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Functional Insights into the Active Site of Purified Seed Acid Phosphatase AP-I from Erythrina Indica: Role of Key Amino Acid Residues †

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Abstract

In acidic environments, acid phosphatases (EC 3.1.3.2) play a crucial role in hydrolyzing phosphate ester linkages. Two isoforms of Acid phosphatases namely AP-I and AP-II were purified to homogeneity from the seeds of *Erythrina indica* using a combination of gel filtration and affinity chromatography techniques. The purification process involved multiple steps to ensure the enzymes were free from other seed components, thereby facilitating detailed characterization. We report in this study the active site characterization of acid phosphatase form AP-I. AP-I active site of the purified AP-I was characterized in detail through chemical modification studies, which revealed the presence of one residue each of carboxylate, tryptophan, and serine amino acid. Substrate protection experiments using p-nitrophenyl phosphate effectively prevented the modification of all three residues, suggesting their essential role in the enzyme's active site. These experiments provided strong evidence that these residues are directly involved in the catalytic process. Kinetic studies of the partially inactivated enzyme, achieved through the use of specific modifying agents Dicyclohexylcarbodiimide (DCCD) for carboxylate, N-Bromosuccinimide (NBS) for tryptophan, and Phenylmethylsulfonyl fluoride (PMSF) for serine, further confirmed the involvement of these residues in the catalytic mechanism. The results demonstrated that the inactivation of any of these residues significantly impaired the enzyme's activity, highlighting their critical roles in the catalytic process. The results provide a comprehensive understanding of the active site architecture and the catalytic mechanism of AP-I function.

Keywords: acid phosphatase; active site residues; AP-I; *Erythrina indica*

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1. Introduction

Erythrina indica, commonly known in English as the Indian coral tree, is also referred to as the tropical coral tree, tiger's claw, moochy wood tree, or variegated coral tree. In Sanskrit, it is called Paribadra, and in Hindi, it is known by names such as Ferrud, Dadap, and Pharad [1]. *Erythrina indica* is a prickly shrub found in Indian forests with medicinal value. It is used in Ayurveda, Siddha, Unani, and Homeopathy. Its bark, roots, leaves, and fruits treat fever, skin diseases, and act as astringents [2]. The seeds contain non-volatile oil, fatty acids, and lectins. Although the plant is traditionally used in various

geographical regions, there is a lack of scientific evidence to support its folkloric applications [3]. Phytochemical studies have resulted in the isolation, purification, and identification of lectins from the seeds and leaves of *Erythrina indica* [4]. Additionally, enzyme analysis of seed extracts has revealed the presence of four glycosidases—α-galactosidase, β-galactosidase, α-mannosidase, and N-acetyl-β-D-glucosaminidase—along with acid phosphatase [5]. Acid phosphatases (EC 3.1.3.2) are key enzymes involved in phosphate metabolism, responsible for catalyzing transphosphorylation and breaking down a wide range of orthophosphate esters in acidic environments [6]. The active site of an enzyme is the region responsible for substrate binding and the catalytic process. It usually consists of a small number of crucial amino acid residues that interact specifically with the substrate, enabling the chemical reaction [7].

The aim of this study is to examine the active sites of AP-I from *Erythrina indica* seeds, offering a better understanding of their substrate specificity, structural function, catalytic performance, and potential regulatory pathways.

2. Materials

Phenylmethylsulphonyl fluoride, phenyl glyoxal, N-acetyl imidazole, p-nitrophenyl phosphate, N-bromosuccinimide, diethyl pyrocarbonate, 5,5′-dithiobis (2-nitrobenzoic acid), and dicyclohexyl carbodiimide were sourced from Sigma (USA). All other chemicals and reagents were obtained from commercial suppliers and were of the highest available purity.

3. Methods

3.1. Acid Phosphatase Assay

Enzyme activity was measured using p-nitrophenyl phosphate as substrate at 37 °C for 30 min, following the procedure of Campbell et al. [8]. The released p-nitrophenol was quantified at 405 nm. One unit of activity corresponds to the release of 1 μmol of p-nitrophenol per minute.

3.2. Protein Estimation

Protein concentration was measured by Lowry's method [9] using BSA as the standard. Absorbance at 280 nm was also recorded, applying an extinction coefficient of $A_{1}\%_{(280)}$ = 6.45 to calculate protein levels.

3.3. Fluorescence Measurements

Fluorescence spectroscopy was performed at 25 °C using a Hitachi F-4500 spectrofluorimeter with 5 nm slit widths. Native and modified AP-I enzymes (5 μM in 0.05 M citrate buffer, pH 4.6) were analyzed with and without 50 μM p-nitrophenol. Excitation was set at 295 nm to target tryptophan residues, and emission was recorded from 300–400 nm. Tryptophan environment was probed through quenching studies using acrylamide, cesium chloride, and potassium iodide [10].

3.4. Chemical Modification Studies

Specific amino acid-modifying reagents were used to identify active site residues in the purified isoenzyme [10].

3.5. Modification of Tryptophan Residues

AP-I (0.5 μM) in 0.05 M citrate buffer (pH 4.6) was titrated with 1–5 μM N-bromosuccinimide, added in five 5 μL increments followed by recording fluorescence spectra and absorbance at 280 nm after each addition. Enzyme activity was monitored over 0–12

min, and reactions were quenched with 0.1 M borate buffer. The extent of tryptophan modification was calculated using an extinction coefficient of 5500 M^{-1} cm^{-1}. Controls lacked NBS [10].

3.6. Detection of Carboxylate Residues

To assess the role of carboxylate groups in catalysis, K_m and V_{max} values for purified AP-I were measured across pH 3.0 to 6.0. pKa_1 and pKa_2 values were determined from a plot of $\log(V_{max}/K_m)$ versus pH [11].

3.7. Modification of Carboxylate and Serine Residues

AP-I (0.5 μM) was treated with 1–5 μM DCHC and PMSF in methanol to modify carboxylate and serine residues. Enzyme activity was monitored over 0–12 min, and reaction arrested with 0.1 M borate buffer. Inactivation was analyzed based on time and reagent concentration. Control lacked DCHC and PMSF [11].

3.8. Determination of Tryptophan Environment

AP-I (5 μM) in 0.05 M citrate buffer (pH 4.6) was titrated with acrylamide, potassium iodide, and cesium chloride. Fluorescence at λ_{max} was recorded after each addition (5 μL for acrylamide and KI; 2 μL for CsCl) until quenching plateaued. Stern–Volmer and modified Stern–Volmer plots were used to calculate K_{sv} and the fraction of accessible tryptophan residues [10].

3.9. Estimation of Surface and Buried Tryptophan Residues

AP-I (5 μM) in 0.05 M citrate buffer (pH 4.6) was titrated with NBS (1.73–5.51 μM) in five 5 μL increments. Absorbance at 280 nm was measured after each addition, and 100 μL samples were withdrawn to assess enzyme activity. Surface-exposed tryptophans were estimated using an extinction coefficient of 5500 M^{-1} cm^{-1}. The same procedure was applied to enzymes denatured with 7 M guanidine hydrochloride to determine total tryptophan content, from which the accessible fraction was calculated [10].

3.10. Thermal and Chemical Denaturation Studies

Structural changes in AP-I induced by guanidine hydrochloride and elevated temperatures were monitored through enzyme activity and fluorescence spectroscopy [12].

4. Results and Discussion

Table 1 shows that NBS, PMSF, and DCHC treatments caused near-total loss of AP-I activity, indicating that tryptophan, serine, and carboxylate residues are critical for its active site.

Table 1. Effect of modifying agents on acid phosphatase activity.

Reagents (5 mM)	Amino Acid Modified	% Relative Activity (AP-I) (Minor Form)
NBS	Tryptophan	0.0
PMSF	Serine	0.0
DTNB	Cysteine	97.00
PG	Argenine	97.00
NAI	Tyrosine	95.00
DEPC	Histidine	92.38
DCHC	Carboxylate	09.00

4.1. pH Dependence of AP-I Activity

DCHC reduced AP-I activity by 90%, highlighting the role of a carboxylate residue with peak activity at pH 4.8. Lineweaver–Burk analysis across pH 3–6 using p-NPP gave pK_a values of 4.2 and 5.25 indicating involvement of carboxyl groups in catalysis (Figure 1).

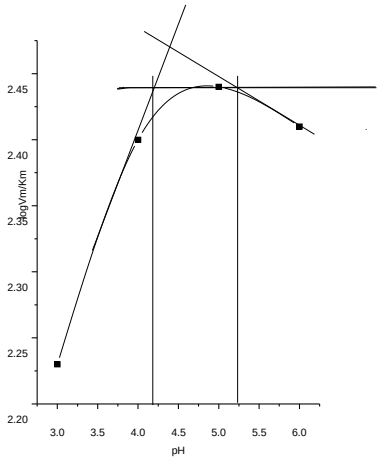


Figure 1. pH dependence of kinetic parameter.

4.2. Substrate Protection Studies

The loss of enzyme activity caused by modification of tryptophan (NBS), serine (PMSF), and carboxylate (DCHC) residues was significantly reduced when 2 mM substrate was added before the modification. Substrate protection results are summarized in Table 2.

Table 2. Substrate protection studies.

(1) For Tryptophan residue	
Control	**% Activity of AP-I**
Enz. + Sub.	100
Enz. + 20 mM NBS	29
Enz. + 2 mM Sub. + NBS	83
(2) For Serine residue	
Control	**% Activity of AP-I**
Enz. + Sub.	100
Enz. + 5 mM PMSF	27
Enz.+ 2 mM Sub. + PMSF	76
(3) For Carboxylate residue	
Control	**% Activity of AP-I**
Enz. + Sub.	100
Enz. + 1 mM DCHC	37
Enz.+ 2 mM Sub. + DCHC	87

4.3. Kinetics of Partially Modified Enzyme

Partial inactivation with NBS, PMSF, and DCHC reduced V_{max} without affecting K_m, indicating that tryptophan, serine, and carboxylate residues are involved in catalysis, not substrate binding.

Table 3. Kinetics of partially modified enzyme.

Residue Modified	Reagent Used	K_m (mM)	V_{max} (Units)
None	Unmodified	86.95	7.40
Carboxylate	DCHC	80.00	6.25
Tryptophan	NBS	83.30	5.30
Serine	PMSF	87.42	4.00

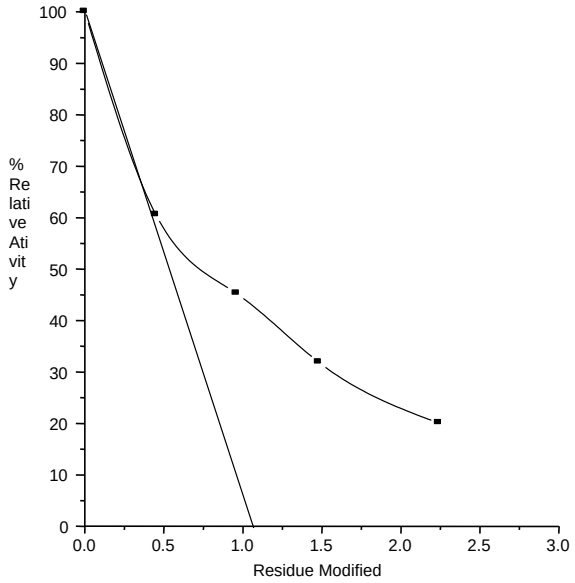


Figure 2. Plot of percent residual activity versus number of tryptophan residues modified in AP-I.

4.4. Modification of Carboxylate Residue

Modification of AP-I with DCHC indicates the involvement of carboxylate residues in catalysis. Inactivation was both time- and concentration-dependent, with a log K vs. log [DCHC] plot showing a slope of one, suggesting that binding of one mole of DCHC per enzyme leads to inactivation (Figure 3). Further evidence comes from pH-dependent kinetic studies: Lineweaver-Burk plots show constant K_m values for pNPP across pH 3.0–6.0, while V_{max} varies, indicating that substrate binding remains unaffected by pH, but catalytic efficiency changes.

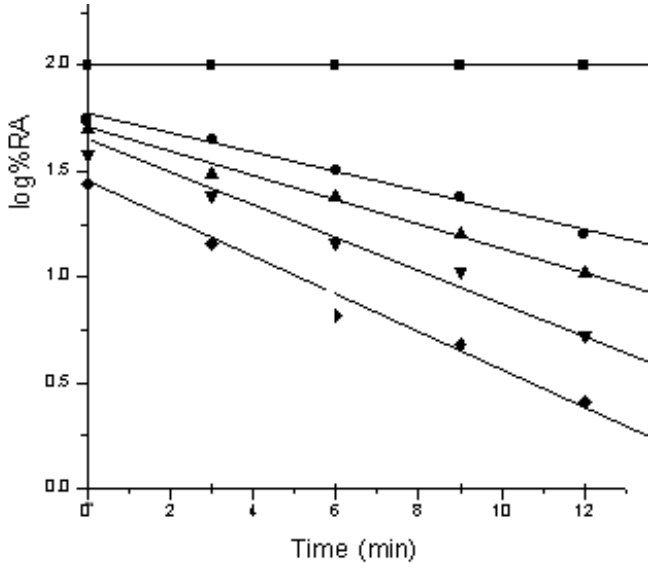


Figure 3. Kinetics of inactivation of AP-I by DCHC.

4.5. Fluorescence Measurements

Fluorescence studies showed Trp residues, buried in the hydrophobic core, contribute to AP-I emission (λ_{max} 310–313 nm). NBS quenched Trp fluorescence without shifting the peak, while pNPP partially protected against quenching, indicating tryptophan's involvement in the active site (Figure 4).

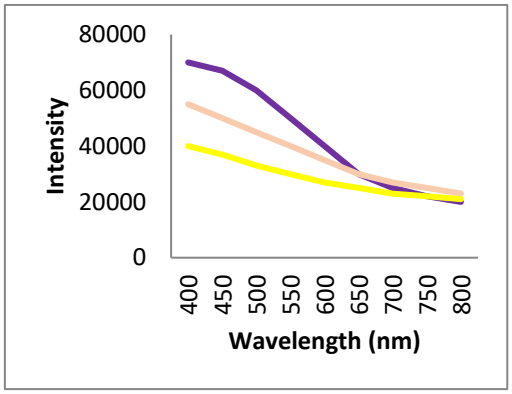


Figure 4. Fluorescence spectra of native enzyme AP-I in presence of NBS and enzyme in presence of substrate and NBS.

4.6. Determination of Tryptophan Environment by Quenching Experiments

Fluorescence characteristics of tryptophan residues depend upon microenvironment surrounding the tryptophan residues. Fluorescence quenching was observed only with acrylamide and cesium chloride as given below (Figures 5–7) indicating a strongly negatively charged environment supported by the modified Stern—Volmer plot (Figure 9).

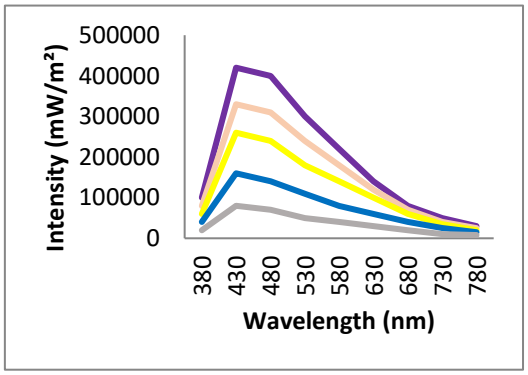


Figure 5. Spectra of Fluorescence quenching by acrylamide of AP-I.

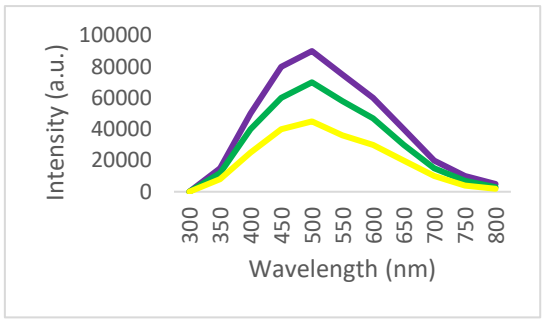


Figure 6. Spectra of Fluorescence quenching by cesium chloride.

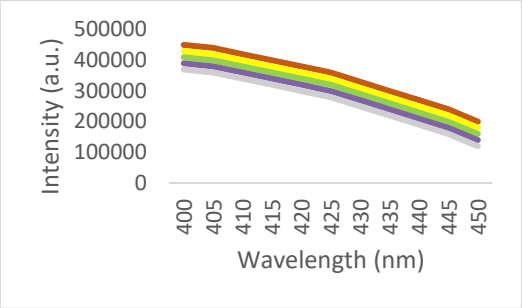
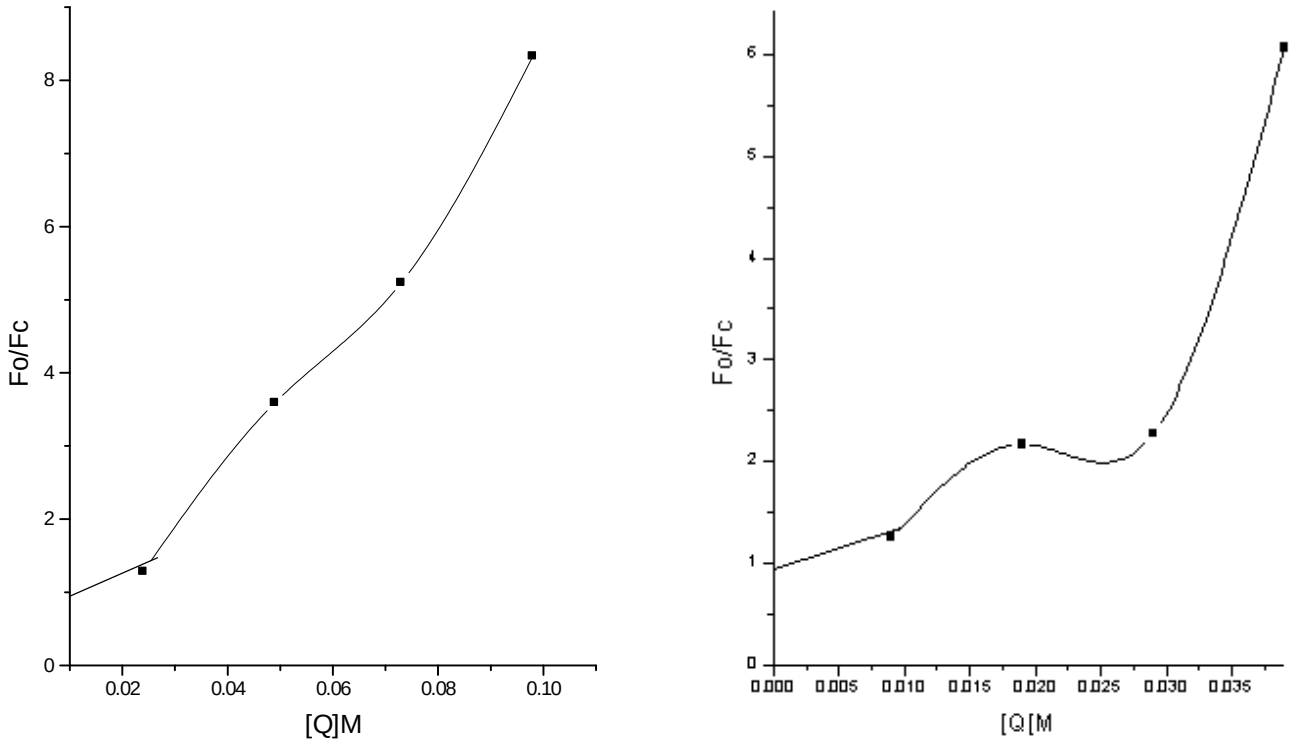


Figure 7. Spectra of Fluorescence quenching by potassium iodide.

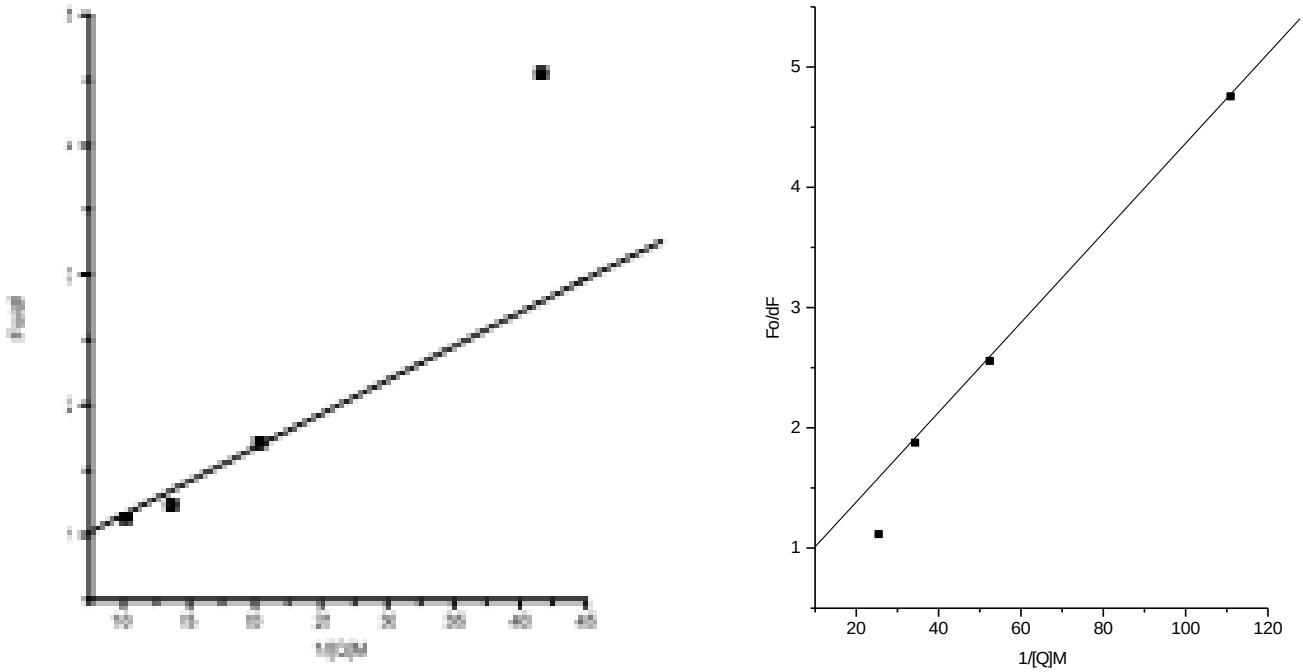


(**a**) Acrylamide (**b**) Cesium Chloride

Figure 8. Stern—Volmer plots of fluorescence quenching.

Table 4. Values of Stern—Volmer and modified Stern—Volmer constants for AP-I.

Constants	AP-I
Using acrylamide	
Kq	22.00
Fa	0.99
Using cesium chloride	
Kq	27.00
Fa	0.99
Using potassium iodide	
Kq	91.00
Fa	0.10



(**a**) Acrylamide (**b**) Cesium Chloride

Figure 9. Modified Stern—Volmer plots of fluorescence quenching studies of AP-I.

The upward curvature in Stern–Volmer plots (Figure 8) suggests both dynamic and static quenching, with CsCl showing two tryptophan populations—one initially quenched, followed by rapid quenching of the other. While full accessibility to acrylamide is common, the unusually high accessibility to Cs^+ ions further supports the presence of a negatively charged microenvironment.

4.7. Thermal and Chemical Denaturation Studies

Acid phosphatase underwent denaturation through heat stress and guanidine HCl–induced unfolding. Thermal denaturation results correlated with activity loss observed during temperature studies, where increasing temperature led to decreased fluorescence intensity (310–313 nm) with a red shift in emission maximum to 333 nm. Chemical denaturation with guanidine HCl caused a sharp drop in activity even at 0.2 M, highlighting the importance of carboxylate residues in catalysis. Titration of the denatured enzyme with NBS, monitored by absorbance decrease at 280 nm, was used to estimate total tryptophan content using an extinction coefficient of 5500 M^{-1} cm^{-1}. For AP-I, 4 tryptophan residues were modified—2 surface-exposed and 2 buried—indicating that denaturation exposes previously inaccessible residues.

5. Discussion

Distinct isoforms of *Erythrina indica* acid phosphatase persist after purification, suggesting genetic or post-translational origins [13]. Chemical modification identified a conserved catalytic triad—carboxylate, tryptophan, and serine essential for catalysis, consistent with findings in other plant phosphatases [14]. PMSF inhibition and substrate protection confirmed their direct catalytic role [15]. Fluorescence quenching studies indicated a strongly negatively charged environment around tryptophan residues. These features suggest form AP-I contribute to phosphate mobilization, with isoform diversity enhancing adaptability during seed germination and stress [16,17].

6. Conclusions

This study on *Erythrina indica* acid phosphatase AP-I revealed a conserved catalytic mechanism involving essential carboxylate, tryptophan, and serine residues. Selective modification and kinetic analysis confirmed their role in catalysis without affecting substrate binding. These findings enhance understanding of plant acid phosphatases and support future structural and applied research.

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