PHENOL-CONTAINING ADSORBENTS FOR THE AFFINITY CHROMATOGRAPHY PURIFICATION OF SOME NAD(H)-DEPENDENT DEHYDROGENASES

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1. Introduction

The use of NAD^+ or AMP derivatives bound to insoluble polymers for the affinity chromatography purification of NAD(H)-dependent dehydrogenases has been described [1–4].

Good adsorbents have proved those containing derivatives of AMP and products of this kind are commercially available.

Elution of the biospecifically adsorbed enzymes is obtained by the addition of the oxidized or reduced cofactor to the eluting buffer [5] or by the use of ionic strength, pH- and temperature-gradients [6,7].

We have purified the isoenzyme H_4 of lactate dehydrogenase (EC 1.1.1.27) and cytoplasmatic (soluble) malate dehydrogenase (EC 1.1.1.37) by a new approach based on the inhibitory properties toward these NAD(H)-dependent dehydrogenases of some substituted phenols [8]. The inhibitory strength of these compounds depends on the substituents present in the phenolic ring [9].

We have selected *o*-phenylphenol for our investigation because this compound is known to inhibit some dehydrogenases [10] and because both *o*-phenylphenol and its sodium salt are currently in use as fungistatics for limes [11].

4-((3′-Phenyl, 4′-oxy)phenylazo)benzoyl-β-alanine was also prepared as a 'soluble' parent compound of the immobilized ligand for kinetic studies.

2. Materials and methods

2.1. *Chemicals and biochemicals*

These were obtained as follows: Affi-Gel 10 (Bio-Rad Laboratories S.R.L., Milan, Italy), *o*-phenylphenol (Carlo Erba S.p.A., Milan, Italy), ethylenediemine, *p*-nitro-benzoylchloride, sodium pyruvate, EDTA disodium salt, NaN_3 (Bracco Industria Chimica S.p.A., Milan, Italy), NAD^+, NADH (Biochemia S.p.A., Milan, Italy), 2-mercaptoethanol (β-ME), oxalacetic acid (Fluka, Buchs-Switzerland), Tris (hydroxymethyl) aminomethane (Tris), nitro blue tetrazolium, phenazine methosulfate (Sigma Chemical Co., London, England). All other chemicals were reagent grade.

2.2. *Enzymes*

Lactate dehydrogenase ($LDH\text{-}H_4$) and cytoplasmatic malate dehydrogenase (s-MDH) were prepared from pig heart by slight modifications of published procedures [12,13] and their activity assayed as described therein.

2.3. *Disc-gel electrophoresis*

The method of Davis [14] was used except for the omission of the spacer-gel. Proteins were stained with Coomassie Brilliant Blue R-250 (0.05% solution in acetic acid/propan-2-ol/water = 3/5/12) and dehydrogenase activities revealed as described by Gabriel [15].

2.4. *Synthesis of the 'soluble' inhibitor*

This compound, 4-((3′-phenyl-4′-oxy)phenylazo) benzoyl-β-alanine, was obtained by reacting *o*-phenylphenol with diazotated *p*-aminobenzoyl-β-alanine (fig.1). The latter compound was prepared by reduction (H_2 and catalyst) of *p*-nitrobenzoyl-β-alanine obtained, in turn, as described by Colles [16] for the parent D- and L-α-alanine derivatives. The 'soluble' inhibitor was purified by preparative chromatography on a silica-gel column. The pK_A of the phenolic group was spectrophotometrically determined as 8.01.

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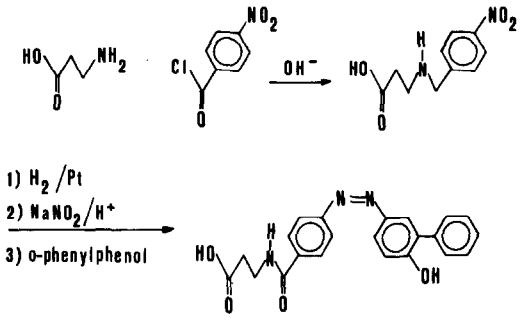

Fig.1. Schematic pathway of the synthesis of the 'soluble' derivative of *o*-phenylphenol. This compound was prepared in order to ascertain whether the chemical modification of the phenol, required for preparing the biospecific adsorbent, would interfere with its inhibitory effectiveness. The β-alanine moiety was introducted in order to increase the similarity with the bound inhibitor and to calculate the dissociation constant of the phenolic group without the interference of the carboxylic group of the benzoic acid residue.

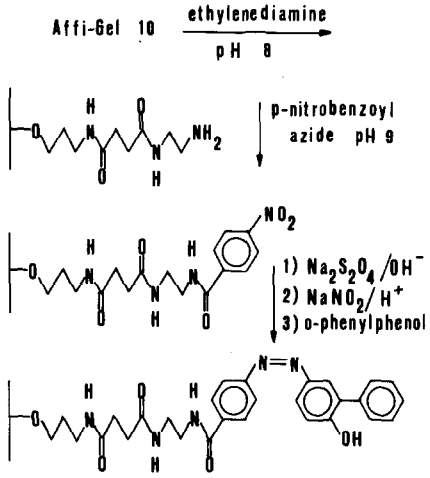

Fig.2. Schematic pathway of the synthesis of the bioadsorbent used for the affinity chromatography purification of LDH-H_4 and s-MDH from pig heart. The degree of substitution of the gel was calculated as 13.4 μmol bound inhibitor/ml packed gel. The ligand is about 20 Å far from the matrix-backbone.

2.5. *Preparation of the biospecific adsorbent*

Affi-Gel 10 was reacted with a large excess of ethylenediemine at pH 8. The reaction product was treated with a 0.1 M solution of *p*-nitrobenzoylazide in 0.1 M borate buffer, pH 9 (50% dimethylformamide v/v). The azide was prepared from *p*-nitrobenzoylchloride and NaN_3 [17]. The nitro group was reduced to amino group on the gel as described by Cuatrecasas [18]. The gel was thoroughly washed with distilled water, diazotated and the diazo compound was then reacted with a 10 mM solution of *o*-phenylphenol in 0.1 M borate buffer, pH 9.3 (50% DMF v/v) for 5 h at 4°C (fig.2). The degree of substitution on the gel was determined spectrophotometrically, at pH 10, using the molar absorptivity calculated for the 'soluble' inhibitor at 472 nm under the same conditions, since the chromophore is identical in both cases.

3. Results and discussion

Elution of the enzymes was carried out at pH 6 and pH 10. Under these conditions the immobilized inhibitor was essentially present in the phenol or phenate form respectively, since the phenol pK_A was found to be 8.01 (pH − pK = ± 2).

A mixture of pre-purified LDH-H_4, s-MDH and human hemoglobin (CO-form) was not adsorbed by the gel at pH 10. Both LDH and MDH were retained at pH 6. Hemoglobin was retained also as a narrow band on the top of the column at this pH. This binding suggests that hydrophobic interactions might be involved in retaining the dehydrogenases.

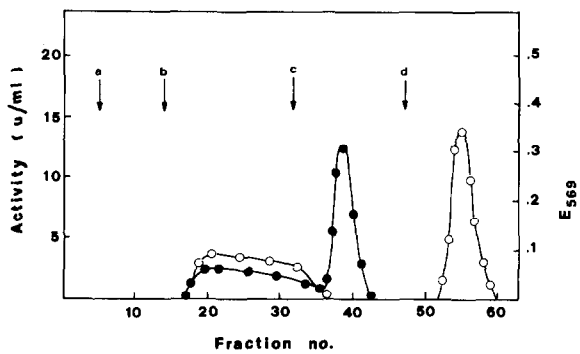

Fig.3. Chromatography of a mixture of LDH and human hemoglobin (CO-form) using different eluting systems. A mixture of 3 mg of LDH and 1.6 mg of hemoglobin in 2 ml 10 mM phosphate buffer, pH 6 (containing 1 mM EDTA and β-ME) was applied to a 12 × 1.2 cm column, filled with the biospecific adsorbent. Successive elutions were carried out with the initial buffer plus: (a) 10 mM pyruvate (b) 10% DMF (v/v), (c) 1 mM NADH. Arrow (d) shows where the buffer was changed to a 50 mM Tris–HCl, pH 10. (•) LDH activity, (○) Hb-CO.

The conditions for the separate elution from the gel of the three components adsorbed at pH 6 was determined as follows, using mixtures of hemoglobin and LDH or MDH. The column was eluted successively with the initial equilibrating buffer containing pyruvate or oxalacetate, DMF and NADH. The elution profile of the LDH-hemoglobin mixture is shown in fig.3. Pyruvate had no effect on the adsorbed proteins, whilst DMF eluted a little LDH and hemoglobin. This finding provides confirmatory evidence to the afore mentioned possibility that the matrix–protein interactions have partial hydrophobic character. Column elution using 1 mM NADH resulted in a quick release of LDH, whereas hemoglobin retention was not affected. Hemoglobin was eluted in a concentrated fraction having pH 7.2 by the use of a 50 mM Tris–HCl buffer, pH 10. NAD^+ was also effective in eluting LDH, whilst MDH was eluted by NADH only.

Therefore, a mixture of the three proteins could be easily separated by successively eluting the column with the buffer, pH 6, containing NAD^+, NADH and then with Tris–HCl buffer, pH 10 (fig.4).

The two dehydrogenases were pure as shown by disc-gel electrophoresis (fig.5). The chromatography of a partially purified preparation containing 20–30% of a mixture of the two enzymes gave the same degree of purification as in the case of the prepurified enzymes.

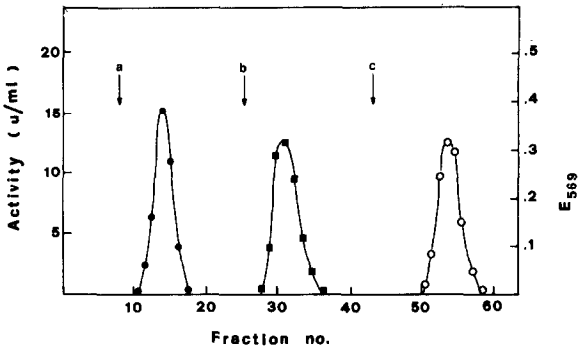

Fig.4. Chromatography of an artificial mixture of LDH, MDH and carbomonoxy-hemoglobin. LDH and MDH, 500 units each, and 2 mg of hemoglobin in 4 ml 10 mM phosphate buffer, pH 6 (1 mM EDTA and β-ME). were applied to the gel. Initial conditions were as described in fig.3. The arrows show where the following changes were made: (a) initial buffer plus 1 mM NAD^+, (b) initial buffer plus 1 mM NADH, (c) 50 mM Tris–HCl buffer, pH 10. (•) LDH activity, (▪) MDH activity, (○) Hb-CO.

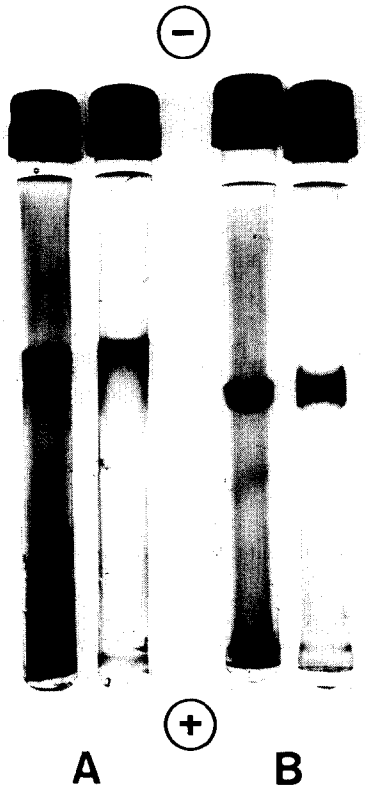

Fig.5. Disc-gel electrophoresis of the enzymes purified as described in fig.4. Active fractions were concentrated by addition of dry Sephadex G-25. Gels were stained for proteins (left) and for activity (right). (A) LDH-H_4, (B) s-MDH.

The capacity of the bioadsorbent as determined in the experimental conditions used (pH 6) was found to be 1 mg/ml and 3 mg/ml of packed gel for LDH and MDH respectively when the enzymes were eluted separately; the capacity for LDH was a little diminished in the presence of MDH. These findings may be related to the fact that s-MDH interacts with a higher number of ligand molecules on the adsorbent, by having a molecular weight about half that of LDH [19,20]. Indeed, Indeed, it has been reported [21] that MDH can interact with NAD^+ coupled to Sephadex G-100, contrarely to LDH.

Mosbach et al. [1] reported the elution of LDH and glyceraldehyde-3-*P*-dehydrogenase from an AMP-containing adsorbent, by means of a linear-gradient made with salicylate (up to 0.15 M). However, the

separation between the two dehydrogenases was very poor, in comparison with the separation obtained using NAD^+ and NADH as eluting agents.

Our results show that by reversing the conditions, i.e., using a phenol derivative as a ligand and cofactors as eluting agents, the affinity chromatography purification of NAD(H)-dependent dehydrogenases is much improved. In fact, the synthesis of our adsorbent is much easier than the synthesis of any NAD^+- or AMP-containing adsorbent.

We are currently investigating the possible use of our adsorbent for the affinity chromatography purification of other NAD(H) dependent dehydrogenases and the improvement of the capacity of the gel by the use of other phenols.

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