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GENERAL CHROMATOGRAPHIC PURIFICATION PROCEDURE BASED ON THE USE OF HETEROBIFUNCTIONAL AFFINITY LIGANDS

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SUMMARY

A new method for affinity purification is described. A heterobifunctional ligand in a soluble form is added to the sample containing the substance to be purified. After binding of the substance with one of the functional groups of the ligand, the complex formed is isolated by passage through a sorption column having affinity for the second part of the heterobifunctional ligand. In a stepwise elution process, first the substance of interest is isolated, followed by the ligand which can then be reused. Model studies on the purification of lactate dehydrogenase using a heterobifunctional ligand containing Cibacron Blue and soy bean trypsin inhibitor are described. The affinity matrix used was trypsin immobilized on Sepharose.

INTRODUCTION

Affinity chromatography has been a very successful technique on the laboratory scale[1,2], but so far very few large scale applications have been described[3]. The procedure used today is, in essence, the same as that described 15 years ago. Few attempts to develop modified affinity purification procedures have been published. The affinity purification of protein by partitioning in aqueous two-phase systems has been studied with great interest and has high potential[4,5]. Affinity precipitation has been attempted and seems to be an attractive alternative when specific interactions are used and the concentration of the compound to be purified is known[6]. Affinity interactions in conjunction with membrane technology have also been studied[7].

However, if affinity interactions are to be useful on a large scale at a competitive cost, then systems for sanitation of the affinity system and, when applicable, for recycling of the solid phase must be developed. Sanitation often involves exposure to extreme conditions, *e.g.*, temperature or chemicals. Autoclaving is normally out of the question in these systems. Chemical treatment has to be harsh in order to be effective. A recommended treatment is the use of 0.5 *M* sodium hydroxide for periods of 5 h[8]. Today there are gels that will withstand this treatment, but the ligands used in affinity chromatography are often much too sensitive.

A step towards achieving a system with higher stability and potential to withstand the sanitation conditions was attempted by us by use of heterobifunctional

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affinity ligands which could first bind a substance in solution and then interact with a solid support. After elution of the substance to be purified, the heterobifunctional ligand was eluted from the column. By this approach it is possible to keep the sensitive ligand in solution and remove it before sanitation.

MATERIALS AND METHODS

Sepharose CL-4B, Sephadex LH-20, Dextran T40 and T500 were obtained from Pharmacia (Uppsala, Sweden), Reactive Blue 2 (Cibacron Blue F3G-A), soy bean trypsin inhibitor (Type II), trypsin (Type IX) (E.C. 3.4.21.4) and $NaBH_3CN$ from Sigma (St. Louis, MO, U.S.A.). Purified bovine heart lactate dehydrogenase (E.C. 1.1.1.27) was a generous gift from P. Wikström, Lund. All other chemicals used were of reagent grade.

Preparation of trypsin-Sepharose

Sepharose CL-4B was activated with cyanogen bromide according to the general procedure[9]. The immobilization reaction was performed for 1 ml of matrix with 8 mg trypsin in 1 ml of 0.1 *M* sodium bicarbonate, pH 8.3 and 0.5 *M* sodium chloride. The suspension was gently mixed for 4 h at 20°C after which the remaining active groups were blocked with ethanolamine (1 *M*, pH 8.0). The adsorbed trypsin was removed by washing in 0.1 *M* acetate buffer, pH 4.0, 0.5 *M* sodium chloride and coupling buffer, respectively.

Synthesis of the heterobifunctional affinity ligand Cibacron Blue–dextran–soy bean trypsin inhibitor

Cibacron Blue F3G-A was purified by chromatography on Sephadex LH-20 as described[10]. The substitution of dextrans with the dye was carried out as described by Ashton and Polya[11]. The amount of dye bound to the dextran was determined spectrophotometrically, assuming no difference in the extinction coefficients of free and covalently bound dye. The dye-substituted dextrans (100 mg dextran in 3 ml of water) were activated with sodium periodate. Solid sodium periodate was added to the dextran solution (ratio of sodium periodate to glucose residues was 0.08 when Dextran T40 was used, 0.03 when Dextran T500 was used) and the solution was stirred for 2 h at room temperature. Soy bean trypsin inhibitor (STI) and $NaBH_3CN$ were dissolved in 1 ml of 10 m*M* phosphate buffer pH 6.0 containing 0.15 *M* sodium chloride, and the solution was added to the activated dextran (STI/Dextran T40 = 1.05; STI/Dextran T500 = 5; $NaBH_3CN$/STI = 30).

After incubation for 14 h at room temperature, 27 μl ethanolamine and 2.7 mg $NaBH_3CN$ in 1 ml phosphate buffer pH 6.0 were added and allowed to react for 3 h. The ligand solution was dialyzed several times against 50 m*M* Tris–HCl, pH 8.0. Dye–dextran without bound STI was removed by passing the solution over the trypsin-Sepharose column. Free STI was not separated from the STI–dye–dextran complex.

Preparation of bovine heart extract

Bovine heart (100 g) was homogenized in a Waring blender with 200 ml phosphate buffer pH 7.0 containing 1 m*M* β-mercaptoethanol and 50 g ice; the homogenate was stirred for 10 min and then centrifuged at 20000 *g* for 15 min. The super-

natant was filtered through glass wool and brought to 30% saturation with ammonium sulphate. After stirring for 15 min, the mixture was centrifuged at 20000 *g* for 15 min. The supernatant was then brought to 65% saturation with ammonium sulphate, stirred for 30 min and centrifuged again for 15 min at the same speed. The pellet was dissolved in 50 ml water and dialyzed against 2 × 2 l of 50 m*M* Tris–HCl, pH 8.0.

Chromatographic procedure

All chromatographic steps were carried out at room temperature. The enzyme solution or homogenate was mixed with the heterobifunctional affinity ligand (STI–dye–dextran) in 50 m*M* Tris–HCl, pH 8.0 containing 2 m*M* calcium chloride, 5 m*M* magnesium chloride, 0.4 m*M* EDTA and 2 μM β-mercaptoethanol and the solution was stirred for 0.5 h. The mixture was applied on the trypsin-Sepharose column (5.6 cm × 1.5 cm) which had been equilibrated with the same buffer at a flow-rate of 1 ml/min. After washing the column with coupling buffer, the bound enzyme was eluted with either (0.5 m*M* NADH, or 0.5 *M* sodium chloride in 0.1 *M* ethylenediamine hydrochloride buffer pH 6.0 containing 2 m*M* calcium chloride. Thereafter, the column was washed to remove sodium chloride. The STI–dye–dextran was then eluted with a pulse of 10 m*M* hydrochloric acid. Fractions were assayed for enzyme activity[12].

Assay of enzyme activity

Lactate dehydrogenase (LDH) was assayed by following the oxidation of NADH at 340 nm in the presence of pyruvate. The assay mixture, in a total volume of 3 ml, contained 0.2 *M* Tris–HCl, pH 7.3, 1.0 m*M* sodium pyruvate and 0.22 m*M* NADH.

Gel electrophoresis

Electrophoretic analyses of the enzyme preparations were carried out on 12.5% polyacrylamide gel in the presence of sodium dodecyl sulphate according to the method of Laemmli[13]. Proteins were stained by the basic silver stain procedure[14,15].

RESULTS AND DISCUSSION

The traditional affinity chromatographic procedure for the preparation of LDH involves the use of an affinity matrix with the ligands bound to the polymeric backbone, or spacer arms attached to the polymer. Upon passage of the homogenate through the column, binding takes place. In a sequential process, washing and elution as well as reconditioning take place in the column. The procedure is thus very much based on interactions between macromolecules in free solution and the immobilized ligand. This traditional process is usually carried out with an excess of immobilized ligands. Because of steric hindrance, only a fraction of these ligands can be utilized. Furthermore, the ligands are attached to the sorbent, which means that when the sorbent has to be treated for sanitation then also the ligands are so treated. When the ligands lose their activity a new sorbent has to be produced. Exceptions are when reversible immobilization is used[16].

The present paper deals with an alternative procedure in the sense that the technology described makes it possible to operate with the subtle affinity interactions

in free solution. Furthermore, the ligands may be removed from the solid phase when harsh treatment is used. The principles of the heterobifunctional affinity chromatography are demonstrated. In a subsequent paper, some theoretical background and experimental evaluations will be presented.

The synthesis of the heterobifunctional affinity ligand was carried out in two stages. First, Cibacron Blue was attached to the dextran molecule. After separation of the complex from unreacted dye, the dextran molecule was activated using periodate and STI was added. After coupling, the complex containing STI was separated from unreacted dye–dextran by means of affinity chromatography on a column of trypsin-Sepharose. A typical elution pattern is shown in Fig. 1. To purify further the complex from unreacted STI, it was possible to pass the complex over a column of concanavalin A-Sepharose. However, no harm due to the presence of traces of free STI was foreseen, hence this latter purification step was not included in the process. The degree of derivatization was measured for the final heterobifunctional complex. The amount of dye bound was measured spectrophotometrically, assuming that the specific absorptivity was not changed when the dye molecules were immobilized. STI was quantified using the absorbance at 280 nm.

The heterobifunctional affinity ligand was then tested in model experiments. Addition of the complex to a solution of purified LDH from beef heart with a subsequent pass over a trypsin column revealed that the heterobifunctional ligand functioned as predicted. Fig. 2 shows a typical elution pattern. The first peak is a result of a slight overloading of the column. When no more protein was present in the effluent, the elution conditions were changed. The addition of 0.50 *M* sodium chloride has been reported to be an efficient way to dissociate the Cibacron Blue–lactate dehydrogenase complex[17]. This was shown to be the case in our experiment as well. It should be stressed that this treatment is specific in the sense that the heterobifunctional ligand is still bound to the column. To rinse the column, a shift in pH was used. In blank experiments when the heterobifunctional ligand added to LDH was passed over an unmodified Sepharose column, the elution pattern was as shown in Fig. 3b. Addition of Cibacron Blue–dextran to LDH and passage over a trypsin column was also tested (Fig. 3). Fig. 2 clearly demonstrates the feasibility of using an heterobifunctional affinity ligand together with an affinity column to isolate spe-

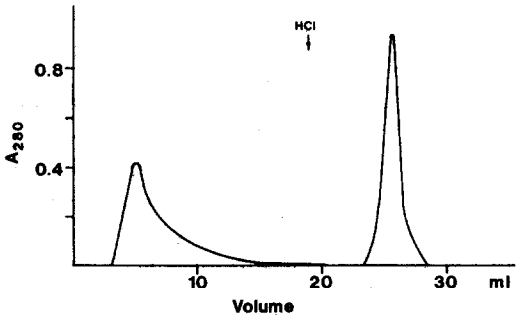

Fig. 1. Separation of STI–dye–Dextran T40 from dye–Dextran T40 on trypsin-Sepharose. Sample applied: 31.5 mg dextran. Flow-rate: 2.0 ml/min. Washing buffer: 50 m*M* Tris–HCl, pH 8.0 containing 2 m*M* calcium chloride, 5 m*M* magnesium chloride, 0.4 m*M* EDTA and 2 μM β-mercaptoethanol. Elution buffer: 10 m*M* hydrochloric acid.

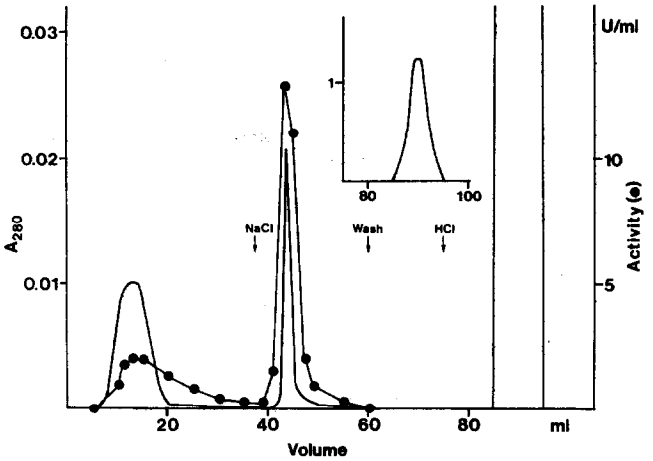

Fig. 2. Chromatography of a pre-equilibrated mixture of LDH and STI–dye–Dextran T40 on trypsin-Sepharose. Sample applied: LDH (88 units) and STI–dye–Dextran T40 (32.5 μM dye). Flow-rate 1.0 ml/min. LDH was eluted with a pulse of 0.5 M ethylenediamine hydrochloride buffer pH 6.0 containing 2 mM calcium chloride. The ligand was eluted by a pulse of 10 mM hydrochloric acid.

cifically a certain molecule.

A crucial point in such a procedure will, of course, be the yield of the substance to be isolated as well as the recovery of the heterobifunctional ligand. The recovery of the ligands when using Dextran T40 as the core onto which the two different affinity ligands were attached is shown in Table I. It is seen that the percentage recovery increased on repeated use. This was interpreted in terms of selection of the most suitable heterobifunctional complexes, the less suitable ones being lost in the initial cycles. It is, of course, important to use appropriate densities of the ligands on the polymer core, both in relation to each other and in absolute terms.

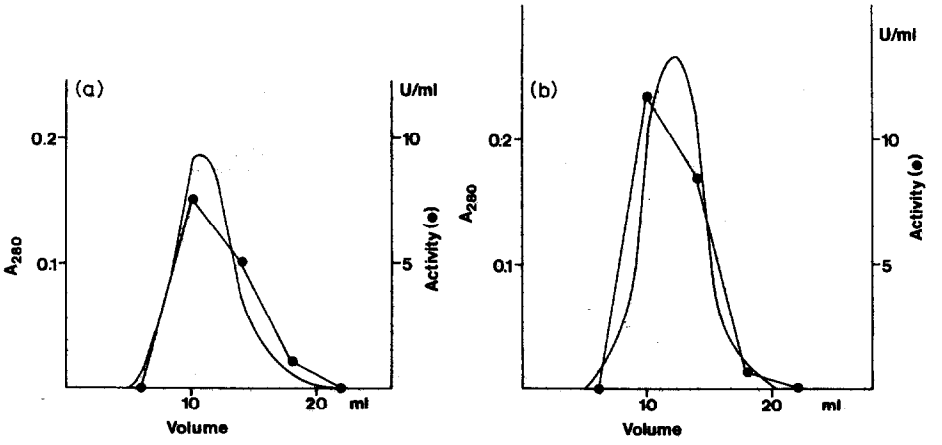

Fig. 3. (a) Chromatography of a pre-equilibrated mixture of LDH (50 U) and dye–Dextran T40 (0.11 μmol dye) on Sepharose-immobilized trypsin. Flow-rate 2.0 ml/min. (b) Chromatography of a pre-equilibrated mixture of LDH (75 U) and STI–dye–Dextran T40 (0.17 μmol dye) on Sepharose CL-4B. Flow-rate 2.0 ml/min.

TABLE I

RECOVERY OF STI-DYE-DEXTRAN AND YIELD OF LACTATE DEHYDROGENASE

Dye concentration (μM)	*LDH (units)*	*Recovery of ligand (%)*	*Yield of enzyme (%)*
Dextran T40			
35.3	65.0	81	72
18.9	43.3	81	59
15.9	32.5	81	52
19.3	21.7	78	44
16.0	21.7	89	37
Dextran T500			
22.3	103	76	63.0
16.8	75	68	92.5
13.2	100	67	58.7
11.2	50	70	52.0
12.1	25	88	44.0

It could be argued that Dextran T40 with a molecular weight of about 40000 is too small and that a larger dextran would have superior ability to bind several of the different ligands, to bind protein in solution and still to be able to expose one or more STI molecules for interaction in the column chromatographic step. In Table I are given data for recovery of the ligand as well as of the enzyme when LDH was isolated from a buffer solution with Dextran T40 and T500.

The yield of the enzyme was also measured. In Fig. 4 are plotted measured values of the yields as well as calculated values. The latter were obtained by calculating the yields in the binding reactions. The dissociation constant between LDH and Cibacron–dextran was set at $6.7 \cdot 10^{-6}$ M[11].

The same preparation of ligands was used to purify LDH from a crude homogenate. As expected, the yield of enzyme was lower (23% as compared to the calculated value of 73%). Since the dye is not very specific for LDH but merely a general ligand for many dehydrogenases and kinases and other proteins as well, one has to take this into consideration and not expect too high a purity in one single early step[18], Fig. 5. In a series of experiments, Dextran T500–Cibacron Blue–STI was used to isolate LDH from a crude homogenate. In Fig. 6 it is seen that the observed yield

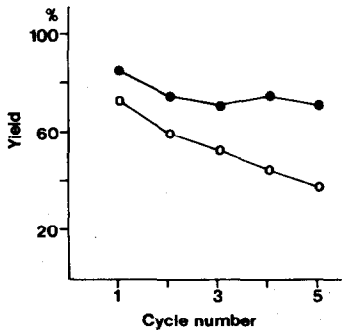

Fig. 4. A plot of measured (○) and calculated (●) yields of LDH when using STI-dye-Dextran T40. Data in Table I were used.

is closer to the theoretical values than when Dextran T40 was used.

A prerequisite for a method like the one described here to be successful is that it should be simple, cost effective and general. Simplicity and generality lie in the fact that, provided the appropriate affinity pairs are used, one and the same column can be used for almost any affinity chromatographic process. Selectivity in the elution process is an advantage, but not a necessity. Hence, by reducing the consumption of gel material in the chromatographic procedure there is a substantial reduction in cost. Furthermore, the ligand used in the primary interaction is much more efficiently used in free solution as compared to that immobilized on the solid chromatographic material. The binding strength and yield will be discussed in a subsequent paper[19].

When applying affinity chromatography under normal conditions, a separate column is needed for each substance to be purified. This may turn out to be a very expensive investment for purification on a large scale. One way to avoid this is the use of general ligands, *e.g.*, immobilized NAD^+ or derivatives thereof in the purification of dehydrogenases[20].

Furthermore, on a large scale, sterility is of crucial importance, and this means that column sanitation is a requirement. This, in turn, leads to the use of stable affinity ligands. During recent years there has been a marked increase in the use of semiaffinity ligands. Substances like hydrophobic groups and dyes have been shown to interact, more or less, specifically with certain proteins and have thus been utilized as affinity ligands. Some of these are stable and can thus withstand the sanitation treatment. Another extreme would be to use ion exchange resins —a quite realistic possibility when working with heterobifunctional ligands.

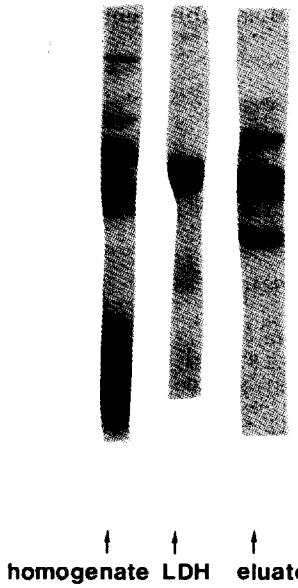

Fig. 5. Gel electrophoresis of crude homogenate. LDH reference and LDH-rich fraction purified with STI–dye–Dextran T500.

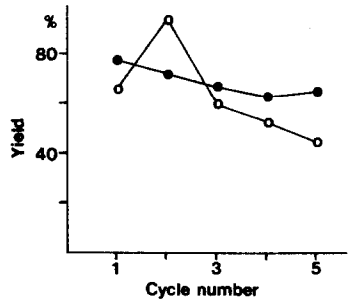

Fig. 6. A plot of observed (○) and calculated (●) yields of LDH when using STI–dye–Dextran T40. Data in Table I were used.

A different line of development in downstream processing has been taken by the genetic technologists. To facilitate the isolation of a target protein, a certain sequence of amino acids has been fused to the protein of interest. The added sequence having extreme properties makes the fused product markedly different from the other protein molecules in the sample. This makes it easy to isolate the fused product from all the other cell components. In a subsequent step the added sequence is split off and the pure protein isolated. The addition of a sequence of arginine residues made it possible to use ion-exchange chromatography[21] and the use of cysteinyl residues enabled the exploitation of covalent chromatography with a subsequent selective elution step[22]. Fusion with protein A made it possible to use an immunoglobulin column as a general affinity column[23]. There are certainly several more posibilities. The technique seems very useful, the only limitation being that the protein has to be a genetically modified one.

When isolating a substance from a complex biological homogenate, affinity purification possesses the highest resolving power. However, it is still characterized by some minor drawbacks in the laboratory scale. On a larger scale, these limitations are even more severe. It would be natural to use affinity interactions as early as possible in a purification process. This is of course governed by a desire to reduce volumes and remove unwanted proteins. Modern affinity chromatographic techniques were mainly developed to serve as a final purification step towards the end of a separation sequence. If, however, more robust systems can be developed, then they will be a natural choic early in the purification process.

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