



# Characterization studies of thermostable alkaline phosphatase from various plant seeds

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## ABSTRACT

**Objective:** The objective of this study is to obtain and characterize thermostable alkaline phosphatase from plant seeds than produced prior from bacterial and animal sources.

**Methodology and Results:** Alkaline phosphatase was isolated and purified from plant seedlings by a streamline method without the use of proteolytic and lipolytic enzymes, butanol, or heavy metals. The study features are the use of a mild nonionic detergent, precipitated with acetone, maintained at low temperature to remove impurity, and the use of ion exchange columns to concentrate the alkaline phosphatase. The enzyme preparation revealed a major phosphatase band of 72 kDa and two minor activity bands. A variety of stability and activity profiles were determined for the purified seedling alkaline phosphatase: pH, temperature, surfactant and effect of UV light.

**Conclusion and application:** The enzyme partially dephosphorylated linearized pUC18 plasmid despite being an alkaline phosphatase and showed potential application as a substitute for alkaline phosphatase from other sources.

**Keywords:** *Vigna unguiculata* (Black-eyed Bean), *Cajanus indicus* (Red Gram), *Arachis hypogaea* (Ground Nut), alkaline phosphatase, Bradford assay, *p*-nitrophenylphosphate.

## INTRODUCTION

Alkaline phosphatases (Stadtman T.C, 1961); EC 3.1.3.1 are enzymes that catalyze the hydrolysis of a variety of phosphate esters and appear to exhibit pH optima above 8. The enzymes (Hollander, V.P and Boyer P.D, 1971) are widely found in nature and are usually rather nonspecific. Alkaline phosphatase is apparently ubiquitous in nature, having already been identified in numerous organisms and tissues. In general, the alkaline phosphatases occur in very small quantities, are unstable in dilute solution, and are subject to surface denaturation in the pure state. These properties, and their tendency of structure and regulation (Cohen P, 1989) of Alkaline

Phosphatase to occur in multiple forms, often make the isolation of alkaline phosphatases difficult. Studies on pure alkaline Phosphatase have so far focused mainly on those of clinical significance (Remaley A T and Wilding P, 1989) from animal sources. Among possible functions of alkaline phosphatases might be the role of providing inorganic phosphate for metabolic excretory and some secretory purposes in plants. Alkaline phosphatases may have a role in the mammalian reproductive (Henri Alexandre, 2001) process. A possible role of human prostatic alkaline phosphatase may be in the dephosphorylation of esters to liberate fructose



(Hye-Shin C. Park and Robert L. Van Etten, 1986), which serves as an energy source for spermatozoa (Winston A. Anderson, 1968), or in the formation of choline from choline phosphate. In plants, alkaline phosphatase activity in seeds often increases greatly during germination, indicating a possible role in phosphate metabolism and mobilization

## MATERIALS AND METHODS

**Preparation of Enzyme smoothie:** All seeds as listed in table 1 are procured from K R market located in Vijayawada, Andhra Pradesh and they were cleaned thoroughly with distilled water to remove contaminants and dust. The skins of the seeds were peeled out and seeds are taken along with 2-3 ml of Phosphate Buffer Saline. The seeds were made into a "smoothie" (Vemuri Praveen Kumar and Ujjwala Krothapalli, 2009) with the help of a mortar and pestle and collected into a sterile test tube.

**Enzyme assay and protein determination:** The standard assay (Tabatabai M A and Bremner J M, 1969) for Alkaline Phosphatase activity was carried out at 70°C, for 2.5 minutes. The substrate used was 2.5 mM *p*-nitrophenylphosphate (pNPP) (Sommer A.J, 1954) in 50 nM NaOH-glycine (pH 10.0) buffer and containing 0.5 mM CaCl<sub>2</sub>, except as otherwise indicated. The release of *p*-nitrophenol in the reaction mixture (1 ml) was continuously measured at 410 nm using a spectrophotometer (Thermo-Fischer), over the linear period. One unit of enzyme activity is defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol from pNPP in 1 min. Protein concentration was determined by the method of Bradford (Bradford M. M, 1976), using bovine serum albumin as the standard. For routine enzymatic characterization assays, 100 μg of purified Alkaline Phosphatase was added to the 1-ml reaction mixture.

**Purity of Enzyme:** The enzyme extract was dispersed in 3 volumes of acetone (Denis Barritault et. al., 1976) at 0°C and adjusted to pH 7.5 by slow addition of Sodium Hydroxide. After vigorous mechanical stirring for about 30 minutes, the 'homogenate' was centrifuged at 1800g for 40 min in REMI C-24 Centrifuge. The filtrate was incubated at -20°C in a freezer overnight. By this means the ionic strength of the material was lowered, and the last traces of soluble protein were removed. The incubated filtrate was centrifuged to collect the precipitated protein. Aliquots of the enzyme preparation were stored at -20°C. For the enzyme

(Duff SMG, Sarath G, 1994). Thus, in addition to the general goal of carefully studying some properties of a plant alkaline phosphatase, this study describes the isolation and characterization of a pure alkaline phosphatase from various seedlings.

assays in defined buffers, an aliquot of the enzyme preparation was thawed and passed through a DEAE Sephadex column (Biorad) into a 100 mM Tris-HCl (pH 7.5) buffer supplemented with 1 mM CaCl<sub>2</sub>.

To check the purity (Stinson R A and Seargeant L E, 1981) of the enzyme, the samples were run on Polyacrylamide gel electrophoresis. Samples were layered on the stacking gel surface with a small volume of bromophenol blue as a tracking dye. Samples were carried out until the tracking dye was near the bottom of gel taking about 4.5 hours. Following electrophoresis, the gels were incubated in 20% Trichloro acetic acids fixing solution. Protein gels were stained with freshly made 0.2% Coomassie Brilliant Blue R-250 in 40% Methanol-20% for 1-3 hours. Developed gels were stored in 7.5% Acetic acid.

**DNA dephosphorylation:** The process of dephosphorylation (Smith S.W and Weiss S, 1957) was carried out by standard cleavage method. pUC18 was digested using EcoRI producing, cohesive ended linearized vectors. Linearized vectors of 1μg were incubated in dephosphorylation buffer and Alkaline Phosphatase for 2 hours. Commercial calf intestinal Alkaline Phosphatase (Genei) was used as a positive dephosphorylation control in its commercial dephosphorylation buffer at 37°C. Following this, Ground Nut Alkaline Phosphatase treated linear vector was ligated in the presence of 2U of T4 DNA ligase along with its commercial buffer (Genei) to give a final volume of 20 μl, which was incubated overnight at 16°C.

### Temperature, pH optima, Surfactant Parameters:

The apparent optimum temperature (Michelle E. Peterson et. al., 1951) was determined by running the standard assay at temperatures ranging from 20 to 100°C in PBS buffer (0.2 M; pH 8.0). The apparent optimum pH (Ross M H and Ely J. O, 1951) of the enzyme was determined by running the standard assay, using Tris-HCl buffer for the pH ranges 6.0 to 12.0, respectively. Triton X-100 was used as the



surfactant (Lalitha J and Mulimani, 1997) and carried out the standard assay in PBS buffer (0.2 M; pH 8.0) ranges from 0.01 to 0.10%. Samples were taken at 30 minutes interval, and the residual activity was

determined by the standard assay. All assays are determined with initial rate of pNPP hydrolysis under the assay conditions with NaOH, using a 1X substrate.

## RESULTS AND DISCUSSION

Although the alkaline phosphatase is not present in large amounts in few seeds, the use of ion exchange chromatography steps made it possible to purify the enzyme. Acetone precipitation appeared to be unavoidable in order to remove lipid from the extract,

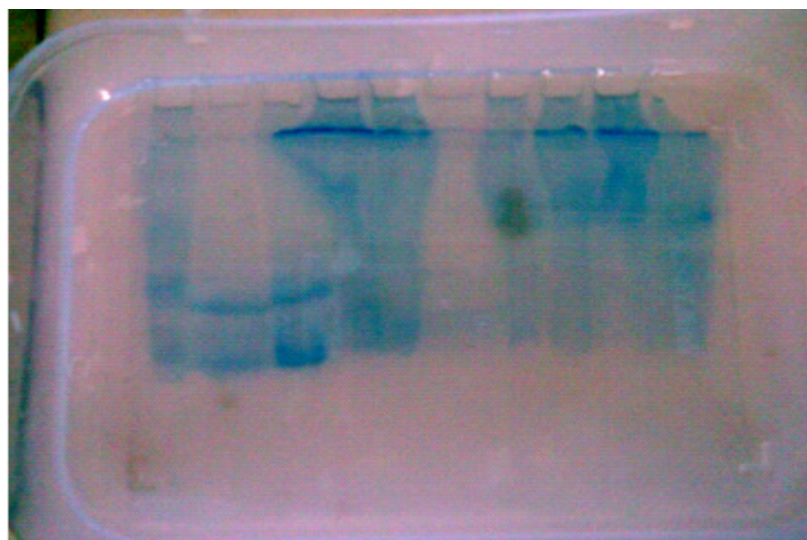
despite the low recovery in this step. Table 1 summarizes the enzyme activity assays and protein determinations done spectrophotometrically for each extract.

**Table 1: Enzyme Activity and Protein concentration of Alkaline Phosphatase**

S. No	Common Name	Botanical name	Enzyme Activity at 410nm	Bradford Assay (mg)
1	Yellow pea	<i>Pisum sativum</i>	0.354	1.2
2	Ladies finger	<i>Abelmoschus esculentus</i>	0.132	1.25
3	Chick pea	<i>Cicer arietinum</i>	0.114	1.15
4	Groundnut	<i>Arachis hypogaea</i>	1.151	1.4
5	Soya bean	<i>Glycine max</i>	0.019	1.22
6	Bitter gourd	<i>Momordica charantia</i>	0.077	1.3
7	Black eyed bean	<i>Vigna unguiculata</i>	0.055	1.33
8	Red gram	<i>Cajanus indicus</i>	0.717	1.55
9	Green gram	<i>Vigna radiatus</i>	0.252	1.26
10	Black gram	<i>Phaseolus mungo</i>	0.239	1.28

Moreover, ion exchange chromatography on a DEAE Sephadex column, calibrated using standard proteins, showed that alkaline phosphatase had an unexpectedly high alkaline content. For each fraction containing alkaline phosphatase, PAGE was done to check the

separation of the proteins. Preparative PAGE separated the two bands of enzyme. Figure 1 exploits that denaturing PAGE shows the purity of these proteins.



**Figure 1:** Purity of enzyme on 12% SDS- PAGE Alkaline Phosphatase presents high pH, temperature and Surfactant concentration

Apparent optimum pH of the Alkaline Phosphatase was determined to be 11.0 in 0.2 M PBS buffer for *Cajanus indicus*. The enzyme exhibited at least 60% of its optimal activity over a rather narrow pH range, from 10 to 11 (Figure 2A). Further more, thermal activation studies enabled the determination of an apparent optimum temperature of 100°C for *Vigna unguiculata* in 0.2 M PBS buffer (Figure 2B). Moreover, the enzyme exhibited its optimal activity over a range of temperature

from 40 to 80°C (data not shown). The influence of surfactant was tested. Increasing concentrations of Triton-X had an inhibitory effect on enzyme activity. At 0.09% of triton-x concentration *Arachis hypogaea* exhibited an optimal activity (Figure 2C). *Vigna unguiculata* is considered as thermostable enzyme which also exhibits alkaline pH and apparent activity towards surfactants (Table 2).

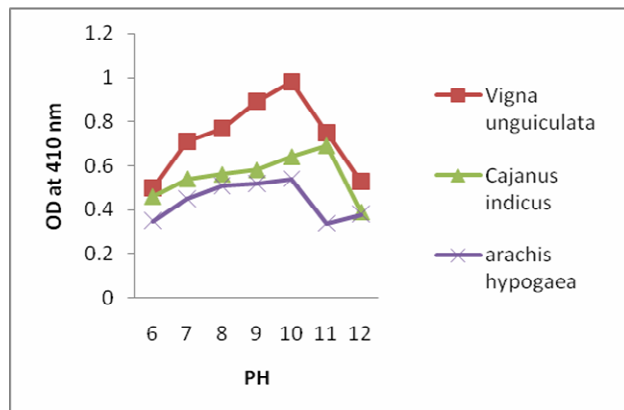


Figure 2A: Optimum pH activity of enzyme

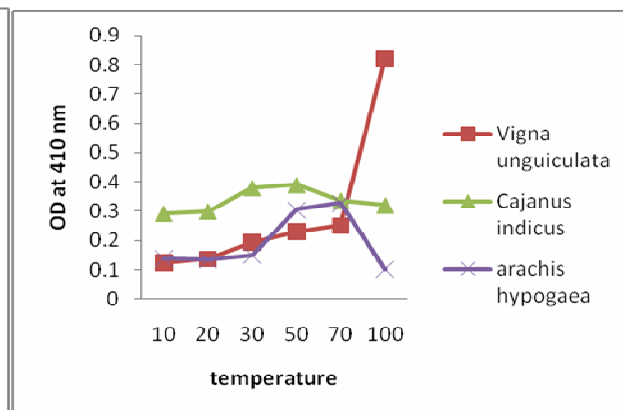


Figure 2B: Optimum temperature activity of enzyme

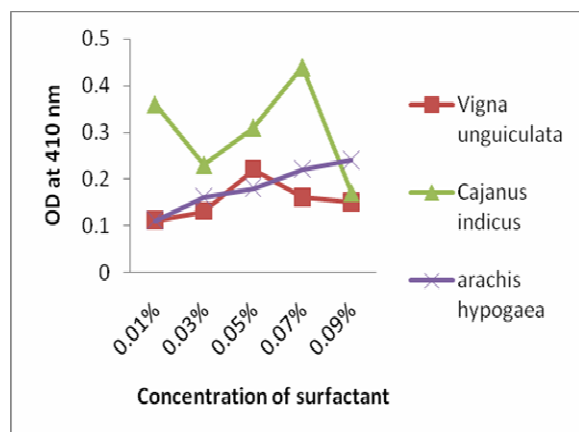


Figure 2C: Optimum concentration of surfactant on enzyme activity

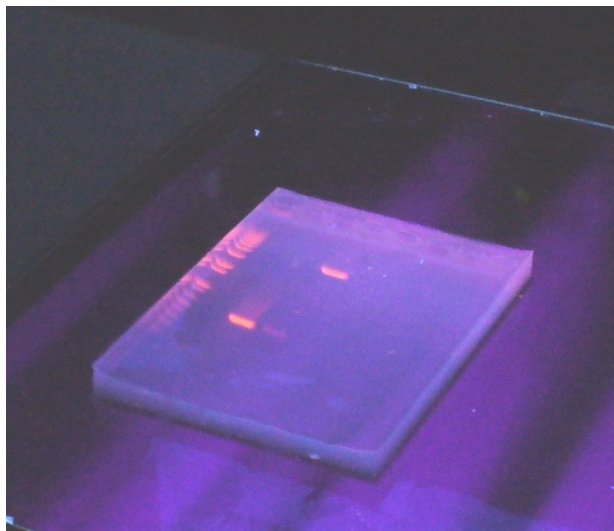
Table 2: Optimum activity of Alkaline Phosphatase

S.No	Botanical name	PH	Temperature	Surfactant
1	<i>Vigna unguiculata</i>	10	100°C	0.05%
2	<i>Cajanus indicus</i>	11	40°C	0.07%
3	<i>Arachis hypogaea</i>	10	70°C	0.09%



**Dephosphorylation of DNA:** Alkaline Phosphatase is classically used in molecular biology to remove 5'-terminal phosphate from DNA fragments. As such, the enzyme was tested against cohesive *EcoRI*-linearized pUC18. The efficiency of dephosphorylation was estimated by comparing non-dephosphorylated

plasmids and 1Kb ladder. Complete ligation of non-Alkaline Phosphatase treated and Alkaline Phosphatase treated fragments was observed when the experiment was carried out using cohesive ends on agarose gel (Figure 3).



**Figure 3:** Enzyme activity on agarose gel stained with ethidium bromide. Lane 1: 1 Kb marker, Lane 2: non-ligated DNA and Lane 3: Circular Plasmid.

## CONCLUSION

The present work reports the first characterization of an Alkaline Phosphatase from a seed as plant source. Alkaline Phosphatase activity has already been found in Sunflower seed, Horse gram seed but the enzyme has not been well characterized for its dephosphorylation activity. Alkaline Phosphatase is commonly used in molecular biology for the construction of recombinant plasmids. They remove 5'-terminal phosphate from DNA fragments and, as such, enzyme treated linearized plasmids are unable to exhibit self-ligation and are more likely to integrate an exogenous gene insert. Plant seed enzyme was tested under plasmid dephosphorylation conditions using a

linear cohesive ended pUC18 vector. Bovine Alkaline Phosphatase was found to be more effective than the plant Alkaline Phosphatase; however; dephosphorylation conditions of the latter were not optimized. Although most Alkaline Phosphatases have been isolated and characterized, only commercial *E. coli* Alkaline Phosphatase (bacterial) and Calf Intestine Alkaline Phosphatase (animals) are routinely used in molecular biology. As plant seed Alkaline Phosphatase presented great thermostability and good efficiency in DNA dephosphorylation, further studies may be required to elucidate its potential use in such applications.

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