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SUBUNIT EXCHANGE CHROMATOGRAPHY: A TOOL FOR THE PURIFICATION OF OLIGOMERIC AND SELF-ASSOCIATING PROTEIN SYSTEMS

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SUMMARY

Subunit exchange chromatography exploits the observation that subunits immobilized on a solid matrix retain the capacity to recognize the subunits in solution in a highly specific way. Hence, oligomeric and self-associating proteins can be extracted in a simple and efficient way from a multicomponent mixture under conditions that favour subunit association; subsequently, the protein is eluted under conditions that favour its dissociation into subunits. The method lends itself also to the isolation of homologous proteins capable of forming hybrids with the immobilized subunits.

INTRODUCTION

Subunit exchange chromatography is a bioaffinity method that allows the purification of oligomeric and self-associating protein systems by means of their subunits immobilized on a solid matrix. The method is based on the specificity of subunit recognition and exploits the observation that properly immobilized subunits retain the capacity to interact in a reversible and specific way with soluble subunits of the same or of homologous proteins. Hence, the experimental conditions that cause the establishment of a finite association–dissociation equilibrium in solution will promote the establishment also of a new equilibrium between immobilized and soluble subunits. As a result, subunits will be exchanged between the liquid and the solid phase, and part of the protein that was initially in solution will be bound to the matrix. The amount of matrix-bound oligomer thus formed is governed by the law of mass action and is a function of several parameters, i.e. the concentrations of immobilized and soluble protein, the association constants in solution and in the solid phase,

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and the stoichiometry of association of the system [1–3].

The possibility of shifting the subunit association–dissociation equilibrium by changing the experimental conditions enables one to achieve an effective purification of a self-associating or oligomeric protein in two simple steps. First, the protein is extracted, for example, from a tissue homogenate and bound to the immobilized subunits under conditions that favour polymer formation; subsequently, the protein is eluted under conditions that favour dissociation. Thereby, the immobilized subunits are regenerated and become ready for a new purification cycle after equilibration with the associating buffer [1—4]. It follows that the use of subunit exchange chromatography is rendered easier if the experimental parameters (pH, composition and ionic strength of the medium, specific cofactors, etc.) that influence the association–dissociation behaviour are known. This type of knowledge is helpful also for the choice of optimal coupling conditions of the protein to the matrix. In the case of oligomeric proteins, it is best to immobilize the protein under conditions that stabilize the oligomer. In this way, coupling to the resin through residues that are located at or near the intersubunit contact regions is avoided and immobilized subunits that display minimal heterogeneity in their interaction with soluble subunits are generated. Immobilized subunits can then be obtained by dissociation of the immobilized oligomer.

A variety of systems have been purified successfully by means of immobilized subunits of the same protein or of a homologous subunit capable of forming hybrids with the protein to be isolated [5—13]. Immobilized subunits have been used also as an analytical tool to study the effect of the coupling step on their associating capacity. Detailed frontal analyses of the elution profiles obtained in subunit exchange chromatography experiments with immobilized human haemoglobin A [14], α-chymotrypsin [15] and glucagon [8] have shown that insoluble subunits have essentially the same associating capacity as the protein in solution. Moreover, the insoluble subunits are quite homogeneous with respect to this property provided multipoint attachment to the matrix and/or coupling of the protein via more than one subunit is minimal. It is therefore advisable to use relatively low concentrations of activated groups and of protein during the coupling step.

EXPERIMENTAL

Preparation of immobilized subunits

Insoluble subunits are best obtained by coupling the protein to the activated matrix.

Highly porous and hydrophilic gels like Sepharose 4B have been employed extensively also because they allow lyophilization of the immobilized subunits.

The standard cyanogen bromide activation method [16] has proved suitable. For the reasons given above, the cyanogen bromide and protein concentrations should be kept low (1–5 mg/ml of settled gel and 1—10 mg/ml, respectively). For immobilization of oligomeric structures, further precautions may prove advantageous to reduce steric hindrance effects: reduction of the concentration of activated groups by washing the matrix with 0.1 *M* sodium bicarbonate before addition of the protein [13]; presence of ethanolamine during the

coupling step in roughly equimolar amounts with respect to the potentially reactive groups on the protein [14]; indirect coupling of the subunits to the matrix via a spacer arm of appropriate length [6]. The drawback of the cyanogen bromide method lies in the lack of chemical selectivity which may lead to heterogeneity in the covalent attachment of the protein. However, preferential binding to the matrix via the α- or ϵ-amino groups may be achieved by varying the pH of the reaction [16]. It should be recalled, in any case, that pH can affect the state of association of oligomeric proteins, which are best immobilized as oligomers since the intersubunit contact regions are protected from reaction with the matrix [14]. A comparison of the amount of protein in the solid phase before and after treatment with strong dissociating agents, such as concentrated urea or guanidine hydrochloride, shows that the average size of the molecule attached covalently to the matrix varies even when the coupling step is performed under conditions that stabilize the oligomer. Thus, proteins may be immobilized as oligomers or subunits of different states of association [1, 9, 13, 14, 17] or as monomers [6].

At the end of the coupling reaction it is necessary to remove any non-covalently-bound protein. The matrix, therefore, is washed exhaustively under conditions that favour dissociation of the protein, and immobilized subunits are generated.

Measurement of the associating capacity of the immobilized subunits

The capacity of a preparation of immobilized subunits to interact with soluble subunits can be assessed very easily with a small chromatographic column ($\approx$5 ml of settled immobilized subunits) equilibrated with associating buffer. For a qualitative indication of the associating capacity, it suffices to apply to the column a small volume (0.5—1 ml) of soluble protein in the same buffer. If there is interaction, elution of the protein will be retarded with respect to that of a non-interacting protein, i.e. to the void volume of the column. For a quantitative measure of the associating capacity, the volume of protein solution percolated through the column should be large enough to allow the establishment of a steady state. This situation is realized when the concentration of the effluent equals that of the inflowing solution. Under these conditions, the amount of soluble protein retained on the column ($\overline{Y}$) is given by the product of the protein concentration in solution (c) and the increase in elution volume with respect to the void volume (ΔV) [1—4]. Fig. 1 shows the relationship between the value of $\overline{Y}$, the increase in elution volume, ΔV, and protein concentration in solution predicted by the law of mass action for a monomer—dimer equilibrium [1—3]. It can be seen that the elution volume is inversely related to the concentration of the soluble protein and that the increase in elution volume with decrease in protein concentration is more marked the higher the association constant.

Use of immobilized subunits for protein purification

Immobilized subunits can be utilized in batch procedures, in closed reactors [8, 13] or in columns [1, 6, 12, 15] for many purification cycles, as their stability is higher than that of soluble protein.

The use of columns offers the advantage that, as shown by Fig. 1, retarda-

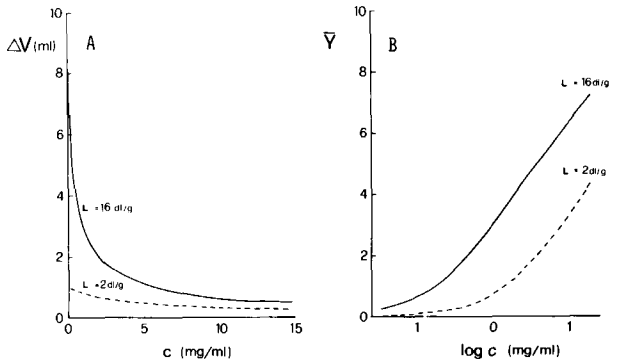

Fig. 1. Elution volume from a subunit affinity column (A) and amount of protein bound under steady-state conditions (B) as a function of protein concentration in solution. The data were calculated for a system undergoing reversible dimerization with the same association constant, L, in solution and in the solid phase. The concentration of immobilized protein was taken as 10 mg/ml. For the derivation of the relevant equations, see refs. 1 and 3.

tion in the elution of the interacting protein is highest when its concentration in solution is low, i.e. in the situation encountered usually during the purification of proteins.

RESULTS

Bovine α-chymotrypsin is known to undergo a pH-dependent dimerization reaction: dimer formation is at a maximum at pH 3.9 in 0.02 *M* acetate buffer containing 0.18 *M* sodium chloride, while monomer formation is induced at pH 2.5 [18]. Bovine α-chymotrypsin was immobilized on cyanogen bromide-activated Sepharose 4B at pH 8.0 by using 30 mg of cyanogen bromide and 7 mg of protein per ml of settled gel. The unreacted groups were quenched by addition of ethanolamine. Subsequently, the matrix was washed exhaustively under dissociating conditions (0.01 *M* acetate buffer at pH 2.5) to remove any non-covalently-bound protein. The amount of α-chymotrypsin immobilized on the matrix was determined spectrophotometrically at 280 nm on the basis of the extinction coefficient $E^{1\%}_{1\,cm} = 20.7$; the turbidity of the material was minimized by using a protein-free gel in the reference beam [15].

Immobilized bovine α-chymotrypsin has been employed for the separation of the enzyme from its zymogen and for the purification of porcine α-chymotrypsin from a pancreatic extract, since immobilized bovine α-chymotrypsin is capable of forming hybrids with the porcine protein [7].

Fig. 2 illustrates the separation of α-chymotrypsin from its zymogen and, in particular, the effect of the concentration of the interacting protein on the efficiency of the subunit affinity column. When the two proteins are at the same concentration in the mixture applied to the column, which is equilibrated with the associating buffer at pH 3.9, the difference in their elution volume is relatively small and part of the α-chymotrypsin is eluted with the associating buffer. Upon dilution of α-chymotrypsin, the difference in elution volume between the two proteins is increased dramatically and α-chymotrypsin is

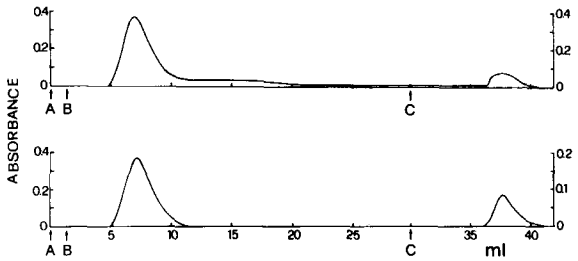

Fig. 2. Subunit exchange chromatography of a mixture containing bovine α-chymotrypsin and chymotrypsinogen on a column of Sepharose-bound bovine α-chymotrypsin. The column (5 ml of settled gel) was equilibrated with 0.02 *M* acetate buffer + 0.18 *M* sodium chloride at pH 3.9. A 1-ml pulse of a protein mixture was applied in both experiments; in the upper one, chymotrypsinogen and α-chymotrypsin were at a concentration of 1.8 mg/ml; in the lower one, chymotrypsinogen was at the same concentration of 1.8 mg/ml while α-chymotrypsin was at 0.5 mg/ml. Arrows indicate the application of protein solution (A), equilibrating buffer (B) and dissociating buffer, i.e. 0.02 *M* acetate buffer at pH 2.5 (C). The flow-rate was 15 ml/h; the absorbance was monitored at 280 nm.

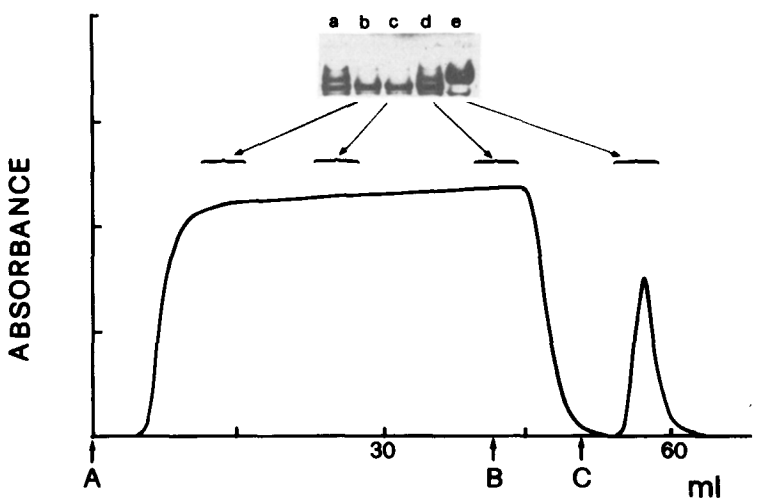

Fig. 3. Purification of α-chymotrypsin from a porcine pancreatic extract on a column of Sepharose-bound bovine α-chymotrypsin. The column (5 ml of settled gel) was equilibrated with 0.02 *M* acetate buffer + 0.18 *M* sodium chloride at pH 3.9. Arrows indicate the application of 2 g of acetone powder dissolved in the same buffer after ammonium sulphate precipitation (A), of the equilibrating buffer (B) and of the dissociating buffer, i.e. 0.02 *M* acetate buffer at pH 2.5 (C). The flow-rate was 30 ml/h; the absorbance was monitored at 280 nm. The inset shows the polyacrylamide gel electrophoretic patterns obtained at pH 4.3 [19] of the pancreatic extract stock solution (a) and of the fractions indicated on the elution profile (b—e).

eluted only after application of the dissociating buffer at pH 2.5.

The purification of porcine α-chymotrypsin from a pancreatic acetone extract provides an example of the use of subunit exchange chromatography as a preparative tool. A solution containing 2 g of porcine pancreatic extract in the associating buffer at pH 3.9 was percolated through a column of immobilized bovine enzyme equilibrated with the same buffer (Fig. 3). When

the capacity of the column is exceeded, α-chymotrypsin is no longer retained, its elution being indicated by the clear rise in the absorbance of the plateau region and by the concomitant appearance of the α-chymotrypsin band in the electrophoretic patterns of relevant fractions of the column eluate (e.g. fraction d). After washing the column with the equilibrating buffer, desorption of α-chymotrypsin was obtained at pH 2.5. The quality of the purified protein is shown in the inset of Fig. 3.

CONCLUSIONS

Subunit exchange chromatography represents a versatile, effective and inexpensive means of purifying self-associating and oligomeric proteins. Major advantages of the method are the very low aspecific binding of extraneous proteins or other components of the system (at ionic strength $\geqslant 0.01\ M$) and the fact that the immobilized protein does not need to be purified to homogeneity unless the contaminants are themselves associating—dissociating systems. Moreover, a protein from a different species may be immobilized in case it forms hybrids with the protein to be isolated. Difficulties may arise due to coupling of the protein through residues near the intersubunit contact regions and/or to heterogeneity of covalent attachment within a preparation of immobilized subunits. In general, such problems may be solved by appropriate adjustment of the coupling conditions.

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