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PURIFICATION OF PROTEINS BY REVERSIBLE SALTING OUT ON UNSUBSTITUTED AGAROSE GELS: GENERAL METHODOLOGY AND USE OF VARIABLES[1]

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INTRODUCTION

In a previous communication it was demonstrated that proteins in general can be precipitated onto the surface of an unsubstituted hydrophilic gel, e.g., Sepharose 4B, at a salt concentration significantly lower than that needed for precipitation out of solution (1). The same phenomenon was independently discovered for the more special case of proteins from halophilic bacteria (2). This method, based on the reversibility of the salting out by lowering the salt concentration, can serve as an alternative to the more traditional protein purification procedures, e.g., ion exchange chromatography.

The principle of using a continuous decrease in the concentration of the precipitating agent in order to extract individual proteins from a precipitated protein mixture was recognized quite early. Several attempts have been undertaken to convert this principle into a practically useful method. During the first approaches, a protein precipitate was stabilized by addition of silica gel (3,4), Sephadex (5), or celite (6), and the extraction of individual proteins was attempted by a decreasing salt gradient. Mayhew and Howell (7) observed that proteins can be precipitated onto DEAE-cellulose at an ammonium sulfate concentration slightly lower than that needed for precipitation out of solution. This effect was interpreted as being due to the increase in ionic strength by the ionic group of the polymer. Later it was reported that proteins can be precipitated onto the surface of so-called

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amphiphilic gels which are hydrophilic gels substituted with lipophilic groups (8–10). The binding forces in this case were ascribed to a hydrophobic interaction, which should be greatly increased by the high salt concentration.

In the light of the results obtained with unsubstituted gels (1,2), this interpretation remains doubtful. A more likely interpretation is that binding is due to a decreased solubility of the proteins caused by an interaction between the solvation sphere of the insoluble polymer and the protein. Hence a monomolecular layer of protein can be built upon the gel, and this is the limiting factor for the capacity of the polymer (1). This working hypothesis, which is substantiated by the work of Wilchek and Miron (11), served as a rationale for setting up procedures that use the salting out phenomenon for protein purification. In the following discussion, the methodology worked out in our laboratory will be given in detail. In addition, the influence of several variables useful for the separation process is investigated.

EXPERIMENTAL SECTION

Materials

All salts and reagents were of the highest purity commercially available. Reinzuchthefe, *Saccharomyces cerevisiae*, was purchased from Asbeck Hefewerke, Hamm, Germany. Sepharose 4B was obtained from Pharmacia, Uppsala, Sweden. Activity tests for aminoacyl-tRNA synthetases were performed as described by Schlimme et al. (12). The percentage ammonium sulfate concentrations given below are relative to saturation at room temperature (4.1 M at 25°C). All operations were performed at 4°C.

Enzyme Fractions Used for Salting Out Experiments. (*a*) To obtain a crude extract, 3 kg yeast cells were lysed as described recently (1). Debris was removed by centrifugation at 20,000 *g* for 30 min, and 250 g of ammonium sulfate per liter of supernatant was added with stirring (47% saturation). The precipitate was centrifuged down at 53,000 *g* for 1 h. The supernatant obtained was used to investigate salting out with the crude extract. (*b*) The pH dependence of the salting out procedure was investigated with protein fractions obtained from different earlier experiments. (*c*) All other experiments were performed with the protein fraction enriched in phenylalanyl-tRNA synthetase (EC6.1.1.20) and isoleucyl-tRNA synthetase (EC6.1.1.5) obtained as described previously (1).

Buffers. Buffer A: 0.03 M potassium phosphate (KH_2PO_4), 10^{-3} M dithioerythritol, 10^{-5} M phenylmethylsulfonylfluoride, and ammonium

sulfate at a concentration specified in the text. The actual pH of the buffer, specified in the text, was adjusted by the addition of a few drops of either concentrated aqueous ammonia or glacial acetic acid, respectively.

Buffer B: 4 M potassium phosphate was adjusted to pH 9.4. This stock solution was diluted to the desired concentration and the pH readjusted as specified in the text.

Methodology

Preparation of the Column. The capacity of Sepharose 4B was determined as being 40–45 mg protein bound per ml bed volume (1). Therefore, in order to ensure that a given amount of protein is quantitatively adsorbed to the resin, we never applied more than 30 mg protein per ml bed volume. The resolution was optimal in a range from 0.5 to 20 mg protein per ml bed volume, and no dependence of resolution on the column size could be observed. This is to be expected, because the resolution should only be dependent on the solubility of an individual protein at any point on the column. For this reason short fat columns can be used with a relatively high flow rate (up to 200 ml per h). If the actual load of protein on the column exceeds 20 mg protein per ml bed volume, then the resolution gradually decreases. If large amounts of protein have to be processed, however, a column load of even 30–35 mg per ml bed volume may be used with a reasonably good resolution.

An appropriate amount of Sepharose 4B, determined according to the criteria given above, is suspended in water and poured into the column. After the resin has settled, the column is equilibrated by washing it with 1–2 column volumes of starting buffer.

Adsorption of Protein to the Column. The only critical point of the procedure is to start with a salt concentration high enough to achieve salting out, but low enough to avoid precipitation of the protein out of solution. Three ways have been found useful to arrive at this point.

(*a*) If the solubility of a protein to be purified in a particular salt solution is known, it is best to saturate the protein solution with salt to just below the concentration where it starts to precipitate. This can be achieved by addition of solid salt or preferably by the addition of a saturated salt solution or dialysis. Any precipitate formed during this procedure is removed by centrifugation and the clear supernatant is poured over the column, which is equilibrated with an appropriate buffer equimolar in salt to the protein solution. Large volumes of protein solution can be processed in this way, and the procedure may even be used to concentrate dilute protein solutions.

(*b*) If the solubility of the protein under investigation is unknown, a batchwise procedure has been found to be useful. In this case the necessary amount of Sepharose 4B is suspended in the protein solution and salt is added to a concentration that brings about the adsorption of the protein to the resin. The extent of binding of an individual protein may be followed by assaying the supernatant, while binding of total protein can be monitored by UV spectroscopic measurement. In this respect, it is of interest that at a 70% saturation with ammonium sulfate almost all soluble proteins from baker's yeast are bound.

The suspension of the resin coated with protein can then be poured into a column and the gradient can be started immediately. For the reasons discussed above, the resolution obtained with this method is as good as with method (*a*).

(*c*) If relatively small amounts of protein in a small volume have to be processed, then the following procedure has been found to be useful. A column is equilibrated with a salt solution sufficiently concentrated to achieve binding. Saturated salt solution is added to the protein solution until the first turbidity just appears. The solution is then passed over the column. Because the gel in the column is equilibrated with a high salt concentration, the protein solution is steadily concentrated during passage down the column, and a point is reached where it is bound to the gel. A prerequisite for this simple general method is that the ratio of the column volume to the volume of the solute applied is greater than a factor of 10.

The Gradient. As can be seen from the examples given below, an individual protein is eluted within a 3–3.5% decrease in ammonium sulfate concentration. Hence, in the examples given we have used a decrease of 1.25% saturation of ammonium sulfate per 100 ml gradient volume. In this case more than 90% of a particular protein is eluted within 250 ml gradient volume. In addition, individual proteins differing in solubility by only 3% relative salt concentration are almost completely separated. Increasing the steepness of the gradient to 1.75% per 100 ml gradient volume means that a difference in solubility of about 5% saturation is necessary to achieve the same resolution [see example given by von der Haar in ref (1)]. Working with small amounts of protein, a steeper gradient may be advantageous to recover the protein in a more concentrated form. In that case, however, one has to be cautious to avoid inhomogeneities in the gradient arising from the large density differences between the salt solutions used. In addition, working with steep gradients, one has to take care to ensure that the flow rate is slow enough to set up the solubility equilibrium at any given point of the column.

Recovery of the Protein. The easiest way to recover a protein fraction is by salt precipitation and centrifugation. In practice, the addition of one

volume of saturated salt solution to one volume of column eluant is enough to increase the salt concentration to such an extent that the protein is quantitatively precipitated out of solution.

Alternatively, saturated salt solution is added slowly to the column eluant until the first turbidity appears. The solution is then passed over a small Sepharose 4B column equilibrated with a high salt concentration in order to adsorb the protein. From this column the protein can be released by lowering the salt concentration in one step to below that concentration needed for salting out.

Variations in the General Procedure. The method described here is based on the solubility of individual proteins in concentrated salt solutions. Hence, all the variables that were originally used for the isolation of proteins by precipitation from solution should also be useful here [see Green and Hughes (13)]. The most important variables in this respect are temperature, pH, and the type of salt. Whereas the influence of temperature was not further investigated, examples are given below for the variations of pH and of the type of salt.

Regarding the type of salt, it is of interest that using ammonium sulfate in the range pH 5–8, only slight differences are observed. With potassium phosphate at pH 7, a much higher concentration of salt is needed than at pH 9.4. This indicates that the active agent is the PO_4^{2-} ion, which is predominant at pH 9.4, whereas the singly charged phosphate ion, present at pH 7, is much less active in precipitation.

Of the different kinds of salt investigated, ammonium sulfate and potassium phosphate proved to be useful. With high concentrations of sodium citrate, which is also very efficient in salting out proteins on the Sepharose 4B surface, the aminoacyl-tRNA synthetases used in this study lost their activity completely. Hence, no further investigations regarding this salt were performed.

Of practical importance is the fact that protein can be bound to the resin under one set of conditions and salting in can be achieved under a different set of conditions without releasing the protein from the gel merely by changing the solvent. In the examples given below, proteins were always salted out of an ammonium sulfate solution. For salting in with phosphate buffer, for example, the column, which was loaded with protein, was rinsed with half a column volume of starting phosphate buffer, and the gradient was then started. Because of the relatively low solubility of potassium sulfate, a few crystals of this salt appeared in the fractions overlapping the phosphate and sulfate regions. This was, however, not detrimental to the procedure. Since Sepharose 4B does not carry ionic groups, a small amount of the new buffer is sufficient for the reequilibration, even if only small differences in pH or salt concentration exist.

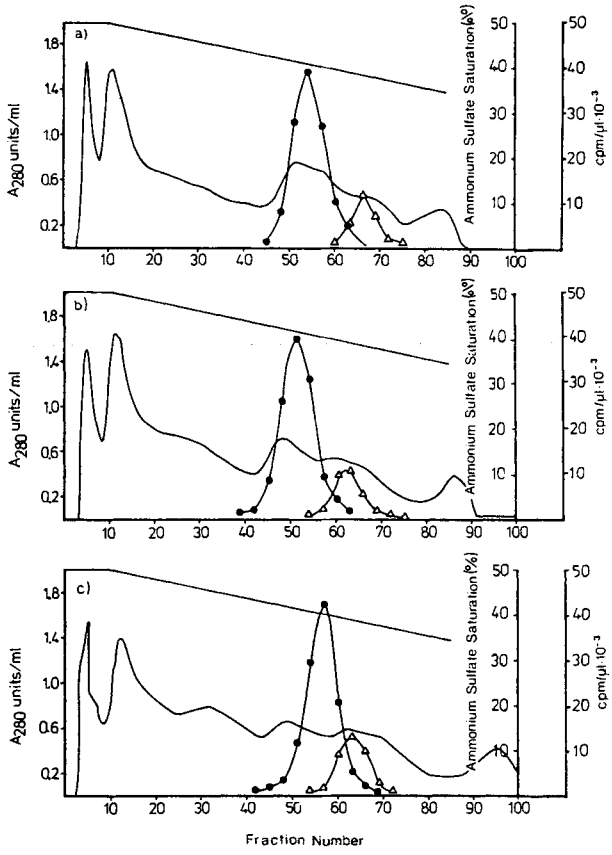

FIG. 1. pH Dependence of salting out with ammonium sulfate; 760 mg protein enriched in phenylalanyl- and isoleucyl-tRNA synthetase was dissoved in 13 ml buffer A saturated with ammonium sulfate to 40%. The solution was then dialyzed against buffer A saturated with ammonium sulfate to 47%. After dialysis it was applied to a 2.5 × 10 cm (50 ml bed volume) Sepharose 4B column equilibrated with buffer A, pH 6, which was saturated to 50% with ammonium sulfate. (a) After adsorption of the protein, the column was rinsed with 50 ml buffer A, pH 5.5, and saturated to 50% with ammonium sulfate. The column was developed with a gradient of twice 1 liter buffer A, pH 5.5, and 50–25% saturated ammonium sulfate solution. The absorption at 280 nm (——), and the activity of phenylalanyl- (●) and of isoleucyl-tRNA synthetase (△) were monitored. Fractions of 16.5 ml were collected. (b) Column was run as in (a) except that buffer A at pH 6.5 was used. (c) Column was run as in (a) except that buffer A at pH 7.5 was used.

Examples

Influence of pH. Figure 1 shows the influence of three different pHs on the salting out of an identical protein mixture using ammonium sulfate. As far as the total protein is concerned (monitored by UV absorption at 280 nm), slight but significant differences are observed. In particular, the maximum of the well-resolved final protein peak is shifted from fraction 84 at pH 5.5 to fraction 86 at pH 6.5 and fraction 105 at pH 7.5. This specific protein is much better resolved at pH 7.5 than at pH 5.5. In contrast, phenylalanyl- and isoleucyl-tRNA synthetase are much better separated from each other at pH 5.5 than at pH 7.5. At pH 6.5 phenylalanyl-tRNA synthetase appears in the same position as at pH 5.5, whereas isoleucyl-tRNA synthetase is salted in somewhat earlier. At pH 7.5 phenylalanyl-tRNA synthetase is salted in at a slightly lower concentration than at pH 6.5, while the position of isoleucyl-tRNA synthetase remains unaltered on going from pH 7.5 to pH 6.5. These activities therefore overlap to a significant extent at pH 7.5. The reproducibility of the procedure is so great that small effects as shown in Fig. 1 are repeatedly observed during rechromatography (see, for comparison, Fig. 3 run at pH 5.5 and Fig. 5 run at pH 7.5). In a second experiment (Fig. 2), the respective phenylalanyl- and isoleucyl-tRNA synthetase fractions obtained at pH 5.5 are rechromatographed individually at pH 7.5. The results are consistent with those given in Fig. 1, in that phenylalanyl-tRNA synthetase shifts to lower salt concentration by less than 1% saturation, whereas the isoleucyl-tRNA synthetase appears at about 2% higher ammonium sulfate saturation. The results of the two subsequent chromatographies are summarized in Table 1. In both cases a 2.5-fold further purification is achieved by rechromatography at the higher pH with good recovery of material.

Use of Different Kinds of Salt. Figure 3 shows salting out of a protein fraction, different from that used in Figs. 1 and 2, with ammonium sulfate at pH 6.0. Whereas the protein profile looks different, phenylalanyl- and isoleucyl-tRNA synthetase are resolved as well as in the previous case. This indicates that the presence or absence of other proteins cannot influence the salting out or the salting in of an individual protein greatly. Leucyl-tRNA synthetase activity was also determined in this case. It coelutes with the phenylalanyl-tRNA synthetase. The phenylalanyl- and isoleucyl-tRNA synthetase fractions were rechromatographed individually using potassium phosphate (Fig. 4). In each case the protein fraction seems to split into two groups. As seen from Table 2, a good further purification is obtained on rechromatography with the second salt.

Combined Effects of pH and Type of Salt. To investigate the possibilities offered by a combination of differences in pH and of a different kind of salt, a crude extract was used instead of a partially purified protein fraction.

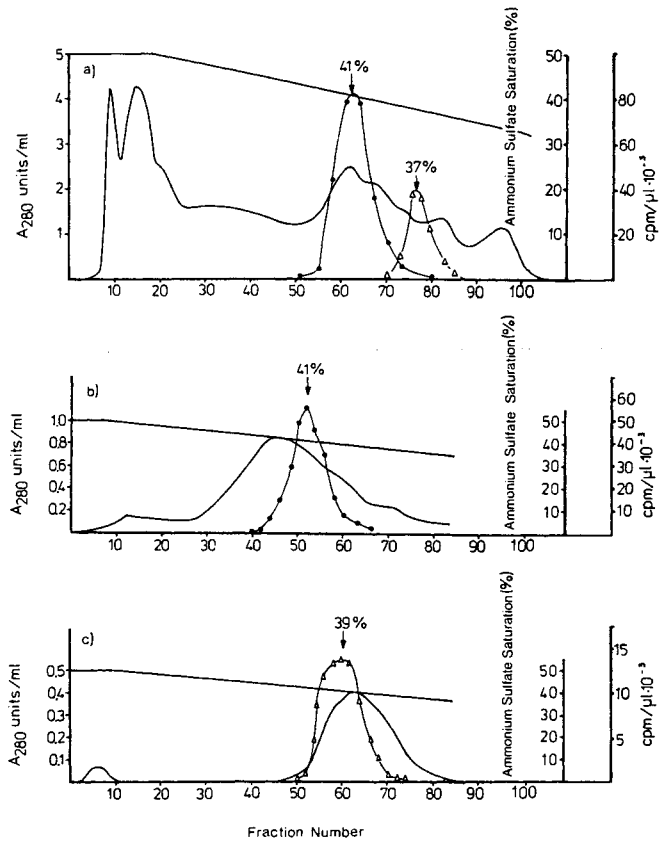

FIG. 2. pH Dependence of salting out with ammonium sulfate. (a) 2,174 mg protein was dissolved in buffer A, pH 6, saturated to 40% with ammonium sulfate. The solution was dialyzed against buffer A, pH 6, saturated to 47% with ammonium sulfate, and applied to a 3 × 14 cm (100 ml bed volume) Sepharose 4B column equilibrated with buffer A, pH 6, saturated to 50% with ammonium sulfate. The column was rinsed with 50 ml buffer A, pH 5.5, which was saturated to 50% with ammonium sulfate. An ammonium sulfate gradient of twice 1 liter buffer A, pH 5.5, and 50–25% ammonium sulfate was then applied. The absorption at 280 nm (——), and the activity of phenylalanyl- (●) and isoleucyl-tRNA synthetase (△), were monitored. Fractions of 16.5 ml were collected. (b) Fractions 57–69 of the column in (a) were recovered by precipitation with ammonium sulfate and dissolved in a minimum amount of buffer A, pH 7.5, saturated to 47% with ammonium sulfate. The column was developed as in (a), using buffer A, pH 7.5, instead of pH 5.5. (c) Fractions 73–82 from the column in (a) were rechromatographed in the same way as described for (b).

TABLE 1. **Salting out of Phenylalanyl- and Isoleucyl-tRNA Synthetase at pH 5.5 and pH 7.5**

	Enzyme	Total protein (mg)	Spec. activity (units/mg)	Total activity (units)	Purification (-fold)
Starting material	Phe	2,174	51	110,000	1
	Ile		14	30,000	1
Chromatography with $(NH_4)_2SO_4$ at pH 5.5	Phe	339	378	128,000	7.4
	Ile	187	105	19,600	7.5
Chromatography with $(NH_4)_2SO_4$ at pH 7.5	Phe	84	947	80,000	18.5
	Ile	56	248	14,000	17.5

As a first step, a column was run with ammonium sulfate at pH 7.5 (Fig. 5). Phenylalanyl- and isoleucyl-tRNA synthetase are quantitatively adsorbed to the column and are salted in at the same salt concentration at which they are eluted, using partially purified protein fractions. This again indicates that, contrary to precipitation of proteins out of solution (13), the level of purity has almost no influence in this process.

From the UV absorbance at 280 nm and 260 nm it can be seen that proteins as well as nucleic acids are bound to the column at pH 7.5. This is in contrast to conclusions drawn from work with tRNA, which pointed to the fact that nucleic acids should only be salted out at pH 4.5 or even less pH value (14). Nucleic acids (predominant UV absorption at 260 nm) tend to be salted in at significantly lower salt concentrations than proteins and are therefore partially removed from proteins.

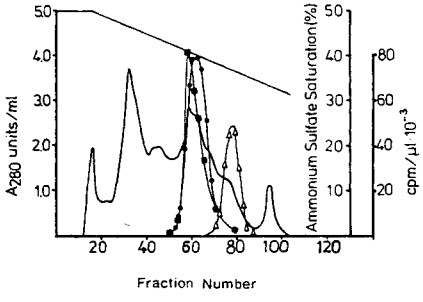

FIG. 3. Salting out of phenylalanyl-, leucyl-, and isoleucyl-tRNA synthetase with ammonium sulfate at pH 6. Protein, 2500 mg, was dissolved in buffer A, pH 6, and dialyzed against buffer A, pH 6, saturated to 47% with ammonium sulfate. A slight precipitate occurring during dialysis was centrifuged down and the supernatant was applied to a 3 × 14 cm (100 ml bed volume) Sepharose 4B column equilibrated with buffer A, pH 6, saturated to 50% with ammonium sulfate. The column was developed with a linear ammonium sulfate gradient of twice 1 liter of buffer A, pH 6, at 50–25% saturation. The absorption at 280 nm (——), and the activity of leucyl- (■), phenylalanyl- (●), and isoleucyl-tRNA synthetase (△), were monitored. Fractions of 16.5 ml were collected.

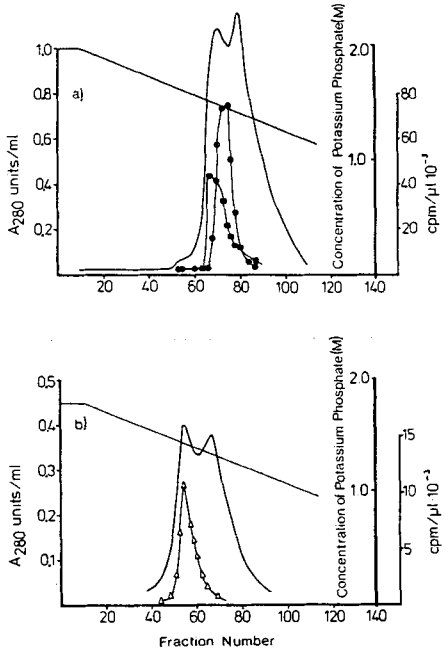

FIG. 4. Salting out of phenylalanyl-, leucyl-, and isoleucyl-tRNA synthetase with potassium phosphate. (a) Protein of fractions 55–67 (445 mg) of the chromatography described in Fig. 3 was recovered by ammonium sulfate precipitation and centrifugation. The protein was dissolved in buffer A saturated to 47% with ammonium sulfate. It was then applied to a 3 × 14 cm Sepharose 4B column equilibrated with buffer A, pH 6, saturated to 50% with ammonium sulfate. After application of the protein, a potassium phosphate gradient of twice 1 liter potassium phosphate, from 2 M to 1 M, pH 9.4, was run. The absorption of 280 nm (——), and the activity of phenylalanyl- (●) and leucyl-tRNA synthetase (■), were monitored. Fractions of 16.5 ml were collected. (b) Protein of fractions 73–83 (140 mg) of the chromatography described in Fig. 3 was subjected to the same procedure as given in (a), except that the potassium phosphate gradient was from 1.8 M to 0.8 M, and the activity of isoleucyl-tRNA synthetase (△) was monitored.

TABLE 2. **Salting Out of Phenylalanyl- and Isoleucyl-tRNA Synthetase with $(NH_4)_2SO_4$ and K_2HPO_4**

	Enzyme	Total protein (mg)	Spec. activity (units/mg)	Total activity (units)	Purification (-fold)
Starting material	Leu	2,500	24	61,000	1
	Phe		60	150,000	1
	Ile		26	65,000	1
Chromatography with $(NH_4)_2SO_4$ at pH 6.0	Leu	445	135	60,000	5.6
	Phe		225	99,900	3.7
	Ile	140	198	27,800	7.6
Chromatography with K_2HPO_4 at pH 9.4	Phe	111	739	82,000	12.3
	Ile	35	534	18,700	20.5

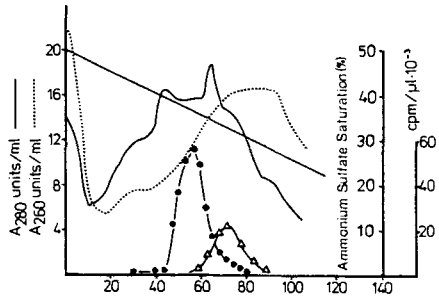

FIG. 5. Salting out of a crude extract from baker's yeast. Three kg of yeast was broken and the debris was removed as described in ref. (1). The supernatant was saturated to 47% by the addition of solid ammonium sulfate. The precipitate formed was removed by centrifugation, and the supernatant (2,000 ml) was poured over a 500 ml Sepharose 4B column equilibrated with buffer A, pH 7.5, which was saturated to 50% with ammonium sulfate. To develop the column, an ammonium sulfate gradient of twice 1.5 liter from 50 to 15% saturation was run. The adsorption at 280 nm (——) and at 260 nm (· · ·), and the activity of phenylalanyl- (●) and isoleucyl-tRNA synthetase (△), were monitored. Fractions of 16.5 ml were collected.

TABLE 3. **Variation of pH and Kind of Salt for Purification of Phenylalanyl-tRNA Synthetase**

	Total protein (mg)	Ratio of absorption at 280 and 260 nm	Spec. activity (units/mg)	Total activity (units)	Purification (-fold)	Yield (%)
Supernatant after removal of debris	70,000	0.63	1.6	114,000	1	100
Supernatant after saturation with 47% $(NH_4)_2SO_4$	38,000	0.63	2.9	113,000	1.8	99
Chromatography with $(NH_4)_2SO_4$ at pH 7.5	3,500	1.00	17.3	60,000	10.7	53
Chromatography with $(NH_4)_2SO_4$ at pH 5.5	1,570	1.35	48.7	70,000	30.2	67
Chromatography with K_2HPO_4 at pH 9.4	594	1.56	45.5[a] 104.0[b]	27,000 62,000	65	54

[a] Value obtained immediately after chromatography.
[b] Value obtained after dialysis.

The phenylalanyl-tRNA synthetase fraction obtained from the first chromatography with ammonium sulfate at pH 7.5 was rechromatographed with ammonium sulfate at pH 5.5 (chromatogram not shown). It was then rechromatographed again with potassium phosphate at pH 9.4 (chromatogram not shown). Results obtained during the subsequent steps are summarized in Table 3. A 65-fold overall purification is achieved with good recovery of enzymatic activity. Additional interesting information can be drawn from the final assay after chromatography on the potassium phosphate column. If the assay is carried out immediately after the chromatography, a rather low activity is found, pointing to a denaturation of the enzyme. Activity returns, however, after removal of the potassium phosphate by dialysis, indicating that the denaturation was reversible.

CONCLUDING REMARKS

Salting out chromatography on unsubstituted hydrophilic gels is based on a principle entirely different from ion exchange and gel permeation

chromatography. Hence it serves as a real alternative to these traditional procedures. Proteins from different organisms and of different kinds have been successfully subjected to the procedure (F. von der Haar, unpublished results), and hence it seems justified to conclude that it is applicable to proteins in general. One of the most impressive examples of this is perhaps that antibodies against a specific protein isolated by affinity chromatography could be further subfractionated according to their specificity against different antigenic determinants on the antigenic protein by the salting out procedure (G. Stöffler, Berlin, personal communication).

Its main advantages are its easy and cheap operation together with an outstanding reproducibility (see the various examples given). Another advantage is that protease attack is largely suppressed at the high salt concentration. Furthermore, like other proteins, proteases appear as well-resolved peaks. This is in contrast to ion exchange chromatography, where proteases often smear out across the whole elution profile. As a consequence, loss of protein during the salting out procedure due to protease attack is almost negligible.

As far as we have observed, two practical difficulties may arise during work with this method. First, with salt sensitive enzymes, especially at low protein concentrations, it may be difficult to locate the enzyme position in the elution profile, because too much salt is introduced into the assay. In this case aliquots have to be desalted prior to the assay. This however, is not a very serious drawback, since due to the high reproducibility of the system, the location of the enzyme need only be made once. In subsequent runs a protein may be isolated with only a very few or even without any tests.

Second, with several proteins a reversible loss of activity during salting out was observed (see, for example, Table 3). The reason for this is not clear, since a particular protein may be denatured in one case but not in the other (compare Tables 2 and 3). For one enzyme, however, alkaline phosphatase from *E. coli*, the reason for the reversible loss of activity could be explained. In this particular case activity dropped to only a very few percent during chromatography and returned only slowly during prolonged dialysis (F. von der Haar, unpublished results). Since alkaline phosphatase is a Zn^{++} enzyme from which the Zn^{++} is relatively easy to remove, the Zn^{++} seemed a likely candidate for removal by competition with the high concentration of cations in the solvent. We therefore added a constant concentration of 10^{-4} M $ZnSO_4$ to the ammonium sulfate gradient and found that with this treatment no loss of activity occurred. This demonstrates that difficulties that may arise during work with the salting out system can be overcome by an appropriate modification of the system.

Finally, a few remarks regarding the nature of the binding processes should be made. Obviously, the solubility of the protein is reduced on

interaction with the surface of the hydrophilic resin. In general, one can imagine two reasons for this. Either the binding process is due to a hydrophobic interaction with the hydrophobic parts of the sugar residues or the hydroxyl groups of the carbohydrate surface displace water molecules from the hydration sphere of the protein and in this way cause the reduction in solubility. For reasons discussed elsewhere (15), the second alternative seems more attractive. It remains to be established, however, whether, and to what extent, either of the reasons are responsible for binding, or whether both types of interactions may exist in a cooperative manner.

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