THE POTENTIAL OF ULTROGEL®, AN AGAROSE–POLYACRYLAMIDE COPOLYMER, AS A MATRIX FOR AFFINITY CHROMATOGRAPHY

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1. Introduction

The selection of the inert support in the synthesis of adsorbents for affinity chromatography is constrained by several factors. It should be stable to chemical and biological degradations, hydrophilic, easily substituted and uniform to enhance the chromatographic process. The comparative advantages of polyacrylamide, glass, cellulose, cross-linked dextrans and beaded agarose have been reviewed [1–3]; currently the most popular support materials are polysaccharides, especially beaded agarose.

Recently the microscopic structure of these polysaccharide matrices has been investigated both with respect to their ability to withstand various activation procedures, in particular cyanogen bromide [4], and also the distribution of the immobilised ligand throughout the matrix following coupling [5,6], which in itself may be a function of the CNBr activation [7]. In a previous communication [4] we reported that beaded cross-linked dextrans were susceptible to structural damage during CNBr activation, whilst beaded agarose was not obviously affected provided that vigorous stirring was avoided. However, microscopic inclusions and vacuoles were observed in approx. 5% of the commercially available agarose beads. Subsequently Gribnau et al. [8] reported the presence of similar particles in commercial preparations of agarose. The uncertain effect of these inclusions and vacuoles on the properties of affinity matrices has been commented upon by both authors.

This report describes the potential of a new range of matrices as insoluble supports for affinity chromatography adsorbents. These matrices are copolymers of agarose and polyacrylamide (Ultrogel®), the percentage of each constituent being varied to provide a range of gels with different pore sizes for use in gel filtration. As matrices for affinity chromatography these copolymers would seem to have all the advantages of each individual polymer, plus the availability of both amide and hydroxyl groups for activation thereby leading to the synthesis of wider range of derivatives.

Abbreviations: LDH, lactate dehydrogenase (EC 1.1.1.27); MDH, malate dehydrogenase (EC 1.1.1.37); YADH, yeast alcohol dehydrogenase (EC 1.1.1.1); N^6-AMP, N^6-(6-aminohexyl)-5′-AMP.

2. Materials and methods

Enzymes were purchased from Boehringer Corp., (London) Ltd. Ultrogel® was obtained from L.K.B. Products (Croydon, UK) and Sepharose® 4B purchased from Pharmacia (UK). CNBr was obtained from R. W. Emmanuel Ltd. (Wembley, Middlesex, UK). All other chemicals were obtained from B. D. H. Chemicals (Poole, Dorset, UK).

N^6-(6-Aminohexyl)-5′-AMP was prepared by the method of Craven et al. [9] and coupled to Ultrogel by either the CNBr method of Porath et al. [10] or by a modification of the procedure of March et al. [11]. A solution of CNBr (100 mg/g moist wt. matrix) in 10% (v/v) aqueous *N*-methylpyrrolidone was added to a slurry of Ultrogel, 1 g in 2 m K_2CO_3–$KHCO_3$, pH 11.0 (1 ml) at 4°C, and the mixture gently stirred

for 10 min. The reaction was stopped by rapid filtration and the activated gel was washed with ice-cold distilled water, 5% aqueous acetone (v/v), and 0.1 M $NaHCO_3$–NaOH, pH 9.5. The agarose moiety of Ultrogel was also activated by the periodate method of Sanderson and Wilson [12] with the following modifications: Ultrogel was oxidised with 0.1 M $NaIO_4$ in 10 mM KH_2PO_4–KOH, pH 7.5, washed thoroughly with 10 mM KH_2PO_4–KOH, pH 7.5 and incubated for 72 h at 4°C on a Coulter Mixer with 2 mM N^6-AMP in 0.1 M KH_2PO_4–KOH, pH 6.0. The gel was washed with ice-cold distilled water and 1 M KCl and resuspended in 0.1 M KH_2PO_4–KOH, pH 8.0, containing 1 M $NaBH_4$ at 4°C. After 48 h the gel was washed with ice-cold distilled water, 1 M KCl and 10 mM KH_2PO_4–KOH, pH 7.5.

The polyacrylamide moiety of Ultrogel was activated by either the acyl azide method of Inman and Dintzis [13] or by the glutaraldehyde method of Avrameas and Ternynck [14]. Coupling of the ligand was achieved by incubation for 16 h at 4°C on a Coulter Mixer with 2 mM N^6-AMP in 0.1 M KH_2PO_4–KOH, pH 7.5: the gel was washed in the above manner prior to use.

The ligand concentration was measured by both spectral analysis (267 nm) of the gel washings and phosphate analysis according to the procedure of Meun and Smith [15]. The phosphate content of N^6-AMP–Ultrogel was determined after drying the gel under reduced pressure with methanol and diethyl ether [9].

Crude dogfish (*Squalius acanthius*) muscle suspension was prepared by the method of Kaplan et al. [16]. Columns were prepared and run as documented by Craven et al. [9] and KCl gradients measured on a Radiometer CDM 3 conductivity bridge. Enzymes were assayed by the methods given by Barman [17] and protein measured by the ultraviolet absorption method of Warburg and Christian [18].

3. Results and discussion

The activation of the two constitutive polymers of Ultrogel was examined using procedures previously established for the individual matrices, namely agarose and polyacrylamide. Both Ultrogel moieties could be successfully activated and table 1 shows the percentage coupling of N^6-AMP to AcA 22 and 34 after each selected activation method. These methods show a range of efficiencies, varying from 43% (glutaraldehyde) to 83% (CNBr). The final ligand concentration, however, was not only dependent on the method used, but also on the reaction time of the coupling step and the original concentration of N^6-AMP. By changing the reaction conditions, higher ligand concentrations could thus be obtained.

On analysis of the CNBr activated gels, the method of Porath et al. [10], a discrepancy was observed between the ligand concentration determined by total phosphate content and that determined by spectral analysis of the gel washings, in that the phosphate concentration was 30% greater than expected from optical density measurements. The incorporation of inorganic phosphate promoted by this activation procedure prompted investigations of alternative CNBr procedures and currently we have found that a modification of the method of March et al. [11], detailed in Materials and methods, is the most satisfactory.

In contrast to the agarose matrix [4], microscopic observations of Ultrogel (fig.1) reveal a uniform physical size and a lack of microscopic inclusions and vacuoles. Following CNBr activation there was no

Table 1
The coupling of N^6-AMP to Ultrogel following different activation procedures

Method of activation	Ligand concentration (μmoles/ml)	% Ligand bound
AcA 34 acyl azide [13]	3.3	48
AcA 34 CNBr [10]	1.2	83
AcA 22 Glutaraldehyde [14]	1.5	43
AcA 22 Periodate [12]	1.0	49

Activation and coupling procedures are given in Materials and methods.

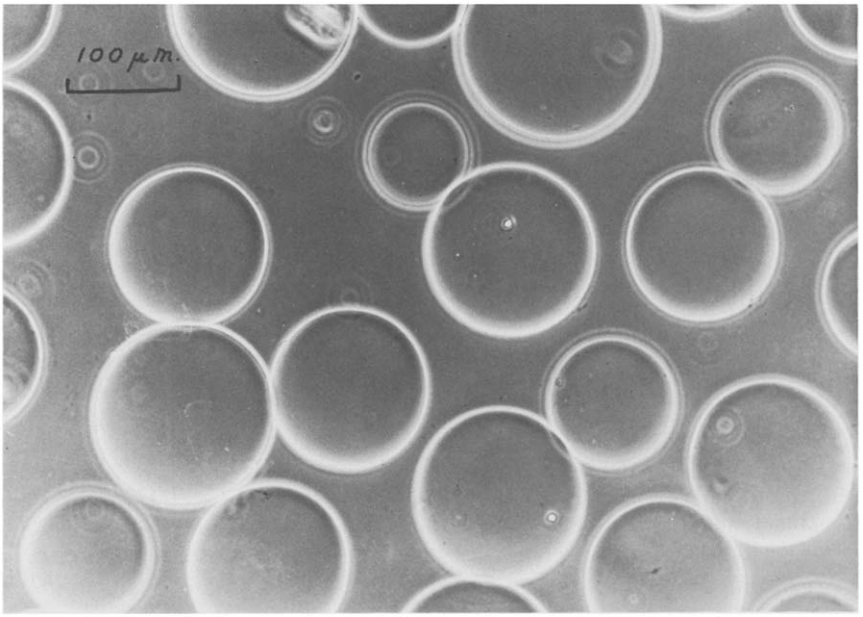

Fig.1. Unsubstituted Ultrogel-AcA-34, washed with ice-cold distilled water. Magnification, X 38, Zeiss Photomicroscope 11, phase contrast.

apparent change in either the size or the structure of the Ultrogel beads or agarose beads; this observation is in distinct contrast to Sephadex matrices [4]. These observations on the stability and structure of Ultrogel have prompted further investigations into the potential of this copolymer as an affinity chromatography matrix.

The binding of LDH-H_4 to a range of N^6-AMP–Ultrogel polymers has been investigated and the results are presented in table 2. Comparison of the Ultrogel polymers containing 4% agarose shows that increasing the polyacrylamide content decreases the strength of binding of LDH. Since increasing polyacrylamide concentration is directly related to a decrease in the pore size of the gel this effect could be due to the gel filtration properties of the polymers. However, this result might be a reflection of the non-uniform distribution of the ligand throughout the bead, especially as considerable trailing was observed in the salt elution of LDH from N^6-AMP–AcA 44 and 54.

Recent reports [5–7] have presented conflicting evidence concerning the distribution of immobilised ligands throughout the beaded agarose matrix. Therefore we cannot eliminate the explanation that a percentage of the immobilised ligand is substituted on the surface of the bead; the extent of this substitution having a direct bearing on the enzyme–ligand interaction.

Quantitative comparison between the four polymers

Table 2
The interaction of LDH-H_4 with a range of N^6-AMP–Ultrogel polymers

Polymer		Ligand concentration (μmoles/ml)	Binding (β)
(A)	AcA 22	1.0	0.50
(B)	AcA 34	1.1	>1.0[a]
(C)	AcA 44	1.2	0.44
(D)	AcA 54	1.7	0.22

Binding (β) is a measure of the enzyme–immobilised nucleotide interaction, equivalent to the KCl concentration (M) at the centre of the enzyme peak, where the enzyme is eluted with a linear KCl gradient. 5 I.U. of LDH (pig heart) was applied to columns containing 1 g moist wt. of Ultrogel. For details of determination of ligand concentration and chromatographic procedures see Materials and methods.
[a]5 mM NADH pulse required to effect elution (250 μl).

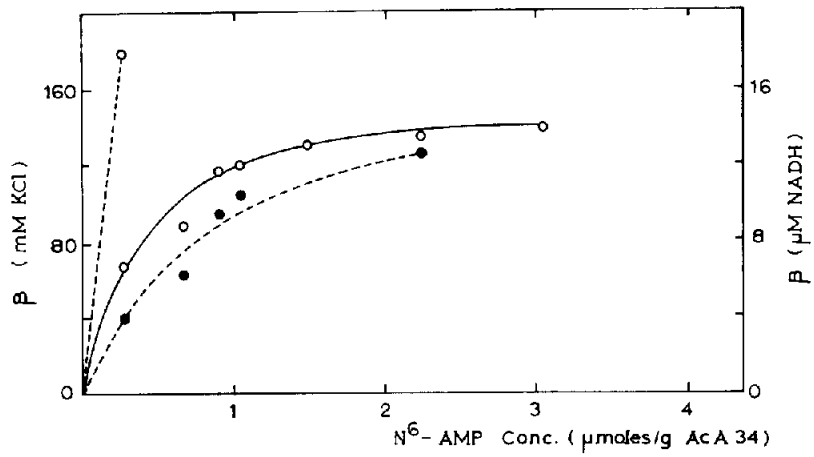

Fig.2. Binding (β) of lactate dehydrogenase in malate dehydrogenase to N^6-AMP–Ultrogel-AcA-34 in relation to ligand concentration. Experimental conditions and procedures are described in Materials and methods and the binding (β) defined in table 2. Elution was effected with linear gradients of KCl (——) or NADH (——) prepared in 10 mM KH_2PO_4–KOH, pH 7.5. (○) Lactate dehydrogenase. (●) Malate dehydrogenase.

was not possible due to variable ligand concentration. The effect of ligand concentration on the binding of dehydrogenases was examined using N^6-AMP coupled to AcA 34. Fig.2 shows that MDH and LDH gave a non-linear, hyperbolic response to increasing ligand concentration and that the form of this response was independent of the eluant employed, viz. KCl and NADH. The non-linear response to ligand concentration could also be a reflection non-uniform distribution of the interacting ligand, in that the observed β values represent the strength of the interaction of LDH with N^6-AMP substituted on the surface of the bead. It could be inferred from fig.2 that above a ligand concentration of 1.5 μmol/g no further enzyme will bind, thus the capacity of this affinity matrix under the same conditions should plateau at this ligand concentration. Preliminary investigations indicate that the capacity continues to increase on raising the

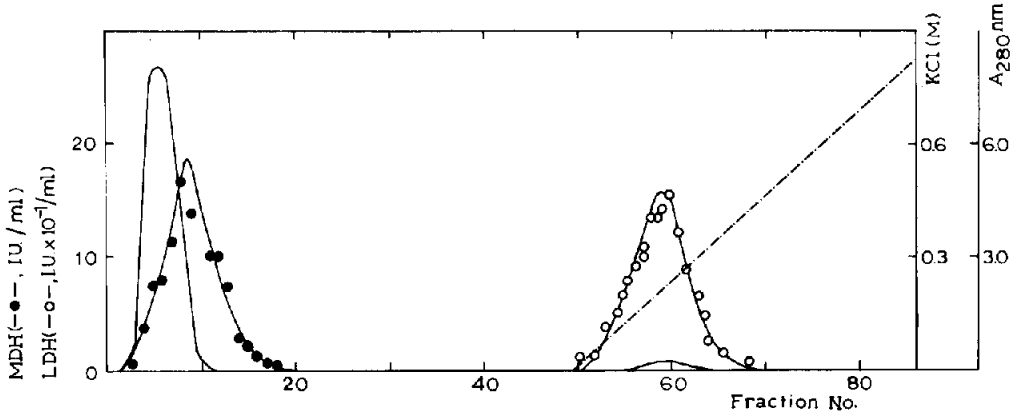

Fig.3. Separation and purification of lactate dehydrogenase from dogfish muscle. Digfish muscle extract (70 g tissue) was applied to a column (0.9 × 30 cm, 17 ml) of N^6-AMP–Ultrogel-AcA-34 in 0.1 M KH_2PO_4–KOH, pH 7.5, and washed with this buffer at a flow rate of 20 ml/h. Fractions (9.6 ml) were assayed for malate dehydrogenase (–●–), lactate dehydrogenase (–○–), protein (——) and KCl concentration (–.–.–) by the procedures given in Materials and methods.

ligand concentration, therefore alternative explanations to these two phenomena must be sought. Under the same experimental conditions we could not observe any interaction between YADH and N^6-AMP–AcA 34: these results are in contrast to the previous observations on N^6-AMP–Sepharose [9], and thus a further variable is available for the manipulation of enzyme separations from crude systems.

Fig.3 illustrates the separation and purification of LDH from a dogfish muscle homogenate: LDH was eluted with a linear KCl gradient and yielded a peak specific activity of above 690 units/mg protein at 22°C (cf. [16]). MDH was retarded by the affinity matrix and separation from the bulk protein could be achieved simply by increasing the column length. In our hands this purification of LDH is more rapid than the method of Kaplan et al. [16] since the LDH eluted from N^6-AMP–Sepharose, with either KCl or NADH–pyruvate adduct, was contaminated by MDH. Thus it appears that N^6-AMP–AcA 34 offers an increased selectivity over the corresponding beaded agarose and, furthermore, the use of substrate adducts in the purification scheme can be replaced by a simple KCl gradient.

In summary, Ultrogel presents a new affinity matrix, capable of many methods of activation, having a defined and regular structure, and a stability to most accepted practices in affinity chromatography. In addition preliminary observations with group-specific ligands indicate differences in the selectivity of Ultrogel and Sepharose affinity adsorbents.

Acknowledgements

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