AFFINITY GEL FILTRATION: A NEW METHOD FOR THE RAPID DETERMINATION OF APPARENT MOLECULAR WEIGHTS OF ENZYMES

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1. Introduction

In classical gel filtration, the distribution coefficient, α, has values which lie within the range 0–1. Hence a long analytical column is required to resolve two proteins with similar distribution coefficients. The theory of affinity chromatography is well established [1–3] and has been extended recently to multisubstrate enzymes [4]. By linking the affinity ligand to the inside of the gel matrix, the distribution coefficient, α, can be made equal to 0 or 1; if the protein or enzyme is totally excluded then $\alpha = 0$, whilst if partly or wholly included, and consequently bound to the affinity ligand, then α can approach 1. The proportion of the enzyme bound to the column compared with that eluted in the void volume is directly related to the apparent molecular weight, since the latter determines the accessibility to the affinity ligand.

In a previous paper [4] we described the use of insolubilised cofactors for the affinity chromatography of NAD(P)-linked dehydrogenases. This report describes a micro-method for the rapid determination of apparent molecular weights of dehydrogenases based on their exclusion from gel filtration media of various pore sizes.

Abbreviations:

GDH	: L-glutamate dehydrogenase
G6PDH	: D-glucose 6-phosphate dehydrogenase
ICDH	: threo-Ds-isocitrate dehydrogenase
MDH	: L-malate dehydrogenase
LDH	: L-lactate dehydrogenase
TDH	: L-threonine dehydrogenase
GlucDH	: D-glucose dehydrogenase
BSA	: bovine serum albumin

2. Materials and methods

Enzymes were purchased from Boehringer (Mannheim) except for L-malate and D-glyceraldehyde 3-phosphate dehydrogenases, which were obtained from Sigma (London) Ltd. L-Threonine dehydrogenase was obtained from *Ps. oxalaticus* extracts, kindly donated by Dr. J.M. Turner. Sephadex G-25, G-50, G-100 and G-200 (medium grades) were obtained from Pharmacia (G.B.) Ltd. and NAD^+ was coupled directly to these by the CNBr technique of Axen and Porath [5].

The enzymes were assayed according to the methods cited by Barman [6], and protein in the column effluents was assayed by the ultraviolet absorption method of Warburg and Christian [7].

3. Results

Fig. 1 illustrates the affinity gel filtration of synthetic mixtures of LDH and MDH and BSA on NAD-Sephadex of several pore sizes. Both dehydrogenases appear in the void volume on columns of NAD-Sephadex G-25 indicating no significant binding to the exterior of the bead. MDH is included in NAD-Sephadex G-100, whilst LDH is not. It is pertinent to note that in the presence of dehydrogenases, serum albumin is excluded from all sizes of NAD-Sephadex, whilst it shows normal behaviour on Sephadex controls.

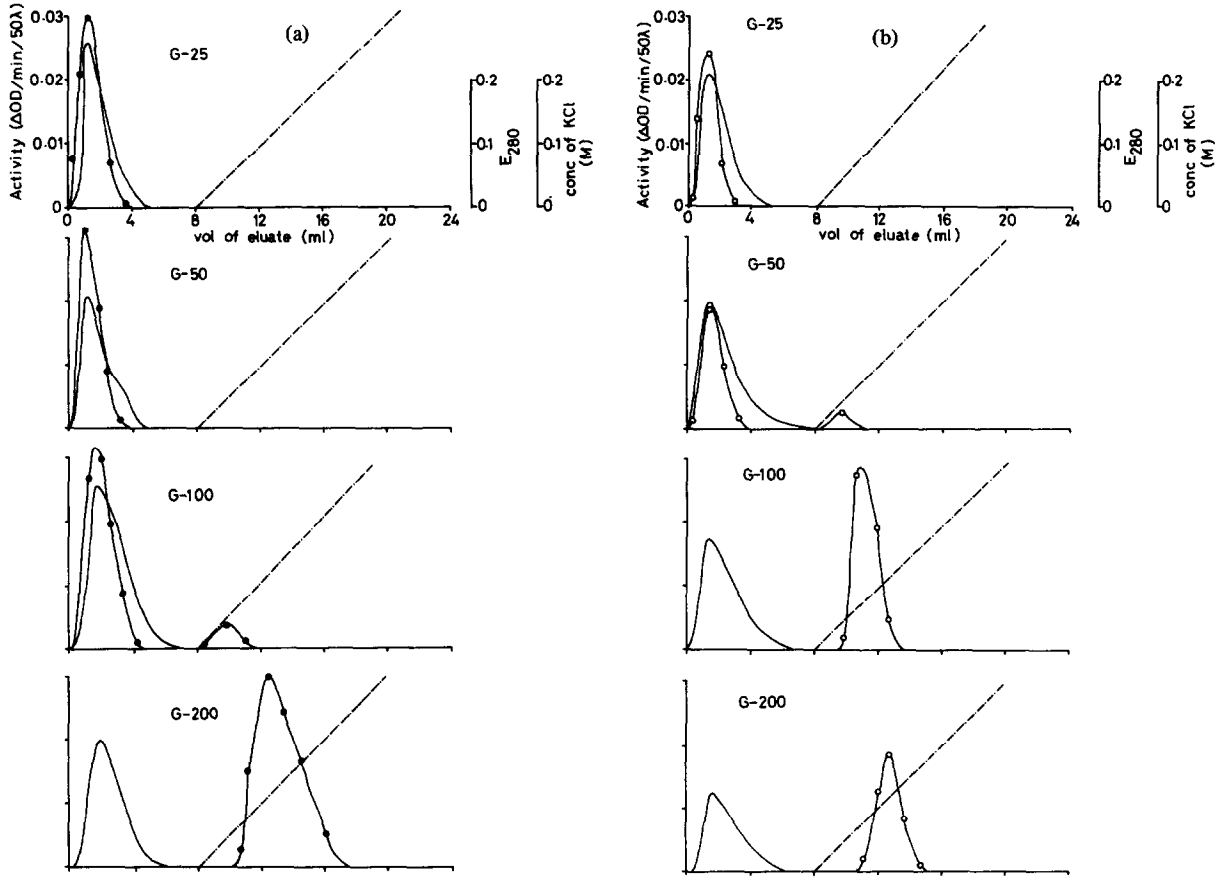

Fig. 1. Affinity gel filtration of synthetic mixtures of (a) lactate dehydrogenase, and (b) malate dehydrogenase with serum albumin on NAD-Sephadex of various pore sizes. A 50 μl sample containing 1.85 units of LDH (or 0.335 units MDH) and 0.8 mg BSA was applied to a 5 mm × 20 mm column of the appropriate NAD-Sephadex equilibrated with 10 mM Pi buffer, pH 7.5. Non-absorbed protein was washed off with the same buffer and the column eluted with a 0–0.5 M KCl gradient in 10 mM Pi buffer, pH 7.5; 20 ml total. LDH (•), MDH (○) and BSA (——) were assayed in the effluent.

An attempt to quantify the data for a range of purified dehydrogenases is shown in fig. 2. The proportion of enzyme eluted on the gradient of KCl (Eg) compared with the total enzyme (Eg + Eo), is plotted against the logarithm of the apparent molecular weight. Using this technique, interpolation gives an estimate of the apparent molecular weight of L-threonine dehydrogenase from crude extracts of *Ps. oxalaticus* as 154,000.

Significantly, however, it will be noted that the apparent molecular weights of GDH and GlucDH are approximately one half of those values generally accepted in the literature. Further complications are introduced when a mixture of MDH and LDH is applied to a similar range of NAD-Sephadexes (fig. 3). Whilst LDH alone is excluded completely from NAD-Sephadex G-100 (fig. 1a), in the presence of MDH both are eluted together at identical concentrations of KCl. The mixture can be resolved, however, on NAD-Sephadex G-200 (fig. 3). Similar results were obtained with the ICDH/GDH diad, when GDH alone is excluded from NAD-Sephadex G-100, whilst in the presence of ICDH, GDH is apparently able to enter the gel.

4. Discussion

In its simplest form, NAD(P) affinity gel filtration can be applied to the rapid determination of apparent

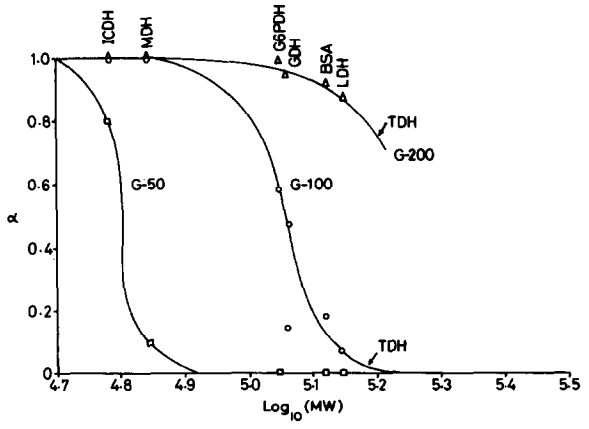

Fig. 2. Affinity gel filtration of several NAD(P)-linked dehydrogenases on NAD-Sephadex of several pore sizes. The ordinate, α, represents the number of units included (and hence retained) in the gel as a proportion of the total number of units recovered from the column.

molecular weights of NAD(P)-linked dehydrogenases. The use of micro-columns coupled with step-wise elution with 0.5 M KCl allows an assessment of the proportion of the enzyme bound to the column. Substitution into the curves shown in fig. 2 gives an approximate measure of the apparent molecular weight. The applicability of this method is illustrated in the case of L-threonine dehydrogenase from crude extracts of *Ps. oxalaticus*, to which an apparent molecular weight of 154,000 has been ascribed.

In the series of curves for each pore size type of Sephadex (fig. 2), the accurate fit of the dehydrogenases examined, and the anomalies such as GDH and GlucDH, suggest some interesting possible explanations. Are the apparent molecular weights of GDH and GlucDH altered in the presence of insolubilized NAD? The steric restrictions imposed by the micro-environment around the insolubilized NAD could have a considerable effect on the dissociation behaviour of multimeric dehydrogenases. The influence of MDH on the elution behaviour of LDH is, as yet, inexplicable. Similar results have been obtained with the other pairs of dehydrogenases ICDH and GDH, and the phenomenon is the subject of further investigation.

Extension of the general method of affinity gel filtration, by linking any ion-exchange or affinity

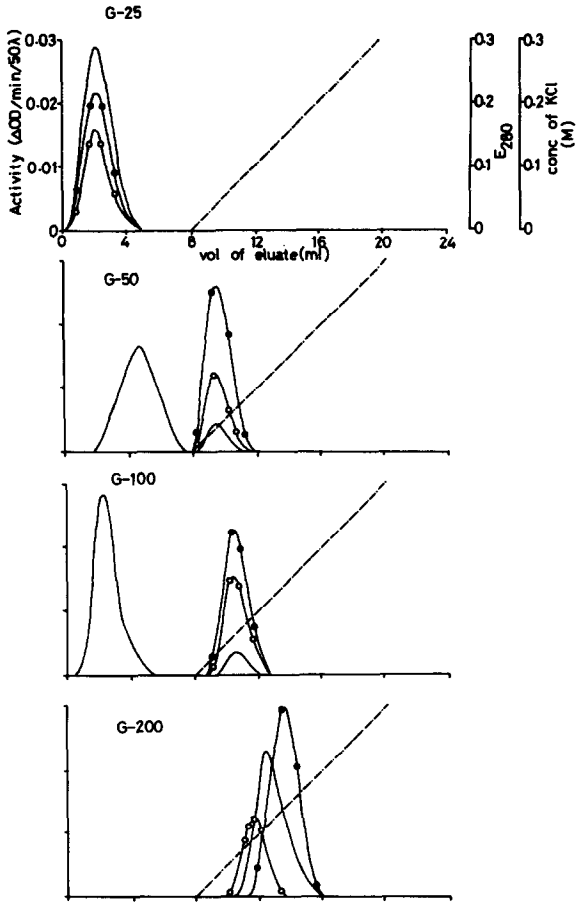

Fig. 3. Affinity gel filtration of synthetic mixtures of lactate dehydrogenase, malate dehydrogenase and BSA on NAD-Sephadex. A 50 μl sample containing 1.85 units LDH, 0.335 units MDH and 0.8 mg BSA was applied to a 5 mm × 20 mm column of the appropriate NAD-Sephadex equilibrated with 10 mM Pi buffer, pH 7.5. Non-absorbed protein was washed off with the same buffer and the column eluted with a 0–0.5 M KCl gradient in 10 mM Pi buffer, pH 7.5; 20 ml total. Malate dehydrogenase (○), LDH (●) and BSA (——) were assayed in the effluent.

ligand to Sephadex (or Agarose) gels of various pore sizes, permits the determination of the apparent molecular weights of many different groups of proteins or enzymes.

Acknowledgements

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