PURIFICATION OF PROTEINS BY FRACTIONAL INTERFACIAL SALTING OUT ON UNSUBSTITUTED AGAROSE GELS

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SUMMARY: Proteins can be precipitated onto the surface of unsubstituted agarose at an ammonium sulfate concentration about 10 % lower than needed for precipitation out of solution. The protein is fractionally redissolved by developing agarose columns with a linear, decreasing gradient of ammonium sulfate. The method is characterized by high reproducibility, good purification factors and high recovery of enzymatic activity. As an example the method is applied to the purification of aminoacyl-tRNA synthetases (E.C. 6.1.1.-) specific for phenylalanine, isoleucine and valine.

INTRODUCTION

Proteins can be salted out from concentrated salt solutions onto so called amphiphilic gels, which are agarose gels substituted with lipophilic groups (1 - 4). The reason for binding of the proteins to the gels was speculated to be hydrophobic interaction in analogy to the so called "hydrophobic chromatography" (5). However, there exists an alternative explanation not yet discussed in the literature. One can imagine that the solvation sphere on the gel surface differs from the solvation in solution such that precipitation of an enzyme occurs in this surface at a lower salt concentration than needed for precipitation out of solution. If this

hypothesis is correct, a salting out should occur also on unsubstituted gels. This happens to be the case as will be shown in the following.

MATERIALS AND METHODS

All salts and reagents were of highest purity commercially available. Reinzuchthefe - Saccharomyces cerevisiae - was purchased from Asbeck, Hefewerk Hamm, Germany. Sepharose 4B and Sephadex CM 50 were obtained from Pharmacia, Uppsala, Sweden. Polymin P was a product of BASF, Ludwigshafen, Germany. Activity tests for aminoacyl-tRNA synthetases were performed as described (6). Percentual ammonium sulfate concentrations given below are relative to saturation at room temperature. All operations have been performed at 4° C.

Buffers used: Buffer A: 0.2 M Tris·HCl pH 9, containing 0.3 M NH_4Cl, 0.02 M $MgSO_4$, 10^{-3} M EDTA and 3 % Glucose. Buffer B: 0.06 M Potassium phosphate pH 7.2, containing 10^{-3} M dithioerythritol and 10^{-5} M phenylmethylsulfonylfluoride. Buffer C: 0.03 M Potassium phosphate pH 7.2, containing 10^{-3} M dithioerythritol, 10^{-5} M phenylmethylsulfonylfluoride and 10 % glycerol (v/v); Buffer D: 0.03 M Potassium phosphate pH 6.0, containing 10^{-3} M dithioerythritol, 10^{-5} M phenylmethylsulfonylfluoride and an ammonium sulfate concentration specified in the text; the buffer has to be readjusted prior to use to pH 6.

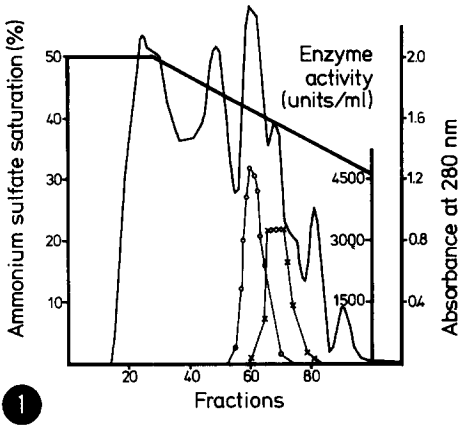
a) Preparation of enzyme fractions used for interfacial salting out

2 l of buffer A were added to 6 kg of yeast stored frozen at -20° C and the mixture was allowed to thaw overnight. The suspension was then passed once through a Gaulin homogenizer. Debris was centrifuged off at 17 000 g. 240 ml of aqueous 10 % (w/v) Polymin P (adjusted to pH 6 with concentrated HCl) was added to the supernatant. This precipitated all the fine debris and more than 95 % of the nucleic acids. The precipitate was removed down at 17 000 g. 430 g of solid ammonium sulfate were added per l of supernatant (70 % saturation). pH was maintained at 7.0 during this operation. The precipitate was collected by centrifugation at 17 000 g, dissolved in buffer B, and dialysed overnight against 5 l of buffer B. The dialysate was diluted with 1 volume of water and passed over a Sephadex CM 50 column of 1 l bed volume. The column was washed with buffer C containing 0.05 M KCl to remove unbound protein.

Phenylalanyl-tRNA synthetase (E.C. 6.1.1.20.) and isoleucyl-tRNA synthetase (E.C. 6.1.1.5.) were desorbed from the column with buffer C containing 0.15 M KCl. Valyl-tRNA synthetase (E.C. 6.1.1.9.) was desorbed from the column with buffer C containing 0.3 M KCl. The respective fractions were saturated to 70 % (430 g/l) with solid ammonium sulfate and the precipitate was collected.

b) Interfacial salting out

The ammonium sulfate pellets obtained from the Sephadex CM 50 column were dissolved in a minimum amount of buffer C and dialysed against buffer D saturated to 47 % with ammonium sulfate. The precipitate was removed by centrifugation. The supernatant was applied to a 4.5 x 17 cm Sepharose 4B column equilibrated with buffer D saturated 50 % with ammonium sulfate. The column was developed by a decreasing gradient from 50 % to 15 % ammonium sulfate in buffer D 1 l each. Fractions of 17 ml were collected.



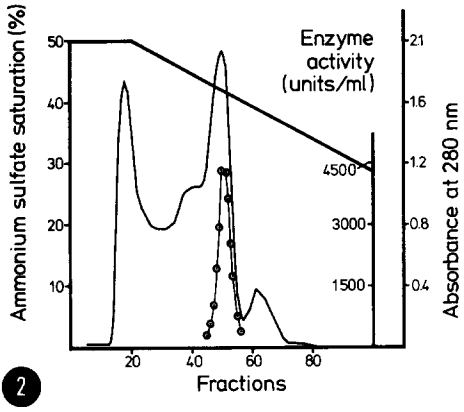


Fig. 1: Fractional salting in of phenylalanyl-tRNA synthetase and isoleucyl-tRNA synthetase: ——, absorbance at 280 nm; o-o, activity of phenylalanyl-tRNA synthetase; x-x, activity of isoleucyl-tRNA synthetase.

Fig. 2: Fractional salting in of valyl-tRNA synthetase: ——, absorbance at 280 nm; o-o, activity of valyl-tRNA synthetase.

RESULTS AND DISCUSSION

Adsorption of the protein to the Sepharose column can be followed visually since the originally transparent gel becomes opaque when coated with protein. From the amount of gel coated with protein in response to a certain amount of protein applied the capacity of the column was estimated to be 40 $\pm$ 3 mg protein per ml bed volume. This value is identical to that determined for amphiphilic Sepharose (1). The identity of these values indicates that the capacity of the amphiphilic gels is not limited by the degree of substitution. More probably for both substituted and unsubstituted Sepharose the monomolecular layer of protein sets the limit of capacity.

The absorbance for total protein eluted and the activity for the aminoacyl-tRNA synthetases tested are given in Fig. 1 and 2. Phenylalanyl-tRNA synthetase (Fig. 1) and valyl-tRNA synthetase (Fig. 2) are dissolved from the agarose surface at an ammonium sulfate con-

Table I: Purification of aminoacyl-tRNA synthetases.

Enzyme	Step	Total protein amount (A_{280} units)	Enzyme activity (units/mg protein[+])	Purification factor	Total enzyme amount (units)
Phenylalanyl-tRNA synthetase	Dialysate	50 000	2.20	1	110 000
	CM 50	2 700	55	40	148 000
	Sepharose	380	342	155	130 000
Isoleucyl-tRNA synthetase	Dialysate	50 000	1.20	1	60 000
	CM 50	2 700	24	20	65 000
	Sepharose	176	349	290	61 000
Valyl-tRNA synthetase	Dialysate	50 000	1.40	1	70 000
	CM 50	2 000	40	28	80 000
	Sepharose	157	298	212	47 000

[+] one unit is defined as the capacity to aminoacylate 1 nmol of tRNA per minute.

centration of 42 %, isoleucyl-tRNA synthetase (Fig. 1) at a concentration of 38 %. All these three enzymes begin to precipitate out of solution at about 50 % ammonium sulfate saturation. At this concentration between 5 and 10 % activity are found in the ammonium sulfate pellet, while 90 - 95 % remain in solution. Hence on the Sepharose surface proteins precipitate at an about 10 % lower saturation of ammonium sulfate than out of solution.

Table 1 gives the purification data for the aminoacyl-tRNA synthetases tested. In contrast to other purification methods (for references see 7) high recovery of total activity and good purification factors are achieved. Related to the activity of homogeneous preparations the phenylalanyl-tRNA synthetase (specific activity of homogeneous enzyme is 1500 units per mg) is 23 % pure, the isoleucyl-tRNA synthetase

(specific activity of homogeneous enzyme is 600 units per mg) is 58 % pure and the valyl-tRNA synthetase (specific activity of homogeneous enzyme is 350 units per mg) is 85 % pure. These relative purities were confirmed by SDS gel electrophoresis (data not shown here). As seen from the absorbance at 280 nm (Fig. 1 and 2) also other proteins of unknown nature elute well resolved. Thus we conclude that this method is generally useful, offering a new alternative in connection with the well established procedures used routinely for protein purification.

In light of the results shown here it does not seem justified to interprete the binding of an enzyme to an amphiphilic gel as an indication that hydrophobic groups are accessible on the enzyme surface (4).

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