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THEORETICAL AND EXPERIMENTAL EVALUATION OF THE USE OF HETEROBIFUNCTIONAL AFFINITY LIGANDS IN GENERAL CHROMATOGRAPHIC PURIFICATION SYSTEMS

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SUMMARY

A new general procedure for purification of proteins is evaluated. The procedure is based upon the interaction in free solution between the protein to be purified and a heterobifunctional ligand having an affinity for the molecule to be purified and also for the solid used as an affinity support. The binding constants needed in the different steps in order to obtain an efficient purification are evaluated on a theoretical basis, and some experimental data are provided to support the conclusions drawn. This new technique is efficient, simple and cost effective.

INTRODUCTION

In a previous paper[1] we demonstrated the feasibility of using a heterobifunctional ligand when purifying an enzyme from a cell homogenate. The system is based on the use of an heterobifunctional ligand that is added to the homogenate. Affinity binding takes place on one of the moieties of the heterobifunctional compound. The mixture is then passed over an adsorption column with affinity for the other moiety of the complex, the idea being that by this latter interaction the protein bound to the first affinity moiety will be trapped on the column. After washing to remove all impurities, a specific elution is carried out and the protein isolated. In a subsequent step the heterobifunctional ligand is dissociated from the column and eluted in a concentrated peak.

This procedure seems to carry many useful properties in that it is easy to operate and is general as the same column may well be used to purify a broad spectrum of substances. One drawback might be its complexity, since two different affinity interactions are involved. The present paper deals with a theoretical treatment of this system and also presents some experimental data to confirm what is shown theoretically.

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THEORETICAL

The primary affinity binding reaction may be represented

$$AB + C \rightleftharpoons ABC \tag{1}$$

where AB is the heterobifunctional affinity ligand and C the protein to be purified. The corresponding equilibrium constant is

$$K_a = \frac{x}{(p - x)(L - x)} \tag{2}$$

where x is the concentration of complex (ABC) formed and p and L denote the initial concentrations of the protein (C) and the ligand (AB), respectively.

The second affinity interaction takes place when the complex formed in free solution is trapped on an affinity sorbent

$$(ABC + AB) + D \rightleftharpoons ABD + ABCD \tag{3}$$

where D is the affinity ligand on the solid support to which the soluble is to be bound. The corresponding equilibrium constant is

$$K_b = \frac{y}{(L - y)(d - y)} \tag{4}$$

where y is the concentration of the complex (ABD) + (ABCD), L is the initial concentration of the ligand (AB) and d is the initial concentration of the ligand D.

A crucial parameter of an affinity purification is the yield. To evaluate the conditions for achieving high yields, some values were chosen in order to investigate whether the system would be able to achieve these values. The yield in the first step was set to 90% and in the last step to 99.9%, *i.e.*, very high values in relation to what is normally achieved in most affinity purification processes. With a 90% yield in the first step, eqn. 2 can be rearranged to

$$K_a = \frac{9}{L - 0.9\,p} \tag{5}$$

and eqn. 4 to

$$K_b = \frac{999}{d - 0.999\,L} \tag{6}$$

$$L = \frac{K_b d - 999}{0.999\,K_b} \tag{7}$$

Multipoint binding of heterobifunctional compound

To increase the amount of bifunctional ligand that is bound to the matrix, it is possible to use more than one ligand on the core. Consider an heterobifunctional

compound A, which contains n sites for the ligand D. Each site has the same microscopic ligand dissociation constant, k, and the sites are assumed to be independent. The equilibria may be written as

$$A_0 + D \rightleftharpoons A_1$$

$$A_{n-1} + D \rightleftharpoons A_n \tag{8}$$

and the macroscopic dissociation constants as:

$$K_1 = \frac{[A_0]\,[D]}{[A_1]}$$

$$K_n = \frac{[A_{n-1}][D]}{[A_n]} \tag{9}$$

Eqn. 9 gives:

$$[A_i] = \frac{[A_0][D]^i}{\prod_{j=1}^{i} K_j} \tag{10}$$

There are $\Omega_{n,i}$ ways to put i ligands on n sites

$$\Omega_{n,i} = \frac{n!}{(n-i)!i!} \tag{11}$$

and the relationship between K_i and k is:

$$K_i = \frac{\Omega_{n,i-1}}{\Omega_{n,i}} \cdot k \tag{12}$$

Rewriting eqn. 10 by use of eqn. 12 gives:

$$[A_i] = [A_0]\left[\prod_{j=1}^{i} (n-j+1)/j\right] \cdot \left(\frac{[D]}{k}\right)^i \tag{13}$$

The unbound fraction of the bifunctional ligand is given by:

$$P_f = \frac{[A_0]}{[A_{tot}]} = \frac{[A_0]}{[A_0] + \sum_{i=1}^{n} [A_i]} \tag{14}$$

Substitution of eqn. 13 into eqn. 14 gives:

$$P_f = \frac{1}{1 + \sum_{i=1}^{n} \left[\prod_{j=1}^{n} (n-j+1)/j \right] \cdot \left(\frac{[D]}{k} \right)^i} \quad (15)$$

The product term in eqn. 15 is identical to $\Omega_{n,i}$, thus eqn. 15 can be rewritten as:

$$P_f = \frac{1}{1 + \sum_{i=1}^{n} \frac{n!}{(n-i)!\, i!} \cdot \left(\frac{[D]}{k} \right)^i} \quad (16)$$

The denominator of eqn. 16 is the binomial expansion of $\left(1 + \frac{[D]}{k}\right)^n$, thus we finally obtain:

$$P_f = \frac{1}{\left(1 + \frac{[D]}{k}\right)^n} \quad (17)$$

MATERIALS AND METHODS

Sepharose 4B, Sephadex G-75, Dextran T10 and T40 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Jack beans, $NaBH_3CN$, trypsin (Type IX from hog pancreas), soy bean trypsin inhibitor (STI) (Type II) and α-methyl-D-glucopyranoside were obtained from Sigma (St. Louis, MO, U.S.A.), sodium periodate and sodium borohydride from Merck (Darmstadt, F.R.G.).

Isolation of concanavalin A (Con A)

Jack beans (500 g) were swollen in 1.25 l of 0.9% sodium chloride solution overnight. The beans were then homogenized in a Turrax mixer in 1.5 l of Tris–HCl buffer (10 m*M*, pH 7.0 also containing 0.9% sodium chloride, 1 m*M* magnesium chloride 1 m*M* manganese chloride and 1 m*M* calcium chloride). The homogenate was stirred for 5 h at 4°C, and was then filtered through cheese cloth. The solution was centrifuged for 15 min at 24 500 *g*. The supernatant was collected and the fat was removed by passage through a layer of glass wool. The extract was then mixed with 400 ml of pre-swollen Sephadex G-75 and gently stirred overnight at 4°C. The gel material was then washed with 5 l of the Tris buffer described above before transferring it to a glass filter where it was washed with the same buffer until A_{280} of the eluate was *ca.* 0.05. The Con A bound to the gel was eluted by a pulse of 1 *M* glucose dissolved in 10 m*M* Tris–HCl, pH 7.5 also containing 0.9% sodium chloride. All fractions with $A_{280} > 0.1$ were pooled and dialyzed against the above mentioned buffer.

Preparation of Con A-Sepharose 4B and trypsin-Sepharose 4B

Sepharose 4B was activated using cyanogen bromide as described[2]. After

thorough washing with 0.1 *M* sodium bicarbonate, pH 8.3, containing 0.5 *M* sodium chloride and 0.1 *M* acetate buffer, pH 4.0, containing 0.5 *M* sodium chloride, coupling was performed by adding 5 mg Con A per ml gel or 8 mg trypsin per ml gel. Coupling proceeded at 20°C for 4 h.

Preparation of STI–Dextran T40

Dextran T40 (100 mg) was dissolved in 2.5 ml of 10 m*M* phosphate buffer pH 6.0 containing 0.15 *M* sodium chloride. Sodium periodate (10.7 mg per 0.5 ml buffer) was added and the reaction allowed to proceed for 2 h at room temperature with gentle mixing. Then, 60 mg STI and 6 mg $NaBH_3CN$ in 1 ml of buffer were added. After reaction at room temperature for 14 h, the pH was adjusted to 9–9.5 with 0.5 *M* sodium carbonate. Sodium borohydride (7.5 mg) was added and the reaction continued overnight at 4°C. The reaction mixture was then dialyzed against 0.05 *M* Tris–HCl, pH 8.0.

Purification of STI–Dextran T40 from the reaction mixture

Underivatized dextran was removed by passing the sample over a column of trypsin-Sepharose (10 ml, diamter 1.0 cm) pre-equilibrated with 0.05 *M* Tris–HCl, pH 8.0. After washing the column with buffer, the STI–dextran was eluted with a pulse of 10 m*M* hydrochloric acid. Unreacted STI was removed by passing the sample over a Con A-Sepharose column (10 ml Con A-Sepharose equilibrated with 50 m*M* Tris–HCl pH 7.0 containing 20 m*M* calcium chloride). The STI–dextran was applied on the column at a flow-rate of 0.25 ml/min. After washing with buffer, the STI–dextran was eluted using 0.1 *M* α-methyl-D-glucopyranoside. The pure ligand was then removed by a second passage over the trypsin column.

Chromatographic procedure

All chromatographic steps were carried out at room temperature. The trypsin-Sepharose was equilibrated with 50 m*M* Tris–HCl, pH 8.0 containing 20 m*M* calcium chloride. Con A and STI–dextran were mixed in the same buffer as above and the mixture was stirred for 45 min before being passed over the trypsin gel at a flow-rate of 0.4 ml/min. After washing with coupling buffer off non-specifically bound protein, the Con A was eluted by a pulse of 0.1 *M* α-methyl-D-glucopyranoside. After complete elution the column was washed with coupling buffer to remove the glucopyranoside. The STI–dextran was eluted by a pulse of 10 m*M* hydrochloric acid.

Determination of K_a for Con A and dextran by zonal affinity chromatography

A column containing 10 ml of Sephadex G-75 was equilibrated with 50 m*M* Tris–HCl pH 8.0 and 20 m*M* calcium chloride containing various concentrations (10–2.5 m*M*) of Dextran T10. A 5-mg amount of Con A in 1 ml of each of the above dextran solutions was applied on the column at a flow-rate of 0.22 ml/min and was eluted with the equilibration buffer.

RESULTS AND DISCUSSION

The heterobifunctional ligand consists of two parts, the STI with affinity for trypsin-Sepharose and the dextran which acts both as a matrix and a ligand with

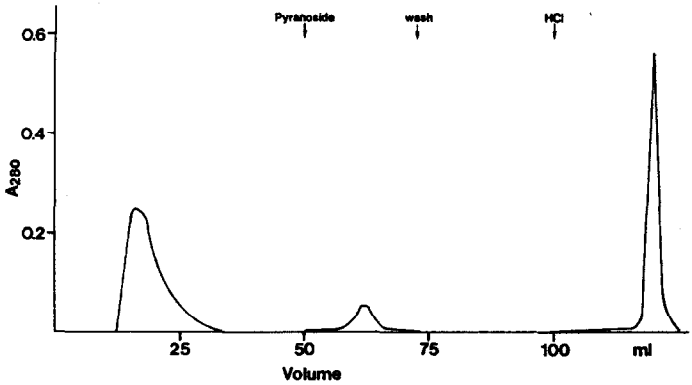


Fig. 1. Chromatography of a pre-equilibrated mixture of Con A and STI–dextran on Sepharose-immobilized trypsin. Sample applied: STI–dextran (25 μM) and Con A (2.7 μM). Flow-rate: 0.4 ml/min. Elution of Con A was performed with 0.1 M α-methyl-D-glucopyranoside and that of STI–dextran with 10 mM hydrochloric acid.

affinity for concanavalin A. When a pre-equilibrated mixture of con A and STI–dextran is passed over the trypsin-Sepharose column a chromatogram like that shown in Fig. 1 is obtained. The first peak is the result of overloading of the column. The affinity bound Con A was eluted with α-methyl-D-glucopyranoside subsequent to the washing of any non-specifically bound protein from the column. The sugar was then removed before the heterobifunctional ligand was eluted from the column. This system is not ideal, but offers certain advantages in this study.

As can be seen in Table I, the major part of the Con A present is not bound by the STI–dextran. Assuming that the affinity binding takes place only during the incubation with STI–dextran and not during the passage through the column, then a value of K_a for the interaction between Con A and STI–dextran can be determined as $6 \cdot 10^3\ M^{-1}$. A value of the equilibrium constant for the interaction between Con A and dextran was estimated using zonal affinity chromatography. In Fig. 2 is shown an elution profile for con A from a column containing Sephadex G-75. The column was equilibrated with buffer containing various concentrations of Dextran T10. Every time the column was loaded with the same amount of Con A, which was then eluted using the equilibration buffer. Increasing concentrations of dextran resulted in reduced elution volumes down to a value V_0, after which there was no further reduction in elution volume with increase in dextran concentration. The elution volume, V_i, at the absorption maximum A_{280}, was measured for the different dextran concentra-

TABLE I

YIELD OF CON A FROM A COLUMN OF TRYPSIN-SEPHAROSE AFTER EXPOSURE OF THE CRUDE HOMOGENATE TO STI-DEXTRAN T40

STI–Dextran T40 *(μM)*	*Con A* *(μM)*	*Yield of Con A* *(%)*
25.0	2.7	13
18.6	0.48	10

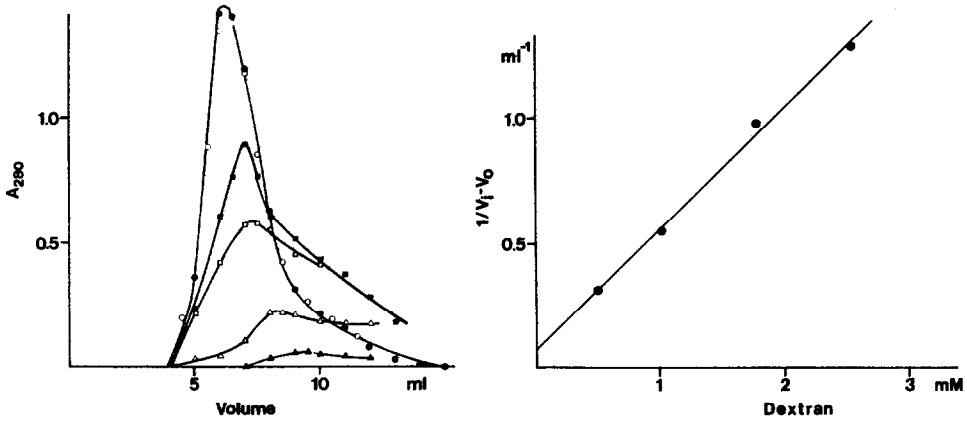


Fig. 2. Chromatography of Con A on Sepharose G-75. Different Dextran T10 concentrations in the eluting buffer were used. The same quantity of Con A (5 mg) was applied to the column, equilibrated with 50 m*M* Tris–HCl buffer containing 0.02 *M* calcium chloride, pH 8.0, and Dextran T10 in the concentrations 10 (●), 7.5 (○), 2.5 (■), 1.75 (□), 1.0 (△) and 0.5 m*M* (▲). Fractions of 0.5 ml were collected while eluting with the corresponding equilibrating buffer.

Fig. 3. Plot of $1/(V_i - V_0)$ (ml^{-1}) *versus* the concentration of Dextran T10 in the eluent. The data in Fig. 2 were used.

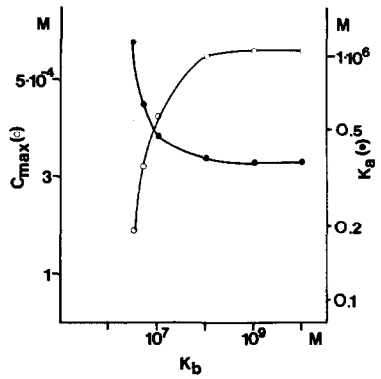
tions. In Fig. 3 is plotted $1/(V_i - V_0)$ *vs.* the dextran concentration. The slope of the line is $1/K_i(V_0 - V_m)(L/K_L)$ and the intercept on the $1/(V_i - V_0)$ axis is $1/(V_0 - V_m)(L/K_L)$, Where V_m is the void volume and K_L is the dissociation constant of the complex of Con A and Sephadex G75. The value of K_a (*i.e.*, $1/K_i$) was derived from the quotient slope/intercept, *i.e.*, $6 \cdot 10^3\ M^{-1}$. This value was obtained by using zonal affinity chromatography, with the assumption that each dextran molecule can bind only one Con A, and is in good agreement with the one calculated earlier (see Table II).

The yield of C, the protein to be purified, depends on several factors: [C], [AB], [D], K_a and K_b. The choice of D (ligand bound to the solid support) and B (the compound with affinity for D) is of great importance, since the concentration of D normally varies between 1 and 10 mg/ml, which in turn leads to the fact that K_b must be very high. This can be seen from eqn. 7 which is derived from eqn. 6, where $K_b[D] > 999$ gives a yield of 99.9%. The calculations are based on interactions in free solution in a batch experiment. A smaller value of K_b is needed when the reaction

TABLE II

CALCULATED YIELDS OF CON A IN THE CHROMATOGRAPHIC PURIFICATION PROCEDURE USING STI–DEXTRAN AND A TRYPSIN-SEPHAROSE AFFINITY COLUMN AT THREE DIFFERENT ASSUMED K_a VALUES

STI–Dextran T40 (*μM*)	*Con A* (*μM*)	K_a (M^{-1})		
		$6 \cdot 10^3$	$12 \cdot 10^3$	$18 \cdot 10^3$
25.0	2.7	13	23	30
18.6	0.48	10	18	25



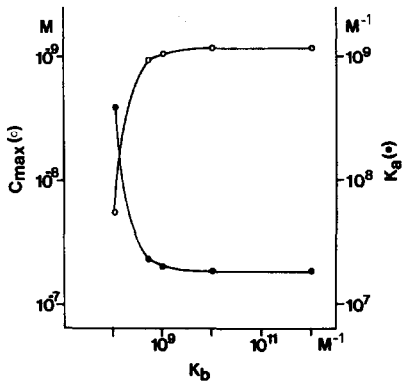


Fig. 4. Plot of $[C]_{max}$ and K_a *versus* K_b for [D] = 0.5 mM. K_a is calculated for [C] = 0.95$[C]_{max}$.

Fig. 5. Plot of $[C]_{max}$ and K_a *versus* K_b for [D] = 10 μM. K_a is calulated for [C] = 0.95$[C]_{max}$.

takes place in a column, but then restrictions due to solid phase–liquid interactions have to be taken into consideration. Normally, K_a is reported only for reactions in free solutions, and this makes such calculations even more approximate, since K_a may decrease for either one or both of the reactants upon immobilization.

Figs. 4 and 5 show K_a and $[C]_{max}$ as functions of [D] and K_b. From Fig. 4 it is seen that K_b cannot be less than $3 \cdot 10^6$ M^{-1} if a 99.9% yield is to be achieved. At high [D], very high concentrations of C can be used with K_a values in the region $2 \cdot 10^5$–10^6 M^{-1}. At lower [D], *e.g.*, when using antibodies, the requirements for a higher K_b ($> 10^8$) are increased. $[C]_{max}$ and K_a decreases and increases, respectively, when decreasing [D]. One way to lower the demands of either a high K_b or a high [D] is to use more than one ligand on the core that could interact with the solid phase, Fig. 6 (it is assumed that [D] $\gg$ [A]).

It is reasonable to desire high recoveries of the heterobifunctional ligand in order to reduce the costs of the process. The use of a heterobifunctional ligand ought to be less expensive than traditional technology, based on several reasons. The need for gel is drastically reduced, since the new method allows the same gel preparation to be used for several different separations. Consequently, the number of columns required is also drastically reduced. Another reason is that the need for ligand is

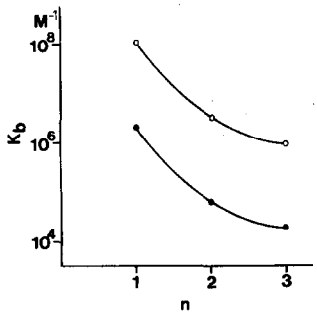


Fig. 6. Plot of the values of K_b needed to achieve a 99.9% yield of bifunctional ligand *versus* the number of ligands interacting with the solid phase. [D] = 0.5 mM (●); 10 μM (○).

reduced, especially when dealing with low-molecular-weight ligands. When using a ligand like 2-iminobiotin in the immobilized state, an excess has to be used in order to obtain a high concentration of ligand under the operating conditions. If, however, the ligand is immobilized on a soluble polymer, then there is no steric hindrance to the interaction with avidin, as observed in the immobilized state[3].

It is difficult to compare the costs of the different methods, due to a lack of relevant information in the literature. A consideration of recycling must deal not only with the ligand as such but also with sanitation of the column. In many affinity chromatographic processes there is a need for good control of the aseptic conditions in the column. It would be possible in many cases to wash the column with, *e.g.*, sodium hydroxide, but such a treatment is often detrimental to the affinity column. The use of heterobifunctional ligands opens up the possibility of using very stable compounds for the affinity interaction on the column, and thus to create systems that can withstand column sanitation.

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