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Note

Efficient purification of a microbial steroid-1-dehydrogenase* by electrophoretic desorption from the affinity matrix on a preparative scale

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Recently an affinity chromatographic method for the purification of the steroid-1-dehydrogenase from *Nocardia opaca* has been developed in our laboratory[1]. By this technique the enzyme was biospecifically retained on affinity matrices prepared by covalent linkage of steroidal ligands to Sepharose gel. The elution of the pure protein has been effected using either a potassium chloride gradient or organic solvents such as ethylene glycol, dimethylformamide or water-soluble steroid derivatives. Such procedures necessitate subsequent removal of these compounds by exhaustive dialysis, and concentration of the eluate. In some cases these last steps resulted in considerable loss of activity. To overcome these difficulties we have desorbed the biospecifically adsorbed steroid-1-dehydrogenase from the affinity matrix into polyacrylamide gel by means of an electric field[2]. These techniques have been recently described for purification of steroid-specific antibodies[5,6], ferritin[7] and steroid-binding globulin[8]. Electrophoretic desorption of other substances from affinity matrices has been summarized in refs. 3 and 4.

We now describe the application of this method to a steroid-transforming enzyme, both analytical and preparative, and to the investigation of the enzyme–affinity support interaction.

EXPERIMENTAL

Methods

Enzyme extract. An ammonium sulphate preparation of the cell free extract from *Nocardia opaca* was used. Specific activity: 0.5 units/mg. Enzyme activity was estimated with 4-androstene-3,17-dione as substrate and 2,6-dichlorophenol indophenol as artificial electron acceptor[9].

Protein determination. The protein content of solutions were assayed according to the method of Hesse *et al.*[10].

Affinity chromatography. Binding of the enzyme to the affinity matrix was performed using the conditions described previously[1]. The following affinity matrices

* 4-en-3-oxosteroid: (acceptor)-1-en-oxidoreductase (E.C. 1.3.99.4).

were investigated: N-(4-androsten-3-on-17β-oxycarbonyl)-ε-aminocaproyladipinic acid dihydrazide-Sepharose 4B (affinity matrix I), N-(4-androsten-3-on-17β-oxycarbonyl)-ε-aminocaproylaminododecyl-Sepharose 4B (affinity matrix II), and N-(4-androsten-3-on-17β-oxycarbonyl)-ε-aminocaproylaminohexyl-Sepharose 4B (affinity matrix III).

The ammonium sulphate preparation of the steroid-1-dehydrogenase (15 units) was adsorbed on 3 ml of matrix. Then the matrix was washed thoroughly with 0.01 *M* phosphate buffer, pH 8.0. The enzyme can be eluted either as described previously[1] in buffer containing 30% ethylene glycol or in an electrophoretic system by the action of an electric field.

Electrophoretic desorption

For preparative purposes the washed affinity gel (1.5 ml) complexed with the steroid-1-dehydrogenase was placed on top of an electrophoresis tube (14.0 × 1.6 cm) containing 10% polyacrylamide gel (2.6% cross-linked). Tris–glycine, pH 8.3, was used as electrode buffer. Negatively charged ions moved from the matrix into the gel towards the anode (top electrode: anode, bottom electrode: cathode, isoelectric point of the enzyme: 4.7). Electrophoretic desorption was carried out for 6–12 h (150 V, 4°).

The visualization of the steroid-1-dehydrogenase was performed by contacting a small strip of the surface of the polyacrylamide gel with filter paper containing a solution of the following composition: 0.3 m*M* nitrotetrazolium blue chloride, 0.08 m*M* phenazine methosulphate and 0.08 m*M* 4-androstene-3,17-dione in phosphate buffer, pH 8.0, at 25°.

The sharp zone of the pure enzyme was sliced from the gel, and used for further experiments or stored. The principle of this method is shown in Fig. 1.

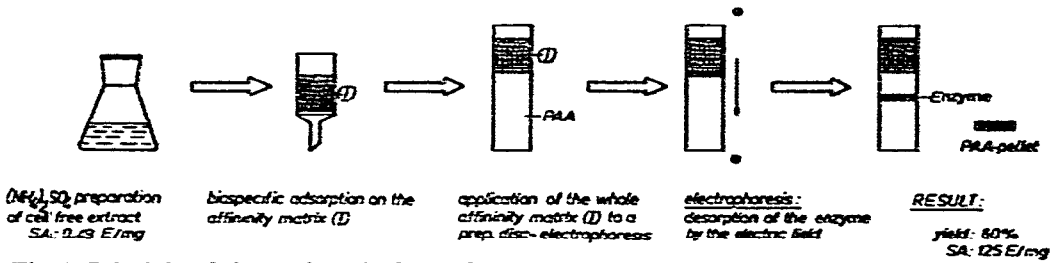


Fig. 1. Principle of electrophoretic desorption.

The influence of the electric potential was investigated by the electrophoretic desorption of a small amount of matrix (0.25 ml, 1.25 units) complexed with the enzyme. The procedure was carried out in an electrophoresis tube for 2 h at 4°. The gel was 10 × 0.45 cm. The electric potential was changed from 80 to 320 V.

After this procedure the electrophoretically desorbed enzyme activity was estimated. The enzyme activity not electrophoretically desorbed was determined after elution with 30% ethylene glycol–0.5 *M* KCl.

RESULTS AND DISCUSSION

The principle of the method is shown in Fig. 1. The results, summarized in

TABLE I

PURIFICATION OF A STEROID-1-DEHYDROGENASE FROM *NOCARDIA OPACA* BY DIFFERENT TECHNIQUES OF THE BIOSPECIFIC AFFINITY CHROMATOGRAPHY INCLUDING THE ELECTROPHORETIC DESORPTION

1.5 ml affinity matrix I and III, respectively, ammonium sulphate preparation of the cell free extract (7.5 units, 15.2 mg protein).

Method	*Specific activity (U/mg)*	*Enrichement*	*Yield (%)*
Affinity matrix I:	125	250	80
Electrophoretic desorption		(625)*	
	24	49	96
Elution by 70% ethylene glycol[10]		(120)*	
Affinity matrix III:	100	200	75
Affinity chromatography after pre-chromatography on aminododecyl-Sepharose		(500)*	
Elution by KCl gradient[10]			

* Related to the cell-free crude extract.

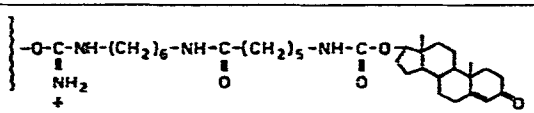
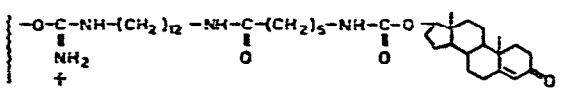
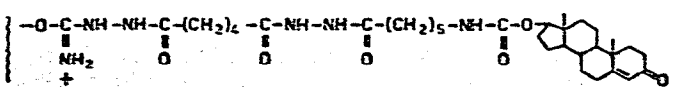
Tables I and II, show that the steroid-1-dehydrogenase can be removed from an immobilized enzyme steroid ligand complex on the affinity matrix by an electric field. Affinity support I with adipic acid dihydrazide as the spacer component has been shown to be the most effective one. Table I shows the results in comparison to our earlier findings obtained by classical methods[11]. Bye lectrophoretic desorption the enzyme was enriched 250-fold, that means 625-fold related to the cell-free crude extract with a yield of 80%.

In comparison to the ethylene glycol elution of the same matrix and to a

TABLE II

INFLUENCE OF THE CHEMICAL NATURE OF THE AFFINITY MATRIX ON THE ELECTROPHORETIC DESORPTION OF THE STEROID-1-DEHYDROGENASE

The electrophoresis was performed at 4° for 2 h at 80 V using 0.25 ml affinity gel complexed with 1.25 units of the enzyme. Ligand density of gels: 2 μmoles/ml.

Matrix	*Desorption (%)*
 III	83.7
 II	96.0
 I	100

ionic character

combined procedure including chromatography on aminododecyl-Sepharose and affinity chromatography, the direct combination of biospecific adsorption on the affinity matrix with electrophoresis results in higher purity and efficiency. After treatment of the affinity matrix I with 30% ethylene glycol–0.5 *M* KCl no enzyme activity was detected, indicating the complete removal from the affinity support by the electric field. The advantages of this technique are: it is not necessary to add chaotropic reagents or free steroid ligands to remove the specifically adsorbed material. A subsequent dialysis or concentration of the protein is not needed. The polyacrylamide slice containing the highly concentrated enzyme can be used directly for further experiments[12]. Within 12 months the activity loss of enzyme stabilized in the slices was about 10%. The technique described above can be used on a larger, preparative scale.

The influence of the chemical nature of the affinity matrix used in analytical experiments on the part of electrophoretically desorbed steroid-1-dehydrogenase under standard conditions is shown in Table II. Each used support contains the same steroid ligand concentration of 2 μmoles/ml detected by application of a 4-[^{14}C]-steroid derivative.

From affinity matrix I all of the initially adsorbed enzyme activity was eluted, but only 80% from affinity matrix III. Fig. 2 shows the relation of desorption to the applied electric potential. Affinity matrix III indicates a strong potential dependence, matrix II a medium one, and no potential dependence was found for affinity matrix I.

The different behaviour of the matrices is to be related to their ionic character or "effective ionicity". Ionic groups, *e.g.* positively charged iso-urea residues, diminish the degree of an electrophoretic desorption. Both matrices II and III contain an aliphatic diamine as spacer component. Hofstee[13] has shown that the ionic or hydrophobic properties of supports containing CNBr-linked aliphatic amines are dependent upon the chain length. Hence, matrix III is more ionic than matrix II. This is an explanation for the strong potential dependence of desorption of the steroid-1-dehydrogenase on matrix III. Supports based on CNBr-linked hydrazides studied by Wilchek[14] are extremely stable and show no effective charges. In agreement with these

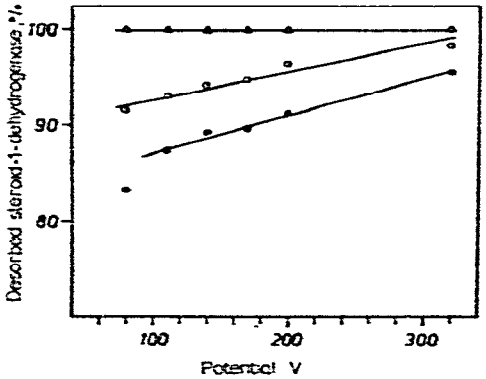


Fig. 2. Degree of electrophoretic desorption of the steroid-1-dehydrogenase from affinity matrix as a function of electric potential. Electrophoresis was carried out at 4° for 2 h in 10 × 0.45 cm test tubes (inner diameters) using 0.25 ml affinity gel complexed with 1.25 units of the enzyme. △, Affinity matrix I; ○, affinity matrix II; ●, affinity matrix III.

results the electrophoretic desorption of the enzyme from the non-ionic matrix I is independent of the potential. These examples may suggest the method of electrophoretic desorption for the characterization of the affinity matrix as well as for the quantification of the nature of the enzyme–matrix interaction.

The strong potential dependence of electrophoretic desorption of the steroid-1-dehydrogenase from affinity support III indicates an attachement of the protein by both biospecific (hydrophobic) and ionic interactions.

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