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Production and Purification of Tartrate Dehydrogenase

Role of Aqueous Two-Phase Extraction

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ABSTRACT

Tartrate dehydrogenase (TDH) is a stereospecific intracellular enzyme produced by *Pseudomonas putida*. Several methods for separation of nucleic acids from the proteins in cell homogenate were compared in this study. These methods included precipitation (using streptomycin sulfate, manganous sulfate, and protamine sulfate) and aqueous two-phase extraction. Under optimal conditions of separation, a single-step aqueous two-phase extraction followed by back-extraction of the enzyme from enzyme-rich PEG-phase resulted in 77% recovery of enzyme. This compared favorably with 50% enzyme recovery using protamine sulfate treatment. Furthermore, the remaining enzyme activity was accounted in the nucleic acid-rich dextran phase and the spent-PEG phase, suggesting that a multistep extraction process would increase enzyme recovery even more. Under the conditions of aqueous two-phase extraction, the selectivity of proteins over nucleic acids was 30, indicating a high degree of separation of proteins and nucleic acids in this process. The experimental data and their implications are presented.

Index Entries: Nucleic acids; proteins; bioseparation; precipitation; *Pseudomonas putida*.

INTRODUCTION

Production of intracellular enzymes involves a series of operations that start with centrifugation to concentrate the cells, followed by cell disruption to release the intracellular components, removal of nucleic acids,

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concentration and purification of the enzyme, and finally, polishing. Each of these operations results in some loss of the product; the net result is that the final yield of the product is very low *(1)*. In an analysis of a purification scheme of intracellular enzyme tartrate dehydrogenase produced by *Pseudomonas putida*, the total loss in enzymatic activity ranged from 72 to 84%; 52–65% of the enzymatic activity was lost in the initial stages in which the enzyme was separated from nucleic acids and other undesirable enzymes *(2)*. These steps typically involve sequential precipitation of undesirable components that are somewhat nonspecific for their target molecules. This is in keeping with the standard practice of using low cost/volume, but low-resolution, methods in operations involving large volumes and using higher unit-cost but very specific techniques for final stages of purification *(3)*. Yet, it will be desirable to use methods that minimize the enzyme losses while achieving the target of removing the nucleic acids and the undesirable enzymes. Because of the large losses of enzymatic activity in these initial phases, it was decided to investigate methods of precipitation of nucleic acids from the homogenates of *Pseudomonas putida* and their impact on losses of tartrate dehydrogenase. Three different precipitation agents and aqueous two-phase extraction involving PEG and dextran were evaluated and compared.

LITERATURE SURVEY

The methods for removal of nucleic acids from cell homogenates involve precipitation, extraction, and enzymatic degradation. Precipitation is induced by addition of polycationic-complexing agents that interact with the phosphate residues of nucleic acids. Cetyltrimethyl ammonium bromide *(4,5)*, streptomycin sulfate *(6)*, protamine sulfate *(7)*, manganous sulfate *(8)*, and polyethyleneimine *(9,10)* are some of the chemicals used as precipitating agents. The complexing between nucleic acids and the precipitating agents is nonspecific and results in simultaneous precipitation of many proteins as well. In general, conditions promoting increased precipitation of nucleic acids cause an increase in loss of enzyme too *(8)*.

Oxenburgh and Snoswell *(6)* examined the conditions for precipitation of nucleic acids in extracts of *Lactobacillus plantarum* using streptomycin sulfate. The optimum pH range was established to be 6.0–8.0 and the best results were obtained with a ratio of streptomycin to proteins of 10.0 (w/w). At a protein concentration of 10 g/L, 10% (w/v) streptomycin sulfate, pH 7.0, solution conductivity of 0.38 ms/cm, protein losses were determined to be 24%. This compared very favorably with other methods. Use of protamine sulfate caused considerably higher losses of proteins. The streptomycin-sulfate precipitation was highly reproducible. Because of the interference caused by salts, Oxenburgh and Snoswell *(6)* found it necessary to to dialyze the ammonium-sulfate extracts before addition of streptomycin sulfate. Toxicity of streptomycin sulfate for the operators

and the possible development of streptomycin-resistant microbes, were pointed out as potential disadvantages of this method. In another study involving recovery of catalase and oxaloacetate decarboxylase from the homogenates of *Micrococcus lysodiekticus*, protamine-sulfate treatment again resulted in considerable loss in enzyme activity, whereas the removal of nucleic acid was still poor. In comparison, with polyethyleneimine treatment, 90% of the nucleic acids were removed and recovery of the enzymes was 70% *(9)*. Polyethyleneimine was effective in removing large quantities of both DNA and RNA *(9)* and in selective purification of restriction endonuclease EcoRI *(10)*. Its drawbacks include potential carcinogenicity of the unreacted monomer. Higgins et al. *(8)* studied the use of heat treatment and manganous sulfate for precipitation of nucleic acids in production of β-galactosidase from *E. coli*. RNA precipitation was dependent on the concentration of manganous sulfate, but the losses in β-galactosidase activity were as high as 70%.

Precipitation of nucleic acids by the complexing agents is affected by solution pH, salt concentration, and the ratio of nucleic acids to complexing agent *(6,11,12)*. Guerritore and Bellelli *(5)* found that sodium salts (chloride, sulfate, and citrate) interfered with precipitation of nucleic acids by cetyltrimethyl ammonium bromide when present at concentrations above 0.2 *M*. On the other hand, glycine, glucose, and urea had no effect. In an extract of *E. coli* EM 20031, the effectiveness in precipitating nucleic acids decreased in the following order: polylysine > polyethyleneimine > cetyltrimethyl ammonium bromide > streptomycin sulfate > protamine sulfate > $MnCl_2$ > spermine > spermidine.

Aqueous two-phase extraction has been used for the separation and large-scale purification of enzymes from *Klebsiella pneumoniae (13)* and *E. coli (14)*. These methods depend upon incompatibilities of aqueous solutions of polymers such as polyethylene glycol (PEG) with solutions of salts (such as ammonium or potassium phosphate) or dextran. This results in formation of two separate phases in appropriate ranges of polymer concentrations. Proteins and nucleic acids partition differently in these two phases because of differences in surface charges and hydrophobic/hydrophilic character. As a result, proteins generally prefer the aqueous phase rich in PEG, whereas nucleic acids partition preferentially into dextran-rich phase *(15)*. Other parameters that influence partitioning of a given molecule in the two phases, include its molecular weight, the concentrations and molecular weights of the phase-forming polymers, temperature, pH, ionic strength of the mixture, and presence of polyvalent salts in the mixture. The operating conditions in terms of pH and salt concentration may be manipulated to fractionate proteins as well *(16,17)*. By coupling a ligand to PEG, partitioning of a specific protein in the PEG-rich phase can be enhanced *(18)*. An advantage of this method is that the polymers stabilize the tertiary structure and biological activities *(19)*. Despite these advantages, aqueous-phase

partitioning has not become very popular because of the high cost of phase-forming polymers. Use of crude dextran and other less-expensive polymer systems has also been explored with success *(20)*.

Hydrolysis of nucleic acids by nucleases, followed by ammonium-sulfate precipitation of proteins and dialysis of the dissolved precipitate, has been found to be an effective method for removal of nucleic acids *(21–23)*. The hydrolysis can be conducted by ribonucleases and by deoxyribonucleases. The cost of nucleases is a major hindrance in use of this method in removal of nucleic acids from mixtures during protein purification.

MATERIALS AND METHODS

Tartrate dehydrogenase (TDH)-producing strain *Pseudomonas putida* ATCC 17642 were maintained and cultured in a basal salt medium prescribed by Kohn et al. *(24)*. The cells were maintained on agar plates containing the basal medium, stored at 4°C and transferred once a month. The cells were cultured according to the methods described by Tipton and Peisach *(25)*. The cells were centrifuged and homogenized by sonication. Prior to disruption, the cell paste was suspended in an equal weight of 20 m*M* phosphate buffer (pH 7.2) containing 1 m*M* dithiotreitol. Protease inhibitors, phenyl methanesulfonyl fluoride in acetone and N^{α}-β-tosyllysine methanesulfonyl fluoride in water, were added to the suspension to a final concentration of 0.5 m*M* just before cell disruption. The suspension was sonicated thrice, each time for a duration of 2 min with a 2-min cooling period between each sonication. The disrupted-cell suspension was centrifuged at 10°C, 25000*g* for 30 min, the aqueous phase was recovered, stored at 4°C, and used for studies involving nucleic-acid removal.

Details of the precipitation studies are described along with the results. All the experiments were conducted at 4°C.

Protein concentration in the samples was measured using Bradford Coomassie blue method *(26)* with bovine serum albumin as a standard. The activity of TDH was measured by the method described by Tipton and Peisach *(25)*. The activity is defined as the rate of oxidation of (+) tartrate, one unit being the amount of enzyme that catalyzes the formation of 1 μmol of oxaloglycolate per minute under the standardized conditions of assay *(16)*. The oxidation rate was determined by monitoring the formation of $NADH.H^+$ at 340 nm in the reaction mixture using a spectrophotometer. For determination of the relative contents of proteins and nucleic acids in a mixture, Christian and Waarburg's method *(27)* was used.

All the chemicals used in this study were obtained from Sigma (St. Louis, MO). All salts were of analytical grade. Polyethylene glycol 8000 and dextran 70 were used to form the aqueous two-phase system.

RESULTS AND DISCUSSION

Separation of nucleic acids from proteins in the cell homogenate was studied using three precipitating agents (streptomycin sulfate, manganous sulfate, and protamine sulfate) and in an aqueous two-phase system. The homogenate used in all the experiments came from the same fermentation run. The concentrations of proteins and nucleic acids, and the activity of TDH was monitored in each phase.

Precipitation by Streptomycin Sulfate

According to Linn and Lehman *(28)*, 0.48 g of streptomycin sulfate should be added per gram of RNA in the crude homogenate. Based on the RNA content of the cells (7% of cell dry weight; ref. *29*), a value of 4.09 mmol of streptomycin sulfate per L of cell homogenate was calculated. The actual amount was varied by ±20% and by ±50% from this value to study the effect of this variable. Streptomycin sulfate was added to the crude homogenate as a powder and stirred for 10 min. pH of the solution was adjusted to 7.0–7.1 by addition of 1 *N* KOH. The precipitate was removed by centrifugation in a cold room and the supernate was analyzed for TDH activity and the relative contents of proteins and nucleic acids. The results are presented in Fig. 1A.

Based on these results, addition of 4 mmol streptomycin sulfate per L of crude homogenate is optimal for precipitation of nucleic acids. At this value, 74% of the tartrate dehydrogenase was still in solution. Increasing the ratio of streptomycin sulfate caused more precipitation of enzyme without increasing the precipitation of nucleic acids. More enzyme remained in solution at lower ratios, but so did more nucleic acids. As shown in Fig. 1B, the ratio of loss in enzyme activity to nucleic acid precipitated had a minimum value of 0.4 under the optimal condition. No attempt was made to recover the precipitated enzyme, as it was presumed to have lost its activity.

Precipitation by Protamine Sulfate

Protamine sulfate has been used by Tipton and Peisach *(25)* in their recovery process to precipitate the nucleic acid from cell homogenate. These authors recommended using 5 mg of protamine sulfate per gram of wet paste used for homogenization. In experiments conducted by Smith and Serafozo *(2)* TDH activity was reduced 23–30% in this process. However, nucleic-acid removal was not measured. Hence, experiments were conducted in which protamine sulfate was added to the homogenate in increments of 3 mg/g wet paste. The mixture was stirred for 10 min, after which the precipitate was removed by centrifugation and the supernate was analyzed for activity of TDH and concentrations of nucleic acids and proteins. These results are presented in Fig. 2A.

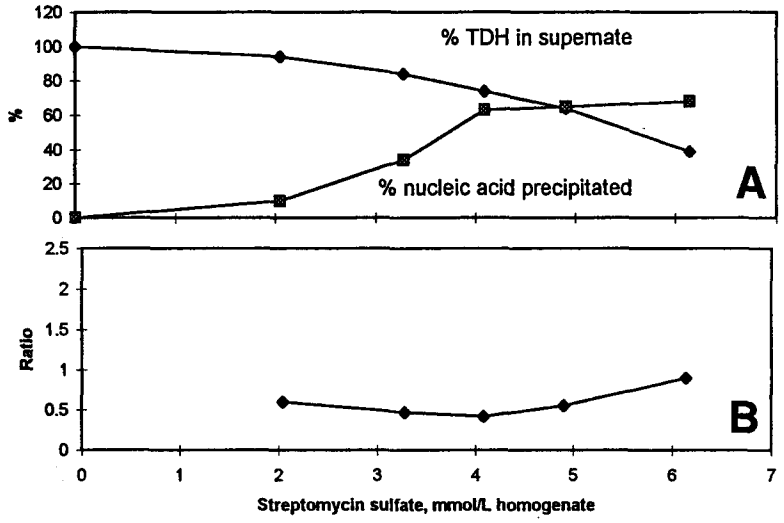

Fig. 1. Precipitation of nucleic acids with streptomycin sulfate. **(A)** Effect on nucleic acid precipitation and residual TDH activity in the supernate. **(B)** Effect on ratio of TDH lost to nucleic acid precipitation.

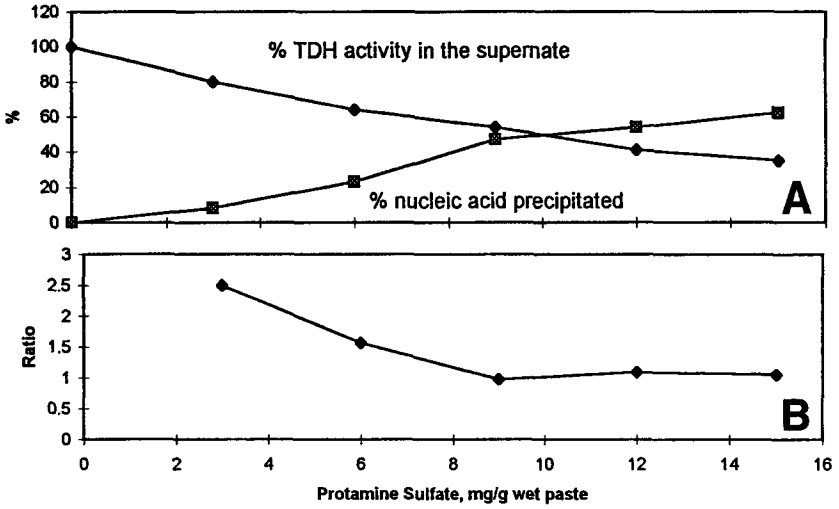

Fig. 2. Precipitation of nucleic acids with protamine sulfate. **(A)** Effect on nucleic acid precipitation and residual TDH activity in the supernate. **(B)** Effect on ratio of TDH lost to nucleic acid precipitation.

These results essentially duplicated the observation of 23 to 30% loss of TDH activity at 5 mg/g loading of protamine sulfate. At this value, only approx 20% of the nucleic acids would have been precipitated. Obviously, it was not the best choice of protamine-sulfate loading. The ratio of enzyme loss to nucleic acid precipitation has been plotted in Fig. 2B. This ratio

decreased as the amount of protamine sulfate in the homogenate was increased and it stabilized at approx 1.0 at a 9 mg/g wet-paste loading of salt. Under these conditions, approx 50% of the nucleic acid precipitated with a concomitant loss of enzyme activity.

Precipitation by Manganous Sulfate

Melling and Atkinson *(23)* have referred to the high cost and other dangers of using streptomycin and protamine sulfate as complexing agents. Hence, manganous sulfate was used as a precipitating agent, as suggested by Higgins et al. *(8)* $MnSO_4$, H_2O was added to the crude homogenate at different concentrations ranging from 0.05 to 0.5 mol/L. The mixture was stirred for 10 min followed by centrifugation and analyses of proteins, nucleic acids, and TDH activity in the supernate. The results have been plotted in Fig. 3A. The results show that the loss in TDH activity paralleled that of nucleic acids in the whole range of concentrations of manganous sulfate. As found by Higgins et al. *(8)* manganous sulfate had little selectivity for nucleic acids over the desired enzyme. Even at the concentration (100 mmol/L) recommended by Higgins et al. *(8)* only 25% of the nucleic acids were precipitated. About the same percentage of TDH activity was lost because of the treatment. The ratio of loss in TDH activity to nucleic acids precipitated remained almost constant at approx 1.0 for manganous sulfate addition between 0.1 to 0.5 mol/L homogenate (Fig. 3B).

Based on these results, it can be concluded that streptomycin sulfate is the best precipitating agent, not only because it precipitated most nucleic acids but also because the associated losses of enzyme were the least.

Aqueous Two-Phase Extraction

Bajpai et al. *(16)* have investigated the partitioning of proteins in PEG 8000-dextran 70 aqueous two-phase system and established the effect of nucleic acids on the partition coefficients of proteins (defined as the ratio of protein concentration in PEG-rich phase to that in dextran-rich phase). Their protocol was used in this work to optimize the partitioning of TDH and nucleic acids in the two-phase system. The two-phase system was created by mixing 480 g dextran solution (21.78% w/w) with 133 g PEG solution (45% w/w) and 387 g of phosphate buffer to produce a mixture containing 10.0% dextran and 5.1% PEG. The volume ratio of the two aqueous phases thus produced (PEG-rich phase:dextran-rich phase) was 3:7. pH of the system could be changed by changing the pH of phosphate buffer. In partitioning experiments, phosphate buffer was replaced by the cell homogenate.

The results of preliminary experiments are shown in Table 1. Here, pH of the system was varied between 5.5 and 7.2 and the partition coefficients of total protein, TDH, and of nucleic acids in the two phases have

Table 1
Partitioning of Proteins, TDH, and Nucleic Acids from Crude Homogenate in Aqueous Two-Phase Extraction System

pH	NaCl Concentration (M)	Partition Coefficients			% TDH recovered in PEG phase
		Proteins	TDH	Nucleic acids	
7.2	0	5.28±0.15	2.03±0.38	0.91±0.03	47
6.5	0	9.30±0.07	4.26±0.21	0.72±0.04	65
5.5	0	11.03±0.56	4.61±0.03	0.49±0.08	66
6.5	5	9.51±0.81	9.20±0.04	0.29±0.02	80

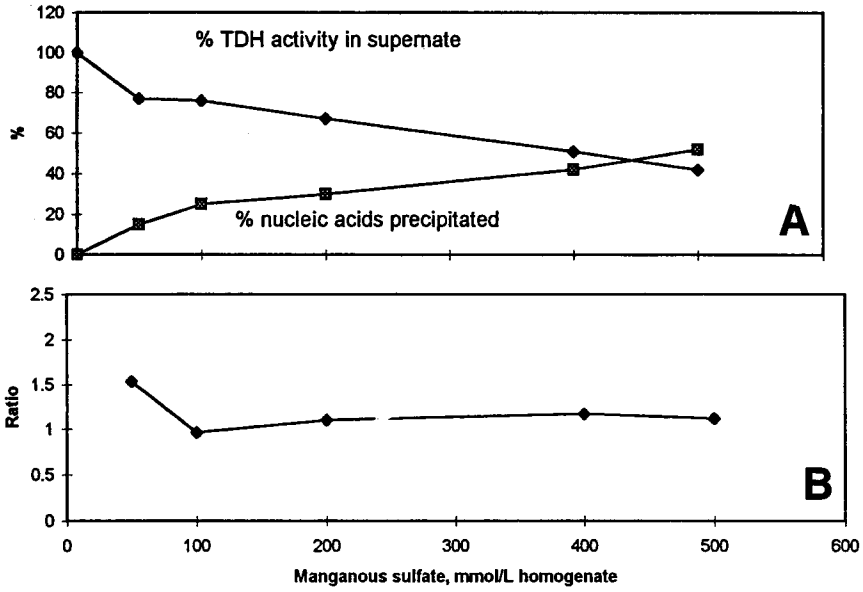

Fig. 3. Precipitation of nucleic acids with manganous sulfate. **(A)** Effect on nucleic acid precipitation and residual TDH activity in the supernate. **(B)** Effect on ratio of TDH lost to nucleic acid precipitation.

been reported. Clearly, the partitioning of proteins and of TDH in the PEG phase improved as the pH was reduced. The partitioning of nucleic acids in the PEG phase decreased at the same time, pointing to improved separation of proteins and nucleic acids. Reducing pH from 6.5 to 5.5 resulted in increased losses of total enzyme activity. Moreover, from the partition coefficients, it was clear that the total proteins partitioned more favorably in the PEG phase than did TDH. Earlier work with partitioning of different proteins had suggested that this could be improved by increasing the molarity of NaCl in the solution. An experiment at pH 6.5 and 5 *M* NaCl concentration, showed significant improvement in this category. Under this condition, partition coefficients of total protein and of TDH were somewhat identical and the partition coefficient of nucleic acids reduced even

Table 2
Aqueous Two-Phase Extraction of Cell Homogenate (pH 6.5, 5 *M* NaCl) and Back Extraction of PEG Phase with Phosphate Buffer (pH 7.2); Protamine Sulfate Control

Total activity added to the biphasic system	32 units
Activity in PEG phase	27 units
Activity in dextran phase	5 units
Activity in buffer back-extracted from PEG phase	25 units
% yield in the back-extracted buffer	77 %
Total activity used for protamine sulfate control	32 units
Activity in supernate after precipitation	16 units
% yield	50%

(Loading 9 mg/g wet paste); wt of wet paste: 48 g, dry weight: 10.7 g.

further. The selectivity (ratio of partition coefficient of proteins to that of nucleic acids) was 30, suggesting a high extent of removal of nucleic acids from the crude homogenate.

In another experiment, TDH partitioned in the PEG phase (at pH 6.5, 5 *M* NaCl) was back-extracted by phosphate buffer at pH 7.2. These data are shown in Table 2. Under these conditions, 77% of the activity of TDH was found in the phosphate buffer back-extract. Moreover, the TDH present in dextran phase was also active. Thus the total activity that could be eventually extracted in a multistage or countercurrent aqueous two-phase extraction system could be even higher. By comparison, only 50% of the TDH activity of the crude homogenate was present in the supernate of protamine-sulfate precipitation, used as control (loading 9 mg/g wet paste).

CONCLUSIONS

Among the complexing agents evaluated for precipitation of nucleic acids from cell homogenate of *Pseudomonas putida*, streptomycin sulfate proved to be most effective. Under optimal conditions, it precipitated 65% of nucleic acids, incurring only 30% losses in the activity of desired enzyme, tartrate dehydrogenase. Protamine sulfate and manganous sulfate were not as effective. In an extraction using aqueous two-phase PEG 8000-dextran 70 system, almost all of the enzyme activity was accounted between the two phases. In a single-pass extraction back-extraction, 77% of the enzyme was recovered in the buffer system, making it potentially a very effective means of removing nucleic acids from the homogenate.

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Tipton's laboratory performed the initial purifications; data from these were used for evaluation of the scheme of purification. The authors gratefully acknowledge this help.

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