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AFFINITY PARTITION OF PROTEINS IN AQUEOUS TWO-PHASE SYSTEMS CONTAINING POLYOXYETHYLENE GLYCOL-BOUND LIGAND AND CHARGED DEXTRANS

ABDELAZIZ CHAABOUNI and EDITH DELLACHERIE

Laboratoire de Chimie-Physique Macromoléculaire, E.R.A. 23 E.N.S.I.C., 1 rue Grandville, 54042 Nancy Cedex (France)

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SUMMARY

The partition of the $\Delta_{5\rightarrow4}$ 3-oxosteroid isomerase of *Pseudomonas testosteroni* in aqueous two-phase systems containing both the macroligand, polyoxyethylene glycol-bound estradiol, and charged (cationic or anionic) dextrans has been studied. If the enzyme is well retained in the upper phase by an adequate amount of macroligand, it is possible to improve the removal of the contaminating proteins by extracting them into a lower phase containing positively or negatively charged dextran. Some multi-step extraction experiments were carried out to purify isomerase, by washing the upper phase successively with cationic and anionic dextran-rich phases.

INTRODUCTION

It is possible to separate a protein from a contaminating medium by using aqueous polymer phase systems to which is added a polymer ligand with a high affinity for a binding site on the protein to be purified. This method, called affinity partitioning, has been used by us to purify $\Delta_{5\rightarrow4}$ 3-oxosteroid isomerase[1] by means of a two-phase system of polyoxyethylene glycol–dextran–water, containing polyoxyethylene glycol-bound estradiol. In this case, the effectiveness of a multi-step partition was strongly dependent on the experimental conditions of each extraction: the partition coefficient of isomerase in fact remained constant in each step, whereas the partition coefficient of the total proteins increased drastically. This means that fractionation of proteins takes place during this process and that the system is progressively enriched in proteins which favour the upper phase. Varying the pH or ionic strength of each extraction was not a very efficient means of extracting the contaminant proteins into the lower phase because, after five extractions, the purification ratio was no greater than 170.

Another means of improving this purification technique is to strongly attract the proteins in the dextran-rich phase while the species to be purified remains in the polyoxyethylene glycol rich phase, owing to its biospecific interactions with the polyether ligand. As it is well known that charged polymers interact strongly with

proteins[2,3], we have studied the distribution of isomerase and of the contaminating proteins in two-phase aqueous systems of polyoxyethylene glycol–dextran–water containing both the macroligand, polyoxyethylene glycol-bond estradiol, and cationic or anionic derivatives of dextran.

In this paper, we report the results obtained for the partitioning of these proteins in such systems and describe the conditions for obtaining an optimal separation of the enzyme.

EXPERIMENTAL

Materials

Polyoxyethylene glycol (POEG, $\bar{M} \approx 6000$) and dextran T110 ($\bar{M} \approx 110{,}000$) were supplied by Roth (Karlsruhe, G.F.R.). The isomerase-containing acetone powder was kindly provided by Pr. E. E. Baulieu and isomerase was extracted according to Weintraub *et al.*[4]. Δ_5-Androstene-3,17-dione was donated by Roussel-Uclaf (Paris, France). Trypsin and benzoyl-D,L-arginine *p*-nitroanilide were obtained from Sigma (St. Louis, Mo., U.S.A.). Polyoxyethylene glycol-bound estradiol was synthesized by a method described elsewhere[1].

Cationic dextran (Fig. 1) was prepared by reaction on dextran, in basic aqueous solution, of the epoxyamine resulting from the action of triethylamine on epichlorhydrin[5,6]. Anionic dextran (Fig. 1) was synthesized according to standard methods[7] by the action of chloroacetic acid on dextran in the presence of sodium hydroxide.

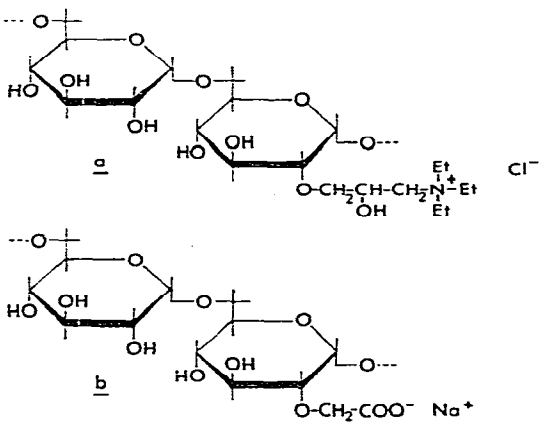

Fig. 1. Partial structures of the polyionic derivatives of dextran. (a) Cationic dextran (D–N^+); (b) anionic dextran (D–COO^-). The charged sites are located at arbitrary positions.

All of the polymers were purified by extensive aqueous dialysis, then freeze-dried. The degree of substitution was determined as the percentage of nitrogen for the cationic dextran, and by an acid–base titration for the anionic dextran.

Methods

Two-phase systems and partition coefficients. Two-phase systems (volume ratio

1:1) were prepared by mixing 0.28 g of POEG ($\bar{M}$ = 6000), 0.48 g of dextran ($\bar{M}$ = 110,000; crude and charged, in a known ratio) and 0.02 *M* phosphate buffer to give a final weight of 3 g. Then, after the polymers had completely dissolved, 1 g of the biological extract, also containing the desired amount of POEG-bound estradiol, was added.

After stirring and centrifugation, aliquots of both phases were collected and assayed for isomerase activity and proteins as described earlier[1]. Partition coefficients were calculated from the enzymatic activity and protein concentration ratios between the upper and the lower phases.

For the partition of trypsin, the system was obtained by mixing 0.28 g of POEG, 0.48 g of dextran and 2.54 g of 0.02 *M* phosphate buffer. After dissolution of the polymers, 0.50 g of a solution of trypsin in 0.001 *M* hydrochloric acid (9 mg/ml) was added. After centrifugation, the trypsin concentration of both phases was measured with benzoyl-D,L-arginine *p*-nitroanilide as substrate at 410 nm[8].

Multi-step extractions. This process was carried out in the same manner as previously outlined[1] and according to the scheme shown in Fig. 2. All of the experiments were carried out at room temperature.

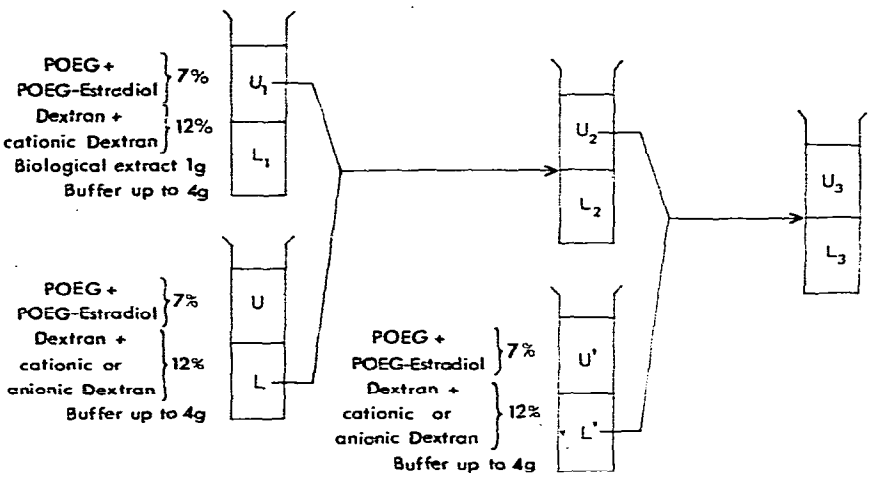

Fig. 2. Schematic representation of the multi-step extraction procedure. The two-phase systems U_1L_1, U_2L_2 and U_3L_3 contain the biological extract, unlike the two-phase systems UL and U'L'.

The two-phasic system U_1,L_1 results from the demixing of the mixture containing, for example, cationic dextran. The upper phase, U_1, is collected and mixed with an equal weight of the lower phase, L, where cationic dextran can be replaced with the anionic derivative, to give the system U_2,L_2, and so on.

RESULTS

Influence of cationic dextran

The polycationic derivative (D–N^+) obtained by the method described under *Materials* contained about 10–15 ammonium sites per 100 glucosyl residues.

Partition of isomerase and proteins. The partition of total proteins and isomerase, which is a negative protein in the pH range used for the experiments ($pH_i = 4.7$), was studied as a function of the amount of charged dextran added to the system (Table I).

TABLE I

PARTITION COEFFICIENTS OF ISOMERASE AND TOTAL PROTEINS AS A FUNCTION OF THE AMOUNT OF D–N^+ (13 CHARGED SITES PER 100 GLUCOSYL RESIDUES)

The biological extract was partitioned in two-phase systems containing 7% POEG 6000, 12% total dextran T110, 25% extract and 0.02 *M* phosphate buffer (pH 8); total weight, 4 g; volume ratio, 1/1.

Partition coefficient	*% of D–N^+ with respect to total dextran*			
	0	*10*	*20*	*50*
Isomerase	3.6	1.6	1.1	0.2
Proteins	0.58	0.20	0.08	0.06

The concentration of cationic polysaccharide in the system strongly affects the partition of isomerase because, without charged dextran, 78% of the enzyme is found in the upper phase, whereas, with 50% of D–N^+ the lower phase contains more than 80% of the enzyme (Fig. 3).

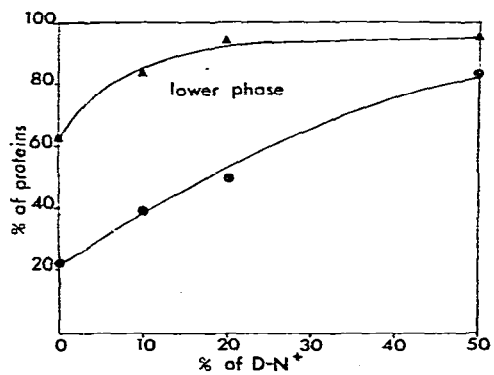

Fig. 3. Partition of proteins (●, isomerase; ▲, total proteins) as a function of increasing concentrations of cationic dextran (see text for details). The content of D–N^+ in the systems is expressed as the fraction (%) of the total amount of dextran.

The effect of charged dextran is less significant for total proteins because in a standard system 62% of the proteins already favour the lower phase; therefore, with 50% of cationic dextran, the lower phase contains about 95% of the total proteins (Fig. 3). Varying the pH had no detectable effect on the partition coefficients.

Affinity partition of isomerase and proteins. Table II shows the partition coefficients of isomerase and proteins in two-phase systems where 50% of the dextran were replaced with the cationic derivative, and containing increasing concentrations of POEG-bound estradiol.

TABLE II

PARTITION COEFFICIENTS OF ISOMERASE AND TOTAL PROTEINS AS A FUNCTION OF THE AMOUNT OF POEG-BOUND ESTRADIOL ADDED TO THE SYSTEM

The biological extract was partitioned in two-phase systems containing 7% POEG 6000 (crude POEG and POEG-bound estradiol), 6% dextran T110, 6% dextran D–N^+ (13 charged sites per 100 glucosyl residues), 25% extract and 0.02 *M* phosphate buffer (pH 8); total weight, 4 g; volume ratio, 1/1.

Partition coefficient	*Amount of POEG-bound estradiol (mg)*				
	0	*4*	*8*	*12*	*20*
Isomerase	0.2	0.8	1.3	1.8	2.2
Proteins	0.06	0.15	0.15	0.20	0.20

It can be seen that the partition coefficient of isomerase is dependent on the macroligand content, and by increasing its concentration in the system it is possible to attract increasingly the enzyme in the upper phase. At the same time, probably owing to low affinity interactions with estradiol, the distribution of proteins is also influenced by the presence of the macroligand and they are shifted, in the upper phase, to some extent. It should therefore be observed that the affinity effect on the isomerase behaviour is more significant than the effect on that of the proteins: in fact, the amount of isomerase in the upper phase increases from 17 to 69%, whereas, at the same time, that of proteins increases only from 6 to 17% (Fig. 4).

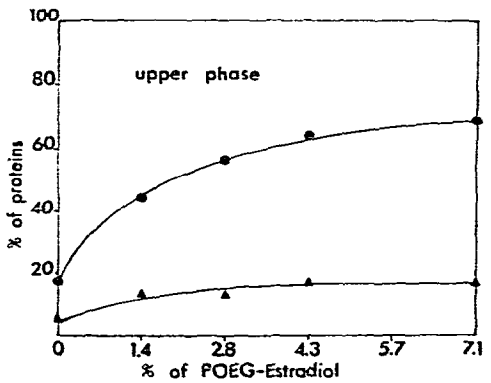

Fig. 4. Partition of proteins (●, isomerase; ▲, total proteins) in systems containing a constant concentration of D–N^+ (50% of total dextran) and increasing concentrations of POEG-bound estradiol. The content of the macroligand is expressed as the fraction (%) of the total amount of POEG.

On the other hand, it must be also noted that the partition coefficient of isomerase is much lower than when the system contains no charged dextran[1]. For this reason, another series of experiments was performed to examine the influence of the amount of polyelectrolyte on the partition of isomerase in the presence of a constant concentration of macroligand (Table III).

Finally, the best separation is achieved when the system contains no more than 10% of cationic dextran, as increasing its concentration substantially affects the partition of isomerase while the contaminant proteins are no longer attracted to the bottom phase.

TABLE III

DISTRIBUTION OF ISOMERASE AND TOTAL PROTEINS IN AFFINITY TWO-PHASE SYSTEMS CONTAINING INCREASING CATIONIC DEXTRAN CONCENTRATIONS

6.4% POEG 6000, 0.6% POEG-bound estradiol, 12% total dextran. Other conditions as usual.

Parameter	*Biological extract*	*U*	*L*	*U*	*L*	*U*	*L*	*U*	*L*
Amount of POEG-bound estradiol (mg)		0		25		25		25	
% of D–N^+ with respect to total dextran		0		0		10		20	
Isomerase activity (U/ml)	773	247	69	394	6.4	342	25	322	57
Proteins (mg/ml)	14	1.45	2.5	1.75	3.6	0.4	3.6	0.5	4
Isomerase specific activity (U/mg)	55	170	28	225	1.8	855	7	644	14.5
Purification ratio		3		4		15.5		11.7	
Partition coefficient of isomerase		3.6		61		13.5		5.6	
Partition coefficient of proteins		0.58		0.50		0.11		0.12	

Influence of anionic dextran

The polyanionic derivative of dextran (D–COO^-; see *Materials*) contained about four charged sites per 100 glucosyl residues. Taking into account that isomerase is a negatively charged protein at a pH higher than 4.7, its partitioning was not modified by adding anionic dextran to the two-phase systems. Therefore, to examine the effect of this polyelectrolyte on the distribution of negatively charged proteins in two-phase systems, trypsin [$pH_i = 10.8$ (ref. 9)] was partitioned in a system in which 50% of the total dextran was replaced with the polyanionic derivative (Table IV).

TABLE IV

PARTITION COEFFICIENTS OF TRYPSIN IN SYSTEMS CONTAINING 7% POEG 6000, 12% DEXTRAN T110 (CRUDE OR MIXED WITH ANIONIC DERIVATIVE), 1.2 mg OF TRYPSIN, 0.02 *M* PHOSPHATE BUFFER pH 7; TOTAL WEIGHT, 4 g; VOLUME RATIO, 1:1

% of D–COO^- with respect to total dextran	*Partition coefficient of trypsin*
0	0.38
50	~0

From these results, it appears that the influence of the charged dextran on the partitioning of trypsin is drastic, as the protein is totally eliminated from the upper phase. Such experiments were carried out on the crude isomerase-containing extract, but the distribution of contaminating proteins was not substantially improved in the presence of the anionic dextran.

Therefore, in order to achieve further separation of isomerase, the multi-step extraction procedure was then used, in which the macroligand-containing upper phase was washed successively with cationic dextran-rich phases, then with an anionic dextran-rich phase.

Multi-step extractions

A preliminary experiment was conducted with a combination of both cationic (first extraction) and anionic (second extraction) dextran (see Table V).

TABLE V

TWO-STEP EXTRACTION OF ISOMERASE

The systems were prepared as described under *Methods*: 6.4% POEG, 0.6% POEG-bound estradiol, 12% total dextran (D–N^+, 16 charged sites per 100 glucosyl residues; D–COO^-, as usual). Total weight, 4 g; volume ratio, ~ 1:1; phosphate buffer (pH 7).

Material	*% of D–N^+ with respect to total dextran*	*% of D–COO^- with respect to total dextran*	*Isomerase activity (U/ml)*	*Proteins (mg/ml)*	*Isomerase specific activity (U/mg)*	*Purification ratio*	*Partition coefficient of isomerase*	*Partition coefficient of proteins*
Biological extract			673	10.5	64			
U_1	20	0	275	0.37	743	11.6	4.5	0.12
L_1			61	3.1	20			
U_2	0	50	259	0.20	1300	22	52	0.80
L_2			4.6	0.25	20			

As expected, the first step leads to a satisfactory purification ratio; on the other hand, in the second step, the partition coefficient is found to be about 0.8, which means that a large amount of proteins still remains in the upper phase, and that consequently, after the first extraction, the bulk of the upper phase proteins are negatively charged.

This was confirmed by another experiment in which the first two successive extractions were carried out with a cationic dextran-rich phase (Table VI). In this instance, it can clearly be seen that the partition coefficient of total proteins has decreased from 0.8 to 0.44, leading to a higher purification ratio.

The separation achieved with a third extraction by means of an anionic dextran-rich phase is then markedly better than that achieved by a three-step standard

TABLE VI

THREE-STEP EXTRACTION OF ISOMERASE

Systems prepared as described in Table V.

Material	*% of D–N^+ with respect to total dextran*	*% of D–COO^- with respect to total dextran*	*Isomerase activity (U/ml)*	*Proteins (mg/ml)*	*Isomerase specific activity (U/mg)*	*Purification ratio*	*Partition coefficient of isomerase*	*Partition coefficient of proteins*
Biological extract			773	11	70			
U_1	10	0	379	0.4	950	13.5	11	0.15
L_1			34	2.6	13			
U_2	10	0	357	0.14	2550	36	13.7	0.44
L_2			26	0.32	81			
U_3	0	50	350	0.04	8750	125	87	0.57
L_3			4	0.07	57			

procedure[1], as here the purification ratio reaches 125, whereas in the previous two experiments it was about 15 and 30, respectively, after the third extraction.

DISCUSSION

The partition of proteins in liquid–liquid aqueous two-phase systems depends strongly on the composition of the phases. Firstly, it is known that the presence in a phase of a polymer ligand with a high affinity for a substance to be purified attracts it into this phase. On the other hand, when one of the layers contains a charged polymer, the proteins carrying opposite net charges are selectively directed to this phase. Thus, a real separation of proteins can occur according to their isoelectric points and provided that the protein–protein interactions are not too strong. Such experiments were previously performed by Johansson *et al.*[3] with dextran–polyoxyethylene glycol–water two-phase systems containing positively charged polyoxyethylene glycol.

A combination of these two phenomena (affinity partition and electrostatic effects) was used by us to achieve further purification of a steroid-binding protein, the $\Delta_{5\rightarrow4}$ 3-oxo steroid isomerase. In this instance, while the enzyme is almost completely restricted to the macroligand-containing upper phase, at the same time the contaminating proteins can be transferred to a charged dextran-rich layer, owing to ionic interactions.

When charged dextran is added to a two-phase system, its re-partition between the two phases depends on the degree of substitution: a small amount of dextran carrying many charges will separate like a polyelectrolyte substance, but if its concentration increases, the system can be completely altered. In contrast, dextrans with very low substitution retain their affinity for the lower dextran-rich phase and are always found in it.

The positively and negatively charged dextrans that were synthesized and used for the preparation of the systems contained no more than 15 charged sites per 100 glucosyl residues; this corresponds to an average of one substituted residue in seven. Replacement of crude dextran by these polyionic derivatives in the two-phase systems never leads to any alterations to the volumes of the phases.

When both polyoxyethylene glycol bound-estradiol and cationic dextran are present in the systems, it should be noted that the affinity of isomerase for its macroligand is markedly lower than in the absence of charged dextran. In fact, as at pH higher than 4.7 isomerase is a negatively charged protein, it interacts both with the polycationic derivative and the polyether ligand. Then, in order to render this method, sufficiently efficient it is necessary to increase the concentration of the macroligand in the medium. On the other hand, when the affinity extraction is carried out with an anionic dextran-rich layer, the partition of isomerase is not affected, as in a standard system (Table III, 0.6% POEG-bound estradiol, no anionic dextran), 98% of the enzyme favour the upper phase, just as in a system containing anionic dextran (Table V).

A striking feature of the results of the multi-step experiments performed on the isomerase-containing extract is that for the second operation the extraction with a cationic dextran-rich phase leads to a better purification ratio than with the anionic derivative, suggesting that most contaminating proteins which remain in the upper phase after the first partition have a negative net charge.

If we compare the purification yield obtained in a three-step extraction by means of affinity material and ionic dextrans with that obtained previously in standard systems[1], it is found that some substantial improvements have been achieved in the procedure. Moreover, the purification ratio could probably be enhanced further by increasing simultaneously the concentrations of the macroligand and the cationic dextran in the first step, and possibly also by varying the pH and/or ionic strength to optimize the experimental conditions.

In conclusion, the results indicate that, by taking into account both the affinity characteristics of a protein for a polymer ligand and the properties of the contaminating proteins, especially their ionic properties, it is possible to select for every particular case the nature and the concentration of the charged dextrans added to the systems and so render the standard affinity partition procedure more effective.

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