Pertanika 3(1), 1–4 (1980)

Immobilised Glutamate Dehydrogenase: Possible Use in Automated Analysis.

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Key words: Immobilised, Glutamate dehydrogenase

RINGKASAN

Glutamat dehydrogenase dilekat ke dalam saluran 'nylon' dan aktiviti enzim yang terlekat dikaji. Dua jenis molekul 'spacer' dan dua teknik penyambung digunakan untuk perlekatan enzim ini.

Aktiviti yang agak rendah diperolehi di dalam saluran tetapi bila saluran mengandungi enzim ini disambungkan ke dalam sistem pengaliran terus bagi menganalisa larutan piawaian ammonia, aktivitinya mencukupi untuk menghasilkan tindakan yang boleh diukur.

Kajian ini menunjukkan kemungkinan penggunaan 'immobilised' glutamat dehydrogenase di dalam kaedah analitik biasa.

SUMMARY

Glutamate dehydrogenase was immobilised onto nylon tube and the activity of the enzyme tube investigated. Two different spacer molecules and two coupling techniques were utilised for immobilising the enzyme.

Relatively low activity was retained on the tube, but when the enzyme tube was incorporated into a continuous flow system, and used to assay standard ammonia solution, there was adequate response for measurement.

The study indicated the potential use of immobilised glutamate dehydrogenase in routine analytical operation.

INTRODUCTION

Present methods for the analysis of blood ammonia and urea still utilise chemical reagents for colour formation (Gutman and Bergmeyer, 1974; Szasz, 1974), which may render the methods to problems of unspecific reactions, resulting in inaccuracy in estimating the levels of these compounds. The advantages of using enzymes in clinical analysis have been discussed (Bergmeyer, 1965). Enzymatic methods for estimation of ammonia and urea have been developed (Kun and Kearney, 1975, Gutman and Bergmeyer, 1975). The enzymatic reactions are as follows:

$$\text{Urea} + H_2O \xrightarrow{\text{urease}} CO_2 + 2NH_3$$

$$NH_3 + \text{2-oxoglutarate} + \text{NADH} \xrightarrow{\text{GDH}} \text{glutamate} + \text{NAD} + H_2O$$

A recent advance in enzymatic methods is the use of immobilised enzymes, which can be incorporated into a continuous flow system resulting in highly precise, efficient and economical operations (Hornby and Noy, 1976). Urease has been successfully immobilised into a tubular reactor, and used in continuous mode operations, coupled to the Berthelot reaction for analysis of urea (Filippusson *et al*, 1972).

GDH = glutamate dehydrogenase (L-glutamate : NAD(P) oxidoreductase (deaminating) (EC 1.4.1.3)
DAE = diaminoethane. A.F.U. = Arbitrary Fluorescent Unit.

Glutamate dehydrogenase (GDH) has been immobilised onto collagen membrane, but was used mainly for kinetic studies (Julliard *et al*, 1971). Preliminary studies on the immobilisation of GDH in a tubular reactor as a possible replacement for the soluble enzyme in clinical analysis were carried out and the results are reported in this paper.

MATERIALS AND METHODS

Activation of nylon tube

A nylon tube (2 m length, 1 mm internal diameter), purchased from Portex, Hythe, England, was activated by triethyloxonium tetrafluoroborate by the method developed by Morris *et al* (1975).

Introduction of Spacer Molecules

The activated nylon tube was filled with 4% (w/v) solution of adipic dihydrazide in formamide or with solution of diaminoethane (DAE), and incubated for 2h. The tube was washed with distilled water.

Coupling of GDH

Before coupling, the nylon-spacer tube was reactivated. Two procedures were adopted. The nylon-spacer tube was perfused with 5% (w/v) glutaraldehyde in 0.2 M borate pH 8.5 for 10 min, or with 4% (w/v) dimethyl suberimidate in N-ethylmorpholine for 10 min (Morris *et al*, 1975).

GDH (bovine from Boehringer Manheim Germany) was dissolved in 0.1 M phosphate buffer pH 7.5. The activated nylon tube was filled with the enzyme solution and incubated for 3h at 4°. The tube was washed with the coupling buffer and buffer containing 0.2 M NaCl.

Assay of enzyme activity

Soluble GDH

Soluble enzyme activity was assayed as described in Biochimica Information II (Boehringer) using 0.1 M phosphate pH 7.5 as the assay buffer.

Immobilised GDH

Immobilised GDH was either assayed by the recirculation method (Ford *et al*, 1972) or by incorporation into the Technicon Autoanalyser 1 flow system as shown in Fig. 1.

In the recirculation method, the concentration of reactants was similar to that in the soluble assay. A total volume of 10 ml was used in the assay.

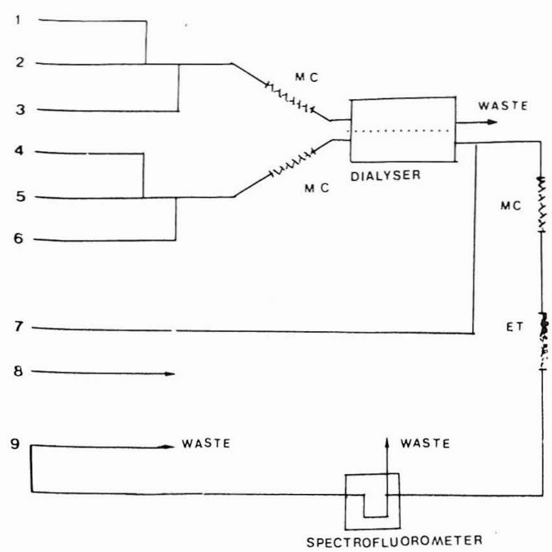

Fig. 1. Flow system for assaying ammonia standards using nylon-immobilised GDH.

Keys to the figure.

Line	*pumping rate (ml/min)*
1. Air	*0.6*
2. Buffer, 0.1M phosphate, pH 7.5 5mM EDTA 0.2M NaCl 0.05% Triton X100	*1.6*
3. Sample, aqueous NH_4Cl	*0.16*
4. Air	*0.6*
5. Buffer (as in line 2)	*1.6*
6. Buffer (as in line 2)	*0.16*
7. NADH, 1mM 2-oxoglutarate, 20mM	*0.32*
8. Sample wash	*1.0*
9. Flow cell	*1.6*

MC – mixing coil
ET – enzyme tube

The pump and dialyser units are standard Technicon AutoAnalyser AA1 modules. NAD was monitored at 365nm excitation and 465nm emission wavelength by the PerkinElmer 1000 spectrofluorometer (fitted with a 1.6 ml flow cell) and recorded on a Kipp and Zonen DB8 recorder.

The mixing coils and the dialyser were maintained at 37°.

RESULTS

The data shown in Table 1 represent typical results obtained when a relatively low percentage

TABLE 1

Coupling data for the immobilisation of GDH onto nylon tube. The data are obtained from nylon-adipic tube. A volume of 0.8 ml enzyme solution was required to fill 1m tube.

Coupling technique	Activity (U/ml)		tube activity U/m	% activity coupled	% activity retained
	coupling solution	post-coupling solution			
glutaraldehyde	2.04	1.12	0.12	45	5.9
imidate	2.04	1.69	0.17	17	8.3

of enzyme activity was retained on immobilisation. The difference in the overall activity coupled and retained, using two coupling techniques, was apparent in all experiments.

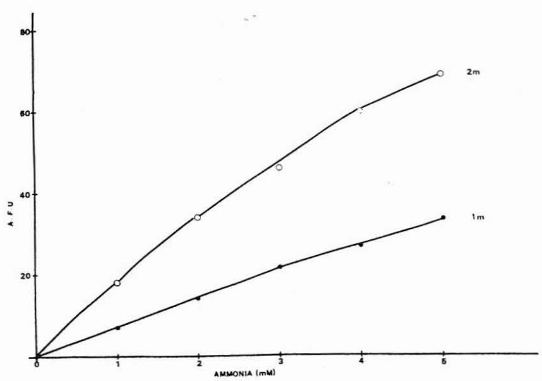

Fig. 2. Calibration curves obtained using 1m and 2m immobilised GDH tube. The tube was incorporated on to the flow system shown in Fig. 1.

Fig. 2 and 3 show results when immobilised GDH tubes were incorporated into a continuous flow system. Fig. 2 shows the calibration curves obtained when aqueous ammonia standards were assayed using 1 m– and 2 m– tube length. Fig. 3 shows the response when tubes prepared by using different spacer molecules and coupling techniques were incorporated into the flow system. Adipic dihydrazide as spacer molecules seemed to produce higher activity tubes. Imidate coupling also resulted in better activity retention, as also shown in Table 1.

The pH profile of the immobilised and soluble enzyme is shown in Fig. 4. There seemed to be no apparent variation in the pH optimum of the enzyme.

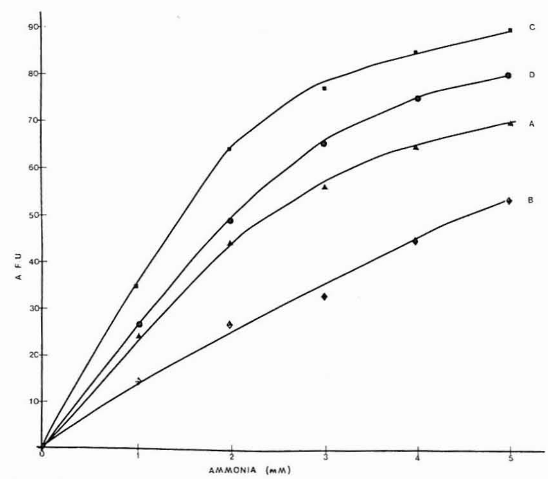

Fig. 3. Calibration curves obtained using 2m tube with different spacers and coupling techniques. GDH was attached to nylon-adipic tube by glutaraldhyde (A) and imidate (C) coupling and to nylon-DAE tube by glutaraldehyde (B) and imidate (D) coupling.

DISCUSSION

The results show fairly low retention of enzyme activity on immobilisation. The loss in activity was not unexpected. The enzyme com prises subunits which may be dissociated into inactive subunits (Frieden, 1959). The immobilisation procedure may contribute towards this dissociation. Covalent attachment usually produces low activity retention. Glutaraldehyde randomly reacts with free amino groups and thus may react with certain active site amino groups or amino groups essential for comformational structure. Imidate, though less efficient in protein coupling, actually retained higher enzymes activity.

The hydrophobicity of the support material may further enhance the enzyme denaturation,

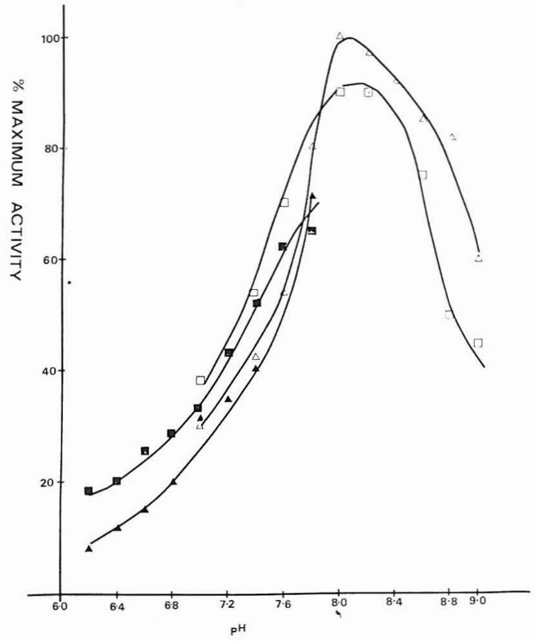

Fig. 4. pH profiles of soluble and immobilised GDH. The soluble (triangles) and immobilised (squares) enzyme were assayed in phosphate buffer at lower pH and TRIS-HCl buffer at the higher pH. The relative activities were calculated by comparing the activities of soluble and immobilised GDH with the highest activity obtained in the experiment for the soluble and immobilised enzyme respectively.

although the use of appropriate spacer molecules may overcome some of this effect. To a large extent, the right spacer has to be discovered by experimentation.

The applicability of immobilised GDH for use in continuous analysis was investigated by its incorporation into a continuous flow system. The calibration plots show that the response for the range of ammonia concentration used seemed to curve when a longer tube was used. This effect was probably due to the carry-over or sample interaction effect of the flow system, accentuated by exposure of the substrate to the long enzyme tube. It can be rectified by using a shorter enzyme tube (and thus the necessity of producing higher activity immobilised enzyme). The range of ammonia concentration used corresponded to that of urea in blood, and linking of urease and GDH in the immobilised form for urea analysis should be the ultimate objective.

The pH profiles indicate that the support and coupling procedures have not imposed a new ionic environment to the enzyme molecules nor does the resulting environment affect the catalytic activity as to cause any shift in the profile.

For its acceptance as a replacement for the soluble counterpart, studies on the stability of the immobilised GDH are essential. The whole analytical system has to be optimised for accuracy and precision as outlined by Broughton *et al* (1969). However, the present studies have shown the possibility of utilising these immobilised GDH for such a purpose.

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(Received 6 December 1979)