve pool" of the coenzyme was created in the paste, retaining high sensitivity. Such biosensors are however difficult to miniaturize.

The use of more stable enzyme, such as thermophilic dehydrogenases can improve shelf and operational stability of biosensors if the enzymatic reaction is the rate-limiting step or stability determining. Thermophilic glutamate dehydrogenase has been purified from different microorganisms such as *Sulfolobus solfataricus,*[28] *Pyrococcus furiosus,*[29] archaebacteria AN1,[30] and *Thermococcus litoralis*.[31] The enzyme demonstrates high stability at elevated temperatures, for instance, the half life of GLDH from *Thermococcus litoralis* at 80^oC is 15 h, from *Pyrococcus furiosus* at 100^0C is 12 h, and from AN1 at 90^oC is 12.5 h. Thermophilic enzymes can be successfully employed in the construction of biosensors, so glutamate carbon paste biosensors operating in the range 40-60^oC constructed using GLDH from AN1 cells have been reported.[32,33]

In this work we report on our efforts to solve the problems associated with NAD^+ dehydrogenase electrodes. Namely, we use an efficient NADH oxidizing mediator and we show that it is sufficiently catalytic to permit reagentless dehydrogenase electrodes. We achieve such reagentless sensors by immobilizing NAD^+ for continuous and disposable operation. We achieve stable and long shelf life sensors by using thermophilic enzymes.

RESULTS AND DISCUSSION

Methods for the fabrication of reagentless glutamate biosensors. The glutamate biosensors described in the present article are based on the oxidation of L-glutamate by NAD^+ through mesophilic and thermophilic glutamate dehydrogenase (GLDH) according to the reaction:

$$\text{L-glutamate} + NAD^+ + H_2O \overset{\text{GLDH}}{\rightleftarrows} \alpha\text{–ketoglutarate} + NADH + NH_4^+$$

with the equilibrium constant shifted to the formation of L-glutamate, but the reoxidation of the formed NADH by the Os-phendione-PVP mediator, which is oxidized in its turn at the graphite surface of a working electrode at low positive potential of 150 mV *vs*. Ag/AgCl/KCl displaces the equilibrium to the formation of α–ketoglutarate. This principle of operation is shown schematically in Figure 1. The immobilization of GLDH, NAD^+ and mediators is the necessary condition for design of reagentless glutamate biosensors. The group of Lo Gorton reported reagenless glutamate biosensors based on the immobilization of thermophilic $NADP^+$ dependent glutamate dehydrogenase in carbon paste[32,33] in which NADH was reoxidized electrochemically by a polyethylenimine Toluiden Blue O redox mediator at applied potential 100 mV *vs.* $Ag/AgCl/KCl_{sat}$. Unfortunately, carbon paste electrodes can not be easily miniaturized. Our purpose was to construct cheap reagentless glutamate electrodes which can be miniaturized hence we opted for thermophilic GLDH from *Pyrococcus furiosus* which can use NAD^+ as cofactor and developed in this work two new methods to produce reagentless biosensors. The methods rely on NADH oxidation by a new polymer Os-phendione-PVP.[39] The 1,10-phenanthroline-5,6-dione moieties in this polymer impart it the capacity for the reversible regeneration of coenzyme. This fact was confirmed by the electrochemical conversion experiment, described in the experimental

part, in which 100 % conversion of NADH by the mediator to enzymatically active NAD^+ was obtained. This conclusion was drawn by comparing spectrophotometric and coulometric data produced by bulk electrolysis of NADH with those recorded during spectrophotometric determination of the enzymatic reconversion of NAD^+ by glutamate dehydrogenase in the presence of L-glutamate, and taking into account the spontaneous hydrolysis of NADH under the experimental conditions. This result is in good agreement with the result of similar study into electrochemical conversion of NAD^+/NADH couple by the monomeric osmium complex [Os(4,4'-dimethyl-2,2'-bipyridine)$_2$(1,10-phenanthroline-5,6-dione)]$(PF_6)_2$, which showed 100 % conversion too.[25]

The first method consisted in physical adsorption of Os-phendione-PVP, GLDH, and polymeric form of NAD^+ (NAD^+-alginate) on the surface of a graphite electrode. Nakamura and co-workers[38] published the procedure for modification of alginic acid with NAD^+ through carbodiimide reaction, which yields a water-soluble polymer.

The second method was more complicated because, a graphite electrode was, first, pre-modified with Os-phendione-PVP, then GLDH and the coenzyme were entrapped in the hydrogel formed *in situ* on the electrode surface. The hydrogel was created by crosslinking poly(vinyl pyridine) (PEGDGE) bearing amino groups ("binder" polymer) with active epoxide functionalities of poly(ethylene glycol) diglycidyl ether. The immobilization of oxidases in hydrogels based on PEGDGE and redox polymers bearing amino groups has been utilized in fabrication of a number of biosensors.[40,41]

Electrocatalytic oxidation of L-glutamate. Reagentless glutamate biosensors of these two configurations demonstrated change in the cyclic valtammetry when glutamate solution was injected into the cell (Figure 2). In the presence of saturating glutamate concentration the reagentless configurations demonstrated clear electrocatalytic waves reaching almost a plateau at potentials more negative than 200 mV *vs.* Ag/AgCl/KCl_{sat}. Our previous study of Os-phendione-PVP proved that the

potential of 150 mV *vs.* Ag/AgCl/KCl_{sat} was sufficient for electrocatalytical oxidation of NADH[39] hence the same potential was used in this work.

Effect of pH on the response of glutamate biosensors. The effect of pH on the maximum response of reagentless glutamate biosensors fabricated by the two methods using mesphophilic and thermophilic GLDH was studied in steady state mode using 0.1 M phosphate buffer, the pH being adjusted with aqueous solutions of 1 M NaOH or H_3PO_4. In Figures 3 and 4 it can be seen that biosensors based on NAD^+-alginate prepared from mesophilic and thermophilic GLDH have maximum response at pH 9.0. This result is in good agreement with the pH optimum of 8.5-9.0 for glutamate oxidation by free bovine GLDH in a solution,[42] and pH optimum of 9.0 for GLDH from *Pyrococcus furiosus.*[29] Meanwhile the biosensors based on the immobilization by crosslinking with the "binder" polymer constructed using mesophilic and thermophilic GLDH achieved maximum response at pH 9.5. This change in pH optimum can be explained in by the increased enzymatic stability caused by crosslinking and by the effect of local buffering due to the presence of pyridine moieties and amino groups in the "binder" polymer.

The rate of reaction between Os-phendione-PVP and NADH slows down with the increase in pH[39] because the formal potential of this mediator in alkaline solutions shifts more negative and it loses the capacity of NADH oxidation. Os-phendione-PVP is not stable at pH values higher then 6.5, moreover, the controlled electrodes prepared without GLDH showed increase in non-specific oxidation of glutamate starting from pH 9.0, hence the pH 7.4 was chosen for further experiments, given the fact that this is the physiologic value of pH at which the analysis *in vivo* could be carried out.

Effect of temperature on the response of glutamate biosensors. The effect of temperature on the response of the reagentless glutamate biosensors is presented in Figures 5 and 6. The thermophilic GLDH based biosensors, independently of the immobilization procedure, have shown increase in response to L-glutamate until 88°C (the maximum temperature achieved), still below the optimal

temperature of 95°C of free thermophilic GLDH. Mesophilic biosensors demonstrated maximum response at lower temperatures. Biosensors based on NAD^+-alginate showed the lowest optimal temperature of 52°C, binder polymer biosensors had the highest optimal temperature 56°C possibly because of improvement in thermostability due to crosslinking. This data is in good agreement with the published thermostability study of bovine GLDH according to which this enzyme starts to lose activity at 52°C.[43] The employment of new immobilization methods allowed to avoid the desintegration of electrodes at elevated temperatures, which was reported for carbon paste biosensors based on thermophilic GLDH by the group of Lo Gorton.[32] The activation energies for the glutamate biosensors were calculated from Arhenius plots. The activation energies are listed in Table I. Activation energies of mesophilic GLDH biosensors are significantly higher than the activation energy of free bovine GLDH, 12.9 kJ/mol,[43] and those of thermophilic GLDH biosensors are lower than the activation energy of GLDH from *Pyrococcus furiosus* 79.3 kJ/mol.[29] This indicates that the response of the glutamate biosensors is not limited by the kinetics of glutamate oxidation with NAD^+ through GLDH. The response of mesophilic biosensors to L-glutamate was studied at 30°C, while the response of thermophilic biosensors at this temperature was not reproducible hence it was decided to characterize them at 40°C.

Response curves and operational stability of the glutamate biosensors. The kinetic mechanism of oxidative deamination catalyzed by bovine GLDH has been debated in the literature. The order of binding of glutamate and $NAD(P)^+$ to the apoenzyme was in the centre of this discussion. Engel and coworkers arrived to the conclusion that this reaction could proceed via an ordered mechanism, NAD^+ being the leading substrate.[44-46] A random order mechanism for of L-glutamate oxidation by $NADP^+$ was offered too.[47] Then a random sequential mechanism was shown.[48] Later it was suggested that this reaction proceeds through ordered binding and $NADP^+$ leads in complexation with the apoenzyme.[49] The latest kinetic study into this controversial deamination mechanism confirmed that bovine dehydrogenase has an ordered sequential mechanism of substrate binding preceded by complexation

with NAD^+ [50] therefore the analysis of the response of the glutamate sensors was based on the modified kinetic model[51] for the conversion of substrate B to products Q and R shown in Supporting Information.

The calibration curves and the Eadie-Hofstee plots for the reagentless glutamate biosensors constructed with the use of mesophilic or thermophilic GLDH based on NAD^+-alginate or the "binder" polymer are shown in Figures S-10 and S-11 (Supporting Information). Their basic characteristics are represented in Table II. All reagentless glutamate biosensors have demonstrated the concave Eadie-Hofstee plots (Figures S-10(B), S-11(B)) revealing that the response currents are limited by the rate of NADH oxidation at the electrode surface through Os-phendione-PVP.

The literature value of the Michaelis constant for mesophilic bovine GLDH determined at 30°C is 2.5 mM,[51] and that determined by us for thermophilic GLDH from *Pyrococcus furiosus* in the presence of 26 mM NADH at 40°C and pH 7.4 is 14 mM.

The biosensors based on mesophilic enzyme showed apparent Michaelis constants which are about 6 times greater than that of the free enzyme. The biosensors based on thermophilic enzyme demonstrated Michaelis constants greater by 3-4 times than that for free enzyme under the same conditions. Taking into account the influence of mass transport on the simulated response of biosensors (Figures S-8 and S-9 and Tables S-II and S-III in Supporting Information) one can arrive to a conclusion that the experimental data imply that the response to glutamate is limited by two factors: the rate of NADH oxidation at the electrode surface and the mass transport. The fact that thermophilic biosensors based on the "binder" polymer demonstrated higher apparent current density than those prepared with the use of mesophilic GLDH implies that the thermophilic enzyme is less deactivated than the mesophilic one by crosslinking with PEGDGE.

The biosensors based on thermophilic GLDH were operated at higher temperature, which enhanced the diffusion, and demonstrated shorter response times. The detection limits of thermophilic biosensors were considerably greater than those of the mesophilic GLDH based biosensors, because of higher

specific activity of mesophilic GLDH at low temperatures. The greater operational linear ranges of thermophilic biosensors can be explained by the greater Michaelis constant of thermophilic GLDH. The thermophilic biosensors demonstrated much shorter operational stability than the mesophilic sensors due to higher temperature of operation (40^{o}C) whereas the operational stability of mesophilic electrodes was studied at 30^{o}C. The loss of response in both cases was caused by leaching of NAD^+ from the electrode surface accelerated at high temperatures. This was proven by injection of NAD^+ at the end of operational stability study, which normally lead to the recovery of response current (shown by the sensors of the same batch in the presence on NAD^+ in a bulk solution), hence another operational stability study was performed in the presence of 30 mM NAD^+ in the bulk solution. This study was performed at elevated temperature of 65^{c}C to show that the half life of thermophilic GLDH based biosensors was 16 min whereas mesophilic biosensors instantly and completely lost response at this temperature so the use of thermophilic enzyme instead of the mesophilic one allowed to increase the operational stability of glutamate biosensors at elevated temperatures. Cyclic voltammetry of thermophilic biosensors revealed that 30% of phendione activity was lost in the course of study, on the other hand calibration of biosensors with fresh glutamate solution in the presence of fresh coenzyme under the same conditions did not result in increase of response current, therefore the main reason for the lose of current was the decomposition of Os-phendione-PVP at the electrode surface, the leaching of the enzyme was a less important factor.

Shelf-life study of glutamate biosensors. In order to compare the efficiency of different ways for the improvement of operational stability of glutamate biosensors, screen printed electrodes (Figure S-3 in Supporting Information) were modified by deposition 0.5 μl of a mixture containing GLDH (thermophilic or mesophilic one), NAD^+, stabilizing additive, and soluble mediator for the electrochemical oxidation of NADH [Os(4,4'-dimethyl-2,2'-bipyridine)$_2$(1,10-phenanthroline-5,6-dione)]Cl_2 as described in Supporting Information. It was found that the best procedure to measure the response (when the relative standard deviation for 3-5 electrodes was minimized to 10-15 %) was to

apply 0.5 μL of a sample solution to an electrode heated to 40°C, wait for 20 s, apply the potential of 200 mV and record the current after 30 s. Big batches of glutamate sensors were prepared and kept at 40°C to carry out the accelerated shelf-life study. The response to glutamate was measured as the difference between response currents to pure buffer and 0.6 M glutamate solution in the same buffer. In order to take into account the decrease in response originated from the instability of NAD^+, the response to samples of 0.6 M glutamate containing 0.18 M NAD^+ was registered during this stability study. The results of this study can be found in Figure S-12 and Table S-IV in the Supporting Information.

The common trend for all tested glutamate sensors based on screen printed electrodes was that the response to pure glutamate was higher by 1.5 times than the response to glutamate samples containing 0.18 M NAD^+. The controlled electrodes prepared without enzymes showed the response to 6 mg/mL NADH equal to 0.46 μA whereas the response to NADH of the same concentration in the presence of 0.18 M NAD^+ was 0.307 μA i.e. once again the ratio was 1.5. According to the literature[52] a charge-transfer complex between a mediator and NADH is involved in the mechanism of electrochemical oxidation of NADH:

$$\mathrm{NADH} + \mathrm{M_{ox}} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} [\mathrm{NADH\ M}]_{\text{charge transfer complex}} \xrightarrow{k_{+2}} \mathrm{NAD^+} + \mathrm{M_{red}}$$

The fact that the overall rate of NADH oxidation can be decreased by addition of NAD^+ supports the hypothesis of charge transfer complex and suggests that the parasite complex between NAD^+ and [Os(4,4'-dimethyl-2,2'-bipyridine)$_2$(1,10-phenanthroline-5,6-dione)]Cl_2 is formed too.

In order to obtain more experimental data about the influence of NAD^+ on the rate of electrocatalytic NADH oxidation by the mediator graphite electrodes were modified with [Os(4,4'-dimethyl-2,2'-bipyridine)$_2$(1,10-phenanthroline-5,6-dione)]$(PF_6)_2$ according to.[54] Then the effect of NAD^+ concentration on the response of modified electrodes to 1.3 mM NADH was studied (Figure S-13 in Supporting Information). The effect of NAD^+ is significant starting from 10 mM. In addition the kinetic

constants k_{+2}, K_M and coefficient k of NADH oxidation in the presence of NAD^+ were measured using graphite rotating disk electrodes modified with the above mentioned mediator.[53] Where k is the apparent coefficient of the following overall reaction:

$$\text{Os-phendione-PVP} + \text{NADH} \xrightarrow{k} \text{Os-catechol-PVP} + \text{NAD}^+$$

The determination of the kinetic constants was performed at pH 7.0 under argon by diluting the initial 1.8 mM NADH solution containing 20 mM NAD^+ with deaerated phosphate buffer containing only NAD^+ of the same concentration in order to avoid the influence of possible electrode fouling. The obtained experimental data in the form of the Koutecky-Levich plot can be seen in Figure S-14 in Supporting Information.. The experimental data were treated by the method published elsewhere[55] to yield $k_{[NADH]=0}$ (0.5±0.1)x10^3 $M^{-1}s^{-1}$, k_{+2} 0.6±0.1 s^{-1}, and K_M 1.4±0.2 mM. According to our previous study[54] this mediator has the following constants under the same experimental conditions but in the absence of NAD^+: $k_{[NADH]=0}$ 0.9x10^3 $M^{-1}s^{-1}$, k_{+2} 0.8 s^{-1}, and K_M 4.3 mM. This decrease in the value of kinetic constant k_{+2} is obviously caused by the interaction of NAD^+ with the charge transfer complex between NADH and mediator. The two fold change in $k_{[NADH]=0}$ could be explained by the competition between NADH and NAD^+ for the free mediator hence it should be admitted that the oxidized cofactor is capable to form parasite complex with the molecules of the osmium mediator.

The use of thermophilic GLDH instead of mesophilic enzyme allowed to increase the shelf half life at 40°C by 11 times (from 7 to 75 h) because of intrinsic thermostability of thermophilic GLDH. Gorton with coworkers also reported that carbon pastes based on thermophilic GLDH retain 90-100% of activity at 4°C during 2 weeks.[32]

In addition a number of compounds such as the copolymer of vinyl-pyrrolidone and dimethylamino ethyl methacrylate termed as Gafquat® HS100, poly(ethylene imine), trehalose, and glycerol were tested by us with the respect to improvement of shelf stability (Table S-IV in the Supporting Information). Electrodes prepared with the use of glycerol proved to be very unstable and lost one half of initial

response in less then 6 h probably because of denaturing effect of glycerol which stayed on the electrode surface during the study. Poly(ethylene imine) was not active in improvement of stability of the thermophilic GLDH based sensors prepared utilizing this polymer as additive, these sensors were less stable than the sensors based on pure enzyme by 2 times, supposedly due to oxidation of imino groups. Trehalose had almost no influence on stability of neither mesophilic or thermophilic GLDH based sensors. Only copolymer of vinyl-pyrrolidine and dimethylamino ethyl methacrylate, Gafquat® HS100, significantly improved stability of both mesophilic and thermophilic sensors by 4.6 and 3.3 times respectively. The most important factor contributing to enzyme stability is the control of the relative water activity at the enzyme surface. We suggest that the latter polyelectrolyte, promoting electrostatic interactions, forms a protein-polyelectrolyte complex resulting in mimicking aqueous environment of enzymes.

CONCLUDING REMARKS

Two novel methods for the fabrication of reagentless biosensors based on NAD^+ dependent dehydrogenases have been developed and reagentless glutamate biosensors operating at 150 mV *vs.* $Ag/AgCl/KCl_{sat}$ have been constructed and characterized by studying response curves, operational stability, dependence of response on temperature and pH. Their response is limited by the rate of NADH oxidation at the electrode surface. Use of thermophilic glutamate dehydrogenase and the stabilizing additive Gafquat® HS100 helped to improve operational and shelf stability of these biosensors. The hypothesis of charge-transfer complex formed between NADH and a mediator was confirmed.

ACKNOWLEDGEMENTS

This paper was supported by the grant of European Community, Industrial & Material Technologies Programme (Brite-EuRam III), Ref.: project No. BE97-4511 and by the Spanish Ministry of Education and Culture Ref.: Acciones Especiales MAT98-1413 CE V. P. acknowledges the Ph. D. fellowship from the Department of Chemical Engineering of the University Rovira i Virgili.

Supporting Information Available. Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org

FIGURE CAPTIONS

Figure 1. Schematic diagram of the electron transfer steps for the mediated glutamate biosensors.

Figure 2. Cyclic voltammograms of reagentless glutamate biosensors fabricated with the use of mesophilic bovine GLDH based on NAD^+-alginate **(A)** and the "binder" polymer **(B)**. Experimental conditions: scan rate 0.4 mV s^{-1}, 0.1 M phosphate buffer (pH 7.4), 0.35 M glutamate, temperature 30°C

Figure 3. Effect of pH on maximum response of reagentless glutamate biosensors based on NAD-alginate fabricated with the use mesophilic **(A)** and thermophilic GLDH **(B)**. Experimental conditions: E_{app} 150 mV *vs.* $Ag/AgCl/KCl_{sat}$, 0.1 M phosphate buffer of varied pH, temperature 30°C for mesophilic biosensors and 40°C for thermophilic ones, glutamate concentrarion was 0.3 M.

Figure 4. Effect of pH on maximum response of reagentless glutamate biosensors based on binder polymer fabricated with the use of mesophilic **(A)** and thermophilic GLDH **(B)**. Experimental conditions: E_{app} 150 mV *vs.* $Ag/AgCl/KCl_{sat}$ 0.1 M phospate buffer of varied pH, 0.35 M glutamate, temperature 30°C for mesophilic biosensors and 40°C for thermophilic ones.

Figure 5. Effect of temperature on maximum response of reagentless glutamate biosensors based on NAD^+-alginate fabricated with the use of mesophilic **(A)** and thermophilic GLDH **(B)**. Experimental conditions: E_{app} 150 mV *vs.* $Ag/AgCl/KCl_{sat}$, 0.1 M phosphate buffer (pH 7.4), 0.35 M glutamate.

Figure 6. Effect of temperature on maximum response of reagentless glutamate biosensors based on binder polymer fabricated with the use of mesophilic **(A)** and thermophilic GLDH **(B)**. Experimental conditions are the same as in Figure 5.

Table I. Activation energies of the reagentless glutamate biosensors calculated from Arhenius plots.

Methods of NAD^+ immobilization	**E_a for mesophilic GLDH / kJ mol^{-1}**	**E_a for thermophilic GLDH / kJ mol^{-1}**
NAD^+-alginate	53.9	58.24
"binder" polymer	56.9	62.2

Table II. Basic characteristics of reagentless glutamate biosensors calculated from Eadie-Hofstee plots. Detection limit is defined as the analyte concentration at which the response is three times higher than the background.

Methods of NAD^+ immobilization	**Mesophilic GLDH**							
	J_{max}, $\mu A\ cm^{-2}$	**RSD, %**	**Response time, s**	**K_B app, mM**	**Limit of detection, mM**	**Linear range, mM**	**Sensitivity, $\mu A\ mM^{-1}\ cm^{-2}$**	**Operatianal half-life time, h**
NAD^+-alginate	14.26	21	100	14.3	0.5	1.852-6.0	0.496	1.5
Binder polymer	7.8	5.0	100	10.1	0.3366	1.4-4.3	0.3366	12

Methods of NAD^+ immobilization	**Thermopilic GLDH**							
	J_{max}, $\mu A\ cm^{-2}$	**RSD, %**	**Response time, s**	**K_B app, mM**	**Limit of detection, mM**	**Linear range, mM**	**Sensitivity, $\mu A\ mM^{-1}\ cm^{-2}$**	**Operatianal half-life time, h**
NAD^+-alginate	13.0	14	70	39	4 .0	13-38.5	0.0633	0.4
Binder polymer	19.1	20	70	55	6.0	6.6-34.0	0.214	0.73

REFERENCES

(1) Niwa, O.; Torimitsu, K.; Osborne, P.; Yamamoto, K. *Anal. Chem.* 1996, *68*, 1865-1870.

(2) Engelsen, B. *J. Neurochem.* 1986, *74*, 1634-1641.

(3) Greenamyre, J. T. *Arch. Neurol.* 1986, *43*, 1058-1063.

(4) Rietz, B.; Guilbault, G. G. *Anal. Chim. Acta* 1975, *74*, 191-198.

(5) Fortier, G.; Tenaschuk, D.; Mackenzie, S. L. *J. Chromatogr.* 1986, *361*, 253-261.

(6) Athanasiou-Malakai, E.; Koupparis, M. M. *Analyst* 1987, *112*, 757-761.

(7) Mora, R.; Berndt, K. D.; Tsai, H.; Meredith, S. C. *Anal. Biochem.*1988, *172*, 368-376.

(8) O'Shea, T. J.; Weber, P. L.; Bammel, B. P.; Lunte, C. E.; Smyth, M. R. *J. Chromatogr.* 1992, *608*, 189-195.

(9) Kiba, N.; Moriya, T.; Furasawa, M. *Anal. Chim. Acta* 1992, *256*, 221-224.

(10) Dremel, B. A. A.; Schmidt, R. D.; Wolfbeis, O. S. *Anal. Chim. Acta* 1991, *248*, 351-359.

(11) Cosford, R. J.; Kuhr, W. G. *Anal. Chem.* 1996, *68*, 2164-2169.

(12) Yao, T.; Kobayashi, N.; Wasa, T. *Anal. Chim. Acta* 1990, *231*, 121-124.

(13) Berners, M. O. M.; Boutelle, M. G.; Filinz, M. *Anal. Chem.* 1994, *66*, 2017-2021.

(14) Hale, P. D.; Lee, H. S.; Okamoto, Y.; Skotheim, T. A. *Anal. Lett.* 1991, *24*, 345-356.

(15) Pantano, P.; Kuhr, W. *Anal. Chem.* 1993, *65*, 623-630.

(16) Davies, P.; Mosbach, K. *Biochim. Biophys. Acta* 1974, *370*, 329-338.

(17) Smith, M. D.; Olson, C. L. *Anal. Chem.* 1975, *47*, 1074-1077.

(18) Amine, A.; Kauffmann, J. -M.; Palleschi, G. *Anal. Chim. Acta* 1993, *273*, 213-218.

(19) Laurinavicius, V.; Kurtinaitiene, B.; Gureviciene, V.; Boguslavsky, L.; Geng, L.; Scotheim, T. *Anal. Chim. Acta* 1996, *330*, 159-166.

(20) Leca, B.; Marty, J. L. *Biosens. Bioelectron.* 1997, *12,*1083-1088.

(21) Maines, A.; Prodromidis, M. I.; Tzouwara, S. M.; Karayannis, M. I.; Ashworth, D.; Vadgama, P. *Anal. Chim. Acta* 2000, *408*, 217-224.

(22) Ikariyama, Y.; Ishizuka, T.; Sinohara, H.; Aizawa, M. *Denki Kagaku* 1990, *58*, 1097-1102.

(24) Yoon, H. C.; Kim, H. S. *Anal. Chim. Acta* 1996, *336*, 57-65.

(25) Hedemno, M.; Narváez, A.; Dominguez, E.; Katakis, I. *Analyst* 1996, *121*, 1891-1895.

(26) Alvarez, S. L.; Lobo, M. J.; Miranda, A. J.; Tuñon, P. *Biosens. Bioelectron.* 1997, *12*, 739-747.

(27) Katz, E.; Heleg, V.; Bardea, A.; Willner, I.; Rau, H. K.; Haehnel, W. *Biosens. Bioelectron.* 1998, *13*, 741-756.

(28) Consalvi, V.; Chiaraluce, R.; Politi, L.; Gambacorta, A.; DeRosa, M.; Scandurra, R. *Eur. J. Biochem.* 1991, *196*, 459-467.

(29) Consalvi, V.; Chiaraluce, R.; Politi, L.; Vaccaro, R.; DeRosa, M.; Scandurra, R. *Eur. J. Biochem.* 1991, *202*, 1189-1196.

(30) Hudson, R. C.; Ruttersmith, L.D.; Daniel, R. M. *Biochim. Biophys. Acta* 1993, *1202*, 244-250.

(31) Ma, K.; Robb, F. T.; Adams, M. W. W. *Appl. Environ. Microbiol.* 1994, *60*, 562-568.

(32) Jeffries, C.; Pasco, N.; Baronian, K.; Gorton, L. *Biosens. Bioelectron.* 1997, *12*, 225-232.

(33) Pasco, N.; Jeffries, C.; Davies, Q.; Downard, A. J.; Roddick-Lanzilotta, A. D.; Gorton, L. *Biosens. Bioelectron.* 1999, *14*, 171-178.

(34) Amouyal, E.; Homsi, A.; Chambron, J.-C.; Sauvage, J.-P. *J. Chem. Soc., Dalton Trans.*1990, 1841-1845.

(35) Parellada, J.; Narvaez, A.; Dominguez, E.; Katakis, I. *Biosens. Bioelectron.* 1997, *14*, 267-275.

(36) Lay, P. A.; Sargeson, A. M.; Taube, H. In *Inorganic Synthesis*; Shreeve J. M., Ed.; John Wiley: New York, 1986; Vol 24; pp. 293-295.

(37) Goss, C. A.; Abruña, H. D. *Inorg. Chem.* 1985, *24*, 4263-4267.

(38) Nakamura, Y.; Suye, S.; Kira, J.; Tera, H.; Tabata, I.; Senda, M. *Biochim. Biophys. Acta* 1996, *1289*, 221-225.

(39) Pavlov, V.; Rincón, O.; Solé, S.; Narváez, A.; Domínguez, E.; Katakis, I. Submitted to *Anal. Chem.*

(40) Degani, Y.; Heller, A. *J. Am. Chem. Soc.* 1989, *111*, 2357-2358.

(41) Gregg, B. A.; Heller, A. *J. Phys. Chem.* 1991, *95*, 5976-5980.

(42) Smith, E. L.; Austen, B. M.; Blumenthal, M. K.; Nyc, J. F. In *The Enzymes*; Boyer, P. D., Ed.; Academic Press: New York, 1975, volume XI, pp. 359-360.

(43) Yamamura, A.; Sakaguchi, T.; Murakami, Y.; Yokoyama, K.; Tamiya, E. *J. Biochem.* 1999, *125*, 760-769.

(44) Engel, P. C.; Dalziel, K. *Biochem. J.* 1969, *115,* 621-631.

(45) Engel, P. C.; Dalziel, K. *Biochem. J.* 1970, *118*, 409-419.

(46) Engel, P. C.; Chen, S. S. *Biochem. J.* 1975, *151*, 305-318.

(47) Colen, A. H.; Prough, R. A.; Fisher, H. F. *J. Biol. Chem.* 1972, *247*, 7905-7909.

(48) Silverstein, E.; Sulebele, G. *Biochemistry* 1973, *12*, 2164-2172.

(49) Rife, J. C.; Cleland, W. W. *Biochemistry* 1980, *19*, 2321-2328.

(50) O'Flaherty, M.; McMahon, M.; Forde, J.; Mulcahy, P. *Prot. Express. Purif.*1999, *16*, 276-297.

(51) Albery, W. J.; Bartlett, P. N.; Cass, A. E. G.; Sim, K. W. *J. Electroanal. Chem.* 1987, *218*, 127-134.

(52) Gorton, L. *J. Chem. Soc., Faraday Trans.* 1986, *82*, 1245-1258.

(53) Fahien, L. A.; Strmecki, M. *Arch. Biochem. Biophys.* 1969, *130*, 468-477.

(54) Popescu, I. C.; Domínguez, E.; Narváez, A.; Pavlov, V.; Katakis, I. *J. Electroanal. Chem.* 1999, *464,* 208-214.

(55) Gorton, L.; Torstensson, A.; Jaegfeldt, H.; Johansson, G. *J. Electroanal. Chem.* 1984, *161*, 103-120.

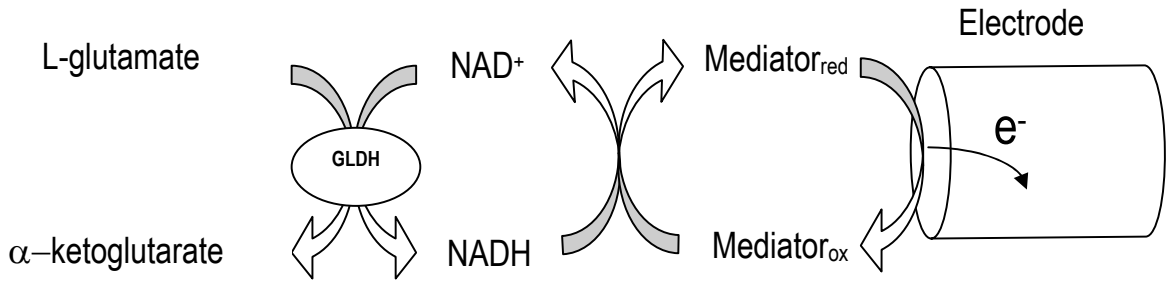

L-glutamate
α–ketoglutarate
GLDH
NAD+
NADH
Mediatorred
Mediatorox
Electrode
e-

Figure 1

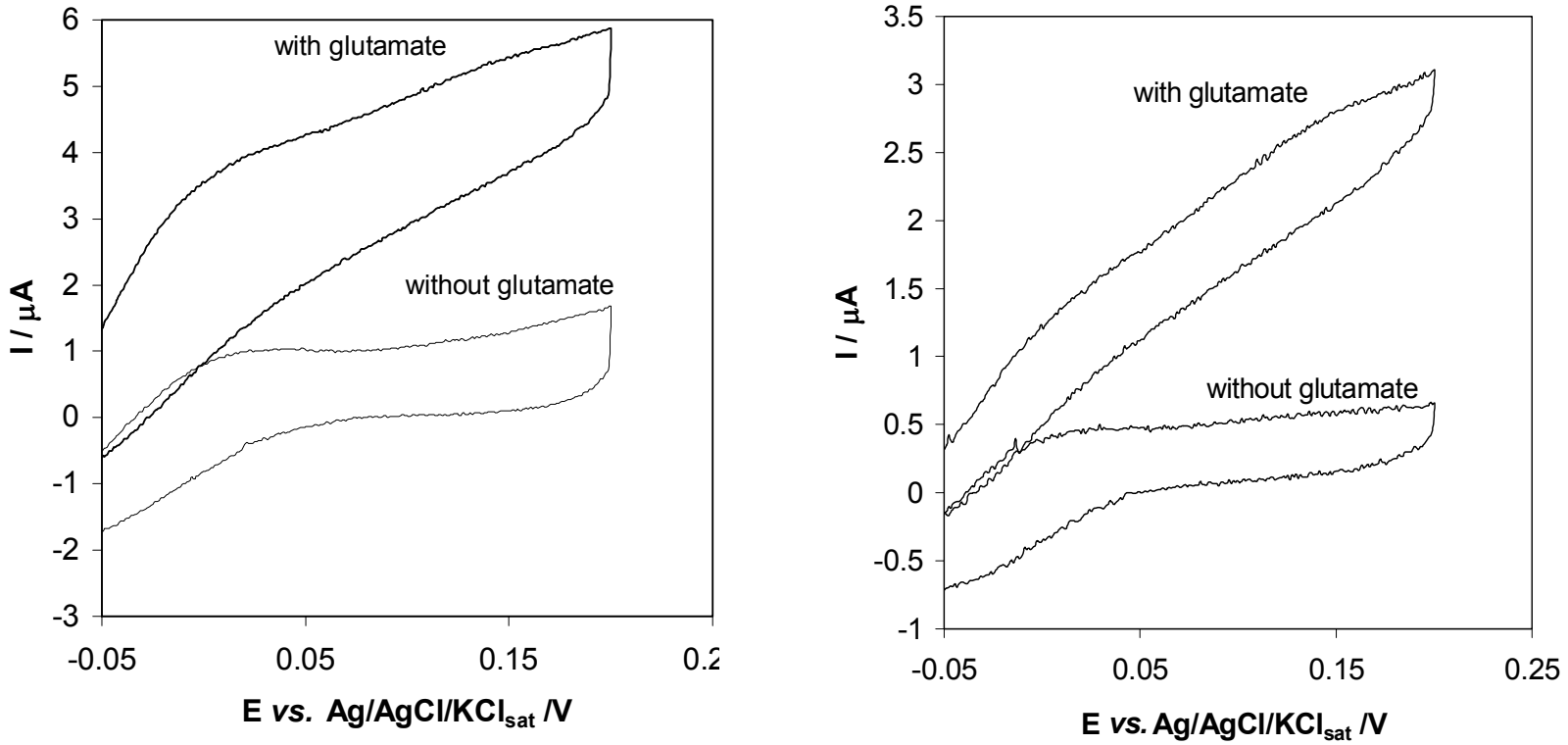
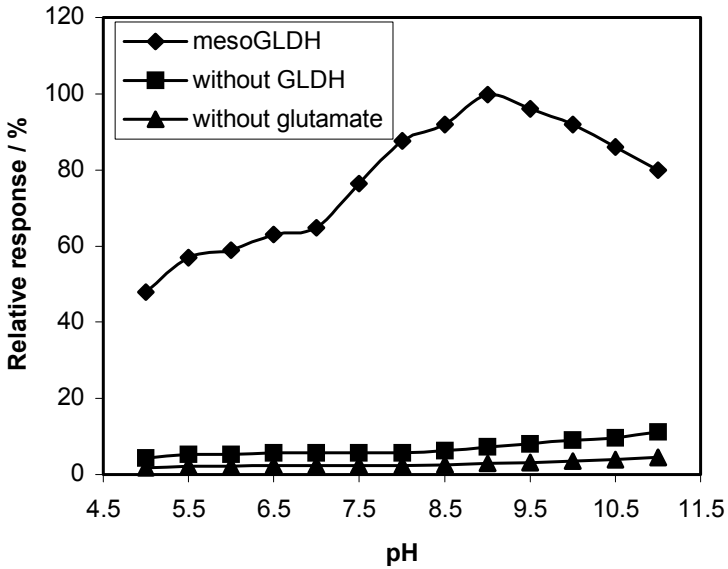
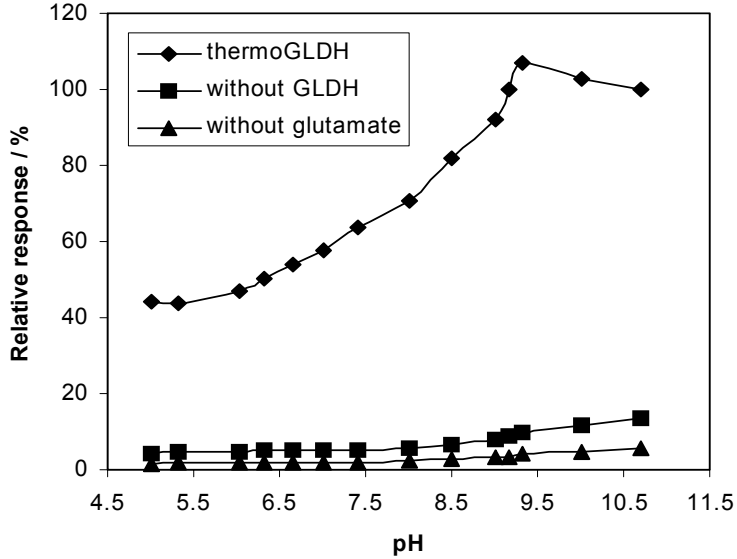

with glutamate
without glutamate
I / μA
E vs. Ag/AgCl/KCl$_{sat}$ /V
(A)
(B)

Figure 2

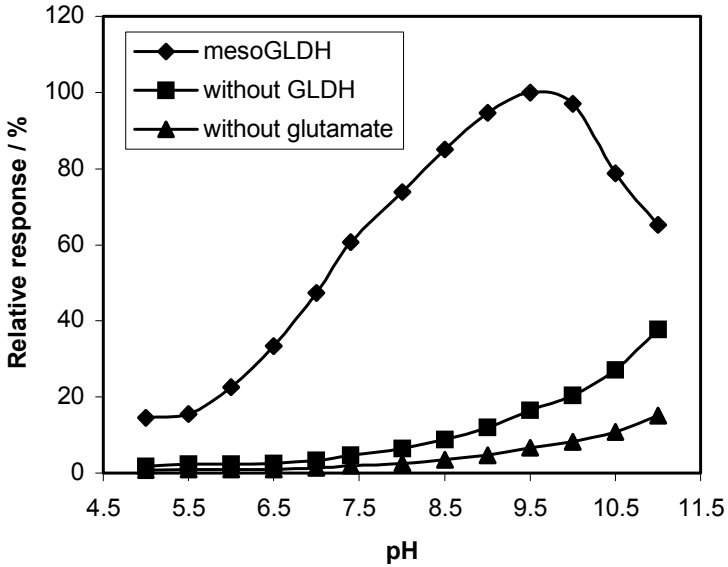

mesoGLDH
without GLDH
without glutamate
Relative response / %
pH
120
100
80
60
40
20
0
4.5
5.5
6.5
7.5
8.5
9.5
10.5
11.5

(A)

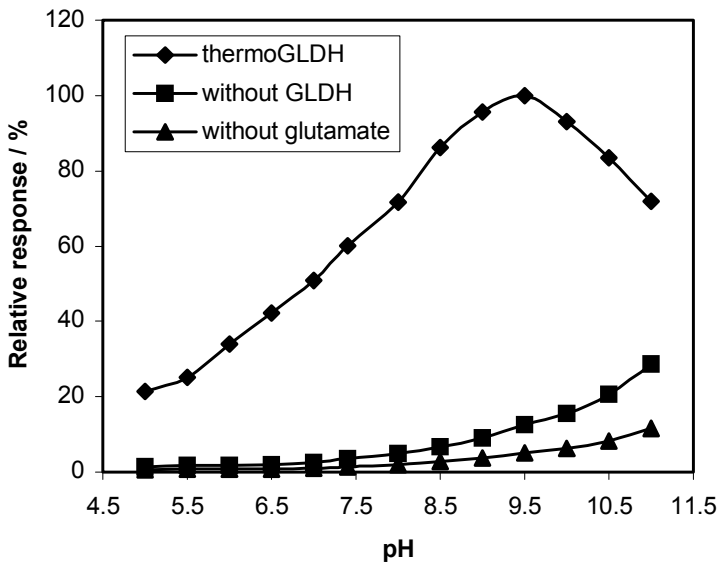

thermoGLDH
without GLDH
without glutamate
Relative response / %
pH
120
100
80
60
40
20
0
4.5
5.5
6.5
7.5
8.5
9.5
10.5
11.5

(B)

Figure 3

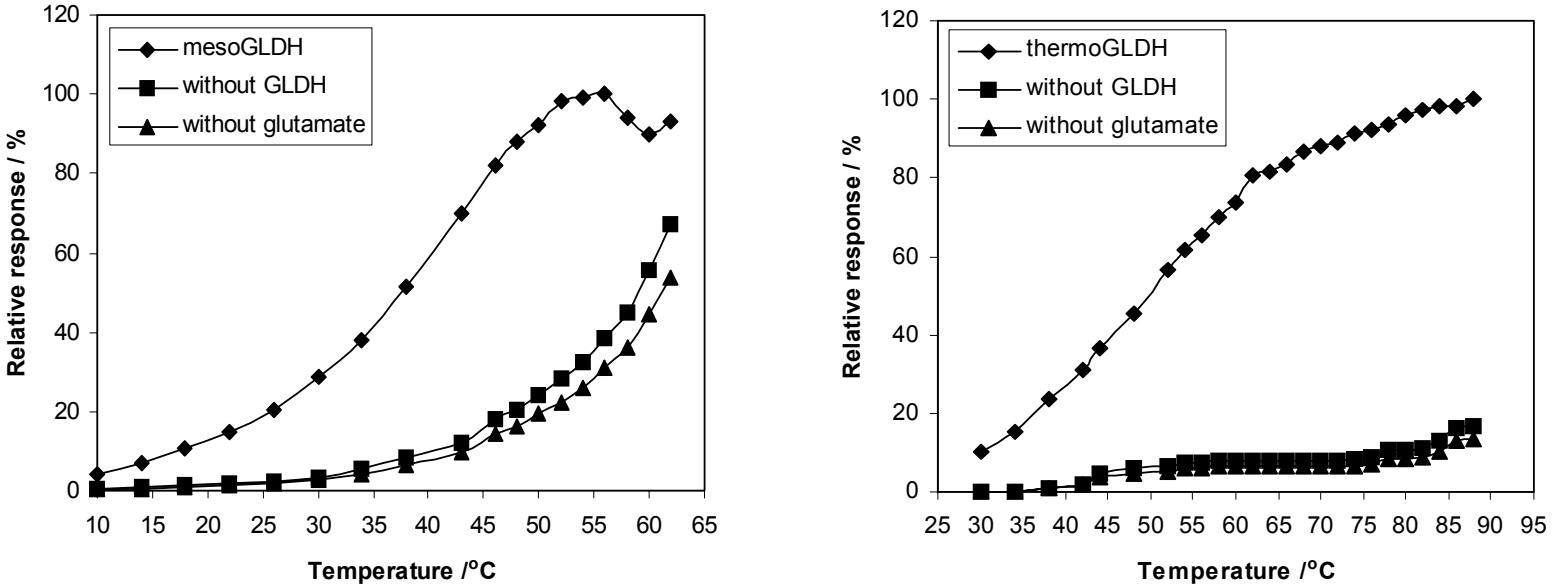

mesoGLDH
without GLDH
without glutamate
Relative response / %
pH
120
100
80
60
40
20
0
4.5
5.5
6.5
7.5
8.5
9.5
10.5
11.5

(A)


thermoGLDH
without GLDH
without glutamate
Relative response / %
pH
120
100
80
60
40
20
0
4.5
5.5
6.5
7.5
8.5
9.5
10.5
11.5

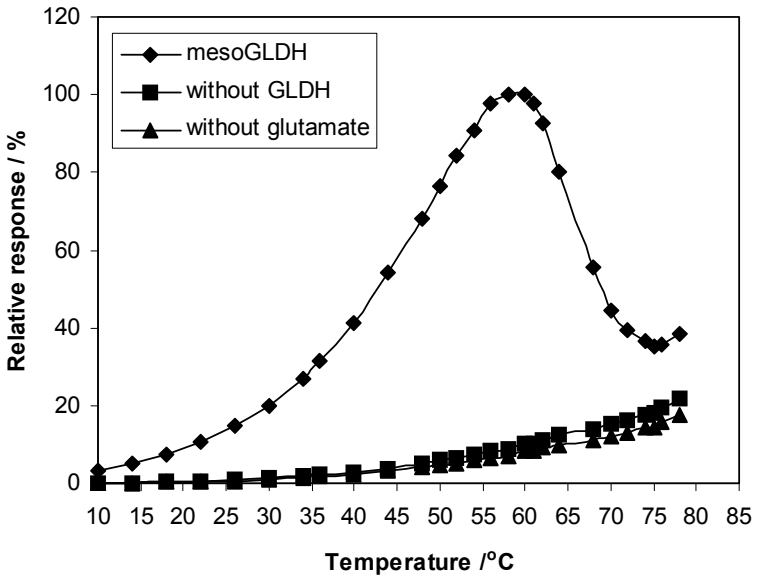
(B)

Figure 4

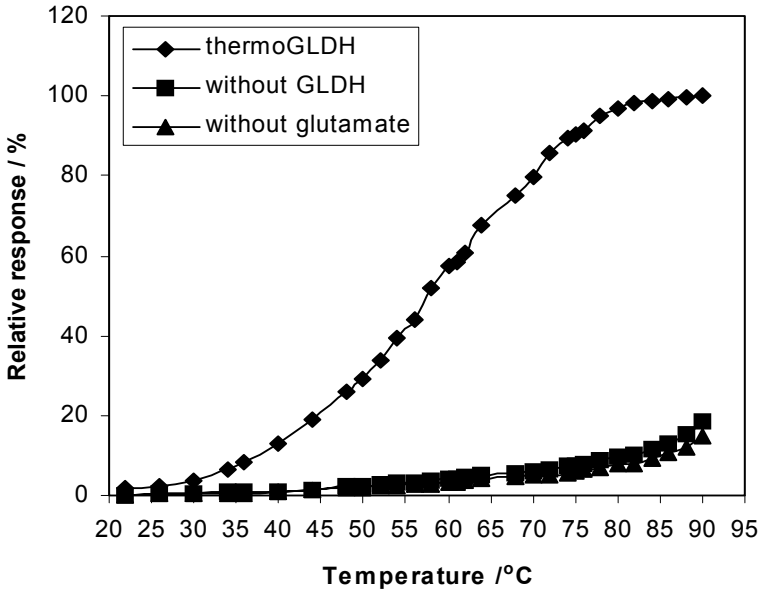

mesoGLDH
without GLDH
without glutamate
Relative response / %
Temperature /°C
thermoGLDH
without GLDH
without glutamate
Relative response / %
Temperature /°C

(A) **(B)**

Figure 5


mesoGLDH
without GLDH
without glutamate
Relative response / %
Temperature /°C
0
20
40
60
80
100
120
10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85

(A)


thermoGLDH
without GLDH
without glutamate
Relative response / %
Temperature /°C
0
20
40
60
80
100
120
20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95

(B)

Figure 6