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EFFORTS TO INTEGRATE AFFINITY INTERACTIONS WITH CONVENTIONAL SEPARATION TECHNOLOGIES

AFFINITY PARTITION USING BIOSPECIFIC CHROMATOGRAPHIC PARTICLES IN AQUEOUS TWO-PHASE SYSTEMS

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SUMMARY

A new affinity-based purification technique is described. By combining the positive properties of affinity partitioning with those of affinity chromatography, a new affinity-based separation procedure was developed. An affinity support carrying the immobilized ligands was mixed with the cell homogenate. After binding had taken place, an aqueous two-phase system was added. The affinity sorbent was recovered from the upper side of the interface, and then packed in a chromatography column. Elution was carried out according to conventional procedures.

INTRODUCTION

Affinity-based purification has been used mainly as a chromatographic step towards the end of a separation process consisting of several steps using various techniques. Traditionally, all the early steps in the purification scheme consist of rather unspecific procedures, e.g. precipitation, centrifugation and ion-exchange chromatography, while affinity chromatography is often used as a highly powerful separation technique for the final "polishing".

A crucial point in the separation of a protein out of a complex mixture, e.g. a cell homogenate, is to quickly separate the protein of interest from proteases present therein. If a high efficiency is achieved in this step, then the basis is set for a high overall yield in the purification process. An efficient separation would need to be based on high-resolving separation technologies. Such procedures are available for the analytical scale, but for large-scale purification very few useful techniques are at hand. The best and most specific technique should be the affinity-based separation where the biological activity of the biomolecule is utilized in the separation process.

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The present work deals with the use of affinity-based purification as a step early on in the separation process. In this way, the enzyme is quickly extracted from the often harmful environment of the homogenate. In this procedures, one has to sacrifice the purity of the product as compared to what is normally achieved from affinity chromatography used later in the separation scheme. This defect may, however, be overcome either by a subsequent affinity or an ion-exchange chromatography step.

Cell homogenates are often dilute solutions with respect to the molecule of interest and the initial steps in conventional separation processes often involve the handling of large volumes of water. Using conventional separation equipment, this will introduce some restrictions on the throughput of the process. However, if the molecules are enriched in a small volume by affinity interaction, then the volume to be processed in all subsequent steps may be reduced.

The present paper deals with an effort to integrate the best of affinity partitioning in aqueous two-phase systems with the well known positive characteristics of affinity chromatography, with a concomitant reduction of the drawbacks of the respective methods.

EXPERIMENTAL

Materials

The following chemicals were obtained from the indicated sources: ethylenediamine, Reactive Blue II (Cibacron Blue), Tris and sodium borohydride from Sigma (St. Louis, MO, U.S.A.); epichlorohydrin, 1,4-diaminobutane and cyanogen bromide from Merck (Darmstadt, F.R.G.); PEG-8000 and PEG-20M from Union Carbide (New York, NY, U.S.A.); Sepharose CL-2B and Dextran T-500 from Pharmacia Fine Chemicals (Uppsala, Sweden); baker's yeast from Jästbolaget (Rotebro, Sweden). Pluronic L-35 was a gift from Atochem, Groupe Elf Aquitaine (Pas-de-Calais, France). Maltrin M-100 (a maltodextrin) was a gift from Grain Processing (Muscatine, IL, U.S.A.). Reppal-PES was a gift from Reppe Glykos AB (Växjö, Sweden).

Phase systems used

Four different phase systems were used: PEG—Dextran (50 g/l PEG-8000 and 130 g/l Dextran T-500); PEG—Maltrin (50 g/l PEG-8000 and 135 g/l Maltrin M-100); Pluronic—Maltrin (144 g/l Pluronic L-35 and 201 g/l Maltrin M-100); PEG—Reppal-PES (50 g/l PEG-8000 and 130 g/l Reppal-PES).

Modification of beads

Sepharose CL-2B (100 ml sedimented volume) was washed and transferred to a beaker containing 500 ml of 0.1 *M* sodium hydroxide, 0.25% $NaBH_4$ and 6 ml of epichlorohydrin. The activation was carried out at 40°C for 1 h. The beads were washed thoroughly and mixed with 70 g of PEG-20M, dissolved in 500 ml water and the coupling was allowed to proceed overnight.

Coupling of Cibacron Blue to beads

Two alternatives for coupling ligands were used, with and without a spacer arm. When no spacer arm was used, coupling was effected by simply mixing 1 g of Cibacron Blue with 100 ml of PEG-modified Sepharose CL-2B in 1% sodium carbonate at 85°C for 2 h.

In the second alternative, 100 ml of PEG-modified Sepharose CL-2B were activated by the cyano-transfer method [1] using 1 mmol of cyanogen bromide. The spacer arm, 1,4-diaminobutane, was added in a ten-fold excess and the coupling was run overnight at 4°C. The gel was washed thoroughly with distilled water and mixed with Cibacron Blue as above. The reaction was allowed to proceed overnight at 40°C.

Purification of alcohol dehydrogenase from yeast

Baker's yeast was homogenized in a bead mill (Dyno Mill Type KDL, Willy A. Bachofen AG Mashinenfabrik, Basel, Switzerland) with glass beads of diameter 0.5—0.75 mm. The homogenization took 5—10 min and was carried out at 4°C. The crude homogenate was used immediately for the binding step in the purification.

The yeast homogenate (corresponding to 200 g of yeast, wet weight) was mixed with the beads (100 ml) in a total volume of 750 ml (pH 6.0), containing 30 m*M* ethylenediamine and allowed to incubate for 10 min before the phase system was added. After phase separation and migration of the beads to the interface, which took 10—20 min, the beads were collected and transferred to a column. The beads were washed with 1 m*M* ethylenediamine (pH 6.0) until absorbance at 280 nm was at the baseline level. The enzyme was eluted by a pulse of 15 ml of 5 m*M* nicotinamide-adenine dinucleotide, oxidized (NAD) and 0.1 *M* Tris—HCl (pH 8.3), giving an undelayed, essentially symmetrical peak. As a reference, the same type of beads were used in conventional affinity chromatography. In this case, however, cell debris had to be removed prior to application of the homogenate to the column.

Measurement of enzyme activity

This was performed according to the method of Vallee and Hoch [2].

Measurement of protein concentration

This was done by the Coomassie Blue binding assay [3].

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

This was run according to a published procedure [4]. The samples were concentrated by precipitation with trichloroacetic acid before application.

RESULTS AND DISCUSSION

Aqueous two-phase partitioning

In the development of new separation technologies, a comparison must be made with already existing techniques. To do this, some characteristics of this new technique, such as time of separation, number of steps involved,

capacity to process large volumes, purity of the final product, etc., were studied in more detail.

Extraction in aqueous two-phase systems is, by now, a well established procedure for protein purification [5]. Spontaneous partitioning in such phase systems has been exploited for purification of some proteins [6—8]. This technique requires knowledge of the partition behaviour of the proteins and may thus involve a number of experiments to set the right conditions. Once this is achieved, there seem to be less problems in scaling up than with many other techniques used for protein purification. The introduction of an affinity ligand in the phase systems made it possible to control the partition more efficiently and thus to reduce the initial work to optimize the conditions. The affinity ligand used was modified by one of the phase polymers to partition preferentially to that phase [9]. When the affinity interaction had taken place, the bound protein would be pulled over to the predetermined phase and then isolated from this phase. In most cases, transport over to the PEG-rich phase is desired. By using conjugates with one PEG molecule bound per ligand, it is possible to transport the affinity-bound material to the PEG-rich phase. However, this is not a general way to control the partitioning of affinity-bound material. To obtain a more predictable partitioning, more highly modified ligands were used. This formed the basis for the partition affinity ligand assay (PALA) [10, 11].

The affinity partitioning so far was based on transfer of one protein to a predetermined phase, often the top phase, while the other proteins remained in the other phase. By transferring this top phase to a fresh bottom phase with a concomitant change to dissociating conditions, the affinity-bound protein is transferred to the bottom phase from where it can be isolated.

The initial step of affinity partitioning was tempting to utilize, but the elution procedure did not seem to be optimal. Instead, chromatography particles carrying affinity ligands were introduced into the system. After collection of the particles, they could be treated with conventional chromatographic elution procedures. To evaluate the potential of this technique, experiments were performed to isolate alcohol dehydrogenase (EC 1.1.1.1) from homogenates of baker's yeast (*Saccharomyces cerevisiae*) using affinity binding to Cibacron Blue bound to PEG-modified Sepharose CL-2B. This system was chosen because it has been used in many other studies and also because the biological material was easily available in large quantities.

The interaction between the dye Cibacron Blue and enzymes in a cell homogenate is only semi-specific, the dye having affinity for most NAD-dependent enzymes. The maximum attainable degree of purity will therefore be rather limited. This also means that the binding capacity of the adsorbent is not efficiently used.

The association constant of the interaction between alcohol dehydrogenase and Cibacron Blue is $10^4\ M^{-1}$, which is in the lower range of utilizable binding strengths. One of the reasons for choosing this binding pair was to investigate the performance of a relatively unfavourable system. The importance of the binding strength of the interaction is discussed further below.

Binding capacity

There are certain requirements concerning the interaction between the

ligand and the molecule to be purified. If the affinity constant is low, there will be an incomplete binding of the protein. The necessary binding strength can be estimated by a simple calculation.

In a binding situation between a protein P and a ligand A, we can write:

$$P + A \rightarrow PA$$

The total concentrations of the ligand and the protein are a and p, respectively. The amount of complex formed is x and we get the common equation for a chemical equilibrium:

$$K = x/(p - x)\,(a - x)$$

Normally, the ligand is present in heavy excess over the protein (often 100-fold or more); $a - x$ can therefore be approximated by a.

The yield of bound protein is defined as x/p

$$\text{Yield} = x/p = Ka/(1 + Ka)$$

It should be noted that the concentration of the protein does not appear on the right-hand side of the equation and, hence, does not affect the yield.

The maximum possible concentration of the ligand is limited by two factors. First, there is a limit to the amount of ligand bound per particle. Secondly, there is a limit to the number of particles in a phase system. Taking these two limitations into account, we obtain a maximum average ligand concentration of ca. 10 μM.

If we want to study an interaction with the association constant of $10^8\ M^{-1}$ and have the maximum concentration of ligand of 10 μM, we obtain a yield of 99.9%. However, if the association constant is $10^5\ M^{-1}$, the yield is only 50%.

It is evident that the attainable yield for low association constants 10^4—$10^5\ M^{-1}$ is unsatisfactory. In the range of this binding strength are many of the interactions frequently used in affinity purification, e.g. interactions between lectins and monosaccharides and also between enzymes and triazine dyes.

It is, thus, in some cases, of interest to find ways to enhance the yield. The equation shows two possibilities for doing this. The amount of ligand can be increased, but for practical reasons this may not be feasible. The only alternative is then to increase the affinity of the interaction. This may be done by choosing conditions that promote multipoint attachment, which can give a considerable increase in the binding strength. In an unrestricted multipoint binding, the overall association constant is $K_{ass}{}^n$, where n is the number of ligands bound and K_{ass} is the individual association constant. Another possibility would be to use a mixed type of interaction using hydrophobic or electrostatic interaction to attract the protein to be purified.

In the initial study, Cibacron Blue was coupled directly to the Sepharose particles, thereby forming a rather inflexible binding structure where the chances for more than one interaction (i.e. multipoint attachment) to take place are extremely small. Consequently, a low yield was achieved (Table I). However, when the ligand is attached via a spacer arm, then a much more

TABLE I

YIELD OF ALCOHOL DEHYDROGENASE

The yield was measured as remaining enzyme activity after elution from Cibacron Blue–Sepharose, run as conventional affinity chromatography.

Source	Yield (%)	
	Without spacer arm	With spacer arm
Alcohol dehydrogenase (Sigma)	35	98
Yeast cell homogenate	10–35*	80

*The lower figure was obtained for excess amounts of homogenate.

flexible unit is formed, which facilitates multipoint attachment. According to the discussion above, a more efficient binding is achieved; this can be seen from Table I.

When the beads with a spacer arm were used, a purification factor of 53 was obtained for a load of 20% (v/v) yeast homogenate. The specific activity of the homogenate was 0.087 U/mg, and that of the eluate from the column was 4.6 U/mg.

Removal of phase components

The approach to exploit aqueous two-phase systems in biotechnology has been studied extensively in recent years [12]. When dealing with purification of proteins by partition or affinity partitioning, it has become obvious that the problem of removing phase polymers may be a real problem. Several attempts to solve this have been presented. Kroner et al. [13] have been operating mainly with potassium phosphate as the bottom phase and, after purification of a certain protein by partitioning to the top phase, this phase is transferred to a fresh salt-rich bottom phase with such conditions that the protein of interest partitions to that phase. In a subsequent dialysis or ultrafiltration stage, the salt is removed. However, a PEG–salt system will have a bottom phase that also contains some PEG. In relation to the amount of protein we are dealing with, the PEG quantity present may appear to be high. PEG is normally not removed on dialysis or ultrafiltration and thus an ion-exchange step has to be utilized as well.

Another approach has been to apply the top phase on reversed-phase high-performance liquid chromatography columns, where the protein is retarded and the PEG washed out. In a subsequent elution step using, for example, propanol, the protein was recovered [14]. The present technique of applying a particulate affinity sorbent offers the possibility of removing all the phase components under mild and gentle conditions before starting the elution of the affinity-bound protein.

It had been foreseen that the purity obtained with the present technique would be lower than that obtained by including an affinity chromatographic step as a final polishing step in a sequence of purification steps. As judged from electrophoretic analysis on SDS-PAGE, this prediction holds (Fig. 1).

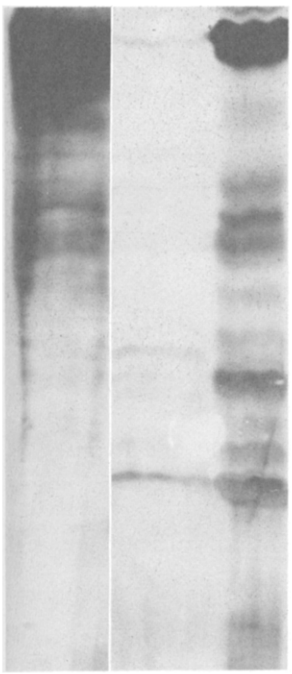

Fig. 1. SDS-PAGE according to Laemmli [4] of homogenate of yeast cells (a) and the alcohol dehydrogenase fraction of the column eluate (b). Molecular weight standards were: bovine serum albumin, ovalbumin, phytohaemagglutinin and myoglobin (c).

However, if the eluted material from this new affinity purification procedure is passed over an ion-exchange column, then an enzyme preparation of equal or better purity is obtained.

Choice of phase components

A factor hampering a more widely spread application of aqueous two-phase systems in biotechnology is said to be the cost of the phase components. PEG and salts have been regarded as acceptable in price, whereas dextran is certainly too expensive. To this aim, we have studied various new two-phase systems to find suitable systems at lower prices. The maltodextrins used as replacements for dextran did work, but only at high concentrations. Furthermore, the tendency of retrogradation was high, which led to systems of low stability on storage.

The other starch-derived polymer, Reppal-PES, gave together with PEG-8000 a phase system similar to that obtained for the PEG-8000—Dextran T-500 system (Fig. 2).

The capacity of the two-phase systems will, of course, also contribute to the total economic evaluation of a purification procedure involving such systems. In this respect, a series of capacity experiments were performed when increasing amounts of homogenate were added to the system. At higher levels of homogenate, PEG had to be added in solid form to avoid unwanted dilution. It was found that yeast homogenate could be added in quantities

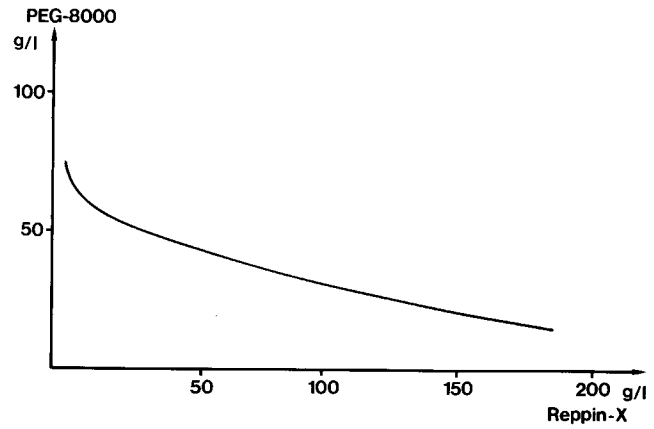

Fig. 2. Phase diagram of a two-phase system consisting of PEG-8000 and Reppal-PES, measured at 20°C.

of up to 25% (v/v) of the total volume of the phase system without any significant changes in the performance.

The polymer concentrations needed for formation of a two-phase system tended to be rather low but because of the density of the beads, a rather dense bottom phase was used to keep the beads floating above the interface. The high polymer concentration also helped to partition most of the proteins, thereby improving the degree of purification in the affinity partitioning step. The behaviour of the beads in the phase separation process is shown in Fig. 3.

A general trend in affinity purification during recent years has been towards the use of group-specific and cheap ligands rather than highly specific, expensive ligands. On introducing affinity interactions as early steps in the purification procedures, a high specificity might be advantageous.

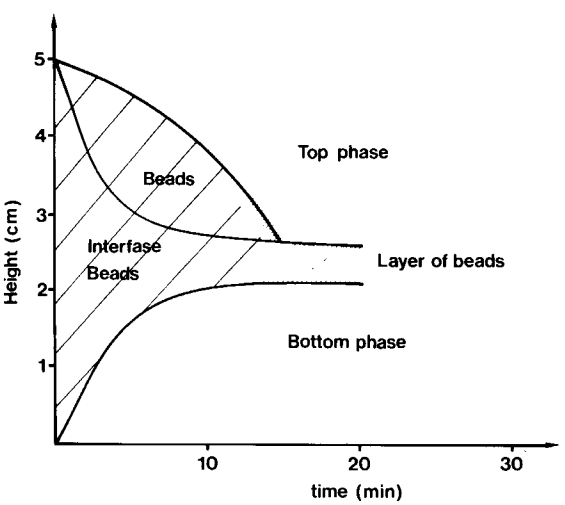

Fig. 3. Time course of a phase separation process shown as the vertical migration in a 5 cm high phase system at 20°C. The separation of the phases is indicated with solid lines and the distribution of modified beads is shown by the shaded area. The phase system consisted of 50 g/l PEG-8000, 130 g/l Reppal-PES and 20% (v/v) yeast homogenate.

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