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PURIFICATION AND CHARACTERIZATION OF ALKALINE PHOSPHATASE FROM *DOLICHOS LAB-LAB* AND ITS *INVITRO* DEPHOSPHORYLATION ACTIVITY ON NUCLEIC ACIDS

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ABSTRACT

Phosphatase serves several functions in plant metabolism including growth governance, phosphorous level control, starch breakdown etc. Alkaline phosphatases, acting at an alkaline pH 8, are a significant class of enzymes that catalyze release of phosphate esters especially. This enzyme study is so far limited only to animal source and partly to microbial sources, in terms of clinical research. Although it has been identified that plant as a source of this enzyme may be exploited, there always has been a challenge on the isolation and characterization of this enzyme and how pure it can be. This paper partly addresses the above problem, where the enzyme has been isolated from the seeds *Dolichos lab-lab* plant characterized and its purity was checked by HPLC. The purity obtained was 98% and the enzyme has been further analyzed for its activity on nucleic acids, which gave promising and positive results.

INTRODUCTION: Phosphorus is an essential element for the growth and development of organisms and the lack of this turns out to be one of the limiting factors of growth in plants, particularly in the development of its organs. Plants obtain phosphorus from the soil [1] as phosphatases are ubiquitous in plants, animals and microorganisms [2]. Alkaline phosphatases [3]; 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.

Alkaline phosphatase [4] has also been reported to be involved in the breakdown and mobilization of starch and sucrose, for the biosynthesis of essential oil in lemongrass. A possible role of human prostatic alkaline phosphatase was proposed in the dephosphorylation of esters to liberate fructose [5], which serves as an energy source for spermatozoa [6], or in the formation of choline from choline phosphate. Alkaline phosphatase also has a role in mineralization [7].

In vertebrates, the enzyme is an ectoenzyme, which is attached to the outer face of the plasma membrane through a phosphatidyl inositol-glycophospholipid (GPI) anchor covalently attached to the C-terminus of the enzyme that facilitates mineralization initiation *in-vitro* [8]. Alkaline phosphatase isolated from plant seeds has better dephosphorylation activity [9] than produced prior from bacterial and animal sources. Also, studies on pure alkaline phosphatase have so far been focused mainly on their clinical significance [10] from animal sources.

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This work is a successful effort in isolation of alkaline phosphatase from plant source with high purity, whose activity was demonstrated on nucleic acids.

MATERIALS AND METHODS:

Preparation of Enzyme Smoothie: All seeds as listed in Table 1 were procured from K R market located in Vijayawada, Andhra Pradesh and they were rinsed thoroughly with distilled water to remove surface contaminants and dust. The seed coats were peeled out and taken along with 2-3 ml of phosphate buffer saline. The seeds were made into a "smoothie" [11] with the help of a mortar and pestle and collected into a sterile tube.

Enzyme assay and Protein Determination: The activity of alkaline phosphatase at pH 8.4 was determined in a reaction mixture of final volume of 2ml containing 0.1ml of enzyme source, 2.5 µmole of 5-bromo-4-chloro- 3'- indolyphosphate/nitro- blue tetrazolium chloride (BCIP/NBT), 190 µmole of a buffer and 4 µmole of $MgCl_2$ Tris-HCl buffer pH 8.4. The reaction mixture was incubated at 37^{o}C. After incubation for 10 minutes, 3 ml of 10% trichloroacetic acid (TCA) was added and centrifuged at 3000rpm for 10 min. The amount of released inorganic phosphate in the filtrate was determined by King's method [12] using Thermo UV Genesys spectrophotometer. Protein concentration was determined by the method of Bradford [13], using bovine serum albumin as the standard.

Ammonium Sulphate Fractionation: Alkaline phosphatase was purified from the crude extract obtained from all the seeds as listed in **Table 1**. The enzyme extract was precipitated by ammonium sulphate by adding varying concentrations of 50%, 60%, 70%, 80% and 90% at 4^{o}C and the mixture was then centrifuged at 15000rpm for 10 min. The pellet obtained was then dissolved in one ml of 1% NaCl to check the purity of the enzyme; the samples were run on SDS-Polyacrylamide gel electrophoresis. The sample with the best band resolution was identified as *Dolichos lab-lab* and was considered for further purification.

Purification of ALP from *Dolichos lab-lab*:

Column purification: The sample was subjected to column chromatography on Bio-scale Mini DEAE Affi-Gel Blue Cartridge (40 mm length × 12.6 mm inner diameter, 5ml bed volume). This was done by equilibrating the cartridge in Bio-Rad application buffer and applying the sample on to it. The column was eluted with 20ml of the elution buffer and the fractions were collected. To check the purity [14] of the enzyme, the samples were run on 12% SDS-PAGE. Samples were layered on the stacking gel surface with a small volume of bromophenol blue as a tracking dye. Protein gels were stained with freshly made 0.2% Coomassie Brilliant Blue R-25O in 40% Methanol-20% water, for l-3 hours.

Purity of Enzyme by HPLC: 20µl of the eluted fraction obtained by DEAE-affinity chromatography was further analyzed by RP-HPLC using C-18 (250mmX 4.6, 5 µm) column (Hibar) with the mobile phase composed of two buffers: [15] Buffer A, containing acetonitrile/ water/ethyl alcohol/acetic acid/O.83M Sodium acetate (800/127/68/2/3) of pH 3.6, and buffer B (400/400/ 68/53/79), pH 3.6.at a flow rate of 1mL min^{-1} detected at 295nm.

DNA Dephosphorylation: The process of dephos-phorylation [16] 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 (Invitrogen) was used as a positive dephosphorylation control in its commercial dephosphorylation buffer at 37°C. Following this, *Dolichos lab-lab a*lkaline phosphatase treated linear vector was ligated in the presence of 2U of T4 DNA ligase along with its commercial buffer (Genei) to make a final volume of 20 µl, which was incubated overnight at 16°C.

Effect of various agents on *Dolichos lab-lab* enzyme: Different agents were tested for their effects on the enzyme activity. The apparent optimum temperature of the enzyme [17] was determined by running the standard assay at temperatures ranging from 37 to 70^{0}C in PBS buffer (0.2 M; pH 8.0). The apparent optimum pH [18] of the enzyme was determined by running the standard assay, using Tris-HCl buffer for the pH ranges 7.4 to 11.0, respectively. Tween 20, SDS and Triton X were used as the surfactants 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 BCIP 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. Ammonium sulphate 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: ASSAY OF ALKALINE PHOSPHATASE

S. No.	Common Name	Botanical Name	Bradford Assay (mg)		
			Smoothie	Salt method	Column method
1	Tomato	*Lycopersicum esculentum*	2.66	1.89	0.76
2	Bottle guard	*Lagenaria siceraria*	2.39	1.09	0.59
3	Ridge gourd	*Luffa acutangula (L.)*	1.81	1.05	0.88
4	Small pea	*Phaseolus Vulgaris*	0.97	0.65	0.07
5	Common pea	*Dolichus lab lab*	3.44	2.24	1.45
6	Brinjal	*Solanum melongena*	3.04	2.54	1.12
7	Long Bean	*Vigna sesquipedalis*	3.2	1.92	0.85
8	Cluster bean	*Cyamopsis tetragonoloba*	1.70	1.08	0.68

Moreover, ion exchange chromatography on a DEAE Sephadex column, calibrated using standard proteins, showed that alkaline phosphatase had a high alkaline content. For each fraction containing alkaline phosphatase, PAGE was done to check the separation of proteins. Preparative PAGE separated the two bands of enzyme. **Figure 1** exploits that denaturing PAGE shows the purity of these proteins.

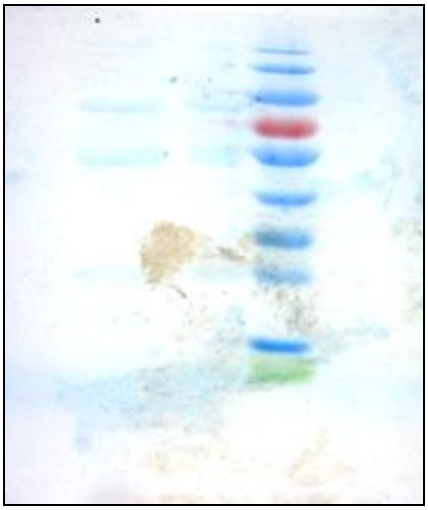

FIGURE 1: PURITY OF ALKALINE PHOSPHATASE ENZYME ON 12% SDS- PAGE REVEALED A MAJOR PHOSPHATASE BAND OF 72 KDA AND TWO MINOR ACTIVITY BANDS

Purity of enzyme: Qualitative analysis of *Dolichous lab-lab* DEAE fraction was carried out using reverse phase HPLC and the chromatographic profiles showing retention time (RT) of *Dolichous lab-lab* and standard ALP were 1.648 and 1.63 respectively (**Figure 2**). The purity of the DEAE fraction shows 99%±0.2 by HPLC analysis. This is a significant purity obtained, which can be used for molecular level studies.

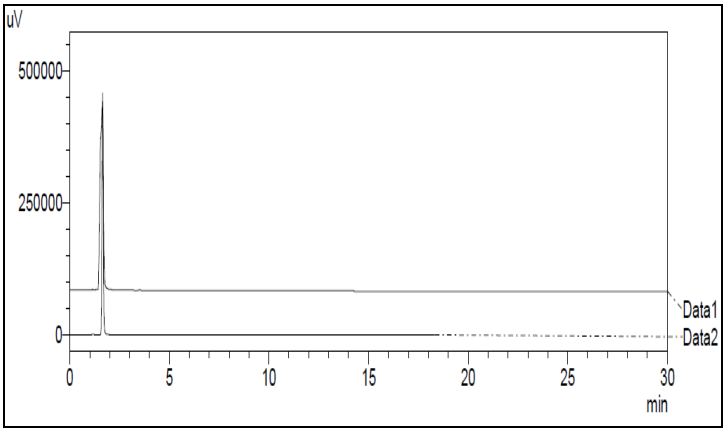

FIGURE 2: RP-HPLC AND UV-VISIBLE ANALYSIS OF ALKALINE PHOSPHATASE ELUTED FRACTION FROM DEAE AFFI-GEL. Data 1 shows the peak obtained by ALP standard. Data 2 shows the peak obtained by purified fraction of Dolichous lab-lab alkaline phosphatase detected at 295nm.

Dephosphorylation activity of Enzyme: Alkaline phosphatase is commonly used in cloning to remove 5'- terminal phosphate from DNA fragments. Hence, the enzyme was tested against cohesive EcoRI-linearized pUC18; the efficiency of dephosphorylation was estimated by comparing non-dephosphorylated plasmids and pUC18.

Complete ligation of non- alkaline phosphatase treated fragments was observed when the experiment was carried out using cohesive ends on agarose gel (**Figure 3**).

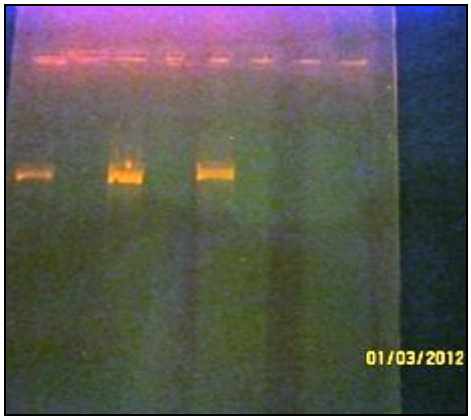

FIGURE 3: ENZYME ACTIVITY ON AGAROSE GEL STAINED WITH ETHIDIUM BROMIDE. Lane 1: pUC18, Lane 2: Dephosphorylated DNA and Lane 3: Cut and resealed DNA

TABLE 2: EFFECT OF VARIOUS AGENTS ON ENZYME ACTIVITY

S.No	pH		Temp		[S] Conc		Tween 20		SDS		Triton X	
	Unit	λ450	^{0}C	λ450	Ratio	λ450	Conc (μg/μl)	λ450	Conc (μg/μl)	λ450	Conc (μg/μl)	λ450
1	7.4	0.056	37	0.114	1:1	0.056	1	0.059	1	0.051	1	0.108
2	8.0	0.109	40	0.113	1:2	0.066	2	0.080	2	0.057	2	0.070
3	9.0	0.077	50	0.090	1:3	0.065	3	0.081	3	0.061	3	0.071
4	10.0	0.094	60	0.153	1:4	0.080	4	0.125	4	0.059	4	0.068
5	11.0	0.085	70	0.127	1:5	0.087	5	0.105	5	0.055	5	0.058

Apparent optimum pH of the alkaline phosphatase was determined to be 8.0 in 0.2 M PBS buffer for *Dolichos lab-lab*. The enzyme exhibited at least 60% of its optimal activity over a rather narrow pH range, from 10 to 11 (**Figure 2A**). Furthermore, thermal activation studies enabled the determination of an apparent optimum temperature of 60°C for *Dolichos lab-lab* in 0.2 M PBS buffer (**Figure 2B**). The influence of surfactant was also tested. Increasing concentrations of Triton-X had an inhibitory effect on enzyme activity. At 1ug of triton-x concentration, 4ug for Tween 20 and 3ug for SDS, *Dolichos lab-lab* exhibited an optimal activity (**Figure 2C**).

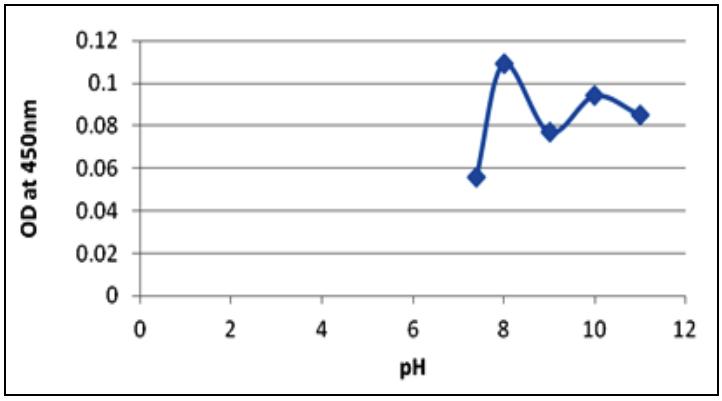

FIGURE 2A: OPTIMUM PH ACTIVITY OF *DOLICHOS LAB-LAB* ALKALINE PHOSPHATASE ENZYME EXHIBITING AT pH 8

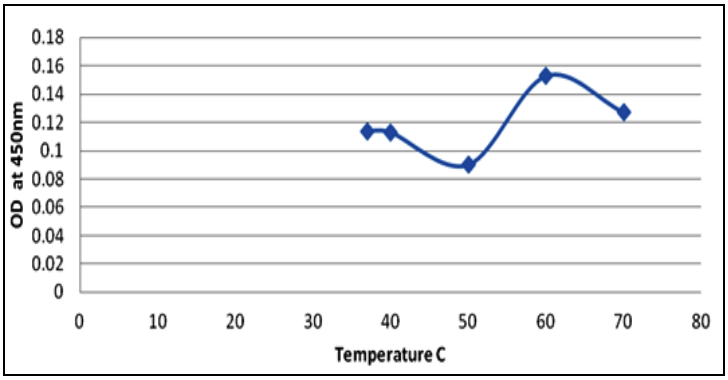

FIGURE 2B: OPTIMUM TEMPERATURE ACTIVITY OF *DOLICHOS LAB-LAB* ALKALINE PHOSPHATASE ENZYME EXHIBITNG AT TEMPERATURE OF 60^{o}C

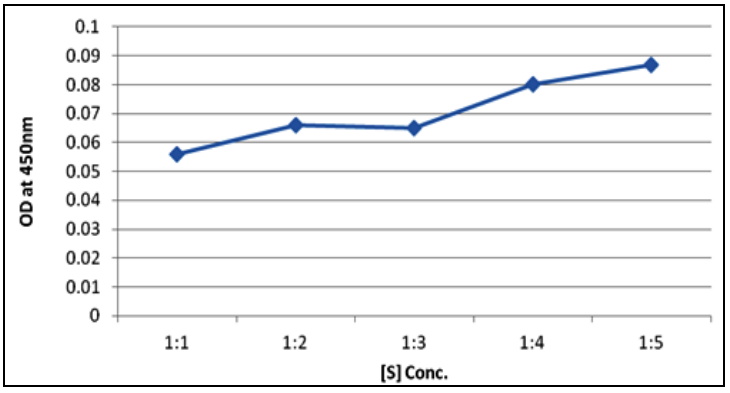

FIGURE 2C: OPTIMUM SUBSTRATE CONCENTRATION ACTIVITY OF *DOLICHOS LAB-LAB* ALKALINE PHOSPHATASE ENZYME EXHIBITING AT 1:5

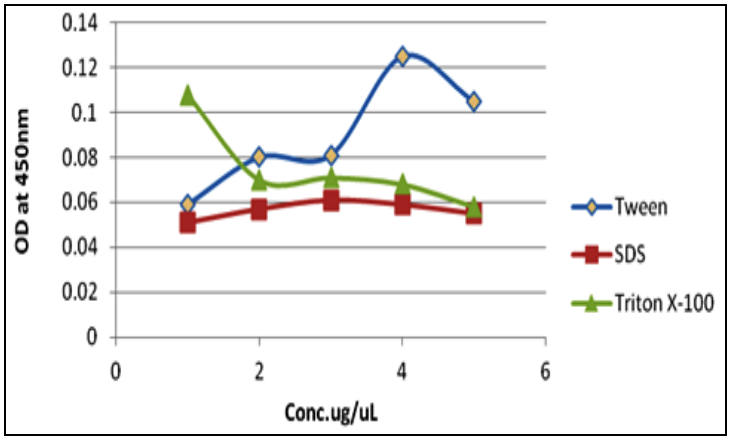

FIGURE 2D: OPTIMUM ACTIVITY OF VARIOUS SURFACTANTS ON *DOLICHOS LAB-LAB* ALKALINE PHOSPHATASE EHIBITING FOR TWEEN 20 AT 4ug, SDS AT 3ug AND TRITON X-100 AT 1ug RESPECTIVELY

CONCLUSION: Alkaline Phosphatase activity has already been found in several seeds 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.

Although most Alkaline Phosphatase have been isolated and characterized, only E. coli Alkaline Phosphatase (bacterial) and Calf Intestine Alkaline Phosphatase (animals) are routinely used in molecular biology. In this endeavor, plant seed Alkaline Phosphatase may have the possibility of use in molecular biology because of their economic importance, thermostable activity, specific activity and benefit the modern world of cloning and as well as recombinant technology.

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