

Alkaline Phosphatase Purification from *Bacillus megaterium*.

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Abstract

The aim of this study was to isolate and purify an alkaline phosphatase (ALPs) from *Bacillus megaterium*. The purified enzyme was established by gel filtration chromatography and high performance liquid chromatography (HPLC). Then, followed by non-denaturing gel electrophoresis to separate the ALPs from described purification technique. The purified enzyme had the following properties: ALPs concentration obtained from enzyme assay culture supernatant was 13.453 μ g and selected purification techniques of gel filtration and HPLC were both 19.74 μ g and 23.52 μ g respectively. The enzyme's activity and specific activity from culture supernatant were both 0.493 U/ml and 36.3 U/mg respectively.

Keyword : *Bacillus megaterium*, alkaline phosphatase, HPLC, non-denaturing gel electrophoresis, gel filtration

1.0 Introduction

Alkaline phosphatase (ALPs) orthophosphoric-monoester phosphohydrolase classified as phosphomonoesterases (E.C. 3.1.3.1) are a group of membrane-bound glycoprotein that limited to the monoesterase activity catalysed through the formation of a phosphoserine intermediate [1]. ALPs however vary in sizes, metal requirements and substrate specificities [2].

Phosphatase is known to be essential in most microorganisms to release phosphate from organic compound when inorganic phosphate is limited [2]. Therefore, the mechanism has practically used by most molecular biologists to removed 5' and 3' monophosphatase (dephosphorylation) from nucleic acid through ALPs *Escherichia coli* [3]. In addition, alkaline phosphatase from calf intestine has proven to be useful in enzyme-immunoassays (EIA) [4].

Since then, studies have been made extensively to find new source with significant properties for commercial purpose.

As recently, species of *Bacillus megaterium* has revealed to meet this requirement, as alkaline phosphatase found within them to be heat stable [5]. Therefore, the aim of this study was to isolate and purify an alkaline phosphatase (ALPs) from *Bacillus megaterium*.

The purified enzyme is established by gel filtration chromatography and high performance liquid chromatography (HPLC), followed by non-denaturing gel electrophoresis. The purified enzyme should obtained higher concentration and enzyme activity accordingly to the following properties: ALPs concentration obtained from enzyme assay culture supernatant was 13.453 μ g and selected purification techniques of gel filtration and HPLC were both 19.74 μ g and 23.52 μ g respectively.

The enzyme's activity and specific activity from culture supernatant were both 0.493 U/ml and 36.3 U/mg respectively. Purified ALPs from gel filtration and HPLC were compared to one another for their activities. Those purified enzyme concentrations were calculated based on Bradford method and Standard Bovine Serum Albumin (BSA) curve. While, the ALPs activity and its specific activity were measured by *p*-nitrophenyl phosphate (*p*-NPP) substrate.

2.0 Materials and Methods

2.1 Sampling and bacterial strains

Bacillus megaterium obtained from Department of Biological Science, University of Essex's storage is cultured overnight and grown in 30°C.

2.2 Aseptic technique

The organism was sub-cultured aseptically from the stock culture in nutrient broth. The cultures were then incubated for 60h at 200 rev min⁻¹. Identification was done by using gram and spore staining based on methods described [6].

2.3 Enzyme assay from culture supernatant

The cultures were poured into a centrifuge tube and spun at 5000rpm for 10 minutes. After centrifugation, the supernatant was decanted into a universal tube and placed into ice. 0.1ml culture supernatant and 0.4ml of distilled water were added into 0.5ml of the substrate, *p*-NPP into a cuvette. The absorbance readings were taken at 405nm within 3 minutes for enzyme activity measurement.

Protein concentration was measured by using the existing published method provided by Bio-Rad manufacturer. This was done by preparing standard protein, bovine serum albumin (BSA) stock in the range of 0 to 25µg/ml. 0.2ml of Bradford dye reagent and 0.8ml of each BSA standard were added and mixed together. The absorbance readings were measured at 595nm after 5 minutes of mixture incubation in room temperature. The calibration curve of BSA, protein concentration (x-axis) against absorbance (y-axis) was then plotted. Diluted culture supernatant was used in order to obtain correspondence value from BSA standard curve treated the same condition. This was done by adding 0.6 ml of distilled water into 0.2ml of culture supernatant. 0.2ml of Bradford dye was added to the dilution supernatant and absorbance readings were taken at 595 nm after 5 minutes of mixture incubation. The protein concentration value was then referred to the standard curve.

2.4 Purification of ALPs

2.4.1 Gel filtration chromatography

The gel filtration column was prepared by using sepharose 6B matrix (according to the manufacturer's instruction). 50ml of Tris-HCl (50mM, pH 8.0) was added to the flask containing the matrix and was degassed for 20 min. 25 ml of the column was packed with the matrix in a retort stand. Then the tap was allowed to let the excess liquid to flow out until the column size was 20ml. The column was left overnight.

0.4ml of blue dextran solution was added to the top of the column and the elution rate was estimated. 0.4ml of the sample supernatant was treated on the same condition and added to the top of the column. The elutions were collected in 50 labelled eppendorf tubes to a total of 50ml and were stored at 4°C.

The alkaline phosphatase activity of each of the 50-eppendorf tubes was estimated. This was done by adding 90µl of *p*-NPP and its absorbance were estimated at 410nm using a microtitre plate reader.

Bradford method was used for protein concentration estimation. Each 140µl of BSA

standard and each eluted fractions were loaded into same microtitre plate and read at 595 m absorbance by adding 35µl of Bradford dye to each wells.

2.4.2 HPLC

4ml of the combined active fractions obtained by gel filtration chromatography was centrifuged for 15 min at 10°C at 5000rpm. HPLC was carried out by using Beckman System gold. 50µl of the concentrated sample were loaded onto the column and were collected in 15 eppendorf tubes.

The alkaline phosphatase activity was estimated at 410 nm for each of the eluted samples from HPLC by adding 90µl of the samples to 90µl of *p*-NPP substrate in a microtitre plate.

The same treatment was applied in protein concentration estimation (according to gel filtration method described earlier).

2.4.3 Non-denaturing gel electrophoresis

The method preparation was followed according to previous described method [7]. The gel was run at 120V until the dye migrated to within 1-5mm of the bottom of the gel. The gel was then vertically cut. One half was stained with *p*-NPP while the other half was stained with Coomassie brilliant blue.

3.0 Result

3.1 Aseptic Technique

Purple rod shapes *Bacillus* were observed by gram staining. Green pore staining coat and red exosporium were observed during spore staining (Figure not included).

3.2 Enzyme assay from culture supernatant

The activity of enzyme within 1 minute is estimated Alkaline Phosphatase activity for 1 ml is 0.493 U/ml. Thus, total volume for 20 ml of the culture supernatant is 9.860 U/ml.

Figure 1: shows an overall activity of ALPs within 3 minutes.

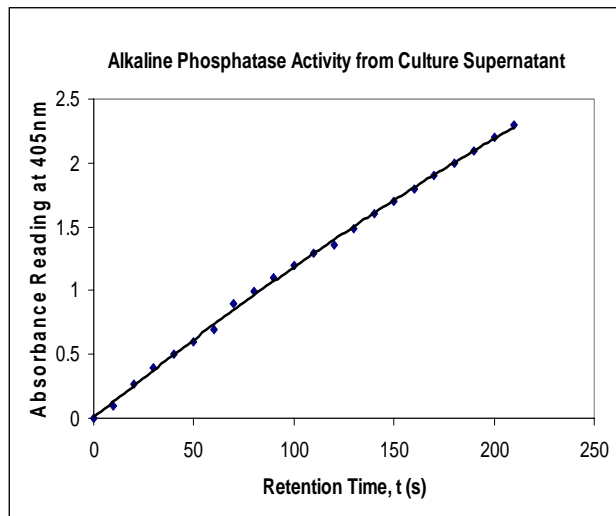


Figure 1: Measurement of ALPs activity from culture supernatant. One unit of alkaline phosphatase was defined as the amount of enzyme necessary to hydrolyse 1 nmol *p*-nitrophenyl phosphate min⁻¹

Standard curve showed the protein concentration after dilution for culture supernatant is 0.363 µg. Thus, the total protein concentration from the supernatant is 13.453 µg. The ALPs specific activity is estimated to be 36.6 U/mg.

3.3 Purification of ALPs

3.3.1 Gel filtration

The elution rate of both, the blue dextran dye and supernatant are estimated to be 0.5 ml per minutes. Each eluted supernatant for 50 tubes from the gel filtration column was measured at 410nm for absorbance reading. This was conducted by using *p*-NPP solution as for the standard scale.

Then for each eluted sample of 50 tubes collected from the gel filtration column was measured for absorbance at 595 nm. From figure 2, number of fractions against the absorbance reading at 595 nm and 410 nm were plotted. It reveals a series of fractions from 13 to 32 have possible active enzymes.

The highest peak shows a fraction of 24 with the reading of 0.246. From the standard curve, protein concentration measured is 19.74µg in total volume.

These series of active fractions from both ALPs activity and concentration procedures were collected and kept for further purification step.

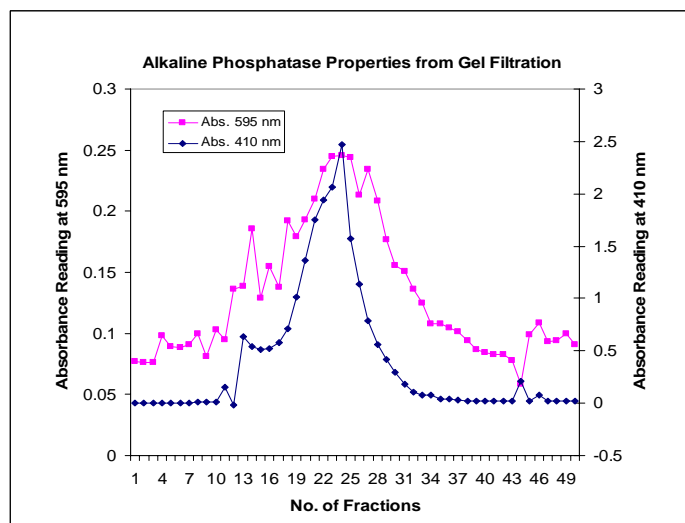


Figure 2: Adsorption spectrum of purified alkaline phosphatase protein from gel filtration.

3.3.2 HPLC

In figure 3, the result generated by the Beckman System Gold shows 3 major peaks.

Each eluted supernatant for 14 tubes obtained from HPLC was measured for absorbance reading at 410 nm. Solution of *p*-NPP used as the standard scale. Each eluted sample for 14 tubes collected from HPLC was then measured for absorbance reading at 595 nm. The result was shown as in picture 4. It indicates that a series of fractions in range of 6 to 9 may have possible active enzymes.

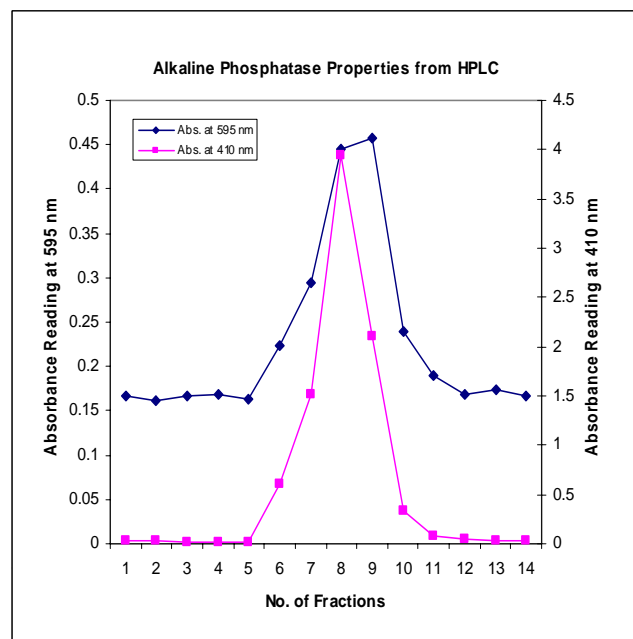


Figure 4: Adsorption spectrum of purified alkaline phosphatase protein from HPLC.

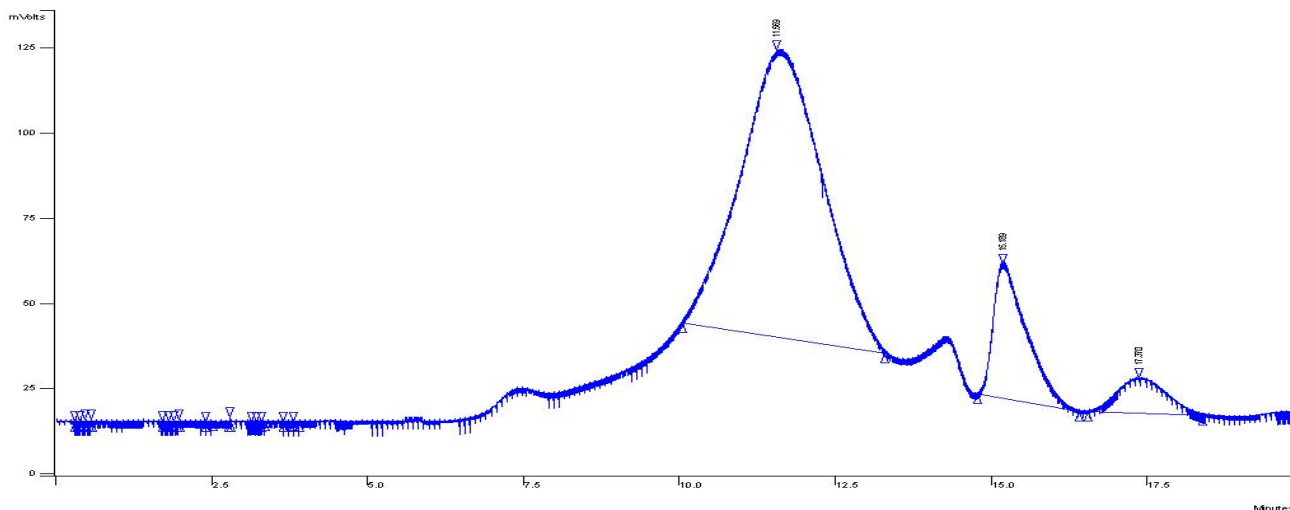


Figure 3: The diagram showing the possible protein bands obtained from HPLC that runs under Beckman System Gold.

3.3.3 Non-denaturing gel electrophoresis

Gel electrophoresis result shows band obtained from the crude supernatant, gel filtration and HPLC after *p*-NPP's (figure 5) and Coomassie blue's treatment (figure is not included). The gel indicates that there is one band observed with better-concentrated color of bands according to type of purification methods. Significantly, the bands are similar in size.

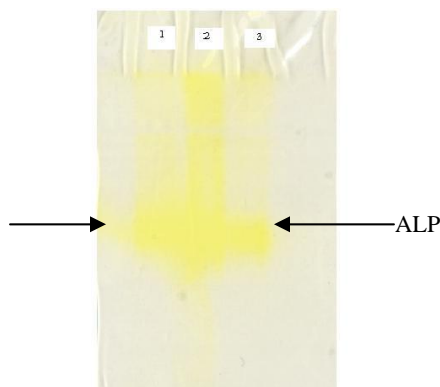


Figure 5: Gel showing activity of ALPs after *p*-NPP's treatment where protein band obtained from lane 1: culture supernatant; lane 2: gel filtration; lane 3: HPLC

4.0 Discussion

Most of protein purification depends on maintaining pure culture of organism by using aseptic technique. Organisms were observed under oil-immersion lenses. The bacteria (exosporium) were stained in purple with 3.0 μm in length and 1.5 μm in breadth. The spores were stained

in green. Based on description found in previous study, a pure culture and spore staining have obtained [8].

Since the concentration of protein might be low in culture supernatant, purification is the essential step in getting concentrated supernatant [9]. Gel filtration chromatography and HPLC are widely used for purification techniques. For instant, gel filtration chromatography is equipped with a porous membrane that filled by buffer (liquid phase) that separate proteins rely on the basis of their size and shape. Sepharose 6B used in this study is a bead-formed gel matrix prepared from agarose. It has 45-164 μm in diameter and 10kDa to 4000kDa range of fractionation. The pores in the gel matrix seem sufficient and suitable with ALPs that 16kDa in size [9]. However gel filtration is a single buffer technique does not require salt concentration or pH changes since protein are not adsorb onto gel surface. When a mixed protein that passes down gel filtration column in buffered aqueous mobile phase, the molecules that are too large to pass through the pores of the stationary phase are excluded from the solvent volume inside the gel beads.

Conceptually, large molecules will be passing more rapidly through the matrix than smaller molecules. This will be observed by smaller elution volumes and longer retention time obtained during the study. However in this study, the behaviour of ALPs could not be quantitatively characterised since no standard plot of known molecular mass were done. The measurement of ALPs activity is based upon the hydrolysis of *p*-NPP to *p*-nitrophenol (*p*-NP) and phosphate. It is known that *p*-NPP is the only substrate used to indicate the presence of alkaline phosphatase. Thus, the rate of this hydrolysis

is determined by observing the increment in absorbance at 410 nm.

On the other hand, High Performance Liquid Chromatography (HPLC) is more likely to develop high degrees of enzyme detection, which easily separate a wide variety of chemical mixture. As final step, HPLC is often employed [7]. This can be seen by the highest protein concentration obtained from overall techniques that have been used (as described earlier in result section). As HPLC detected protein at low-levels in small fraction sizes, allows it to be used as an analytical and preparative technique. This significant application was shown by three peaks observed under a Beckman System Gold indicate that 3 possible proteins may presence. It is known that *Bacillus megaterium* have other type of enzymes such as lipase, proteinase besides of ALPs [5].

Non-denaturing gel electrophoresis or called native gel electrophoresis separates protein based on their sizes and charge properties. It is common to run non-denaturing gel with higher pH buffer of 8.8. This is because, most proteins are negatively charged at higher pH and move towards anode [7]. When each preparation was subjected to non-denaturing electrophoresis, activity was detected by staining for phosphate released with *p*-NPP. Each enzyme migrates differently and only one band was observed at Figure 5 in each lane. This significantly shows that alkaline phosphatase is successfully isolated and indicated in each lane.

5. Conclusion

From the study it shows that higher concentration of enzyme obtained by purification technique rather than culture supernatant itself. The measurement of ALPs activity is based upon the hydrolysis of *p*-NPP to *p*-nitrophenol (*p*-NP) and phosphate. For future quantitative characterization, standard plot of molecular mass is required to study the ALPs behaviour.

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