SPECIFIC HYDROPHOBIC CHROMATOGRAPHY OF ALCOHOL DEHYDROGENASES ON 10-CARBOXYDECYL-SEPHAROSE

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1. Introduction

In recent years purification of proteins by chromatography on Sepharose carrying hydrophobic groups was demonstrated [1–10]. This phenomenon was called 'hydrophobic chromatography' [1], 'hydrophobic salting-out chromatography' [3], 'phosphate induced protein chromatography' [4], or 'repulsion-controlled hydrophobic chromatography' [10]. Different conceptions exist to explain the mechanism of interactions between protein and adsorbent matrix [1,11,12]. It was shown that the observed effect of increasing adsorption with increasing length of aliphatic chain does not only imply participation of interactions of essentially hydrophobic character. Additionally the aliphatic chain may have a local effect on the dielectric constant of the solvent, changing electrostatic interactions [9,11]. Hitherto hydrophobic chromatography was applied for protein fractionation on the basis of 'unspecific' interactions between hydrophobic areas of proteins and a matrix.

Moreover this principle should be applicable to protein purification on the basis of 'specific' interactions between a hydrophobic matrix and enzymes with a hydrophobic active centre, suggesting a special case of true affinity chromatography.

Alcohol dehydrogenases from yeast [13–15] but also from other sources [16–18] possess such hydrophobic active centres, indicated by an increased affinity of alcohols to the enzyme with increasing chain length. Sepharose derivatives with long chain alkyl residues should therefore be suitable to separate alcohol dehydrogenases from other proteins.

2. Materials and methods

10-Carboxydecyl-Sepharose was prepared from Sepharose (Pharmacia, Uppsala) activated with CNBr as described by Cuatrecasas [19]. For the coupling reaction 50 mg CNBr and 0.5 mmoles 11-aminoundecane acid (Fluka AG, Buchs) were used per ml settled Sepharose. The activated Sepharose was shaken with a suspension of aminoundecane acid in water for 18 h at pH 10 and 25°C. Excess of aminoundecane acid was removed by washing with 0.02 N HCl. The end of washing was indicated by an increase of $[H^+]$ concentration below pH 5. Other derivatives of Sepharose were prepared as described by Cuatrecasas [19] using the same Sepharose/alkylamine ratio.

The yeast extract was obtained from dried baker's yeast by the method of Racker [20] including heat treatment. Preparation of cell-free extracts from *Acinetobacter calcoaceticus* was performed by ultrasonic disruption and centrifugation at 15 000 × *g* [21]. In order to remove substances with low molecular weight both extracts are given to a column of Sephadex G-25. Simultaneously the desired phosphate concentration was adjusted.

The activity of the yeast enzyme was measured with ethanol and NAD^+ as substrates [14] and the bacterial enzyme with octanol and $NADP^+$ [18]. Protein was monitored as A_{280}; occasionally it was determined by the method of Lowry et al. [22]. Measurements were carried out with a Beckman DK 2A recording spectrophotometer or with an instrument as described elsewhere [23].

Polyacrylamide-gel electrophoresis was carried out

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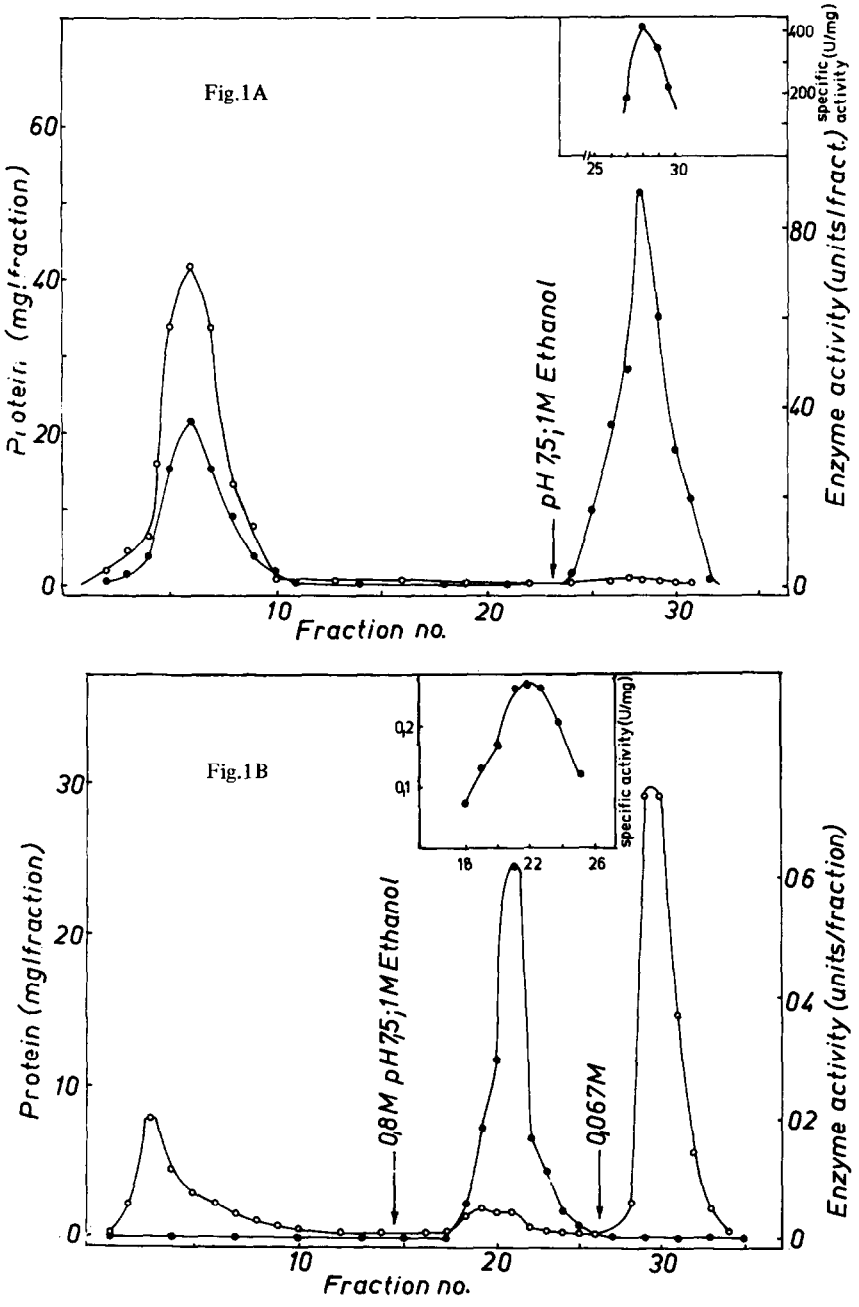

Fig.1. Purification of alcohol dehydrogenase from crude enzyme extracts of *Saccharomyces cerevisiae* (A) and *Acinetobacter calcoaceticus* (B) on 10-carboxydecyl-Sepharose. The extracts were applied to a column (7 × 1.6 cm) containing 8 ml of 10-carboxydecyl-Sepharose. Elution was done at 4°C with a flow rate of 0.7 ml/min. The volume of collected fractions was 3.5 ml or in presence of 1 M ethanol 2.8 ml. (•) Enzymatic activity; (○) protein concentration. NAD^+-dependent alcohol dehydrogenase from *Saccharomyces cerevisiae:* The extract (2 ml) contained 140 mg protein in 0.8 M K_2HPO_4/KH_2PO_4 pH 8.5 (containing 0.1 M glycine). Elution was done with a buffer pH 7.5 of the same composition, containing 1 M ethanol. The fractions were concentrated before using in electrophoresis. $NADP^+$-dependent alcohol dehydrogenase from *Acinetobacter calcoaceticus:* The extract (2 ml) contained 100 mg protein in 1 M K_2HPO_4/KH_2PO_4 pH 8.5. Elution was done with 0.8 M K_2HPO_4/KH_2PO_4 pH 7.5 containing 1 M ethanol. Columns were regenerated by washing with 0.1 M NaOH in 80% ethanol.

in a linear gradient of 5–20% acrylamide using the method of Williams and Reisfeld [24]. Protein was stained with Coomassie Blue G 250 in 12.5% trichloroacetic acid [25]. Active alcohol dehydrogenase bands were demonstrated by incubating the gels at 25°C in a solution containing 570 mM ethanol (or 0.75 mM octanol), 1.5 mM NAD^+ (or 0.67 mM $NADP^+$), 0.32 mM tetrazolium chloride, and 0.054 mM phenazine methosulfate in 0.1 M pyrophosphate buffer, pH 8.7. Sodium dodecyl sulphate electrophoresis was performed as described by Weber and Osborn [26] in a 7% gel over 7 h at 25°C. Substances for electrophoresis were from Serva, Heidelberg, all other chemicals were analytical grade reagents of the highest grades available.

3. Results

We started our investigations with binding experiments of yeast alcohol dehydrogenase to various alkyl-Sepharoses. As has been shown already for other proteins [1,11,12] affinity of alcohol dehydrogenase to the matrix increases with increasing length of aliphatic chain. The same effect was obtained by raising the ionic strength or changing the pH value, e.g., from pH 7.0 to pH 8.5 (table 1). On the other hand in presence of ethanol the affinity was decreased. We tested all of the Sepharose derivatives shown in table 1 for their applicability to purify alcohol dehydrogenase from yeast. In all cases the enzyme could be enriched from a crude extract, if the adsorption was carried out in presence of 1 M potassium phosphate buffer, pH 8.5, and if the elution was made

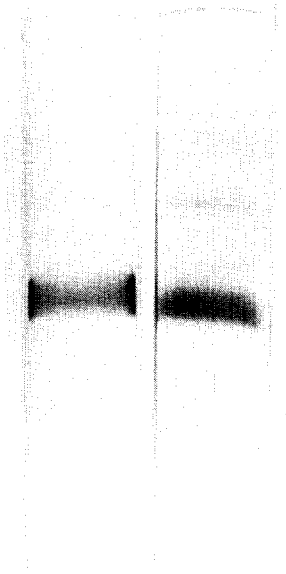

Fig.2. Polyacrylamide-gel electrophoresis of yeast alcohol dehydrogenase after purification on 10-carboxydecyl-Sepharose. The running time was 4 h at 10°C and 200 V (4 mA/gel). A: active staining (25 μg protein); B: protein staining (70 μg protein).

Table 1
Adsorption of yeast alcohol dehydrogenase on derivatives of Sepharose 4 B at 25°C

Sepharose derivative	Adsorbed alcohol dehydrogenase (%)		
	0.067 M K_2HPO_4/KH_2PO_4 pH 8.5	1 M K_2HPO_4/KH_2PO_4 pH 8.5	1 M K_2HPO_4/KH_2PO_4 pH 7.0
10-Carboxydecyl-Sepharose	0	96	87
5-Carboxypentyl-Sepharose	0	51	28
6-Hydroxyhexyl-Sepharose	30	83	35
6-Aminohexyl-Sepharose	37	56	25
Hexyl-Sepharose	50	98	98

The assays (3 ml) contained 7.5 mg of the alkyl-Sepharose and 0.25 μg of alcohol dehydrogenase. The suspensions were equilibrated for 30 min followed by estimation of non-adsorbed protein in the supernatant.

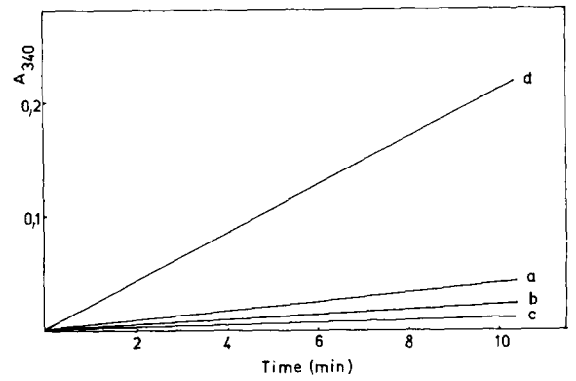

Fig.3. Loss of activity of yeast alcohol dehydrogenase after binding to 10-carboxydecyl-Sepharose. 0.5 μg of alcohol dehydrogenase were stirred immediately in the cuvette with 10-carboxydecyl-Sepharose (15 mg dry weight) in 2 ml 0.5 M K_2HPO_4/KH_2PO_4 pH 8.5 containing 0.82 mM NAD^+. After 10 min ethanol (100 mM) was added without interruption of stirring and A_{340} was monitored continuously (a). In an other experiment the Sepharose was separated by filtration and activity was estimated in the filtrate by addition of ethanol (b). The Sepharose was resuspended in buffer (with NAD^+ and ethanol) and the enzyme reaction was registrated immediately in the suspension (c). Curve (d) shows the activity of the enzyme in absence of 10-carboxydecyl-Sepharose.

with gradients of decreasing phosphate concentration, decreasing pH or increasing ethanol concentration. Most effective results were obtained in a column with carboxydecyl-Sepharose.

Figure 1A demonstrates purification of alcohol dehydrogenase from a crude yeast extract up to a uniform behaviour in polyacrylamide gel electrophoresis (fig.2). Likewise nearly one band is found in sodium dodecyl sulphate electrophoresis after 16 h incubation of the enzyme in 1% sodium dodecyl sulphate at 25°C. The estimated molecular weight of the subunit was double in size in comparison with the enzyme prepared in conventional manner [20,27].

In fig.3 it is shown that yeast alcohol dehydrogenase adsorbed to carboxydecyl-Sepharose is enzymatically inactive. In this point alcohol dehydrogenase differs from other enzymes, which are enzymatically active after adsorption to various alkyl-Sepharoses [28].

Figure 1B shows that also a bacterial alcohol dehydrogenase with entirely different properties [18] than the yeast enzyme can be purified with similar success. Results for both crude extracts are summarized in table 2.

4. Discussion

The behaviour of two different alcohol dehydrogenases on alkyl-Sepharose derivatives indicates that the formation and dissociation of a specific complex between the alkyl residues and the active centres of the enzyme are the basis of an effective purification step. This concept seems to be plausible with regard to the ability of both enzymes to catalyze the oxidation of long chain alcohols with decreasing K_M-values, if the chain length increases [14,16–18]. This assumption is also supported by the fact that yeast alcohol

Table 2
Purification of two alcohol dehydrogenases on 10-Carboxydecyl-Sepharose

Enzyme	Buffer for adsorption	Buffer for elution	Specific activity (μmol/min/mg) of the enzyme in the crude extract	Specific activity (μmol/min/mg) of pooled fractions	Yield in activity of pooled fractions
Yeast alcohol dehydrogenase	0.8 m K_2HPO_4/KH_2PO_4 pH 8.5 containing 0.1 M glycine	0.8 M K_2HPO_4/KH_2PO_4 pH 7.5 containing 0.1 M glycine and 1 M ethanol	31	425 (fraction 27)	20%
Bacterial alcohol dehydrogenase	1 M K_2HPO_4/KH_2PO_4 pH 8.5	0.8 M K_2HPO_4/KH_2PO_4 pH 7.5	0.013	0.28 (fractions 21 to 23)	50%

dehydrogenase adsorbed to carboxydecyl-Sepharose is enzymatically inactive, suggesting an interaction between the active centre of the enzyme and the immobilized substrate analogue. One can assume that hydrophobic interactions are of essential importance because the affinity increases with increasing phosphate concentration [4]. Moreover, electrostatic interactions are involved depending on pH and ionic strength. Such a combination of hydrophobic and electrostatic forces in hydrophobic chromatography was already described earlier [1,4, 11,12,29]. It is arguable in our case that binding forces are predominantly hydrophobic and may be controlled by varying the repulsion between protein and adsorbent as reported by Yon and Simmonds [10].

Positive charges on the end of hydrocarbon arm induce a more tight binding of alcohol dehydrogenase to Sepharose derivatives, especially at low ionic strength, but negative charges weaken the adsorption drastically. This refers to the participation of a negative charged group of the enzyme.

Suprisingly we have found that a value of 78 000 daltons was detected for molecular weight of the subunit of our purified yeast enzyme. A value of 38 000 daltons was found for an enzyme purified in conventional manner [30,31]. This can be probably attributed to the fact that the method requiring a short time is preventing the attack of proteases or other inactivating influences.

Further studies with alcohol dehydrogenase isoenzymes will show, whether this method may be generally applicable for purification of enzymes with substrates of a homologous series similar to the use of NAD^+-Sepharoses for separation of dehydrogenases [32–35].

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