Flow-Injection Procedures for the Determination of L-Glutamic Acid and Glutamate Dehydrogenase using Co-Immobilized Bacterial Luciferase and Oxidoreductase

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Summary: Flow-injection procedures for the indirect determination of L-glutamic acid and glutamate dehydrogenase, based on a coupled reaction with immobilized bacterial luciferase and oxidoreductase, are described. The linear ranges are 1×10^{-6} - 1×10^{-3} M for L-glutamic acid and 0.015 - 0.3 pmol for glutamate dehydrogenase. The reproducibility is less than 5.8% (n = 5) for both assays and the sample throughout is 60 h^{-1}.

Introduction

Glutamate dehydrogenase (GLDH EC 1.4.1.3) is a useful diagnostic indicator of liver diseases and L-glutamic acid is an important parameter in food chemistry[1]. GLDH catalyzes the oxidation of L-glutamic acid to 2-oxoglutaric acid, in the presence of NAD as a cofactor, according to the following reaction:

$$HOOC\text{-}CH(NH_3^+)\text{-}CH_2\text{-}CH_2 = COO^- + NAD^{\pm} + H_2O \text{----} HOOC\text{-}C(=O)\text{-}CH_2\text{-}CH_2\text{-}COO^- + NADH + NH_4^+$$

The rate of formation of NADH can be monitored at 340 nm and is directly related to L-glutamic acid concentration under appropriate reaction conditions[2]. However, the equilibrium position of the reaction favours the formation of L-glutamic acid and for a more sensitive procedure the equilibrium must be shifted to the right by removal of NADH.

The bioluminescent bacterial luciferase/ oxidoreductase system, which requires NADH as a cofactor, can be coupled with the above reaction to provide a very sensitive method for L-glutamic acid. Batch procedures using this method have been reported with a detection limit of 100 pmol[3] and 1 pmol[4]. More recently, the method has been adapted to an airsegmented continuous flow procedure with a detection limit of 0.01 pmol, a sample throughput of 30 h^{-1} and a reproducibility of 5.0% (n = 10)[5].

This paper describes flow-injection procedures for GLDH and L- glutamic acid using light emission from the bacterial luciferase- oxidoreductase system as the end point. The enzymes are immobilized on Sepharose 4B via cyanogen bromide to reduce reagent costs.

Experimental

Reagents and standards

All solutions were prepared in phosphate buffer (0.05 M) and stored at 4°C and all reagents were obtained from Sigma unless otherwise stated. Phosphate buffer (0.05 M) was prepared by dissolving 6.8g of potassium hydrogen orthophosphate (BDH) in distilled, de-ionized water (1l) and adjusting to the required pH with hydrochloric acid (2 M). Stock solutions of NAD (0.01 M), flavin mononucleotide (FMN; 0.01 M) and dithiothreitol (DTT; 0.1 mM) were prepared in phosphate buffer at pH 7.0. A stock of emulsified decanal solution was prepared by solubilizing decanal (0.01% v/v) with Triton X-100 (0.001% v/v) in phosphate buffer at pH 7.0.

A stock solution of GLDH (1×10^{-6} M) was prepared by dissolving 20 mg bovine liver GLDH

(E.C. 1.4.1.3; 390 U mg^{-1}) in phosphate buffer (100 ml) at pH 7.5. Standards covering the range $5x10^{-10}$ -$1x10^{-7}$ M were prepared by serial dilution of the stock in phosphate buffer at pH 7.5. The relative molecular mass of bovine liver GLDH is 2,200,000.

Enzyme immobilization

The co-immobilization of bacterial luciferase/oxidoreductase on Sepharose 4B has been described previously[6]. GLDH was immobilized by the same procedure. A 1 mg amount of GLDH (390 U mg^{-1}) was separately dissolved in 4 ml of sodium hydrogen carbonate solution (0.1 M) at pH 8.0. Cyanogen bromide activated Sepharose 4B beads (0.5 g) were added and the mixture was stirred gently for 16 h at 4°C. The immobilized GLDH was washed with buffer and stored at 4°C.

Instrumentation and procedures:

Enzyme activity determination: Bacterial luciferase and oxidoreductase activity were measured using a batch luminometer (Berthold LB 9500) as described previously[6]. GLDH activity was measured using a flow through spectrophotometer (LKB Ultrospec) fitted with an 8 ul flow cell. The supernatant from the immobilization step containing unbound GLDH (25 µl) was injected into a stream of phosphate buffer (0.05 M) containing NAD (0.01 M) and L-glutamic acid (0.01 M) via a rotary injection value (Rheodyne 5020). The carrier stream was pumped at 1.0 ml min^{-1} using a peristaltic pump (Ismatec Mini S840) and the absorbance was monitored at 340 nm.

GLDH determination: The merging-zones flow-injection manifold for the determination of GLDH is shown in Fig.1. GLDH standards (30 µl) covering the range 0.015-30 pmol and FMN (1 x 10^{-6} M) were simultaneously injected into separate carrier streams using a dual-injection rotary valve, GLDH into a phosphate buffer stream at pH 7.5 containing NAD (5 x 10^{-3} M), L-glutamic acid (5 x 10^{-2} M) and DTT (1 x 10^{-4} M) and FMN into a phosphate buffer stream at pH 7.5 containing emulsified decanal (0.001% v/v). The carrier streams were pumped at 0.8 ml min^{-1} using a peristaltic pump (Ismatec Mini S840) and PTFE tubing (0.5 mm. i.d.) was used throughout the remainder of the manifold. The two zones were merged 12.5 cm downstream and then travelled 2.2 cm before passing into a glass coil (60 x 2.5 mm) containing immobilized bacterial luciferase/oxidoreductase (0.1 g beads). The detector was an end window photomultiplier tube (Thorn EMI 9789 QB) located in a light tight housing[7].

L-Glutamic acid determination: The manifold and procedures were as described above for GLDH. L-Glutamic acid standards (30 ul) covering the range 1 x 10^{-6} - 1 x 10^{-2} M and FMN (1 x 10^{-6} M) were simultaneously injected into separate carrier streams, L-glutamic acid into a phosphate buffer stream at pH 7.5 containing NAD (5 x 10^{-3} M) and DTT (1 x 10^{-4} M) and FMN into a phosphate buffer stream at pH 7.5 containing decanal (0.001% v/v). The glass coil contained immobilized GLDH (0.1 g beads) and co-immobilized bacterial luciferase/oxidoreductase (0.1 g beads), packed in series.

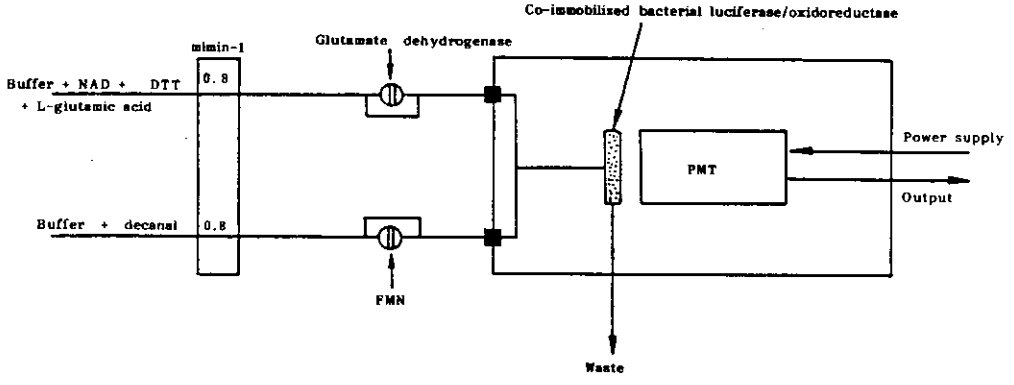

Fig. 1: Flow-injection manifold for the determination of GLDH using co-immobilized bacterial luciferase/oxidoreductase.

The above analysis was also carried out using soluble GLDH in order to compare the performance of the free and immobilized enzymes. For these experiments GLDH (220 mg l^{-1}; 1 x 10^{-7} M) was pre-mixed with the L-glutamic acid carrier stream.

Results and Discussion

Optimization of the flow-injection manifold

The flow rate was shown to be inversely related to sensitivity over the range 0.8 - 3.0 ml min^{-1} per channel due to lower sample dispersion at low flow rates and a relatively slow turnover rate for luciferase[8]. A flow rate of 0.8 ml min^{-1} was therefore used for all subsequent experiments. The concentrations of FMN (1 x 10^{-6} M), NAD (5 x 10^{-3} M) and decanal (0.001% v/v) used were the rate limiting concentrations for both GLDH and L- glutamic acid over the ranges studied.

Calibration data for GLDH

Fig. 2 shows a log-log calibration graph of GLDH injected (0.015 - 3.0 pmol) versus light intensity. The response is linear over the range 0.015 - 0.3 pmol.The sensitivity is shown inTable 1 and relative standard deviations were all less than 5.8% (n = 5). The limit of detection is determined by the magnitude of the blank signal which arises from the presence of contaminating dehydrogenases in the bacterial extract[9].

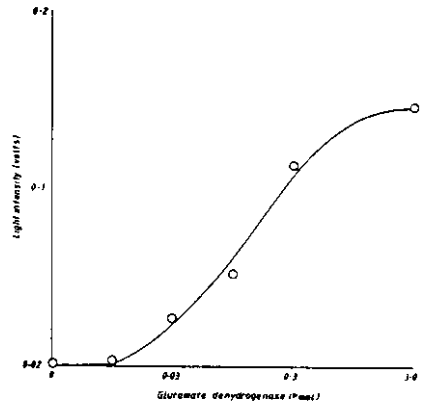

Fig. 2: Calibration graph of GLDH concentration versus light intensity.

Calibration for L-glutamic acid

The protein recovery for GLDH on Sepharose 4B was 60%. Protein recoveries for bacterial luciferase (86%) and oxidoreductase (96%) have been reported previously[6] and were shown to be the major factor in limiting sensitivity.

Fig. 3 shows a log-log calibration graph of L-glutamic acid concentration (1 x 10^{-6} - 1 x 10^{-2} M) versues light intensity using the immobilized GLDH manifold, with a linear range of 1 x 10^{-6} - 1 x 10^{-3} M). The limit of detection is again determined by the background signal from the blank. The sensitivity is shown inTable 1 and is compared with the

Table 1: Calibration data for GLDH and L-glutamic acid

Calibration data for GLDH		Calibration data for L-glutamic acid			
		with immobilized GLDH		with soluble GLDH	
Concentration (pmol)	Output (V)	Concentration (mol l^{-1})	Output (V)	Concentration (mol l^{-1})	Output (V)
0	0.020	0	0.080	0	0.030
0.015	0.022	1 x 10^{-6}	0.130	1x10^{-5}	0.035
0.030	0.026	1x10^{-5}	0.218	1x10^{-4}	0.060
0.15	0.050	1x10^{-4}	0.500	1x10^{-3}	0.123
0.30	0.114	1x10^{-3}	0.856	1x10^{-2}	0.312
3.0	0.145	1x10^{-2}	1.090	1x10^{-1}	0.563

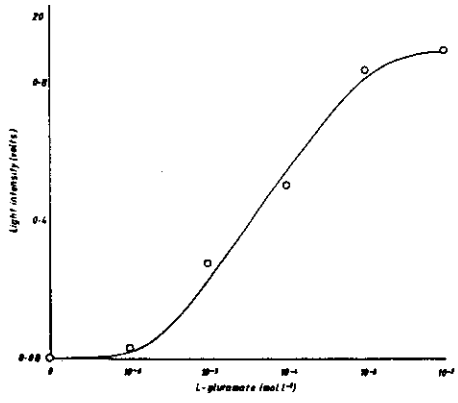

Fig. 3: Calibration graph of L-glutamic acid concentration versus light intensity.

results obtained using free GLDH. Clearly, the sensitivity and reagent cost of the immobilized system are much better than those of the free system under the conditions reported. The relative standard deviations were comparable for the two systems, being less than 4.2% in all cases.

Conclusions

The results show that bacterial luciferase/oxidoreductase co-immobilized on Sepharose 4B within a flow-injection manifold can be coupled with an NADH producing reaction for the determination of L-glutamic and glutamate dehydrogenase.

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