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Note

Cibacron Blue 3G-A-substituted cross-linked agar beads: an inexpensive, fast-flow affinity medium for large scale applications

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Affinity chromatography using immobilized dyes has become a standard technique for rapid purification of proteins, especially of dehydrogenases and kinases. In the case of pig heart nucleoside diphosphate kinase on Cibacron Blue 3G-A–Sepharose CL-4B, batch adsorption followed by elution led to a purification factor as high as 450 in one step[1]. Although the best resolution is obtained using a solid phase comprised of small beads, for the optimization of new purification procedures we required an inexpensive porous affinity material with larger beads. This was necessary to obtain higher flow-rates when enzymes were adsorbed from turbid crude extracts in column procedures and better filtration properties when enzymes were adsorbed in batch procedures. Large agar or agarose beads cross-linked with epichlorhydrin by the standard procedure[2] did not withstand high flow-rates. Since no such material was commercially available we developed a procerdure starting from agar. Much harder particles were obtained by cross-linking with epichlorhydrin in aqueous ethanol.

EXPERIMENTAL

Preparation of Cibacron Blue 3G-A immobilized on beaded, cross-linked agar

A 4% solution of agar (either purified Noble Difco agar or raw agar fibres for bacteriology) was obtained by heating the pre-swollen material in an autoclave for 45 min at 120°C. Agar beads were obtained as described[3] and were subsequently kept in aqueous suspension for at least 3 days at room temperature, for ageing of the agar structure. For cross-linking, the agar beads were recovered by vacuum filtration and suspended in a freshly made solution of 1 *M* KOH containing 10% epichlorhydrin in 95% ethanol. The suspension was slowly warmed to 37°C, kept at this temperature for 3 days, filtered and washed with a large volume of hot distilled water. Cibracron Blue 3G-A (Fluka, Switzerland) was immobilized on the beaded agar as described[4], except that after heating at 80°C the suspension was kept at 37°C for 3 days before washing.

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Analytical procedures

The amount of immobilized Cibacron Blue 3G-A was measured as described[5]. The protein was estimated by the biuret procedure[6] and haemoglobin by measuring the optical density at 576 nm[7].

RESULTS

Properties of the affinity material

The spherical particles of agar had a diameter of 350 μm (standard deviation of the mean 150 μm). Their dimensions did not change during the cross-linking procedure. The affinity material allowed a very high flow-rate for an extended period of time at a very low driving pressure. A packed column of 15 × 3.5 cm withstood a flow-rate of 30 ml/min (or fourteen column volumes per h) under a hydrostatic pressure of only 100 cm of water. No reduction in the flow-rate was noted after 3 days. The concentration of bound dye was 2.3 μmol per ml of packed gel. The loading capacity of the material was 26.2 mg of haemoglobin (at pH 6.2) or 17.5 mg of albumin (at pH 8.3) per ml of packed gel.

Partial purification of yeast nucleoside diphosphate kinase

Dried brewer's yeast was extracted with 0.1 *M* ammonium sulphate pH 8.0 (7 ml/g) for 2 h at 37°C, centrifuged for 30 min at 3000 rpm (1000 *g*) and desalted by gel filtration on a Sephadex G-25 M column equilibrated with 50 m*M* sodium phosphate buffer (pH 7.0) containing 2 m*M* ethylenediaminetetraacetate (EDTA). The turbid solution was adjusted to pH 8.0, then adsorbed at room temperature on a 100 × 1.5 cm column of Cibacron Blue 3G-A-stubstituted cross-linked agar. The weakly bound proteins were washed with 2.5 column volumes of 50 m*M* Tris–HCl (pH 8.0)

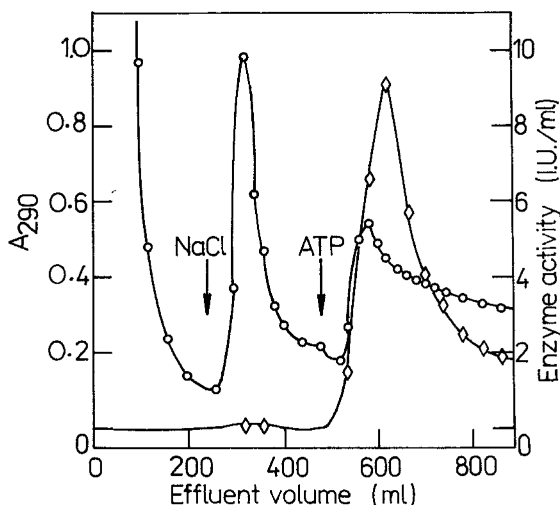

Fig. 1. Elution profile of the partial purification of yeast nucleoside diphosphate kinase, adsorbed on Cibacron Blue 3G-substituted, cross-linked agar. The column (100 × 1.5 cm) was eluted at a rate of 3 ml/min with 50 m*M* Tris–HCl, 2 m*M* EDTA, pH 8.0 buffer, followed by the same buffer supplemented with 0.25 *M* NaCl and with 0.25 *M* NaCl, 1 m*M* ATP. O—O, $A_{290}^{1 cm}$; ◇—◇, enzyme activity.

TABLE I

PARTIAL PURIFICATION OF YEAST NUCLEOSIDE DIPHOSPHATE KINASE BY AFFINITY CHROMATOGRAPHY ON CIBACRON BLUE 3G-A-SUBSTITUTED, CROSS-LINKED AGAR

Nucleoside diphosphate kinase activity was measured by a spectrophotometric assay[8]. The reaction medium, final volume 1.0 ml, at 25°C contained 50 m*M* Tris–HCl (pH 7.4), 75 m*M* KCl, 6 m*M* $MgCl_2$, 1 m*M* phosphoenol pyruvate, 0.1 m*M* NADH, 0.3 m*M* ATP and 2 units of lactate dehydrogenase and pyruvate kinase. The reaction was started by the addition of 0.1 m*M* 8-bromoinosine 5′-diphosphate. The absorbance decrease at 366 nm was recorded.

Stage	*Volume (ml)*	*Protein (mg/ml)*	*Enzyme activity (units/ml)*	*Specific activity (units/mg protein)*	*Yield (%)*	*Purification factor*
Desalted yeast extract	310	11.4	10.4	0.91	(100)	(1)
Eluted from Cibacron Blue 3G-A-substituted agar	300	0.196	4.2	21.4	39	23.5

containing 2 m*M* EDTA and 0.25 *M* NaCl. The nucleoside diphosphate kinase was eluted by adding 1 m*M* ATP to the washing buffer (Fig. 1). Details of the purification are also given in Table I.

DISCUSSION

The affinity material was primarily developed for the affinity chromatography of dehydrogenases and kinases, but may also be useful for other proteins. The hard, spherical particles had very good flow properties and should find applications in purification procedures that employ affinity chromatography as the first purification step, starting with turbid crude extracts. The high purification factor obtained in the partial purification of yeast nucleoside diphosphate kinase demonstrates the efficiency of this material. The yield can however be increased by working at low temperature, or by the addition of glycerol, to stabilize the enzyme. It is worth noting that inexpensive raw agar could be used instead of purified agarose.

It was previously shown that, in several cases, separations on Blue Sepharose involve the ion-exchange properties of the material[9]. This is particularly evident at low pH. The so-called "specific" binding of dehydrogenases and kinases takes place at pH $\geqslant$ 8.0 at which the ionic interactions are either weak or absent. Under such conditions, the effect of the sulphate groups of the agar (present in similar amounts to those of the sulphonic groups of the immobilized dye) is negligible in most cases.

The cross-linking procedure is very efficient, leading to hard particles. In further experiments (not shown) we omitted the relatively complicated beading procedure and obtained irregularly shaped agar with good flow properties. Finally, we obtained spherical magnetic particles by applying essentially the same procedure as that described in the Experimental by inclusion of 40 g magnetite (particle size *ca.* 2 μm) per litre of agar solution.

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