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USE OF WATER-SOLUBLE BIOSPECIFIC POLYMERS FOR THE PURIFICATION OF PROTEINS

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1. INTRODUCTION

Enzymes form specific and reversible complexes with substrates, inhibitors or structurally closely related substances, commonly called ligands. If such a ligand is associated with an insoluble matrix packed in a chromatographic column, then the percolation through it of a solution containing the biological complementary macromolecule results in the formation of a complex that is purified from contaminants by applying an appropriate elution buffer. This is the basis of affinity chromatography, a method that has been used extensively in recent years. However, the use of insoluble biospecific polymers involves difficulties, which are related to the preparation of the affinity gel (binding of the ligand and evaluation of the amount effectively linked, in a heterogeneous medium) and to the formation of a complex between the immobilized ligand and the complementary macromolecule (exclusion effects and diffusional limitations of the matrix).

In our laboratory, we have developed some other purification techniques based on the same principle of bioaffinity, but in which the biospecific polymers are water-soluble. In this review are described the main advantages of these water-soluble macroligands, the different methods used to extract the macrocomplexes formed between the polymeric ligands and the complementary macromolecule from the biological extracts and the characteristic results obtained in the purification of two steroid binding proteins.

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2. PREPARATION AND CONTROL OF WATER-SOLUBLE BIOSPECIFIC POLYMERS

In comparison with the limitations due to the insolubility of the affinity material, the use of water-soluble polymers improves ligand coupling and control of the amount immobilized.

Solubility in water and in some organic solvents makes possible the coupling reactions of the ligand with the polymer in homogeneous solutions. Consequently, the yields are much better and highly substituted macromolecular compounds can therefore be obtained. Thus, to purify the $\Delta_{5\to4}$ 3-oxosteroid isomerase *(Pseudomonas testosteroni)* we synthesized a macroligand by binding estradiol-7α-butyric acid (Fig. 1) on the terminal functions of polyoxyethylene glycol according to the following scheme:

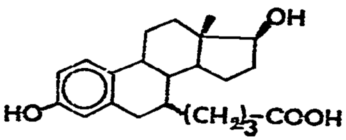
$$\underset{(2)}{H(OCH_2CH_2)_{n+1}{-}OH} + SOX_2 \rightarrow \underset{(3)}{XCH_2CH_2(OCH_2CH_2)_n{-}X}$$

$(X = Cl,Br)$

$$3 + R^1NH_2 \rightarrow \underset{(4)}{R^1NHCH_2CH_2(OCH_2CH_2)_n{-}NHR^1}$$

$$4 + R^2CO_2H \rightarrow \underset{(5)}{R^2CONR^1CH_2CH_2(OCH_2CH_2)_n{-}NR^1COR^2}$$

Halogenation and amination reactions, carried out on a polymer of 6000 molecular weight (hydroxyl group content determined by acetylation = 1.9 per macromolecule) are quantitative. The coupling reaction between derivative 4 and the carboxylic compound was performed in an organic solvent in the presence of peptide condensation reagents (carbodiimide, isobutyl chloroformate, N-hydroxysuccinimide, etc.) and yielded substitution rates as high as 95% if a large excess (20-fold) of the carboxylic compound was used. When performing the reaction with only 4 equivalents of derivative 1 (twice the stoichiometric amount), a macroligand containing 1.3 equivalents of steroid per macromole was obtained[1]. Quantitation can be performed directly on an aqueous solution of the macroligand by simply measuring the absorbance at 285 nm.



(1)

Fig. 1. Estradiol-7α-butyric acid (1).

Other macroligands have been synthesized by coupling derivative 1 with dextrans with different molecular weights, according to the following scheme[2]:

$$\text{Dextran–OH} + \underset{\diagdown O \diagup}{CH_2{-}CHCH_2Cl} \xrightarrow{Zn(BF_4)_2} \underset{(6)}{\text{Dextran–O–}CH_2\underset{\substack{|\\OH}}{CH}CH_2Cl}$$

$$6 + NH_4OH \xrightarrow{\text{pH } 9.5} \text{Dextran-O-}CH_2\underset{\displaystyle OH}{\underset{|}{C}}HCH_2NH_2$$

(7)

$$7 + R^2COOH \longrightarrow \text{Dextran-O-}CH_2\underset{\displaystyle OH}{\underset{|}{C}}HCH_2NHCOR^2$$

(8)

Under the conditions we used, we repeatedly obtained, whatever the molecular weight of the polysaccharide, a chlorine content of 2–3%, corresponding to $5 \cdot 10^{-4}$–$8 \cdot 10^{-4}$ equivalents of Cl per gram of polymer and to a topochemical substitution of one hydroxyl group every tenth glucopyranose unit[3]. Nevertheless, it is possible, using more drastic conditions [higher temperature and larger amounts of $Zn(BF_4)_2$] to increase considerably the chlorine content.

The replacement of the chlorine atom with an amino group takes place with a yield over 90% and the final coupling reaction is carried out either in a water–dimethylformamide mixture in the presence of N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDCI) or in an organic solvent such as dimethyl sulphoxide with benzotriazolyloxytrisdimethylaminophosphonium hexafluorophosphate[4] (BOP) as a condensation reagent. The second pathway gives better results and yielded macroligands containing $1 \cdot 10^{-4}$–$2 \cdot 10^{-4}$ equivalents of steroid per gram of polymer. It should be pointed out that biospecific insoluble matrices commonly contain a few 10^{-6} moles of ligand per gram of dry resin[5–7] and that in attempts to purify estradiol receptor by affinity chromatography, the polystyrenic adsorbents contained 1.3 mg ($4 \cdot 10^{-6}$ mol) of steroid (estradiol-7α-butyric acid) per gram of dry resin, the acrylamide supports 1.25 mg ($4 \cdot 10^{-6}$ mol) per gram of dry powder and the agarose adsorbents about 0.03 mg (about 10^{-7} mol) per millilitre of gel[8].

3. BINDING CAPACITY OF THE LINKED LIGANDS

The homogeneous-phase synthesis of affinity polymers yields high concentrations of specific sites. This results in macroligands with a high affinity that can be easily and directly measured in aqueous solutions. For instance, we have measured the affinity constants of the polymeric ligand 5 ($\overline{M}_w = 6000$) in which R^1NH_2 is 1,3-diaminopropane and which contains 1.3 equivalents of estradiol per macromole, for the $\Delta_{5\to4}$ 3-oxosteroid isomerase of *Pseudomonas testosteroni*, using the Dixon technique[9]. This enzyme is known[10] to bind to free estradiol with $K_a \approx 10^6\ l \cdot mol^{-1}$; in the case of the immobilized steroid, considering the fact that isomerase has only one binding site for estradiol[11], the affinity constant can be estimated to be $1.5 \cdot 10^5\ l \cdot mol^{-1}$, *i.e.*, seven times weaker than for the free estradiol. This decrease is relatively weak, especially if one takes into account the fact that the inhibition efficiency of a 7α-butyric alcohol estradiol is about five times lower than for estradiol whereas the affinity of the corresponding 7α-butyric acid derivative is 18 times weaker[11].

The coupling reaction of estradiol-7α-butyric acid with the amino derivatives of dextran yields macroligands the biospecificity of which has been controlled by measuring their binding capacity with cytosolic calf uterus estradiol receptor[3]. The affinity constant of a dextran ($\overline{M}_w \approx 500{,}000$) containing $3 \cdot 10^{-5}$ equivalents of steroid per gram of polymer is $10^2\ l \cdot g^{-1}$ for the estradiol receptor (determination by the Lineweaver–Burk technique[12]). If one assumes that this receptor contains only one steroid binding site per molecule[13], then the affinity constant of immobilized estradiol for the receptor is $3.3 \cdot 10^6\ l \cdot mol^{-1}$. With estradiol, free and unsubstituted, its affinity constant for the receptor is about $5 \cdot 10^9\ l \cdot mol^{-1}$ [14]. As the value of K_a for estradiol-7α-butyric carboxamide is not known, the decrease in the binding capacity, which should be due only to the immobilization of the steroid, is difficult to establish. Nevertheless, it can be noted that the introduction into estradiol of a 7α-carboxylic 3-carbon chain leads to a very low (undetectable) affinity of the derivative for the receptor, and the affinity constant of the estradiol-7α-butyric methyl ester is about 10^8 and $10^7\ l \cdot mol^{-1}$ for the 7α-butyric alcohol derivative[8].

From a precise knowledge of the binding capacity of the macroligands, it is possible to calculate the minimal amount of polymer to be added to the solution in order to complex completely the species to be isolated.

In comparison with conventional affinity procedures, this approach permits stricter control of the conditions for the binding of proteins and, as the efficiency of the polymer–ligand coupling is generally good, the amount of macroligand to be added is small in absolute terms and it is then possible to reduce, to a large extent, the non-specific interactions between the polymer and the contaminants. For example, it can be calculated that, to complex 99% of the estradiol receptor present in the cytosolic extract with the dextran–estradiol described above, a concentration of only $1\ g \cdot l^{-1}$ will be necessary.

4. EXTRACTION OF THE MACROCOMPLEXES FROM THE EXTRACTION SOLUTION

4.1. Affinity partition

When the biospecific ligand is linked to one of the polymers constituting the aqueous two-phase system described by Albertsson[15], it is possible to attract specifically the macromolecule to be purified into the phase where this polymer is almost totally restricted and to adjust parameters such as pH or ionic strength to direct the contaminants towards the other layer (for a review, see ref. 16).

In Fig. 2, it can be seen that increasing amounts of polyoxyethylene-bound estradiol, added to an aqueous two-phase system composed of polyoxyethylene glycol 6000, dextran 80,000 and water, and containing the bacterial extract, draw up to 95% of $\Delta_{5\to4}$ 3-oxosteroid isomerase into the upper phase containing the polyether. After a five-step extraction, the enzyme was obtained with a purification ratio close to 170 (ref. 17).

This technique, which in principle is non-denaturing as it is performed in homogeneous aqueous solutions, has also been used for the extraction of membranes[18,19] and could be applied with success to the purification of whole cells, a problem for which affinity chromatography has not so far proved very satisfactory.

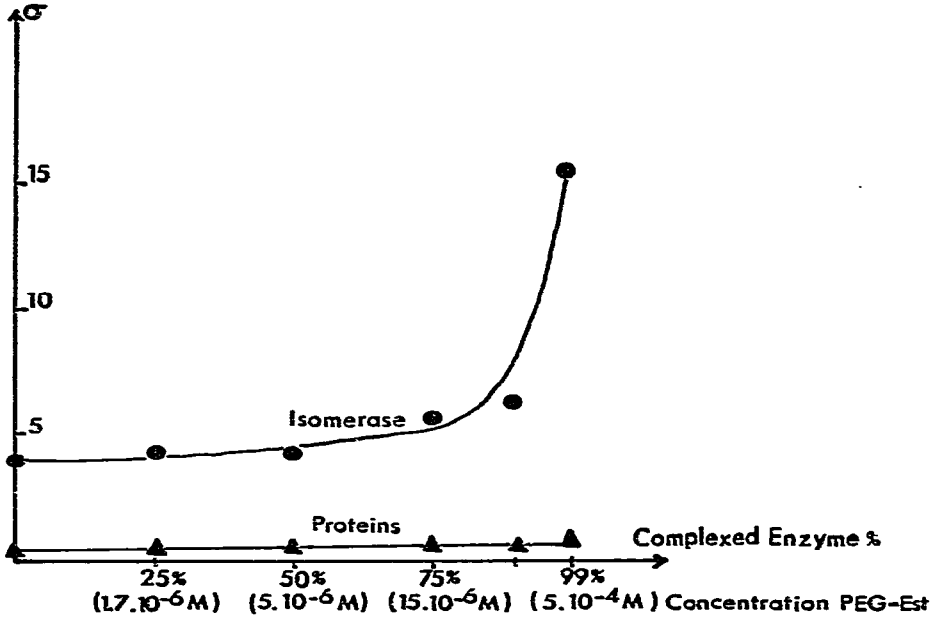


Fig. 2. Partition coefficients (σ) of isomerase (●) and total proteins (▲) as a function of the theoretical percentage of complex formed by affinity between isomerase and polyoxyethylene-bound estradiol (PEG–Est), and of the molar concentration of PEG–Est added to the system [PEG 6000, 7% (w/w); dextran T 80, 12% (w/w); phosphate buffer, 0.03 *M*, pH 7.0; PEG–Est up to 12 mg; crude extract from *Pseudomonas testosteroni* cultures, 0.4 ml; total weight, 4 g].

4.2. Gel filtration of macrocomplexes

When the affinity material is a high-molecular-weight polymer, the macrocomplex is characterized by a high apparent hydrodynamic volume. It then can be separated from the contaminants by fractionation on a gel the porosity of which is chosen so that the macrocomplex, owing to its size, is eluted in the void volume.

This technique has been applied in our laboratory to the purification of the cytoplasmic calf uterus estradiol receptor[3], a protein very unstable and of low concentration in the cytosolic extracts.

The linked estradiol–receptor complex was formed by incubating a cytosolic extract with the biospecific polymer described above ($K_a = 10^2\ l \cdot g^{-1}$) at a concentration of 2.5 $g \cdot l^{-1}$. At this concentration it can be calculated that the receptor is theoretically complexed to the extent of about 99.5%. The macrocomplex could then be separated from the bulk of the proteins present in the crude extract by filtration on an appropriate gel (Fig. 3). All of the receptor was removed from its normal elution region (Fig. 4) and eluted as a complex with biospecific dextran in the void volume.

A final procedure is then necessary to eliminate the polymeric material. During this operation, the macrocomplex, cleared of contaminants and collected in the fractions corresponding to the void volume, was dissociated by exchange with an excess of free estradiol. The mixture was then fractionated on the same gel and

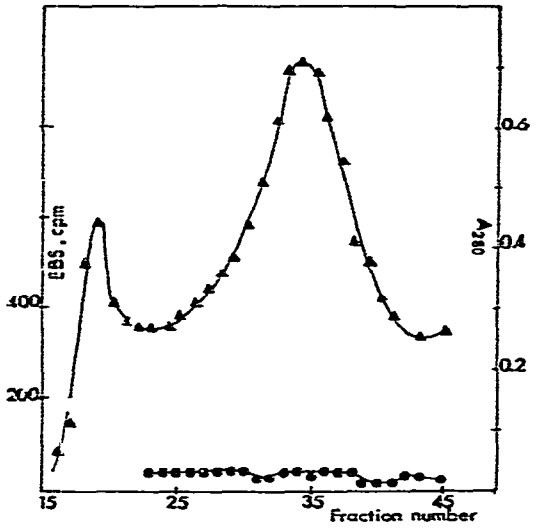


Fig. 3. Gel filtration of a cytosolic extract (10 ml; 4 S trypsin receptor preparation) after treatment with dextran–estradiol conjugate (25 mg) on an Ultrogel AcA-34 column (bed volume, 200 ml, elution buffer, Tris–HCl 50 mmol·l^{-1}, EDTA 1.5 mmol·l^{-1}, pH 7.5). ●, Concentration of specific estrogen-binding sites (EBS) determined as described in ref. 20. These values are related to the specific binding capacity of the receptor to radioactive estradiol and then with the concentration of this protein. Estrogen binding could not be measured in the void volume fractions because of the presence of a large excess of competing macroligand. ▲, Concentration of total proteins determined by their absorbance at 280 nm.

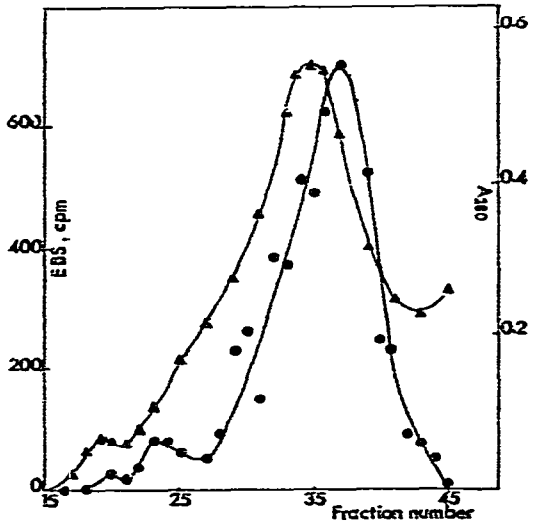


Fig. 4. Gel filtration of a cytosolic extract (10 ml; 4 S trypsin receptor preparation) on an Ultrogel AcA-34 column. Conditions and symbols as in Fig. 3.

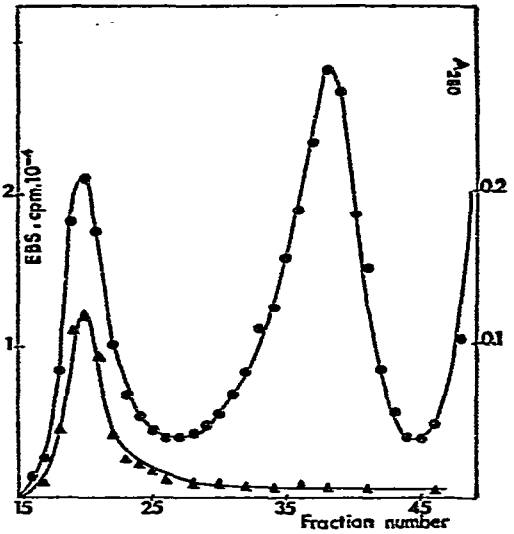


Fig. 5. Gel filtration of the void volume fractions collected in the filtration described in Fig. 3 after exchange with an excess of radioactive free estradiol. Conditions as in Fig. 3. The 4 S trypsin receptor form complexed with free estradiol is found in fractions 35–42. ●, Estradiol binding sites expressed in terms of radioactivity of 0.1-ml portions of the eluted fractions; ▲, absorbance at 280 nm.

the protein was recovered, purified, in the elution volume associated with its normal molecular weight (Fig. 5). Starting with 1 l of cytosol (400 g of calf uterus), prepurified and concentrated to 10 ml, to which were added 25 mg of dextran–estradiol, we obtained 10–20 μg (depending on the quality of the tissue) of the "4 S trypsin" receptor form[3] (yield 50–75%), corresponding to an 80% pure receptor[20].

4.3. Other methods

When the macroligand is characterized by a high molecular weight, it is possible to separate the macrocomplex from the contaminants on ultrafiltration membranes. With this technique, contaminants can be eliminated in the ultrafiltrate while the macrocomplex concentration is kept constant (diafiltration) or even increased (ultrafiltration). This results in protection of the protein to be purified against denaturation, which always increases on dilution and purification. Some experiments have been performed in our laboratory in order to extract the $\Delta_{5\to4}$ 3-oxosteroid isomerase of *Pseudomonas testosteroni* from a crude extract prepared as described earlier[17]. The macroligand was a dextran–estradiol of $\bar{M}_w \approx 2 \cdot 10^6$, containing about $0.7 \cdot 10^{-4}$ equivalents of steroid per gram. The rejection, R, is defined by

$$R = \frac{C_0 - C_p}{C_0} \cdot 100$$

where C_0 is the initial concentration of the species in the crude extract and C_p is their concentration in the permeate.

Without a polymeric ligand, the rejection of isomerase and contaminating

proteins were $R_I = 64\%$ and $R_P = 50\%$, respectively (membrane, Millipore P.S.V.P. 10^6; pressure, 0.2 bar). In the presence of 3 $g \cdot l^{-1}$ of dextran–estradiol it was observed that the isomerase was retained by the ultrafilter ($R_I = 90\%$) whereas the rejection of the other proteins was unchanged. Some other studies on this method are at present in progress in our laboratory, especially in order to improve the release of the contaminating proteins.

5. ADVANTAGES AND DISADVANTAGES OF WATER-SOLUBLE BIOSPECIFIC POLYMERS COMPARED WITH AFFINITY CHROMATOGRAPHY

The results reported above demonstrate the ready applicability of water-soluble biospecific polymers in the purification of proteins. The advantages of these new affinity materials lie in the high yields obtained from ligand coupling, the rapid control of the amount immobilized and the easy complexation with the complementary macromolecule from which follows a high binding capacity. Also, the affinity of the species to be isolated for the polymeric ligand can be determined very exactly. Hence it is possible to complex completely the protein to be extracted with a very low concentration of affinity material and consequently the following practical advantages can be found in the purification processes: firstly, there are little or no non-specific interactions of contaminating proteins with the biospecific polymer because of its low concentration in the extraction solution. Such interactions result in major difficulties in work with affinity columns. Secondly, protein binding takes place under equilibrium conditions, resulting in quantitative complexation, whereas with affinity gels the rate of column loading has to be taken into account. Thirdly, owing to the exact knowledge of the binding capacity of the macroligand and to its low concentration, the dissociation of the macrocomplex and consequently the release of the protein from the polymer are easily achieved by means of a calculated excess of the free ligand.

On the other hand, these water–soluble biospecific polymers do have some limitations. When the ligand to be immobilized has a marked hydrophobic character, the water-soluble polymer has a substitution limit beyond which its solubility properties may be substantially altered. As an example, during the synthesis of macroligands based on dextran and steroids, the attempts to anchor estradiol-7α-butyric acid in amounts greater than $2 \cdot 10^{-4}$ equivalents per gram of polymer led to the insolubilization of the affinity material. On the other hand, it was possible to link a model carboxylic acid, such as *p*-nitrophenylacetic acid, up to $5 \cdot 10^{-4}$–$6 \cdot 10^{-4}$ moles per gram of polymer, without any apparent decrease in the solubility of the resulting material. This phenomenon obviously involves a major limitation when a species with a low affinity towards the immobilized ligand is to be purified. In fact, if one takes as an example a steroid linked with a concentration of $2 \cdot 10^{-4}$ equivalents per gram of dextran, in order to purify a protein the affinity constant of which towards this ligand would be about 10^5 $l \cdot mol^{-1}$ it then would become necessary to add the macroligand at a concentration of 5 g per litre of extract to achieve 99% complexation of the complementary macromolecule. For lower affinity constants the macroligand concentrations to be used would be far too high and could lead to unsatisfactory non-specific adsorption. In contrast, if the ligand is a hydrophilic compound, it is then possible to perform high substitutions on the dextran without

modification of the solubility in water and purifications of low-affinity biological species can be carried out successfully by using the resulting water–soluble biospecific polymers.

6. ACKNOWLEDGEMENTS

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7. SUMMARY

Purification of proteins or other biological macromolecules can be performed by techniques based on the biospecific affinity of these species for ligands linked to water-soluble polymers. The characteristics of such water-soluble biospecific polymers are presented, their applicability in protein purification techniques is discussed and their advantages and limitations are compared with those of affinity chromatography.

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