**Modeling of the thermal behaviour of acid phosphatase from breadfruit (*Artocarpus communis*) seeds: equilibrium model approach**

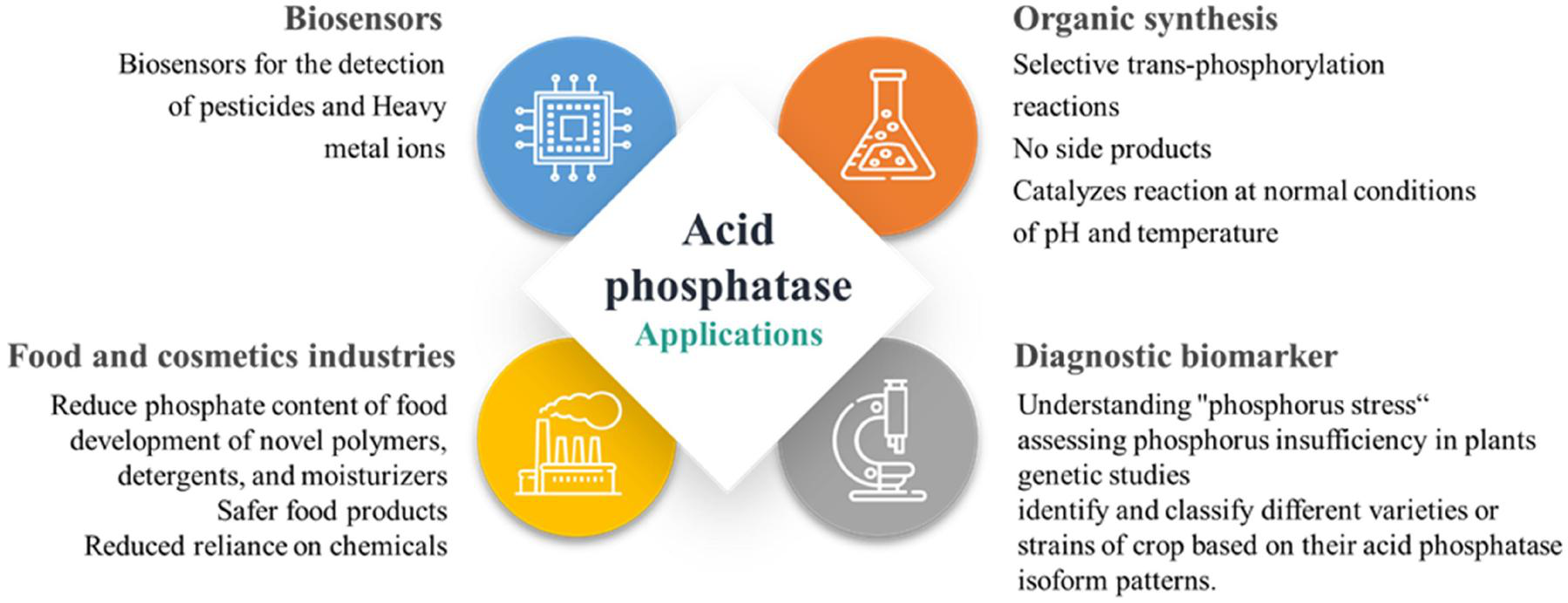
**Abstract**

Stabilization of enzymes is crucial to improve their durability and efficiency in various industrial applications. Thus, the search for new thermostable enzymes is a booming field. The seeds of *Artocarpus communis* are rich in acid phosphatases. Study of these enzymes could also be of interest in different biotechnological applications. Acid phosphatases are the enzymes that catalyze transphosphorylation reactions and promotes the hydrolysis of numerous orthophosphate esters in acidic media, as a crucial element for the metabolism of phosphate in tissues. The catalytic activity of Acid phosphatase from *Artocarpus communis* (*ACP*) seeds has been investigated using *p*-nitrophenylphosphate (*pNPP*) as substrate. Using the Equilibrium Model (*EQM*), the thermal inactivation data were analyzed. *ΔG\*act*, *ΔG\*inact*, *ΔHeq* and *Teq* were found to be (83.37 ± 0.02 kJ mol-1), (101.9 ± 0.2 kJ mol-1), (185 ± 2 kJ mol-1) and (326.90 ± 0.16 K) respectively. These results indicate that the enzyme is relatively stable in its native state, with the inactivation energy exceeding the catalytic energy.

***Keywords***:Acid Phosphatase; *Artocarpus Communis*; Thermodynamic Parameters; Equilibrium Model.

**1. INTRODUCTION**

Enzymes are highly valuable catalysts in sustainable chemistry due to their remarkable properties. Moreover, Enzymes are widely used in various industrial applications due to their efficiency, selectivity, and ability to accelerate reactions. Advances in enzymatic biotechnology have ushered in a new era of enzyme applications in industrial processes. However, enzymes face significant challenges as catalysts in industrial applications due to their solubility, limited reusability, and inherent instability (Mirsalami et al., 2024). Instability not only hinders the adoption of enzymatic reactions under standard conditions but also limits process optimization through strategies such as increasing reaction temperatures (Choi et al., 2023). *Artocarpus communis* is native to Oceania. 3000 years ago, the tree was domesticated for its edible fruit in the equatorial and tropical regions of the globe, including New Guinea, Indomalesia-Micronesia and the Samoan Islands in the 12th century (Zerega et al., 2006). Its economic importance stems from its use in agriculture, folk medicine, and natural products. The ethnopharmacological uses of *A. communis* include the treatment of malarial fever, diarrhea, and infection (Yang et al., 2021).*Artocarpus Communis* seeds contains interesting enzymatic machinery (Ahi et al., 2007). Among these enzymes are phosphatases (acid). Acid phosphatases are a group of enzymes that catalyzed the hydrolysis of several phosphate esters. Acid phosphatases are widely distributed in plants and animals. Proteases, glycosidases, lipases, phosphatases, and other enzymes belong to the main category of enzymes that hold great industrial significance. Enzyme-based phosphorylation technology is increasingly applied in various industries (Auriol et al., 2008; Babich et al., 2012). For example, acid phosphatase is used to reduce the phosphate content of caseins, which mainly consist of phosphoserine residues, thereby reducing phosphate toxicity (Sharma et al., 2023) In the food industry, acid phosphatases are used to produce phosphorylated substances used as flavor enhancers or nutritional supplements (Auriol et al., 2008). Furthermore, acid phosphatases find applications in the development of novel polymers, detergents, and moisturizers that incorporate cosmetic ingredients (Auriol et al., 2008). The applications of acid phosphatases in various fields are summarized in Fig. 1. Owing to their potential industrial applications, acid phosphatases have attracted considerable and sustained attention. Most of the current research works are either focused on finding new resources of acid phosphatases or improving the performance of those already known for more efficient use in biotechnological applications.The effect of heat treatment over a range of temperatures on Acid phosphatase from *A. communis* seeds was investigated (Gnanwa et al., 2014). Thermal inactivation of this enzyme, evaluated by loss in activity, was apparently followed by first-order kinetics. Activation energy was estimated to 65.405 kJ mol-1. According to (Konan et al., 2008) the same enzyme exhibits its maximum activity at pH = 5.5 and T = 328 K. All the results suggest that this acid phosphatase was relatively resistant to long heat treatments up to 333 K. These previous results are accepted in the present work. Gnanwa et al., 2014 investigated also the thermodynamic parameters which are among the important factors in the control of bioprocesses in biotechnology (Kannan et al., 2019; Najafpour 2015; Goswami & Stewart, 2016). They used the classic approach based on application of the well-known Arrhenius equation to describe the temperature dependence of enzyme activity. The weaknesses of this classical model have been mentioned several times in literature (Eisenthal et al., 2006; Peterson et al., 2004; Daniel & Danson, 2010; Daniel & Danson, 2013; Lee et al., 2013). Indeed, the Arrhenius law assumes that an increase in temperature leads to an increase in the reaction rate. But with regard to enzymes, this principle has its limits because beyond the temperature corresponding to maximum activity (optimum temperature) the process of denaturation of the enzyme is triggered, which leads to a decrease in the enzymatic activity of the catalyzed reaction rate. In short, the Arrhenius law only applies strictly to enzymes by influencing the activation phase of the enzymatic reaction (ascending part of the activity curve as a function of temperature). The equilibrium model (*EQM*) (Peterson et al., 2004; Daniel & Danson, 2013; Lee et al., 2013; Lee et al., 2007; Peterson et al., 2007) has been formulated to address these weaknesses and provide a complete and quantitative description of the effect of temperature on enzymes. In our recent works (Kambiré et al., 2021; Kambiré et al., 2022), this model has been used satisfactorily to analyze the thermal inactivation data of the β-glucosidase and β-galactosidase from *Rhynchophorus palmarum* larvae. In the present work, the effect of temperature on *A. communis* phosphatase (*ACP*) in presence of *p*-NitroPhenylPhosphate (*pNPP*), a synthetic substrate is evaluated. Then, experimental data are analyzed using *EQM*. The thermal inactivation data used in this work are taken from previous work undertaken by Gnanwa et al., 2014. The main purpose was to provide a set of reliable thermodynamic parameters which could be used for the enzyme optimization.



**Fig. 1.** Applications of acid phosphatases(Sharma et al., 2023).

**2. MATERIALS AND METHODS**

**2.1 Equilibrium model**

The model proposes that the active form of the enzyme (*E*act) is in reversible equilibrium with an inactive (but not denatured) form (*E*inact), and it is the inactive form that undergoes irreversible thermal inactivation to the thermally denatured state (*X*) ( Kambiré et al., 2021) for more details):

***k*inact**

***K*eq**

***E*act *E*inact *X*** **(1)**

where *K*eq is the equilibrium constant describing the ratio of *E*inact/*E*act, *k*inact is the rate constant for the *E*inact to *X* reaction.

Thus, the variation of enzyme activity with temperature, expressed as *V*max (maximum velocity of enzyme), is given by (Eq. (2)):

where *k*cat is the enzyme catalytic rate constant, *k*inact is the thermal inactivation rate constant, *t* is the assay duration, *E*0 is the total enzyme concentration, *K*eq is the equilibrium constant.Using the *EQM*, the quantitative expression of the dependence of rate on temperature and time can be described by the relationship presented below (Daniel & Danson, 2010; Lee et al., 2013):

:

where *kB* is Boltzmann constant (1.380510-23 J K-1), *h* is Planck constant (6.625610-34 J s), *T* is the absolute temperature and *R* is universal gas constant (8.314 J K-1 mol-1).*EQM* shows four thermal parameters, two well established parameters: *ΔG\*act* (Gibbs free enthalpy of the catalytic reaction) and *ΔG\*inact* (Gibbs free enthalpy of the thermal inactivation process), and two specific parameters: *ΔHeq* (enthalpic change associated with the reversible, temperature-driven interconversion of an enzyme between its active and inactive state or enthalpy of the equilibrium) and *T*eq (the temperature at the mid-point of transition between active and inactive forms of the enzyme or equilibrium temperature). *k*cat, *k*inact and *K*eq at a given temperature *T* are expressed by Eqs. (4), (5) and (6), respectively.

Using Eq. (3), it is possible to fit the experimental data for “zero time” (*i.e*., initial rates) to the Equilibrium model (Eq. (7)). In these conditions, the time-dependent thermal denaturation parameter, *ΔG\*inact* cannot be determined.

At *t* = 0,

**2.2 Determination of Equilibrium Model parameters**

It is possible to fit the experimental data for ‘zero time’ (i.e. initial rates) to the EQM to determine *ΔG\*act*, *ΔHeq* and *Teq*, although the time-dependent thermal denaturation parameter, *ΔG\*inact* cannot be determined. It should be noted that all the *EQM* parameters are derived using active enzymes in the presence of substrate. A stand-alone MATLAB® application (version 7.1.0.246 [R14] Service Pack 3; The Mathworks, Inc.), enabling the facile determination of the *EQM* parameters from a Microsoft® Office Excel spreadsheet of experimental progress curves is available. This application is suitable for computers running Microsoft® Windows *XP* and is for non-commercial research purposes only (Daniel et al., 2008).

**2.3. Non-linear regressions**

Non-linear regressions are done using Sigma Plot version 15.0 software with iterations number of 200, step size and tolerance equal to 1 and 10-12, respectively. 3D plot has been also done by the same software.

**3. RESULTS AND DISCUSSION**

The initial rate *vs*. temperature obtained from thermal inactivation study is fitted satisfactorily using *EQM* (R2 = 0.9821) as showed in Fig. 2. The fit leads to values of *ΔHeq*, *ΔG\*act* and *T*eq (Table 1) used as initial values during the complete optimization (rate *vs*. time *vs*. temperature). Final optimized parameters are listed in Table 2. The high correlation observed (R2 = 0.9828) proves that EQM is suitable for modeling the thermal response of *ACP*. 3D plot of rate *vs*. temperature *vs*. time generated from the obtained parameters is shown in Fig. 3. The value of *ΔG\*cat*is 83.37±0.02 kJ mol-1**,** while *ΔG\*inact* is 101.9 ± 0.2 kJ mol-1. The intrinsic parameters of the equilibrium model, ***ΔHeq***and ***Teq***, have respective values of 185 ± 2 kJ mol-1 and 326.90 ± 0.16 K**.** Given the relatively **high Gibbs free energy** value for the thermal inactivation process (*ΔG\*inact* = 101.9 ± 0.2 kJ mol-1) compared to other enzymes (Lee et al., 2007; Daniel et al., 2008), it can be inferred that acid phosphatase from *A. communis* (***ACP*) exhibits high thermal stability**. This means that a substantial amount of energy is required to cause the denaturation or inactivation of the enzyme. Furthermore, biocatalysts with a high *ΔG\*inact* are often preferred in industrial processes requiring harsh thermal conditions, as they retain their activity for a longer time at elevated temperatures. This is advantageous for various biotechnological and industrial applications. Therefore, the equilibrium temperature (*T*eq) can help to discriminate. Additionally, considering the **equilibrium temperature (*Teq* =** 326.90 ± 0.16 K**), *ACP*** can be classified as a **mesophilic enzyme**, meaning it operates at **moderate temperatures**, close to its optimum.The Gibbs energy of the catalytic reaction (*ΔG\*act*) of *ACP* (83.37 ± 0.02 kJ mol-1) which is close to those of acid phosphatase from Wheat germ (79 kJ mol-1) (Daniel et al., 2008) is among the highest available in literature.Fig. 3 shows the evolution of the enzymatic reaction rate as a function of temperature and assay duration. A variation of the enzymatic activity as a function of temperature is observed. An optimum temperature zone seems to exist around 315-330 K, where the enzymatic activity is the highest. Beyond this optimum temperature, the reaction rate decreases, which may be due to a progressive thermal inactivation of the enzyme. At the beginning (short time), the enzymatic activity is higher, indicating that the enzyme is fully functional. Over time, the activity gradually decreases, suggesting a denaturation or thermal inactivation of the enzyme. The equilibrium temperature is often defined as the temperature where the enzyme maintains a stable catalytic activity before its inactivation. The stability seems stronger around 315-330 K, where the enzymatic activity is maintained for a longer time.

**Table 1.** *EQM* parameters of the inactivation kinetics for *ACP* (at *t = 0*)

|  |  |  |
| --- | --- | --- |
| Parameters |  | Initial rates  (Eq. (7)) |
| *ΔG\*act* (kJ mol-1) |  | 82.620 ± 0.135 |
| ***ΔHeq*** (kJ mol-1) |  | 170.54 ± 3,74 |
| *T*eq (K) |  | 324.37 ± 0.66 |
| *T*opt (K)a |  | 321.43 ± 0.13 |
|  |  |  |

aGraphical optimum temperature of the enzyme at time zero.

**Table 2.** *EQM* parameters of the inactivation kinetics for *ACP* (at t > 0)

|  |  |  |
| --- | --- | --- |
| Parameters |  | Entire time course (Eq. (3)) |
| *ΔG\*act* (kJ mol-1) |  | 83.37 ± 0.02 |
| *ΔG\*inact* (kJ mol-1) |  | 101.9 ± 0.2 |
| ***ΔHeq*** (kJ mol-1) |  | 185 ± 2 |
| *T*eq (K) |  | 326.90 ± 0.16 |
| ***ΔSeq*** (J K-1 mol-1)a |  | 559.1 ± 12.5 |

aEquilibrium entropy deduced from *T*eq and ***ΔHeq***.



*T*opt

**Fig. 2.** The effect of temperature on the initial (zero-time) rate of reaction  
of *ACP* : EQM (Eq. (7)) compared with experimental data.



**Fig. 3**. 3D plots of rate *versus* time *versus* temperature obtained by *EQM* (solid lines) for acid phosphatase from *A. communis* surperimposed with experimental data (full black circles).

**4. CONCLUSION**

The thermal behaviour of acid phosphatase from *Artocarpus communis* (*ACP)* has been performed using a synthetic substrate (*pNPP*) in the temperature range 303 - 353 K. This work has provided for the first-time thermal information of *ACP* using Equilibrium model (*EQM*). Our results showed that this model is suitable for modeling the thermal inactivation of the enzyme. However, further work using continuous assay supposed to give more accurate results, should be undertaken to validate the present results. The set of thermodynamic parameters obtained in this work can be useful for its possible biotechnological applications.

**COMPETING INTERESTS DISCLAIMER**:

Authors have declared that they have no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

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