

PURIFICATION AND CHARACTERISATION OF ALKALINE PHOSPHOTASE ENZYME FROM THE PERIPLASMIC SPACE OF *Escherichia coli* C90 USING DIFFERENT METHODS

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ABSTRACT

Alkaline phosphatase enzyme was purified from bacteria *Escherichia coli* C90 grown in phosphate-poor medium as stationary phase; using an ion exchange column packed with DEAE-cellulose as matrix and size exclusion chromatography using Sepharacryl S-300HR, equilibrated with Buffer A. The enzyme was extracted from the periplasmic space, external to the cell membrane. Previous studies with *E. coli* have shown increase in alkaline phosphatase activity upon phosphate deprivation with much of the enzyme released into the medium during osmotic shock, indicating that the enzyme is located either in the periplasmic space or is loosely bound to the cell wall. Initially, a DEAE column was used leading to 28% yield and 77 times fold purification, followed by Sepharacryl gel filtration column giving 25% yield and 72 times fold purification; indicating loss of enzyme in the subsequent purification step. SDS PAGE analysis was carried out as a control and to compare the results with the spectroscopy. There was no clear decrease in the yield seen in the bands and the loss of enzyme was not observed with the gel analysis. It may, therefore, be assumed that the decrease in yield was due to some pipetting errors in the spectroscopy measurements. The native gel results show clear distinct bands for the 3 alkaline phosphatase isoenzymes, confirming that, the purification procedure for the enzyme was a success.

Key Words: DEAE cellulose, SDS PAGE analysis, Sepharacryl S-300HR

RÉSUMÉ

L'enzyme phosphatase alcaline était purifié de la bactérie *Escherichia coli* C90 cultivé dans un médium pauvre en phosphate comme phase stationnaire utilisant une colonne d'échange d'ion enveloppée avec une cellulose DEAE comme matrice et exclusion de taille chromatographique utilisant le Sepharacryl S-300HR équilibré avec le tampon A. L'enzyme était extrait de l'espace périplasmique externe à la membrane cellulaire. Des études antérieures avec *E. coli* ont montré une augmentation en activité de la phosphatase alcaline sur la déficience en phosphate avec plus d'enzymes éjectés dans le media Durant le choc osmotique, montrant que l'enzyme est situé dans l'espace périplasmique ou simplement lié à la membrane cellulaire. Initialement, une colonne de DEAE était utilisée avec pour rendement 28% et purifiée 77 fois, suivie d'une colonne de gel de filtration Sepharacryl produisant 25% et 72 fois de purification; indiquant une perte d'enzyme au cours de l'étape suivante de purification. L'analyse SDS PAGE était conduite comme control et pour comparer les résultats avec la spectroscopie. Il n'y avait pas une diminution claire du rendement dans les bandes et la perte d'enzyme n'était pas observée par l'analyse du gel. Il pourrait être présumé que la diminution du rendement était due aux erreurs de pipettage lors des mesures spectroscopiques. Les résultats du gel natif montrent clairement des bandes distinctes pour 3 isoenzymes alcalines phosphatases confirmant que, la procédure de purification pour l'enzyme était un succès.

Mots Clés: cellulose DEAE, analyse SDS PAGE, Sepharacryl S-300HR

INTRODUCTION

Alkaline phosphatase (ALP) (EC 3.1.3.1) is an enzyme which removes phosphate groups from nucleotides and proteins, in the process known as dephosphorylation (http://en.wikipedia.org/wiki/Alkaline_phosphatase –). It works best in an alkaline pH. In bacteria, alkaline phosphatase is located in the periplasmic space, external to the cell membrane. Studies in both *Escherichia coli* and cyanobacterium, *Anacystis nidulans* R2, have shown increase in alkaline phosphatase activity upon phosphate deprivation. Much of the activity is released into the medium during osmotic shock, indicating that the enzyme is located either in the periplasmic space or is loosely bound to the cell wall (Golden and Sherman, 1984).

The amino acid sequence of the *E. coli* alkaline phosphatase subunit [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1, isozyme 3] has been determined. The first Isoenzyme has additional arginine residue at the NH₂ terminus that has resulted from variability precursor molecules processing. The enzymatically active alkaline phosphatase is composed of two identical polypeptides, coded by a single structural gene (Bradshaw *et al.*, 1981). Several distinct isoenzyme bands of equal spacing are observed when a purified enzyme sample is analysed by starch or polyacrylamide gel electrophoresis. The isoenzyme showing the slowest mobility on electrophoresis (the least negative charge) of the three isoenzymes is referred to as isoenzyme 1 and the faster and fastest referred to as 2 and 3, respectively.

The first step in purifying intracellular proteins is preparation of a crude extract. The extract contains a complex mixture of all the proteins from the cell cytoplasm, and some additional macromolecules, cofactors and nutrients. Crude protein extracts are prepared by removal of cellular debris generated by cell lysis, using chemicals and enzymes, sonication or a French Press. However, sonication can not be used when the protein required is expressed on the periplasm. The debris is removed by centrifugation and the supernatant is recovered.

Ion exchange chromatography (IEC) is one of the techniques used in the separation of

proteins and other organic compounds based on charge (Hodson and Latner, 1961). Change in pH values from low to high shifts the surface charge of proteins from positive to a negative charge. The higher the net charge of the protein, the higher the ionic strength needed to bring about desorption (Greene *et al.*, 2005). Elution can generally be achieved by increasing the ionic strength (i.e. conductivity) of the buffer to compete with the solute for the charged sites of the ion exchange matrix; or changing the pH and thereby altering the charge of the solute.

Using gel filtration in size exclusion chromatography (SEC), protein is purified on the basis of their molecular size. Separation is achieved using a porous matrix, e.g. Sepharacryl S-300HR; to which the molecules, have different degrees of access. Smaller molecules have greater access and larger molecules are excluded from the matrix. Hence, proteins are eluted from the gel filtration column in decreasing order of size (Shibahara *et al.*, 1985). Both molecular weight and three dimensional shape contribute to the degree of retention time inside the column.

Bradford protein assay involves binding of the dye Coomassie Brilliant Blue to protein in an acidic solution and once it is bounded then there is a simultaneous absorbance shift from 465 nm to 595 nm. However, its bound condition is best detected at the maximal absorbance spectrum of 595 nm. The increase in absorbance at this wavelength shows an increase in binding of protein-coomassie and that relationship is proportional i.e. the higher the amino acid (or protein) content in a sample, the higher the value of the optimal wavelength.

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) is useful for molecular weight analysis of proteins. The SDS detergent binds to the polypeptides to form complexes with fairly constant charge to mass ratios. The electrophoretic migration rate through the gel is therefore determined only by the size of the complexes. Molecular weights are determined by simultaneously running marker proteins of known molecular weight. Native gel is used to check the purity. Proteins are separated based on charge density while maintaining biological activity i.e. enzyme activity. An appropriate activity stain can therefore be used to detect the enzyme activity

and reveal specific enzyme. This can be done by comparing its position on the gel to all the proteins revealed by the total protein stain. The objectives of this study were to (i) purify and characterise alkaline phosphatase enzyme from *E. coli* C90; (ii) evaluate the efficiency of the method used in the purification procedure; and (iii) determine the enzyme activity of the purified alkaline phosphate.

MATERIALS AND METHODS

Preparation of buffers. Buffers A, B and C used in the experiment were prepared as indicated in Table 1. The volume was calculated from the equation: $C_1V_1 = C_2V_2$, where C_1 and V_1 are the concentration and volume of the stock solution, respectively, C_2 and V_2 are the concentration and volume of the diluted sample. The values for either concentration or volume are substituted and calculated for unknown. Buffer A was degassed after preparation. All the buffers prepared, were kept in bottles at room temperature until use.

Inoculation of culture media. Bacteria *E. coli* C90 were inoculated in a 400 ml culture media and was incubated over night at 37 °C with gentle shaking.

Protein purification. Three millilitres of overnight culture (400 ml) was harvested by centrifugation

at 6000x g for 1 min. The supernatant was decanted and pellet washed twice in cold, protease inhibitor-containing 0.01M Tris-HCl (pH 7.5) at 6000x g for 1 min. A total of 0.6 ml cold protease inhibitor-containing 0.01M Tris-HCl (pH 7.5) was then added and the cells were sonicated twice for 10 sec using a 2 mm needle and centrifuged at 13000 x g for 10 min at 4 °C. The supernatant was collected and kept at 4 °C.

The remaining culture, approximately 397 ml, was divided equally in two separate bottles each 198.5 ml and harvested by centrifugation at 5000 x g for 15 minutes. The pellets were re-suspended and washed three times with Buffer A. The pellet was completely suspended in 20 ml of Buffer B after washing, and stirred for 10 mins at room temperature. It was then centrifuged again at 7500 x g for 10 min. The supernatant was decanted, the cells resuspended in 20 ml cold distilled water and again stirred for 10 min. before being centrifuged at 13000x g for 10 min at 4 °C. A total of 0.2 ml of the supernatant was saved for later analysis. It was then loaded into a column packed with 3 ml of DEAE-cellulose equilibrated with Buffer A. The column was washed first, with 5 ml of Buffer A and then 10 ml of Buffer C. The enzyme was eluted with Buffer D. One millilitre fractions in total 10 ml were collected. The amount of protein in the fractions was determined as well as the enzyme activity. Those fractions with high activity were combined in a pool. One hundred microlitres was saved. The pool concentrates

TABLE 1. Buffers' composition and volume

Buffer	Ingredient	Volume (ml)	Total volume (ml)
A	0.01M Tris-HCl (pH 7.5)	50	500
	ddH ₂ O	450	
B	Provided by the biochemistry lab in Bergen		
C	0.01M Tris-HCl (pH 7.5)	10	100
	0.02M NaCl	2	
	ddH ₂ O	88	
D	0.01M Tris-HCl (pH 7.5)	10	100
	0.1 M NaCl	10	
	ddH ₂ O	80	

were later on resuspended on a 1.5 x 20 cm Sepharcryl S-300HR column equilibrated with Buffer A.

Blue Dextran was used to determine the void volume (V_0) of the 1.5 x 20 cm Sepharcryl S-300HR column, potassium ferricyanide ($K_3Fe(CN)_6$) was used for the final volume (V_i). Oval albumin was run as size marker. A volume of approximately 11 ml was taken before the Blue Dextran eluted and then samples were collected in fractions of 1 ml. Saved aliquots were marked and kept in a fridge. The amount of protein was determined by the Bradford Method; the enzyme activity in the fractions between (V_0) and (V_i) was also measured.

The pool of the highest activity from DEAE-cellulose column was then transferred into the Sepharcryl S-300HR column after eluting almost all the Buffer inside the column. The samples were collected in fractions of 1 ml, after 10 ml run through the column. Protein concentration in this sample was determined by direct spectrophotometer measurement at 280 nm, Alkaline phosphatase activity was measured at 400 nm using p-nitrophenylphosphate (sodium salt) in H_2O .

Standard enzyme measurement for Alkaline phosphatase. The enzyme activity was measured in a square plastic cuvette (1cm light-path) at 400 nm in a spectrophotometer. The chemicals used comprised of Solution 1 (0.2 M Tris-HCl, pH 8.0), and Solution 2 (20MM p- nitrophenylphosphate (sodium salt) in H_2O). The measurement was done by taking 0.5 ml of solution 1 and 0.1 ml of solution 2; and double distilled H_2O was added so as to make the final volume 1 ml according to the amount of enzyme, which varied from 10 to 20 μ l. The enzyme was added last. The amount of enzyme which gave an increase in absorbance of 0.1 -0.2 per min was determined. The absorbance was read in 60 sec intervals; the reaction started immediately following the addition of the enzyme.

SDS PAGE. SDS PAGE gel was prepared using 12% SDS running gel and 5% stacking gel. A 10 well-comb was used. The samples loaded were 10 and 5 μ l of each of the water extract, Cell homogenate, DEAE pool, Sepharcryl pool were all prepared in separate 1.5 ml Eppendorf tubes, 3.3 μ l of 5x SDS-PAGE sample buffer was added

in each tube. Ten and 5 μ l of the sample from each tube (12 and 7 μ l of the same sample) was loaded in separate wells on the gel. Five microlitres of diluted low Range Molecular weight Marker from BioRad was loaded in the flanking lanes as Marker. Gel was then run at 100 V for 15 mins and then increased to 200 V for 1 hr 30 minutes. The gel was then stained with Coomassie gel-staining solution for 3 hrs and de-stained overnight. This was followed by gel analysis.

Non-denaturing gel. Non-denaturing gel was prepared by mixing 6.47 ml DDH_2O , 2.7 ml Bis acrylamide, 1 ml 0.1 M Tris-HCl pH 6.8, 25 μ l APS, and 10 μ l TEMED in a 15 ml tube. The mixture was transferred immediately into 4 tubes as soon as TEMED was added, water was added on top of the tube to level the gel and it was allowed to polymerize. The samples to be loaded were prepared by adding, 30 μ l of the each of Water extract sample and sephacryl S-300HR pool in separate Appendorf's tubes and 30 μ l of sample loading buffer was added. The samples were loaded in two tubes, each containing 15 μ l. The gel was run constantly at 200 V, for approximately 2 hrs 15 min and stopped as soon as the bromophenol blue reached the edge of the gel. It was then removed and placed on separate wells for 60 seconds to stain; one in a commassie blue and the other for activity staining. The activity stain was prepared using a solution of 33 mM Tris HCl, pH 9.2, 2.5 mM naphthyl phosphate and 1 mg ml^{-1} Fast Red TR. The reaction was stopped as soon as red bands appeared corresponding the three alkaline phosphate isoenzymes, by rinsing with 7% acetic acid in a well. The gel was then removed and scanned. The commassie blue gel was stained with 0.1% Coomassie Blue in 40% ethanol, 10% acetic acid overnight and destained for 48 hours using 10% acidic acid and 10 % ethanol.

RESULTS

Purification using DEAE cellulose chromatography (Ion exchange). Both enzyme activity and protein content of the 10 ml fractions eluted using the DEAE cellulose chromatography column was measured and the values obtained

were used to plot the graph (Fig. 1). Samples 2 and 3 show highest enzyme activity and protein concentration. They were pooled for further purification using a Sephacryl S-300HR column.

Purification using Sephacryl S-300HR column (Gel filtration). Initially, the volume around the Sephacryl S-300HR column was determined using Blue Dextran and potassium ferricyanide, for void volume and initial volume, respectively. Ovalbumin is used as size marker. The elution profile was measured at 280 nm, shown in Figure 2, the most blue was eluted after 12 ml which means the void volume (the volume around the matrix) (V_0) was 13ml and potassium Ferricyanide (most yellowish) was eluted after 30; which means the (V_i) is 31 ml. Therefore, the volume inside the matrix was $31-12=19$ ml.

The peak of the graph shows elution of oval albumin (indicated by the black arrow). The fraction with the highest protein content was eluted between 11 and 12 ml, indicated as 1 and 2 ml, respectively in the graph. (This was actually eluted between 21 and 22 ml).

The ovalbumin was eluted between the Blue Dextran and potassium as shown in Figure 2 and absorbance was measured at 280 nm.

The DEAE pool obtained from fractions 11 and 12 were added to the 1.5 x 20 cm Sephacryl S-300HR column equilibrated with Buffer A.

Elution profile is shown in Figure 3 and both protein concentration at 280nm and Enzyme activity measured at 400 nm were studied. The enzyme rate measured at 400 nm (or alkaline phosphatase) with the highest values was taken in a pool. The fractions 14 and 15 were pooled (Sephacryl pool); with fraction 15 showing the highest enzyme rate. The highest protein content values were obtained in fractions 14 and 15 measured at 280 nm; fraction 14 shows the highest protein content.

Determination of protein concentration by Bradford assay. The absorbance of the samples: Cell homogenate, Water extract, DEAE pool and Sephacryl pool are all shown in Table 2. The concentrations of the samples were calculated from the absorbance measured at 595nm and deduced from the equation of the standard curve of BSA protein (Fig. 4). The concentrations of the samples were determined by substituting for the values of y in the linear regression equation worked out from the standard curve, with the values of the absorbance of the sample and

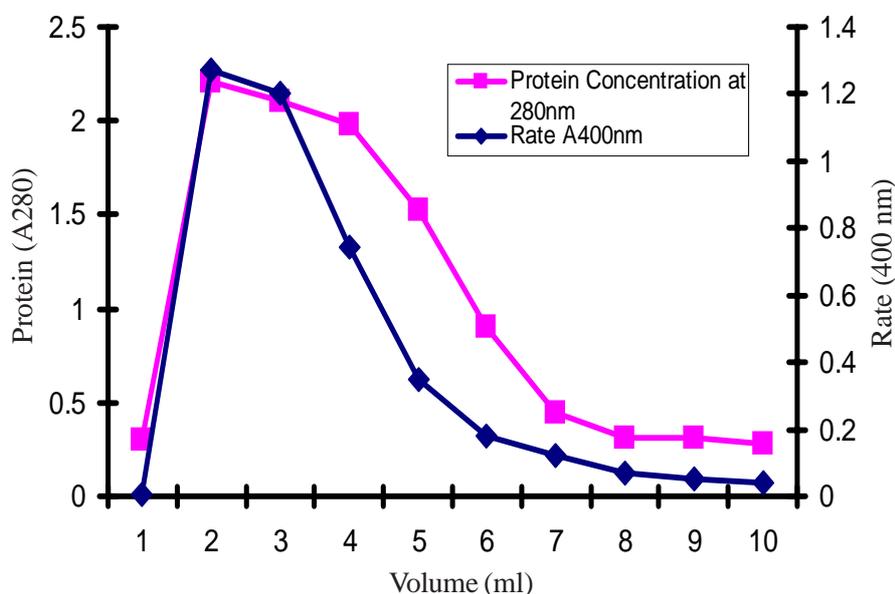


Figure 1. Elution profile of the DEAE fractions' plot of protein content values measured at absorbance of 280 nm and enzyme activity/rate at 400 nm.

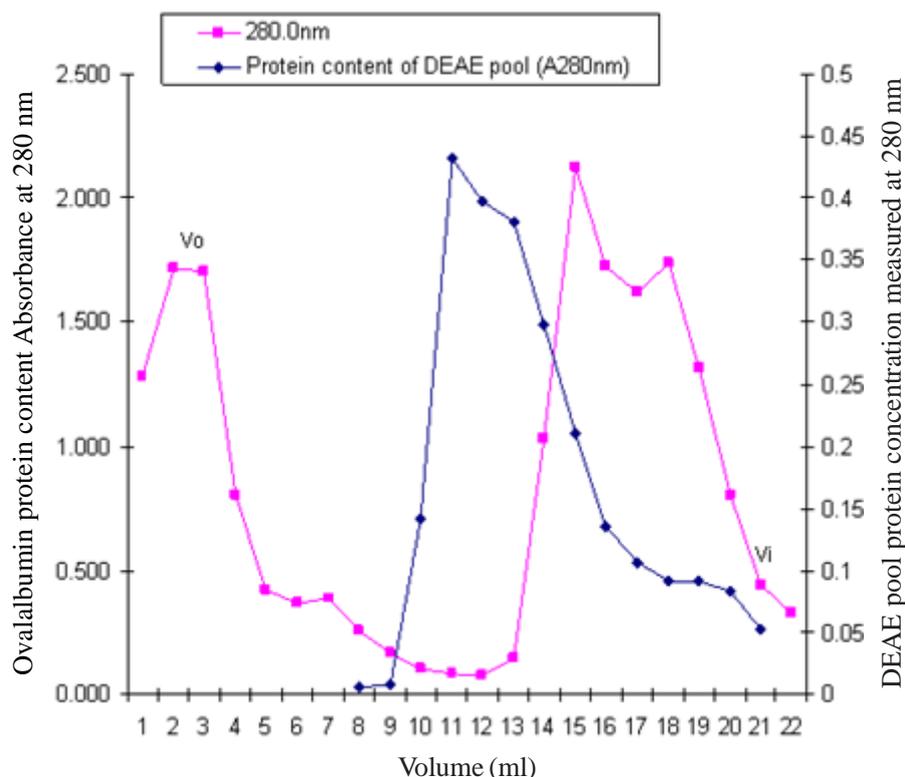


Figure 2. Gel filtration analysis of a mixture of 200 μ l each of Blue Dextran, Potassium Ferricyanide ($K_3Fe(CN)_6$) and ovalbumin run as size marker, Blue Dextran and Potassium Ferricyanide ($K_3Fe(CN)_6$) as V_o and V_i respectively; with DEAE pool all collected as fractions from 1.5 x 20 cm Sephacryl S-300HR column equilibrated with Buffer A and measured at the absorbance of 280 nm. Highest protein elution is between fraction 11 and 12 collected as Sephacryl pool.

calculating for the value of x , which represents the samples concentration.

The concentration of the cell homogenate was 0.82 $mg\ ml^{-1}$, Water extract: 0.557 $mg\ ml^{-1}$, DEAE pool: 0.599 $mg\ ml^{-1}$ and Sephacryl pool: 0.574 $mg\ ml^{-1}$.

The concentrations obtained from the Bradford assay were used to solve the protein purification scheme table. The amount of protein in mg was calculated from the concentration multiplied by the actual volume used. The volume for each of the samples used was indicated in the table. Total enzyme activity (in $\mu mol\ min^{-1}$) was calculated by multiplying the measured activity or rate, times the volume all divided by the extinction coefficient ($1.7 \times 10^4\ M^{-1}\ per\ cm = 0.017\ \mu M$) and the sample used in the measurement - {Total activity (in $\mu mol\ min^{-1}$) = (Enzyme activity*Volume)/Extinction coefficient*sample volume} Specific activity (in $\mu mol\ min^{-1}\ mg^{-1}$) is

calculated by dividing the value obtained above (Total activity) with the protein in mg (second column):

Specific activity (in $\mu mol\ min^{-1}\ mg^{-1}$) = Total activity/ Amount of protein.

To calculate the yield, the Cell homogenate was assumed to contain all the proteins (Enzyme) in the sample; therefore the total activity it showed was considered of 100% yield. The yield of all the other samples was calculated from the percentage yield of the Cell homogenate total activity. Purification fold was also calculated from the original sample which was the Cell homogenate specific activity value designated 1 fold; the rest of the samples were calculated as component of the cell homogenate specific activity.

The amount of protein in each sample, total activity, specific activity and yield are indicated

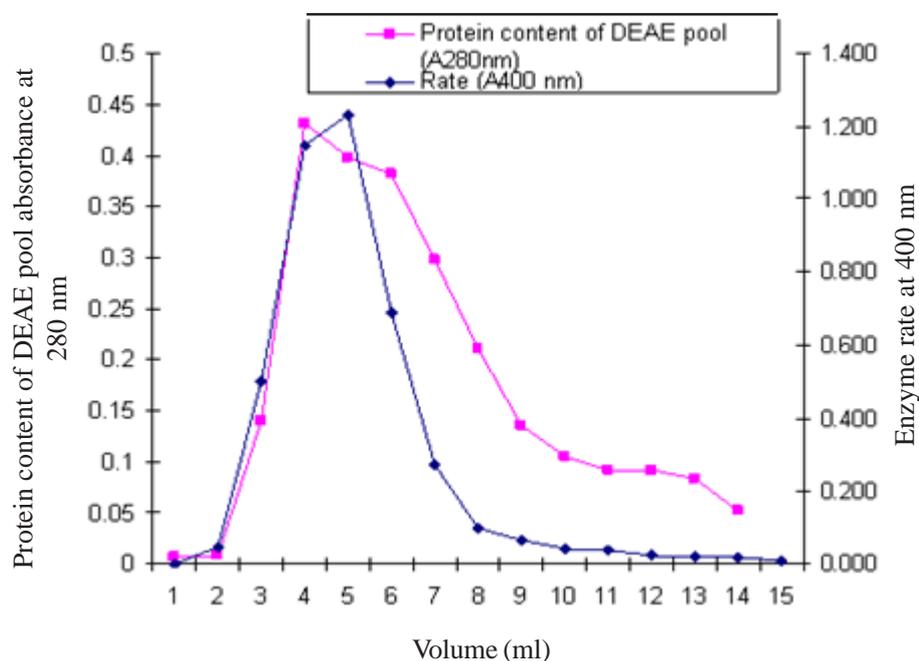


Figure 3. Protein content of DEAE pool measured at 280nm and enzyme rate measured at 400 nm, eluted from 1.5 x 20 cm Sephacryl S-300HR column; the absorbance is read in 60 sec intervals, the reaction started immediately following the addition of the enzyme. (Figure shows positive values only).

TABLE 2. BRADFORD assay results and sample concentration values determined from BSA standard curve *

Standards			Samples				
0.2 mg ml ⁻¹ BSA Sample [μg μl ⁻¹]	A _{595nm}	Sample	Probe	Dilution	A _{595nm}	Final Conc. [mg ml ⁻¹]	
S1	0.01	0.149	S5	Cell homogenate	1:10	0.827	0.82
S2	0.025	0.306	S6	Water extract	1:10	0.559	0.557
S3	0.05	0.495	S7	DEAE pool	1:10	0.601	0.599
S4	0.075	0.736	S8	Sephacryl pool	1:10	0.576	0.574

*The absorption and concentration values for both the standard and protein sample

in Table 3. The initial volume of water extract sample used was 40 ml, and the cell homogenate was 400 ml; the total and specific activity of the purified enzyme were 15.89 μmol min⁻¹ and 13.81 μmol min⁻¹ mg⁻¹ respectively, and the yield was only 25%.

The samples were also analysed by SDS PAGE (Fig. 5). Protein Ladder was used as marker. The band numbered on the left is a picture indicating the different molecular weights for the purified protein used as standard. The band no.1

corresponds to the distinct band on the gel and it is approximately 181.8kDa. The arrows point to clear bands located at the same level on the gel for each of the samples used, indicating the position of the purified enzyme. The marker protein gives an idea about the molecular weight or size of the protein. The band corresponds to no.4 band on the Marker protein on the left and it is approximately 64.2 kDa.

This means that the molecular weight of Alkaline phosphatase is approximately 64.2 kDa.

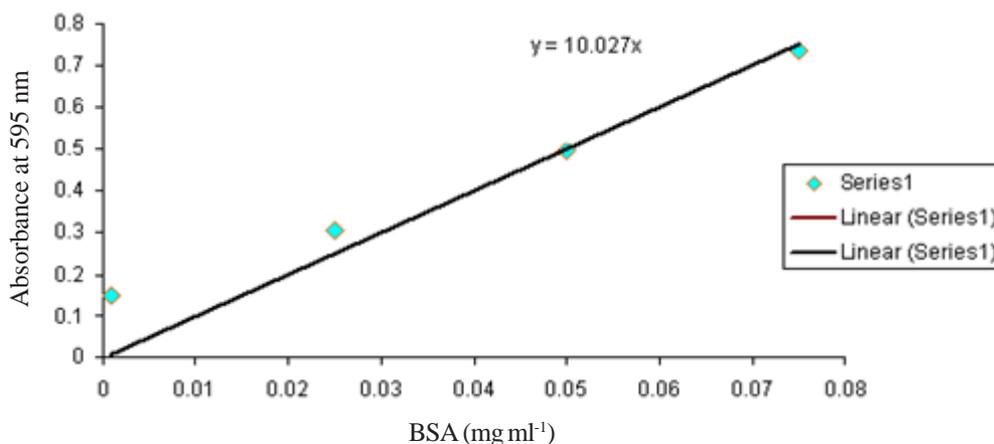


Figure 4. BSA Standard Curve – a plot of absorbance measured at 595 nm vs BSA concentration, a linear regression is deduced with equation of $y=10.027x$.

TABLE 3. Protein purification scheme table

	Volume (ml)	Protein (mg)	Total activity ($\mu\text{mol min}^{-1}$)	Specific activity ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	Yield (%)	Fold purification
Cell homogenate	400	328	62.59	0.19	100	1
Water extract	40	22.28	54.15	2.43	87	13
DEAE pool	2	1.20	17.64	14.7	28	77
Sephacryl pool	2	1.15	15.89	13.81*	25.39	72

The water extract labeled WE shows so many bands but the bands do not have the same intensity as that of cell homogenate. Most of the bands (WE) are not very strong except for the one for the enzyme. Cell homogenate has very strong band for enzyme phosphatase but also has so many other bands which differ in size and intensity. This shows that there were impurities which could be other proteins in the periplasmic space. The bands for the DEAE and SEPH pool are very strong and do not have so many other bands; this means that there was a lot of improvement in the purification process of the enzyme.

Non-denaturing tube gel. The native non denaturing gel for both samples: Water Extract and Sephacryl pool were stained one each with activity staining and coomassie blue (Fig. 6). Three bands were visualised pertaining to the three alkaline phosphatase isoenzymes.

The black arrow points to both Commassie and activity staining tube gels; it is the most negative. The others that follow are 2 and 3, respectively. The bands of the commassie staining tube gel for the water extract did not show good bands, the bands shown are very weak.

DISCUSSION

The results for the DEAE cellulose chromatography column (Ion exchange chromatography) and DEAE pool eluted from 1.5 x 20 cm Sephacryl S-300HR column, for both enzyme activity measured at 400 nm and protein content measured at 280 nm shows almost same results. The curve shown in Figures 1 and 2 appear similar and both have peak at fractions 2 and 3. The DEAE pool eluted from 1.5 x 20 cm Sephacryl S-300HR also have peak from the same fractions measured for both enzyme activity and protein

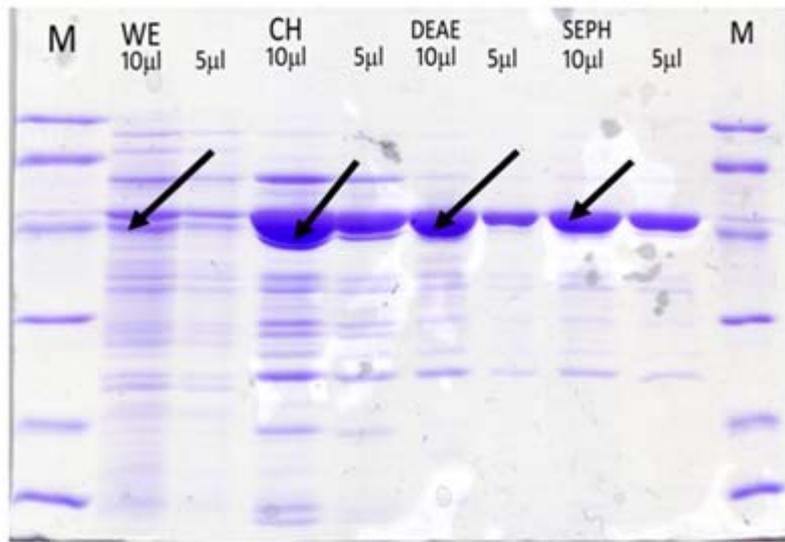


Figure 5. SDS PAGE analysis for the different purification steps of alkaline phosphatase enzyme extracted from *E.coli*C90 grown in phosphate-poor medium as stationary phase and visualized using Commassie gel stain. Marker (M) is the Protein Ladder (250ng), Lane marked WE = Water extract, Lane marked CH=Cell homogenate, the other two Lanes are DEAE pool (DEAE) & Sephacryl pool (SEPH).

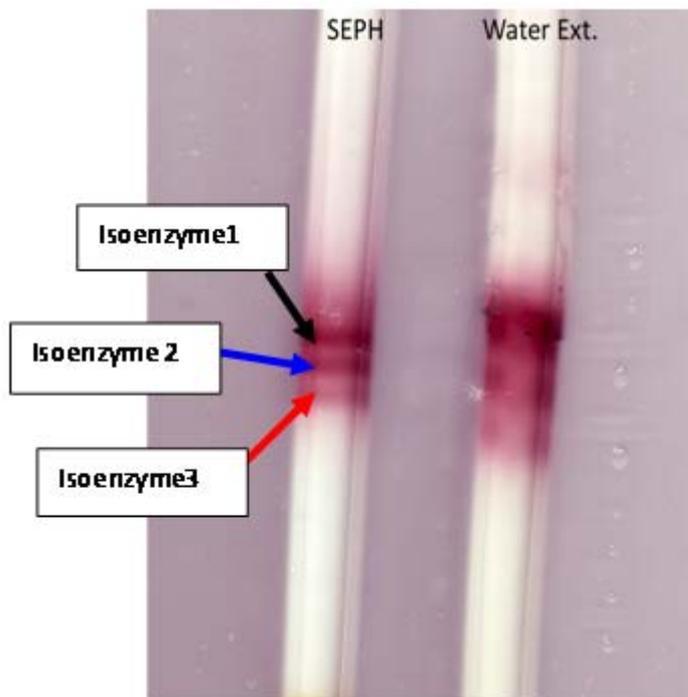


Figure 6. Non-denaturing tube gel electrophoresis activity staining of Water extract and sephacryl pool samples obtained from characterisation and purification of alkaline phosphatase enzyme from *E. coli*C90 grown in phosphate-poor medium as stationary phase and visualized using solution of 33 mM Tris HCl (pH 9.2), 2.5 mM nappthyposphate and 1mg ml⁻¹ Fast red TR. Arrows shows position of the 3 bands.

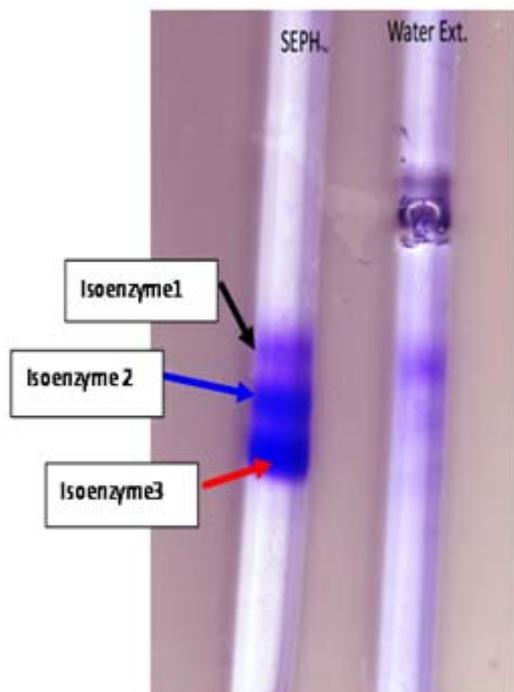


Figure 7. Non-denaturing tube gel electrophoresis of water extract and sephacryl pool samples obtained from characterisation and purification of alkaline phosphatase enzyme from *E. coli* C90 grown in phosphate-poor medium as stationary phase and Coomassie staining done with 0.1% Coomassie Blue in 40% ethanol, 10% acetic acid. Arrows shows position of the 3 bands.

content. The fraction with highest protein content is eluted between 21 and 22 ml. It can, therefore, be concluded that since both protein amount and enzyme activity are highest in the same fractions, then perhaps most of the protein measured in the sample is the enzyme itself, that is why it gives highest absorbance in the same fractions. If there are other proteins in those fractions, it would have shown a peak in other fractions. Elements of the same compound absorb light at the same wavelength but with different intensities, resulting in different absorption spectrum. It may also indicate that there were no other proteins found within the periplasmic space in *E. coli*.

The Bradford assay was used to determine the concentration of the samples, which is based on the BSA standard curve. The assay showed good results, which were used in the calculation of the protein purification scheme table.

The purification table shows increase in total activity in the different sample, with the highest total activity of $62.59 \mu\text{mol min}^{-1}$; and the lowest $15.89 \mu\text{mol min}^{-1}$. The total activity decreases with each purification step. The activity of a substance is actually related to the chemical potential of that substance. This means that as the substance is getting purified most of the other elements are removed, the few remaining elements become less dynamic and, therefore, shows less activity because the elements become wide spread and not bounce or collide frequently producing less energy.

Specific activity of the samples increase steadily but the SEPH pool shows less specific activity, $13.81 \mu\text{mol min}^{-1} \text{mg}^{-1}$, than the DEAE pool $14.7 \mu\text{mol min}^{-1} \text{mg}^{-1}$. Specific activity is actually the activity of the enzyme itself and more of the enzyme should be obtained with each purification step. The amount of enzyme in the SEPH pool is less perhaps because most of the enzyme might have been lost during the last protein purification process. This indicates that much of the enzyme is purified in the DEAE pool and not the SEPH pool. It can, therefore, be concluded that the DEAE pool step was sufficient to purify all the enzyme in the sample and this extra purification step resulted into loss of the enzyme. This is clearly indicated in the percentage yield 25 from 28% DEAE pool, and the fold purification of 72 from 77.

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