UNIVERSITY OF ILLINOIS

May 1 1990

THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

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ENTITLED Purification of Bacillus subtilis Aspartate Transcarbamylase Expressed in Escherichia coli (TB2/pLS2000)

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE DEGREE OF Bachelor of Science in Biochemistry

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HEAD OF DEPARTMENT OF Biochemistry

PURIFICATION OF *BACILLUS SUBTILIS* ASPARTATE TRANSCARBAMYLASE EXPRESSED IN *ESCHERICHIA COLI* (TB2/pLS2000)

BY

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THESIS

for the

DEGREE OF BACHELOR OF SCIENCE

IN

BIOCHEMISTRY

College of Liberal Arts and Sciences

University of Illinois

Urbana, Illinois

1990

ACKNOWLEDGEMENTS

I have many to thank for helping me through this project. First and foremost on the list is Dr. R.L. Switzer, without whose guidance this procedure could not have been elucidated. Although his duties leave him with few free moments, he always found time to work through the rough spots with me.

My salvation in the lab came from all those who work in SwitzerLand, but from three people in particular: Lee Graves, Lee Bussey and Shannon Sheehan. Lee Graves got the ball rolling for me during my initial struggles, which was no small feat. Once that was accomplished, Lee Bussey and Shannon Sheehan kept it rolling by helping me over innumerable obstacles, taking time out of their own busy schedules on a regular basis. I am forever in their debt.

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INTRODUCTION

Purification of aspartate transcarbamylase (ATCase) from *Bacillus subtilis* is a long and tedious process and yields ATCase with contaminating protease activity. This process is no longer necessary because of a new plasmid, pLS2000, bearing *B. subtilis pyrB* (the ATCase structural gene) expressed in a strain of *Escherichia coli* from which the structural gene for *E. coli* ATCase has been deleted. The transformed *E. coli* TB2/pLS2000 strain overproduces *B. subtilis* ATCase, which is easily purified due to the enzyme's high concentrations in the crude extract.

Under certain growth conditions TB2/pLS2000 contains ATCase at specific activities as high as 150 μmol/min/mg of protein in the crude extract. This is compared to *B. subtilis* crude extract specific activities of 1 μmol/min/mg as reported by Brabson, Maurizi, and Switzer (1985). In fact *E. coli* crude extracts can show a specific activity as high as that found after six purification steps of the *B. subtilis* enzyme. Such overproduction of the aspartate transcarbamylase by *E. coli* has obvious benefits in reducing the time required for purification and increasing percent recoveries at the end of the procedure. This thesis describes procedures for practical realization of such benefits.

MATERIALS AND METHODS

I. Bacteriological Methods

A. Bacterial Strains

The *E. coli* strain used in these experiments was TB2 (Hoover *et al.*, 1983), which contains the plasmid pLS2000 constructed by Claude Lerner and Peter Hickman of our laboratory (*pyrBI- argI*). This strain has a deletion of the *pyrB* gene, which is the *E. coli* structural gene for ATCase. The *E. coli pyrB* mutation is complemented by wild type *B. subtilis pyrB* from pLS2000. A deletion of the transcription terminator which normally lies 5' to *B. subtilis pyrB* results in vast overproduction of *B. subtilis* ATCase, which is expressed from the *lacZ lpp* promoter of a pIN vector (Masui, 1983).

B. Growth Conditions

Two different media were employed in separate growths. The media were M9 minimal medium (Miller, 1972) and LB rich medium (Miller, 1972). The LB medium was mixed with 5.0 g NaCl, 5.0 g yeast extract, and 10.0 g Bacto tryptone per liter of double distilled water. The solution was then autoclaved for 30 minutes. Just prior to the start of growth, powdered ampicillin was added at 50 μg/ml. The M9 media used in this experiment and for subsequent experiments

consisted of the following components, in grams per liter of double distilled water: Na_2HPO_4, 6.0; KH_2PO_4, 3.0; NaCl, 0.5; NH_4Cl, 1.0. After autoclaving these dissolved components, 1 ml of sterile 1 M $MgSO_4$ and 10 ml of sterile 0.01 M $CaCl_2$ were added to the solution. Casamino acids were added at 0.2%(w/v), glycerol was added at 0.5%(v/v), and arginine was added at 50 μg/ml. As in the LB medium, 50 μg/ml ampicillin was poured in as a powder at the start of the growth period.

Growth of E. coli was conducted at 37°C in a New Brunswick Series 25 Incubator Shaker. Shaking speed was 300 rpm. A 500 ml fluted sidearm flask was used for smaller growths while 6 L flasks, both fluted and unfluted, were used for the larger 1 L to 3 L growths.

Cells were grown from 8 to 12 h depending on when they reached stationary phase. The gratuitous inducer isopropyl β-D-thiogalactosidase (IPTG) was added at a concentration of 1 mM when the culture turbidity reached between 50 and 100 Klett units using a red #66 filter. Harvests of 10 ml were taken at various points during the growth to test for increase in ATCase activity. All cells were harvested by centrifuging them in an SS-34 centrifuge at 4°C at 12,000 x g. They were taken up to speed and then allowed to slow to a stop. After centrifuging, liquid nitrogen was

used to freeze the cells before storage in a freezer at -80°C.

II. Assay Methods

A. ATCase Assay

The method used (Bond et al., 1983) to determine the ATCase activity in extracts was based on the method modified by Shindler and Prescott (1979) from an earlier procedure developed by Prescott and Jones (1969).

B. Protein Assay

The protein content was determined using the Lowry method (Lowry et al., 1951). A protein standard of 0 5 mg/ml bovine serum albumin was used for this procedure.

C. Procedure For Assay of ATCase

To prepare the primary reaction, 0.4 ml of 1.0 M Tris acetate (pH 8.2), was mixed with 0.1 ml of 0.5 M L-aspartate (pH 8.2) in a 12 x 75 mm tube. An appropriate amount of extract or purified enzyme (usually 5-15 µg) was added to the tube with the volume of double distilled water necessary to bring the total volume to 0.9 ml. The reaction was initiated by the addition of 0.1 ml of freshly prepared dilithium carbamyl phosphate. The mixture was incubated for

20 min at 30°C. After this period had elapsed, the reaction was stopped by the addition of 0.1 ml of 5%(v/v) perchloric acid. The tubes were then chilled on ice for 15 min. If any precipitate formed, it was removed by pelleting in a Dynac Model 0101 clinical centrifuge for 5 min at 80% of full speed. The secondary reaction, or color step, was performed by mixing 0.1 ml of the supernatant from the first reaction, 0.9 ml of double distilled water, 0.5 ml of freshly mixed 0.5%(w/v) 2,3-butanedionemonoxime in 5%(v/v) acetic acid, and 1.0 ml of 0.4%(w/v) antipyrene in 40%(v/v) H_2SO_4. Standards containing 50 to 250 nmoles of carbamylaspartate were also prepared. All samples were incubated at 60°C for 2 h under yellow light produced by two 25 Watt lamps covered with a sheet of yellow cellophane.

III. Polyacrylamide Gel Electrophoresis

The procedure employed was that of Laemmli (1970). Gel electrophoresis was performed with a 3% acrylamide stacking gel and a 10% acrylamide separating gel. The stacking gel consisted of the following: 3% acrylamide, 0.8% bis-acrylamide, 24 mM Tris/HCl, pH 6.8, and 0.1% SDS. The contents of the separating gel were 10% acrylamide, 2.7% bis-acrylamide, 37 mM Tris/HCl, pH 8.8, and 0.1% SDS. The buffer used for electrophoresis contained 14.4 g glycine, 3 g Tris (free base), and 0.1% SDS per liter. The 2X SDS-sample buffer used to solubilize the proteins prior to

electrophoresis was made up of 130 mM Tris/HCl, pH 6.8, 4% SDS, 20% glycerol and 10% 2-mercaptoethanol. Gels were stained with 0.1% Coomassie Brilliant Blue R-250 in 25% isopropanol and 10% acetic acid. The initial destaining was done in Destain I (10% acetic acid and 25% isopropanol) for 30 min. Destaining was completed overnight using Destain II (7% acetic acid and 5% methanol). After destaining, the gels were placed between two sheets of cellophane and dried at room temperature for 24 h.

IV. Purification of ATCase

A. Cell Preparation

To prepare the cells for purification, approximately one gram of cells was suspended in 10 ml of Buffer A (50 mM Tris/Cl, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.02% NaN_3, pH 8.1 at 4°C). These cells were separated into 2.5 ml aliquots for more thorough breakage and each was sonicated with one twenty second pulse. Sonication was accomplished using a Heat Systems-Ultrasonics Model W-375 sonicator at a duty cycle setting of 60% and an output setting of 4. Samples were kept chilled on finely crushed ice throughout the process. Once sonication was complete, the aliquots were centrifuged at 12,000 x g and 4°C. The precipitates were discarded and supernatants were combined.

B. Heat Treatment

The enzyme present in the pooled supernatant fractions was stabilized by adding 32.5 g of solid K_3 citrate per liter of solution, making it 0.1M in the salt, while pH was maintained at 8.1. The solution was incubated in a hot bath at 60°C for 30 min with occasional swirling to assure thorough mixing. The resulting precipitate was removed by spinning at 23,000 x g for one hour in an SS-34 centrifuge at 4°C. A large amount of denatured protein was precipitated and was discarded.

C. Ammonium Sulfate Fractionation

Pilot scale ammonium sulfate fractionations were conducted in disposable 12 x 75 mm glass tubes on 0.8 ml samples of extract. Precipitation was effected at 40%, 50%, 60%, and 70% saturation with volumes of 0.530, 0.800, 1.20, and 2.80 ml of saturated $(NH_4)_2SO_4$ (pH 8.2, in distilled H_2O) respectively. All samples were chilled on ice during addition of $(NH_4)_2SO_4$, which required 15 min of slow dripping. Further precipitation on ice was then allowed for another 30 min. Precipitates were removed by centrifugation at 4°C for 30 minutes at 23,000 x g.

Full scale ammonium sulfate fractionation was performed with the heat treatment supernatant placed in an ice bath. The solution was first brought to 50% saturation using an equal volume of saturated $(NH_4)_2SO_4$ at pH 8.1. The salt

solution was dripped in over a period of about 30 min and then allowed to precipitate on ice for at least another 30 min. This 50% salt solution was spun to 23,000 xg for 30 minutes at 4°C. The supernatant was returned to the ice bath and subsequently brought to a 70% concentration with a volume of 0.7 ml of saturated $(NH_4)_2SO_4$ per ml of 50% solution. This solution was treated in a manner identical to that described above, except that the precipitate was saved, and the supernatant was discarded. The appropriate amount of saturated $(NH_4)_2SO_4$ was determined using the following equation:

$$\text{Volume Saturated } (NH_4)_2SO_4 = \frac{(\text{Volume sample})(S2\% - S1\%)}{100} \Big/ \left[1 - (S2\% / 100) \right]$$

where S1% = $\%(NH_4)_2SO_4$ saturation initially

S2% = $\%(NH_4)_2SO_4$ saturation required

The precipitate was dissolved in a small volume of Buffer A at pH 8.1 (5 ml was found to work best).

D. Diethylaminoethyl (DEAE)-Sephacel Chromatography

Before performing column chromatography on the extract, the dissolved pellet was dialyzed against two 4 L volumes of Buffer A (pH 8.2) for 7 h each at 4°C to remove the ammonium sulfate. Small scale column chromatography was carried out

using Pierce disposable polystyrene columns (8 mm I.D. x 102 mm). The miniature columns used for trial experiments had a maximum effective bed volume of 2 ml and a total volume of 6 ml. The columns held 5 ml of Buffer A in addition to the DEAE-Sephacel, and flowed at a rate of 7 ml/hr. Three column volumes of a column wash consisting of 3 M KCl and isopropanol was used to rinse any foreign proteins from the DEAE-Sephacel, followed by 5½ hours of continuous rinsing with pure Buffer A before the actual sample (100 µl) was introduced onto the bed.

A 5 cm x 6 cm column was used for full scale column chromatography (approximately 20 ml of diethylaminoethyl (DEAE)-Sephacel). The column was packed by gravity at a flow rate of 120 ml/h. The 20 ml of DEAE was found to easily adsorb the 5 ml of sample introduced onto the column. The sample was eluted using gravity, also at a flow rate of 120 ml/h. A great deal of contaminating protein was removed by elution with 100 ml of Buffer A brought to 75 mM with KCl (pH 8.1). A gradient of 75 mM to 250 mM KCl in Buffer A was then used to elute the ATCase from the column. The eluent was collected in 5 ml fractions. Fractions 32 to 52 contained the peak ATCase activity. These fractions were pooled in three approximately equal volumes consisting of fractions 32-37, 38-44, and 45-52, which were subsequently analyzed using SDS PAGE. Before analysis, each fraction pool was dialyzed in Buffer A and then concentrated using

two consecutive pressure concentration steps with different size Amicon pressure cells. A 50 ml Amicon was used first to reduce the sample volume to about 10 ml, followed by a 10 ml Amicon, which reduced the volume further to approximately 2 ml. Both steps were performed using an Amicon PM-10 filter with 30 psi of nitrogen. Following dialysis in Buffer A to remove KCl, purity was evaluated by SDS PAGE.

V. Materials

Reagents used in all procedures were reagent grade and obtained from Sigma Chemical Company unless otherwise specified.

RESULTS

I. Growth of *E. coli*

After attempting growths of one, two, and three liters of media, it was determined that smaller volume growths yielded the most ATCase activity. All growths were conducted in 6 liter flasks. The one, two, and three liter growths yielded specific activities of 35, 18 and 6 μmol carbamylaspartate per min per mg of protein respectively. A fluted flask was used in one 2 L growth to test whether higher activity levels resulted from greater aeration. On the contrary, the activity was actually much lower (about 3 μmol/min/mg. An experiment was also conducted on 100 ml and 250 ml growths to test sensitivity to volume of lower quantities. These tests were done in 500 ml flasks. At volumes of 250 ml or less, there was little variance in activity resulting from different volume growths. To maximize quantity of cells and enzyme activity, one liter growths were chosen for future work (see Fig. 1).

The TB2/pLS2000 strain was grown in M9 minimal and LB rich liquid medium to determine which gave the greatest number of cells and the most ATCase activity. Not surprisingly, the rich medium produced more cells, but by far the most activity was produced by cells grown in M9 (see Fig. 2). *E. coli* grown in M9 had nearly ten times the ATCase activity of that found in LB-grown cells. Based on

FIGURE 1. Comparison of E.coli TB2/pLS2000 growth in both fluted and unfluted flasks at various volumes. ATCase activity levels in fluted flasks are denoted with triangles while those in the unfluted flasks are marked with squares.

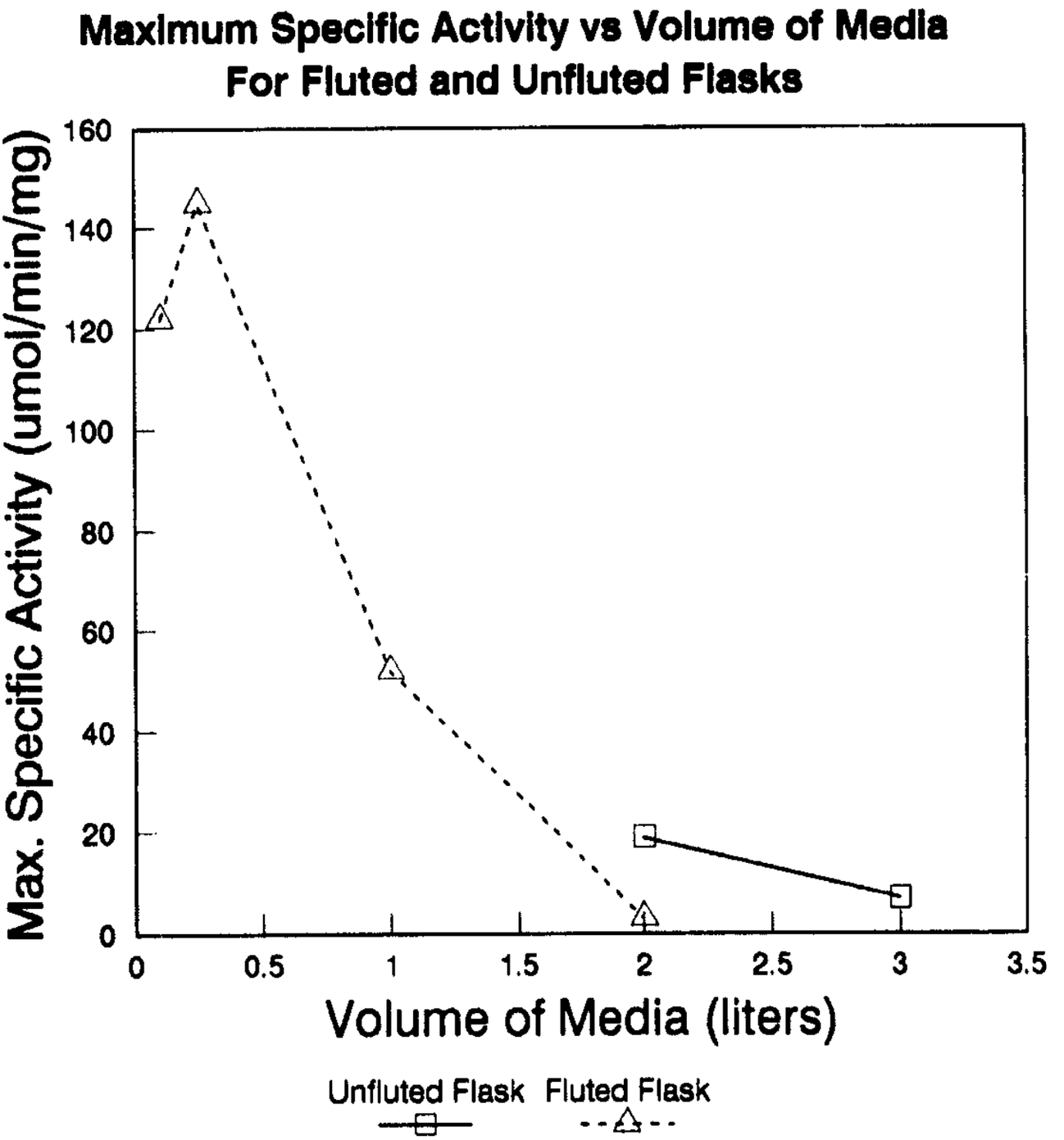
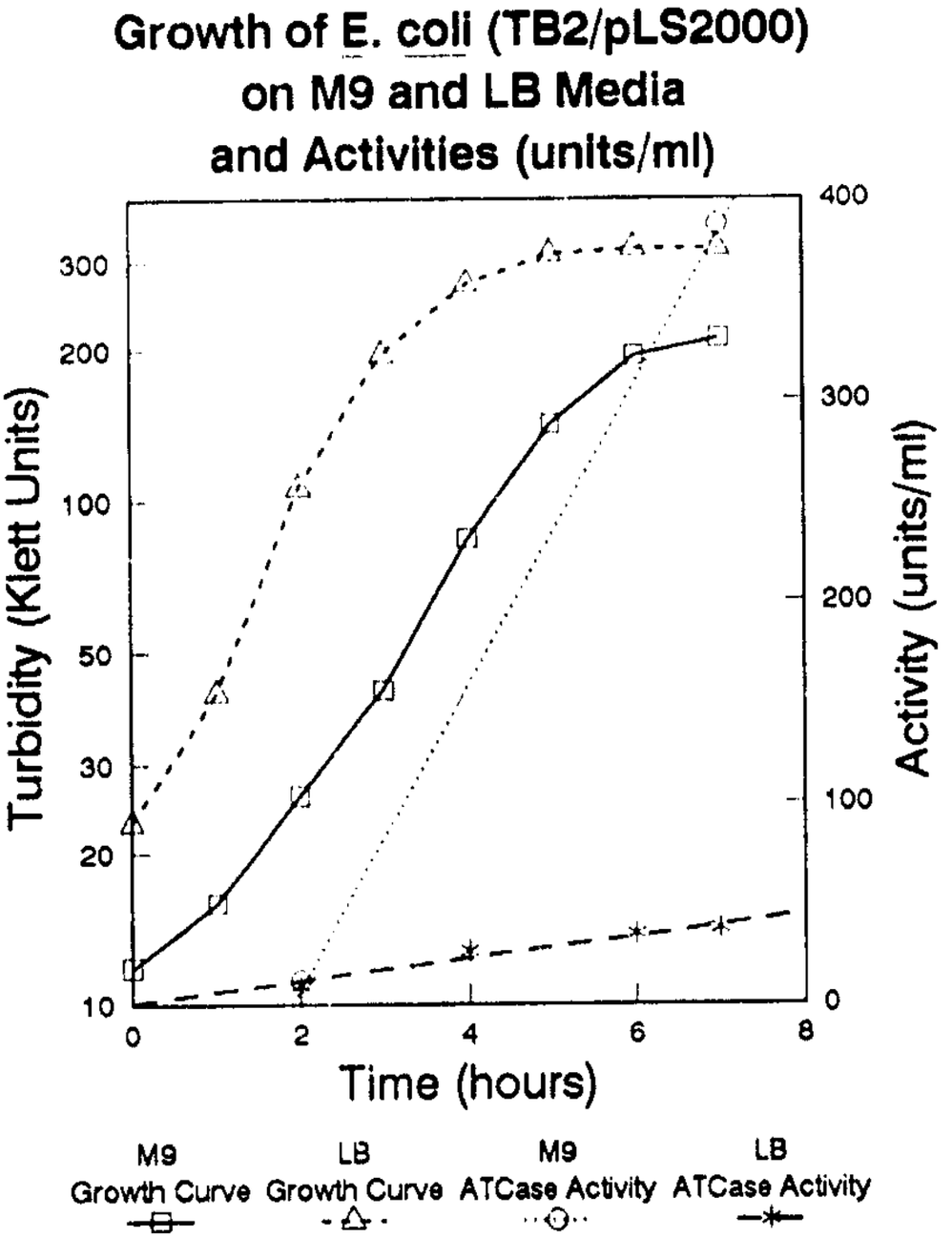

Maximum Specific Activity vs Volume of Media
For Fluted and Unfluted Flasks
Max. Specific Activity (umol/min/mg)
160
140
120
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
3
3.5
Volume of Media (liters)
Unfluted Flask
Fluted Flask

FIGURE 2. Comparison of E. coli TB2/pLS2000 growth on M9 minimal and LB rich media. Curves are indicated for cell density and ATCasc activities. Changes in turbidity of M9 medium are illustrated with squares and LB turbidity is plotted using triangles. M9 ATCase activity and LB ATCase activity are marked by circles and asterisks, respectively.


Growth of E. coli (TB2/pLS2000)
on M9 and LB Media
and Activities (units/ml)
Turbidity (Klett Units)
Activity (units/ml)
Time (hours)
10
20
30
50
100
200
300
0
100
200
300
400
0
2
4
6
8
M9
Growth Curve
LB
Growth Curve
M9
ATCase Activity
LB
ATCase Activity

the somewhat surprising result that more cells did not mean more enzyme activity, all cells from that point on were grown on the M9 minimal medium.

II. Protamine Sulfate Precipitation

The first purification step initially taken was precipitation using protamine sulfate to remove nucleic acids. The procedure attempted was essentially the same as described by Brabson et al. (1985). The effect on the E. coli extracts was not the same as on B. subtilis extracts. Unlike B. subtilis, the E. coli supernatant had no activity, indicating that all the ATCase precipitated with the nucleic acids. This step was accordingly omitted, and the first step of purification became heat treatment. Because purification was adequate after the above procedure had been employed, no other steps from the B. subtilis purification were necessary.

III. Heat Treatment

The heat treatment, which proved to be the most effective in the E. coli TB2/pLS2000 extract, was nearly identical to that used for B. subtilis. The only necessary modification in the procedure was in the incubation temperature, which was lowered to 60°C. When the hot bath was used at a setting of 65°C as in the B. subtilis purification, total enzyme activity actually dropped by 65%,

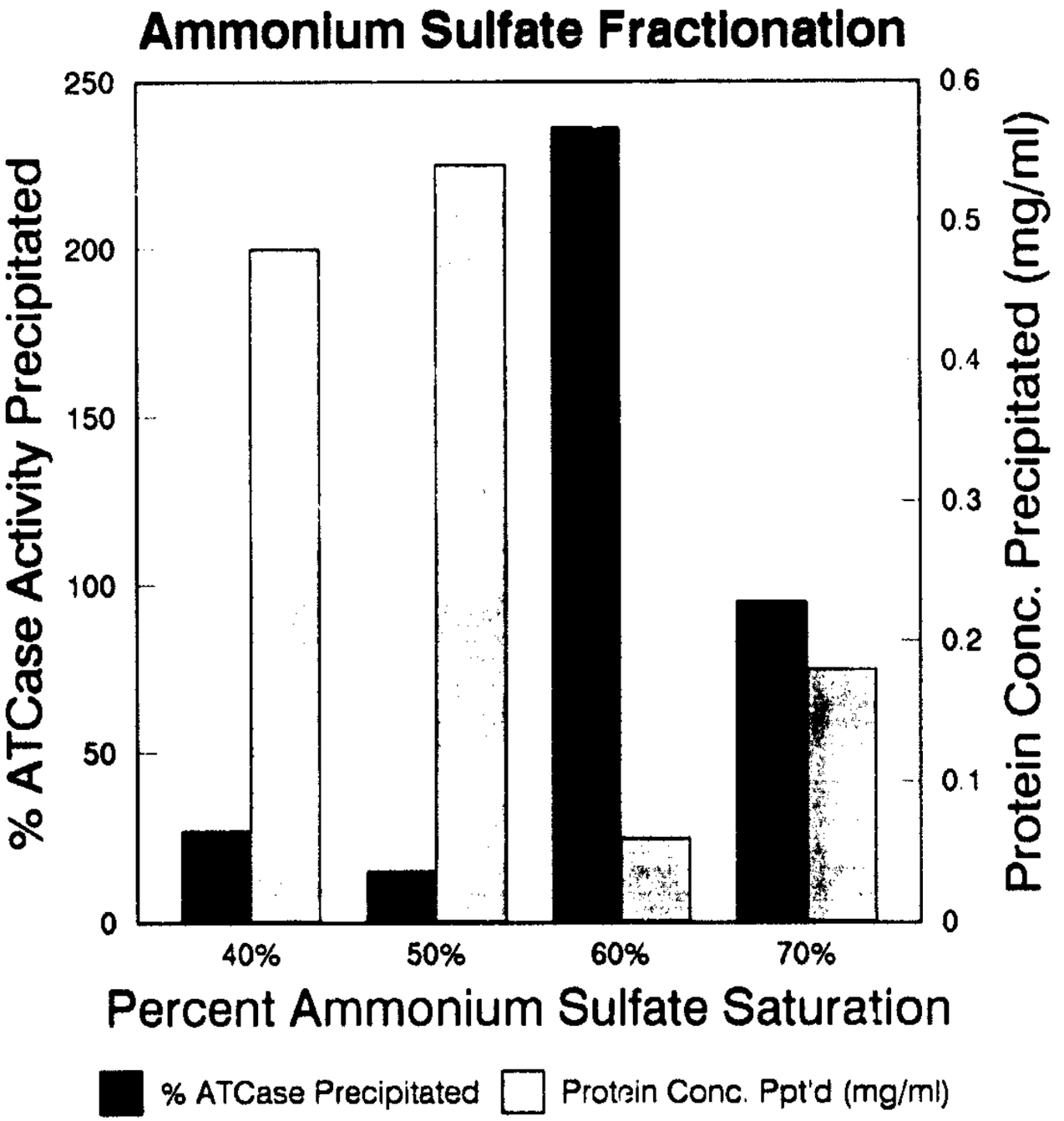
presumably due to heat denaturation of the ATCase. Lowering the hot bath temperature to 60°C yielded an equivalent removal of contaminants without the resultant loss of activity. Heat treatment was a highly effective purification step, as can be seen from increases in specific activity and polyacrylamide gel analysis.

IV. Ammonium Sulfate Fractionation

Pilot scale ammonium sulfate fractionations showed that 50% saturation with $(NH_4)_2SO_4$ precipitated a maximum amount of protein and a minimum amount of ATCase activity. Raising the saturation to a final concentration of 70% precipitated nearly all of the ATCase present in the sample (see Fig. 3).

Although precipitation of contaminants using ammonium sulfate was attempted at various points following the heat treatment, the procedure precipitated significant amounts of contaminating proteins only when used on the post heat supernatant. Any endeavors to use ammonium sulfate precipitation at subsequent stages yielded no further purification. A large amount of purification was evident with little enzyme activity remaining in the supernatant of the 70% cut. It was necessary to check the pH of the saturated ammonium sulfate frequently, because it became quite acidic over short periods of time (one to two weeks). The ammonium sulfate was kept at a pH of 8.1 by addition of concentrated ammonium hydroxide when necessary.

FIGURE 3. The amount of ATCase activity and protein precipitated at varying percent saturation by ammonium sulfate is shown. Although the percent activity exceeds 200% in one instance, the graph is still useful, since it shows the amount of activity in each sample relative to the other saturation levels. Possible explanations for this dicrepancy are elaborated in the "Dicussion" section.


Ammonium Sulfate Fractionation
% ATCase Activity Precipitated
Protein Conc. Precipitated (mg/ml)
250
200
150
100
50
0
0.6
0.5
0.4
0.3
0.2
0.1
0
40%
50%
60%
70%
Percent Ammonium Sulfate Saturation
% ATCase Precipitated
Protein Conc. Ppt'd (mg/ml)

V. Second Ammonium Sulfate Fractionation

A second pilot scale fractionation was carried out to determine what concentration of saturated ammonium sulfate precipitated the ATCase in the cell extract immediately following the first fractionation. The enzyme once again precipitated in the 50%-70% cut. After performing the second full-scale cut, however, little additional purification was apparent either from an increase in specific activity or from SDS-PAGE analysis. Thus, this step was eliminated.

VI. DEAE-Sephacel Chromatography

Initial tests of protein elution carried out using miniature columns showed no ATCase activity was found in the wash collected by elution with pure Buffer A at pH 8.1. Indeed, virtually no protein at all was eluted with Buffer A at a 0 M KCl concentration. It was noted that the majority of ATCase activity were not eluted until a KCl concentration exceeding 100 mM was attained. To be sure no activity was lost in the low salt rinse of the column, a 75mM solution was used, followed by a gradient of 75 mM to 250 mM. The eluted protein contained a high concentration of ATCase with few contaminating proteins remaining. The fraction containing the greatest enzyme activity and fewest contaminants was the fraction made by pooling fractions 32-52. The ATCase activity values obtained for this particular

column purification were too high to get accurate results using the quantity of extract present in the assay mixture. For this reason the elution profile for another purification is shown in Figure 4. This graph illustrates the removal of contaminants by the 75 mM KCl wash and the close agreement between the peak of ATCase activity and protein elution. It is clear that nearly all of the protein eluted by the gradient was ATCase.

VII. Third Ammonium Sulfate Fractionation

An ammonium sulfate precipitation was attempted immediately following the DEAE-Sephacel column purification, but contaminating proteins present in the column fractions precipitated at the same concentration of ammonium sulfate as ATCase. This is obvious from studying the precipitation profile (see Fig. 5). Since no clear cut could be made in the sample, this step was omitted.

Gel 1 shows the purification process at each step. Large amounts of ATCase are evident even in the crude extract. The band becomes more intense with each step as the number of contaminants diminishes. The DEAE-Sephacel fraction is the cleanest sample at a purity of around 95%.

Gel 1. This gel illustrates the purity of the ATCase at each step in the purification. B. subtilis ATCase is located at a MW of 33,500 ± 1000 Da. Lane 1 contains the MW standards, Lane 2 is the crude extract, Lane 3 is the ATCase after heat treatment, Lane 4 is the ATCase after ammonium sulfate treatment, and Lane 5 is the ATCase after being passed over the DEAE-Sephacel column.

Gel 1

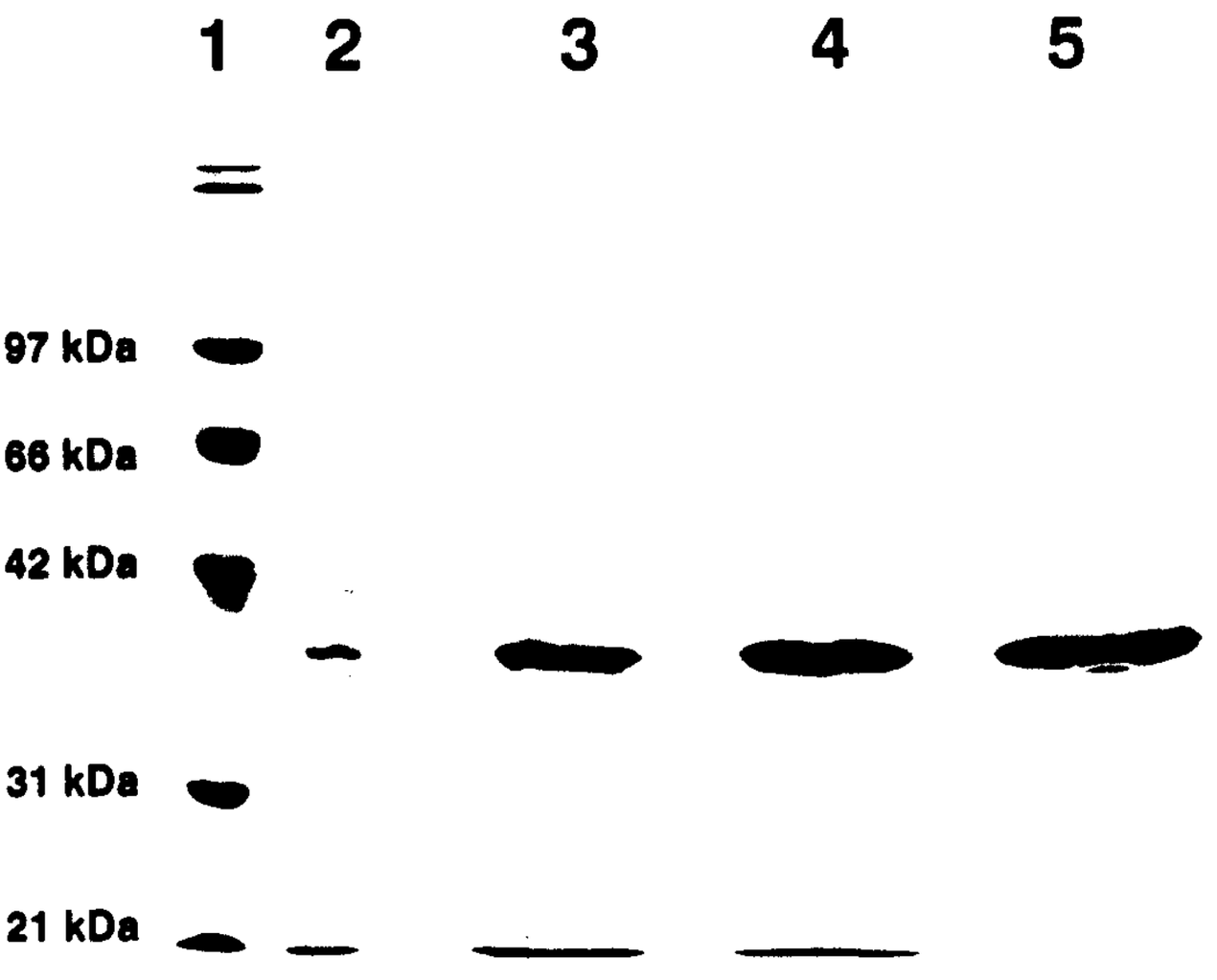


FIGURE 4. The elution curve for the full-scale DEAE-Sephacel column. The protein concentrations in the fractions are depicted using triangles, and the ATCase activities are shown with squares.

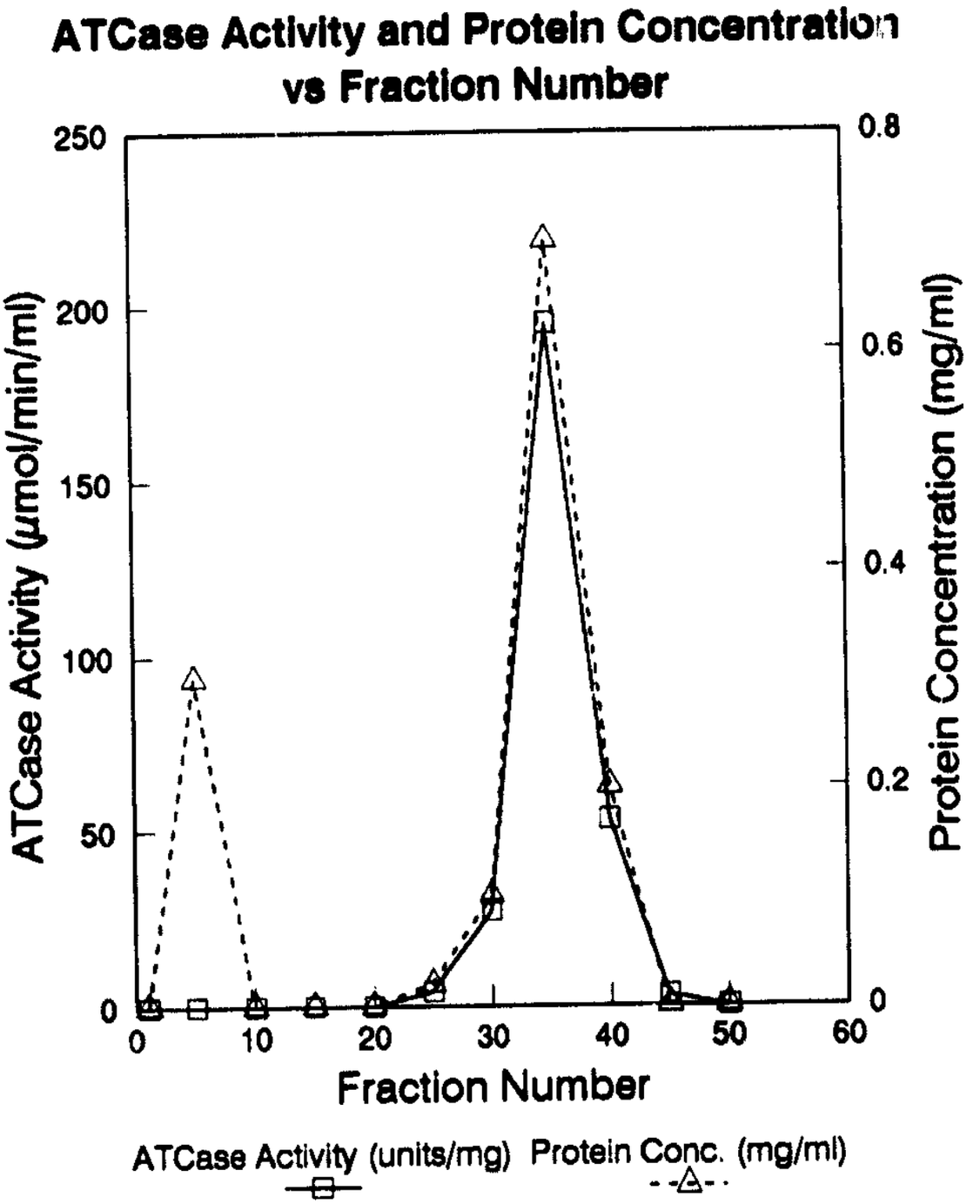
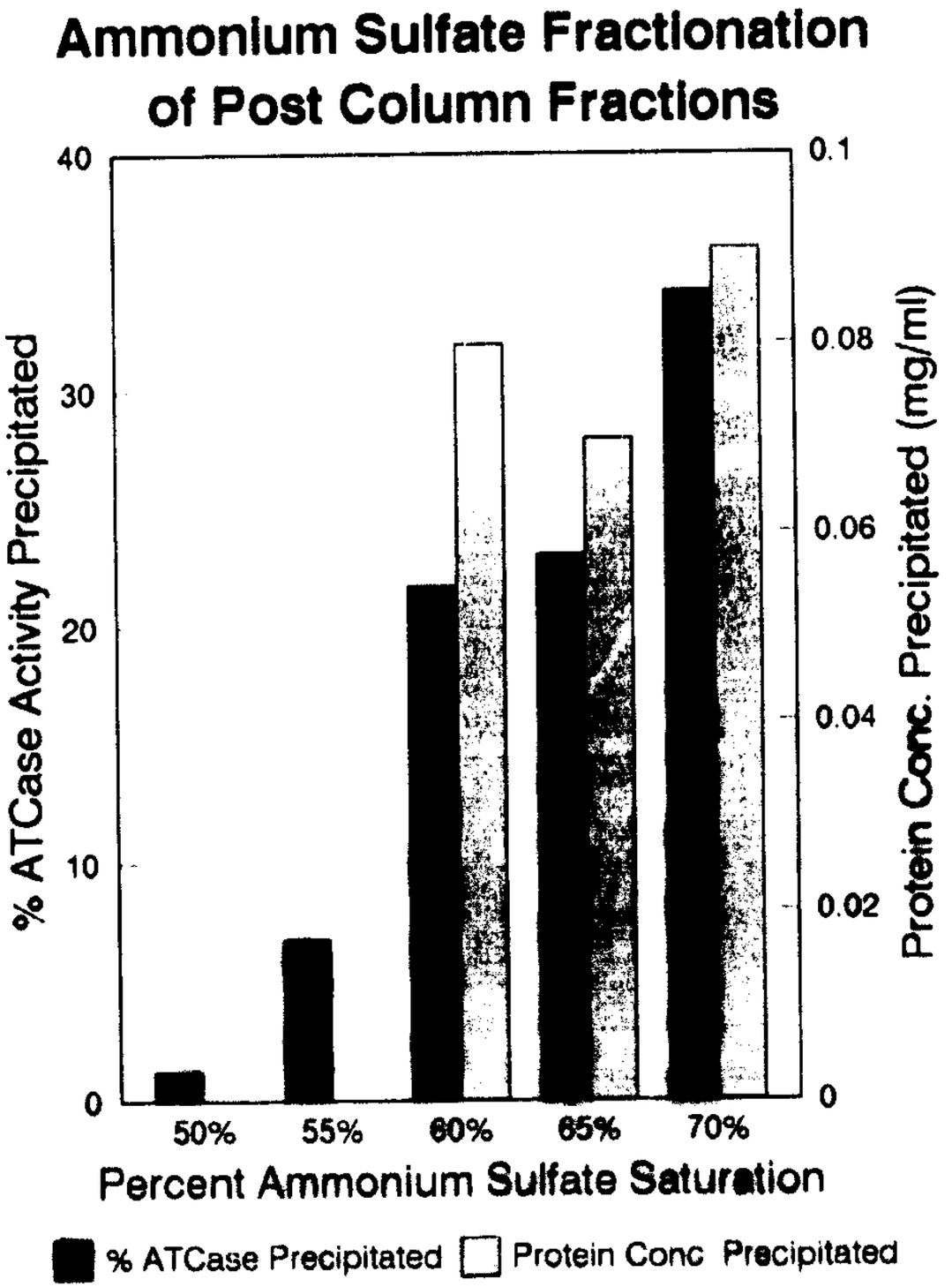

ATCase Activity and Protein Concentration vs Fraction Number
ATCase Activity (µmol/min/ml)
Protein Concentration (mg/ml)
Fraction Number
250
200
150
100
50
0
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
ATCase Activity (units/mg)
Protein Conc. (mg/ml)

FIGURE 5. The ammonium sulfate fractionation after DEAE-Sephacel purification. Nearly all of the protein present fractionates in the same way as ATCase.


Ammonium Sulfate Fractionation
of Post Column Fractions
40
30
20
10
0
0.1
0.08
0.06
0.04
0.02
0
% ATCase Activity Precipitated
Protein Conc. Precipitated (mg/ml)
50%
55%
60%
65%
70%
Percent Ammonium Sulfate Saturation
% ATCase Precipitated
Protein Conc Precipitated

DISCUSSION

I. Growth of *E. coli*

Lower activities at higher levels of aeration as were found in the 2 L fluted flask experiment are not unheard of. Dave Post and Dr. Lee Bussey of our laboratory have shown similar results with several other cloned enzymes in *E. coli* hosts. It has been hypothesized that the increased aeration causes aggregation of the enzyme. The aggregates may be insoluble and precipitate during centrifugation. Thus substantial loss of enzyme could result. Also these aggregated forms may not be active.

II. Purification of ATCase

The purification procedure employed was modified from that of Brabson, Maurizi, and Switzer (1985) for purification of ATCase from *B. subtilis*. A comparison of the steps involved in each of the procedures demonstrates the efficiency of the procedure outlined above as made possible by TB2/pLS2000 (see Table 1). To insure that the final calculation of protein concentration was accurate, it was checked with Beer's Law as well as Lowry determination. *B. subtilis* ATCase at a concentration of 1.0 mg/ml has an absorbance of 0.460 at 280 nm. The value obtained using Beer's Law was 0.6 mg/ml rather than 0.7 mg/ml. The

TABLE 1. Comparison between purification of B. subtilis ATCase from B. subtilis and from E. coli TB2/pLS2000. Note the number of steps required to reach the given specific activity in each case.

	Purification of B. subtilis ATCase	Specific Activity (μmol/min/mg)	Purification of B. subtilis ATCase Expressed in E. coli	Specific Activity (μmol/min/mg)
1.	Crude Extract	1.1	Crude Extract	37
2.	Protamine Sulfate	1.4	Heat Treatment	77
3.	Heat Treatment	3.4	Amm. Sulfate Fract.	185
4.	Amm. Sulfate Fract.	8.5	DEAE-Sephacel	279
5.	Sephadex G-150	50.0		
6.	DEAE-Sephadex	80.0		
7.	2nd Heat Treatment	165.0		
8.	Sephadex G-150	310.0		
9.	DEAE-Cellulose	560.0		

specific activity calculated for the final purified enzyme changes accordingly to 325 μmol/min/mg. This small change in protein concentration thus results in a large change in specific activity. At a value of 325 μmol/min/mg, however, the specific activity is still much lower than 560 μmol/min/mg as reported by Brabson et al. (1985). This is surprising since the purity of both preps appear approximately equal on an SDS-PAGE gel. The discrepancy is most likely due to error in the ATCase assay since the accuracy of the assay is greatly affected after dilution by the DEAE-Sephacel column.

In Figure 3 it is noted that the percent activity exceeds 200% in one instance. This could be accounted for in a few different ways. First, the ammonium sulfate purification may have freed the extract from contaminating inhibitors such as metal ions and proteases. Once these are removed, the ATCase activity increases greatly. Another possible explanation is that the ATCase activity increased due to concentration of the enzyme during the ammonium sulfate step. Aggregation of an enzyme has been observed to enhance its activity in a number of instances. The final explanation is experimental error in the ATCase assay which gave incorrect results. The latter is most probable since yields of ATCase activity were not consistently exceeding 100% after $(NH_4)_2SO_4$ fractionation. The other two explanations suggest that this would have been the case.

CONCLUSIONS

When the steps required to purify ATCase were carried out from start to finish, the entire process was completed in two days. Without any interruptions, the procedure could be completed in a 24 hour period, making it an extremely practical method for purifying B. subtilis ATCase from E. coli TB2/pLS2000. In the past purification of B. subtilis ATCase has required several weeks. Such long purifications allow the native structure of the enzyme to be modified and inactivated by both contaminating proteases and oxidation. Table 2 shows the specific activity of the extract following each step in a typical purification using the new method, as well as percent recovery. After only three purification steps, the ATCase extract reached a specific activity of 279 μmol/min/mg with a percent recovery exceeding 90%.

Starting with approximately 0.5 g of E. coli cells I was able to obtain 4.2 mg of nearly pure ATCase. Because the procedure is so quick and easy, it should prove quite useful. Dr. Lee Bussey of our lab hopes to use the purified enzyme as an antigen for very specific polyclonal antibodies. Attempts at this in the past have proved less than satisfactory because of minor impurities in the ATCase purified from B. subtilis. The antibodies produced react not only with the ATCase, but also with a number of

TABLE 2. This table indicates the activity values found in each step during purification of B. subtilis ATCase from E. coli TB2/pLS2000. Percent recoveries are also indicated.

Purification Table

	Fraction	Volume (ml)	Protein (mg/ml)	Total Activity (μmol/min)	Specific Activity (μmol/min/mg)	Recovery (%)
	Crude Extract	9.5	3.4	1,187	37	100
	Heat Treatment	9.3	1.5	1,079	77	91
	Amm. Sulfate Fract.	8.5	0.6	943	185	79
	DEAE-Sephacel	6.0	0.7	1,170	279	99

contaminating B. subtilis proteins. This results in numerous bands on Western blots and difficult interpretation of colony blots. The use of pure B. subtilis ATCase produced in E. coli should alleviate this problem, especially since any trace contaminants will be E. coli proteins, not B. subtilis proteins.

Another possible use for the purified B. subtilis ATCase would be as a substrate for in vitro degradation. Without the residual protease activity found in previously employed "pure" ATCase samples, newly purified B. subtilis ATCase could be used as a substrate for specific proteolysis. This degradation assay could then be used to characterize specific proteases.

A third important use of this purification procedure lies in its ability to make large quantities of pure B. subtilis ATCase available for crystallization. These crystals could be used to determine the three dimensional structure of the B. subtilis ATCase, which would provide insight into the location and shape of the active site of the enzyme. This is of particular interest, since B. subtilis ATCase is a trimer of catalytic subunits, whereas the E coli ATCase is a more complex structure containing six catalytic and six regulatory subunits.

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