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Kinetic and thermodynamic parameters of four acid phosphatases from edible seeds of oleaginous cucurbit *Lagenaria siceraria* (Molina) Standl.

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ABSTRACT

In Sub-Saharan Africa, *Lagenaria siceraria* is an oleaginous cucurbit widely consumed for its edible seeds. These seeds were previously categorized as good enzyme sources, including four non-specific acid phosphatases isolated and designated as B*Ls*AP1, B*Ls*AP2, R*Ls*AP1, and R*Ls*AP2. In this study, we investigate on the kinetic and thermodynamic characteristics of these biocatalysts in order to evaluate their thermostability. Thermal inactivation was carried out in the temperature range of 55°C to 80°C from 5 to 60 minutes. The results revealed that thermal inactivation of studied acid phosphatases follows first order kinetics. At their optimum temperatures, these enzymes showed high half-lives ranging from 169.06 to 495.10 minutes and *D* values from 561.71 to 1,645.03 minutes. Moreover, they exhibited high activation energies (from 155.08 to 200.55 kJ/mol) and average enthalpy values (from 152.23 to 197.74 kJ.mol^{-1}) suggesting their good thermostability. The comparison of all these values revealed that the two acid phosphatases (R*Ls*AP1 and R*Ls*AP2) from the round-fruited cultivar of *L. siceraria* showed better thermal stability than those (B*Ls*AP1 and B*Ls*AP2) from the blocky-fruited cultivar.

1. INTRODUCTION

Acid phosphatases (orthophosphoric-monoester phosphohydrolase, EC 3.1.3.2) are well known for hydrolysis of phosphomonoesters [1]. These enzymes exhibited many biological roles as metabolism regulation, cellular signal transduction pathways [2], and defence against herbivorous insects [3]. The action of acid phosphatases on phosphorylated compounds transforms organic phosphate into a soluble inorganic form, available for many cellular functions in living organisms [4,5]. Knowing the importance of inorganic phosphate in plants, animals, and microorganisms growth, we would understand why phosphatases are widespread in nature [6]. Indeed, phosphorus is an essential element in all metabolic processes and in synthesis of nucleic acids and membranes [7].

Like most biocatalysts, acid phosphatases are applied as valuable tools for various biotechnological and bioindustrial purposes [8]. Their uses in environmental bioremediation of polluted soils (e.g., removal of heavy metals) has been reported by several studies [9–11]. In the context of sustainable agriculture, phosphatases could increase soils fertility by phosphate solubilization, and thus, minimize the chemical fertilizer application [12]. Moreover, phytate-specific phosphatases called phytases were added to the diet of monogastric animals to improve phosphate absorption from phytate (myo-inositol hexakisphosphate) which is the major storage form of phosphate in plant seeds [13]. Another key area of acid phosphatases interest in biotechnology is their exploitation to produce phosphoconjugates as nucleotides. These nucleotides are highly sought after in the food and pharmaceutical industries for their taste and flavor potentialities [14–16]. However, temperature is a key variable in bioprocesses using enzymes [17]. Due to the fact that many industrial processes require stable and sturdy biocatalysts, it is essential to know their thermal stability and thermodynamic properties. According to Ward and Moo [18], temperature is the best optimized physical variable in chemical reactions. Generally, thermostable enzymes have several advantages in terms of strength, reactivity, and process yield. Hence, this could translate the ability

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of enzyme to maintain maximum activity over time, since enzyme stability represents a critical point in most bioprocesses [19,20].

To our knowledge, despite the various applications mentioned above, very few reports detailed thermodynamic characterization and thermal stability of acid phosphatases. In this paper, we report on the thermostability parameters of non-specific acid phosphatases B*Ls*AP1, B*Ls*AP2, R*Ls*AP1, and R*Ls*AP2 [21,22], isolated from edible seeds of *Lagenaria siceraria* (Molina) Standl., an oleaginous cucurbit largely consumed in Sub-Saharan Africa [23].

2. MATERIALS AND METHODS

2.1. Enzymes

The phosphatases B*Ls*AP1, B*Ls*AP2, R*Ls*AP1, and R*Ls*AP2 (EC 3.1.3.2) originated from the purified enzyme collection of "Laboratoire de Biotechnologies, UFR Biosciences, Université Felix Houphouët-Boigny (Abidjan, Côte d'Ivoire)." B*Ls*AP1 and B*Ls*AP2 were isolated from seeds of *L. siceraria* blocky-fruited cultivar, while R*Ls*AP1 and R*Ls*AP2 were isolated from seeds of the round-fruited cultivar as described previously by Koffi et al. [21,22].

2.2. Enzyme assay

The acid phosphatase activity was carried out in a total volume of 250 µl, containing 100 mM sodium acetate buffer (pH 5.6), substrate (*p*NPP, 5 mM), and the enzyme solution (25 µl). The reaction mixture was incubated at 37°C for 10 minutes. Then, 2 ml of Na_2CO_3 2% (w/v) were added to stop the reaction and absorbances were measured at 410 nm by using a spectrophotometer GENESIS 5. *para*-Nitrophenol (*p*NP) was used as standard.

One unit of activity was defined as the amount of enzyme that hydrolyzes 1 µmol of substrate per min under the enzyme assay conditions. The specific activity was expressed as a unit of activity per mg of protein.

2.3. Protein estimation

Protein concentrations and elution profiles from chromatographic columns were determined by the Folin method [24]. Bovine serum albumin (BSA) was used as the standard protein.

2.4. Thermal Inactivation

The thermal inactivation of each purified acid phosphatase was determined at various constant temperatures from 55°C for R*Ls*AP2 (its optimum temperature) and 60°C for B*Ls*AP1, B*Ls*AP2, and R*Ls*AP1 (their optimum temperature) to 80°C. Enzymes in sodium acetate buffer (pH 5.6) were exposed to each temperature for a period of 5 to 60 minutes. Then, aliquots were withdrawn at intervals (5 minutes) and immediately cooled at 4ºC for 20 minutes. Residual activities, determined at 37°C under the standard enzyme assay conditions, were expressed as percentage of activity of zero-time control of untreated enzymes.

2.5. Residual activity

$$\text{Residual activity (\%)} = (A_t / A_0) \times 100 \qquad (1)$$

where A and A_0 are the activities of the treated and untreated enzyme solution, respectively.

2.6. Kinetic data analysis

2.6.1. Denaturation constants of the enzyme fraction k

Thermal inactivation of each phosphatase can be described by a first-order kinetic model [25]. The integral effect of an inactivation process at constant temperature, where the inactivation rate constant is independent of time, is given in Equation /(1) as follows:

$$A_t/A_0 = e^{-kdt} \qquad (2)$$

$$\ln (A_t/A_0) = -kt \text{ (first-order kinetics)}$$

where A_t is the residual enzyme activity at time t (minutes), A_0 is the initial enzyme activity, k (minute^{-1}) is the inactivation rate constant at a given condition. k values were obtained from the regression line of ln (A_t/A_o) versus time as slope.

2.6.2. Determination of half-life of the enzyme solution $t_{1/2}$

The $t_{1/2}$ was obtained from the following equation:

$$t_{1/2} = \ln 2/k \qquad (3)$$

2.6.3. Calculation of activation Energy Ea

The *Ea* was obtained from the Arrhenius equation:

$$\ln (k) = Ae^{-EaR/T} \qquad (4)$$

where k is the first-order rate constant of thermal inactivation of the enzyme activity, obtained through the slopes of the plots described above.

2.6.4. Calculation of D values of the enzyme fractions

D-value is defined as the time needed, at a constant temperature, to reduce the initial enzyme activity (A_0) by 90 % (or decimal reduction time or time required to preincubate the enzyme at a given temperature to maintain 10% residual activity). For first-order reactions, the *D*-value is directly related to the rate constant *k* (Eq. 2) [26,27]:

$$D = \ln (10/k_d) = 2.303/k \qquad (5)$$

2.6.5. Calculation of z values of the enzyme fractions

The *z* value (°C) is the temperature increase needed to induce a 10-fold reduction in *D* value [26]. This *z* value follows the Eq. (6):

$$\log (D1/D2) = (T2 - T1)/z \qquad (6)$$

where *T*1 and *T*2 are the lower and higher temperatures in °C or K. Then, *D*1 and *D*2 are *D* values at the lower and higher temperatures in minute, respectively.

The *z* values were determined from the linear regression of log (*D*) and temperature (T).

2.6.6. Thermodynamic parameters

The enthalpy of reaction ($\Delta H^{\#}$), which is the amount of energy required to bring the enzyme to the activated state for the subsequent denaturation at a given temperature, Gibbs free energy ($\Delta G^{\#}$) and the entropy ($\Delta S^{\#}$) were calculated through the following equations [28,29].

$$\Delta H^{\#} = Ea - RT \qquad (7)$$

where $\Delta H^{\#}$ = Change in enthalpy; Ea = activation energy of inactivation; R = general gas constant; T = temperature.

$$\Delta G^{\#} = -RT \times \ln\left(k \times h / K_b \times T\right) \qquad (8)$$

where $\Delta G^{\#}$ = free energy change of inactivation; h = Plank's constant; K_b = Bolztman constant.

$$\Delta S^{\#} = (\Delta H^{\#} - \Delta G^{\#}) / T \qquad (9)$$

$\Delta S^{\#}$ = Change in entropy.

3. RESULTS AND DISCUSSION

As reported by Koffi et al. [21,22], acid phosphatases purified from *L. siceraria* seeds were mesophilic enzymes. Thus, B*Ls*AP1, B*Ls*AP2, and R*Ls*AP1 showed maximal hydrolytic activities at 60°C, while R*Ls*AP2 has an optimum activity at 55°C. The present study shows that when submitted to heat treatment, these enzymes are completely inactivated between 75°C and 80°C after 30 minutes (data not shown). It is well known that enzymes are proteins with three-dimensional structure which are essential for their activities [30]. It seems that after a long-term exposure to heat, this enzyme three-dimensional structure was destabilized and then, inactivated. So, a biocatalyst capable to resist against thermal unfolding in absence of substrate is categorized as thermostable, while an enzyme molecule able to catalyze reactions (that means substrate presence) at high temperatures (beyond 70°C) is qualified of thermophilic [31].

The thermal inactivation of the four studied acid phosphatases represented in Figure 1 shows that these enzymes followed a first-order kinetic. This is explained by the logarithmic linear relationship between the phosphatase activities and time of treatment for the temperatures ranged from 55°C to 80°C (Fig. 1). Similar results have been reported for α-amylases from sprouted pearl millet [32]. Moreover, this linear appearance for the graph ln (A_t/A_0) suggests that each reaction mixture contains only one enzyme that catalyzes the substrate hydrolysis. Indeed, the combined activities of several phosphatases (in the same reaction mixture) would have resulted in a curve with many phases instead of a straight line.

From the slope of the graphs in Figure 1, the inactivation rate constants (k) were estimated. Then, k values and half-life ($t_{1/2}$) of studied acid phosphatases are presented in Table 1. These results show that k values increase with increasing pre-incubation temperature for the four enzymes. This confirms the instability of these enzymatic proteins at high temperatures as reported by Marangoni [33]. This author argues that higher value of k constant means that the enzyme was less thermostable. As concerned the half-life of acid phosphatases from *L. siceraria* seeds, they appear to be too longer at optimum temperatures, then they decrease beyond. At their optimum temperatures, acid phosphatases B*Ls*AP1, B*Ls*AP2, R*Ls*AP1, and R*Ls*AP2 showed half-lives of 169.06, 223.60, 495.11, and 495.10 minutes, respectively. This result suggests that acid phosphatases R*Ls*AP1 and R*Ls*AP2 isolated from the round-fruited cultivar of *L. siceraria* were more thermally stable than those from the blocky-fruited cultivar seeds. However, the half-lives found in this study were higher compared to half-life obtained for beta-glucosidase from land crab (*Cardisoma armatum*) digestive juice which was of 169.02 minutes at 60°C [34].

Figure 2 depicts the effect of temperature on D values for inactivation of acid phosphatases isolated from *L. siceraria* edible seeds. The D values, which are the time required to reduce the enzyme activities by 90%, corroborate results obtained for half-life. Indeed, for the four studied acid phosphatases, D values decreased very sharply with increasing temperature. This

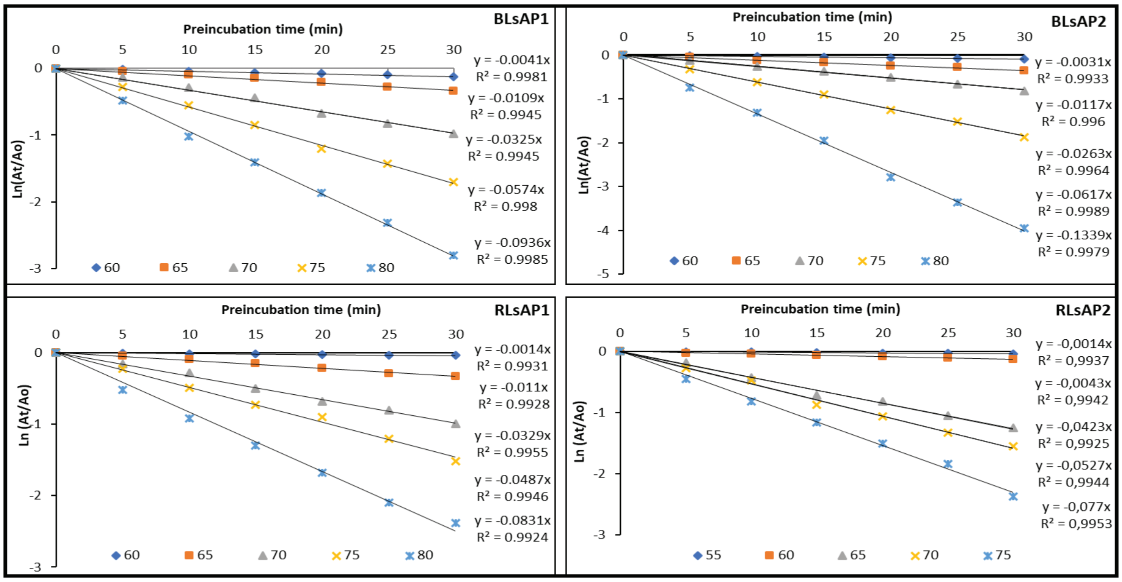

Figure 1: First order thermal inactivation of acid phosphatases B*Ls*AP1, B*Ls*AP2, R*Ls*AP1, and R*Ls*AP2 isolated from *L. siceraria* seeds at different temperatures. A_0 is the initial enzymatic activity and A_t the activity at each holding time.

Table 1: *k* values and half-lives of acid phosphatases B*Ls*AP1, B*Ls*AP2, R*Ls*AP1, and R*Ls*AP2 isolated from *L. siceraria* seeds.

Temperature (°C)	Phosphatases							
	B*Ls*AP1		**B*Ls*AP2**		**R*Ls*AP1**		**R*Ls*AP2**	
	$t_{1/2}$ (minute)	*k* (minute^{-1})	$t_{1/2}$ (minute)	*k* (minute^{-1})	$t_{1/2}$ (minute)	*k* (minute^{-1})	$t_{1/2}$ (minute)	*k* (minute^{-1})
55	nd	nd	nd	nd	nd	nd	495.10	0.0014
60	169.06	0.0041	223.60	0.0031	495.11	0.0014	161.20	0.0043
65	63.59	0.0109	59.24	0.0117	63.01	0.011	16.39	0.0423
70	21.33	0.0325	26.36	0.0263	21.07	0.0329	13.15	0.0527
75	12.08	0.0574	11.23	0.0617	14.23	0.0487	9.00	0.077
80	7.41	0.0936	5.18	0.1339	8.34	0.0831	nd	nd

nd: not determined.

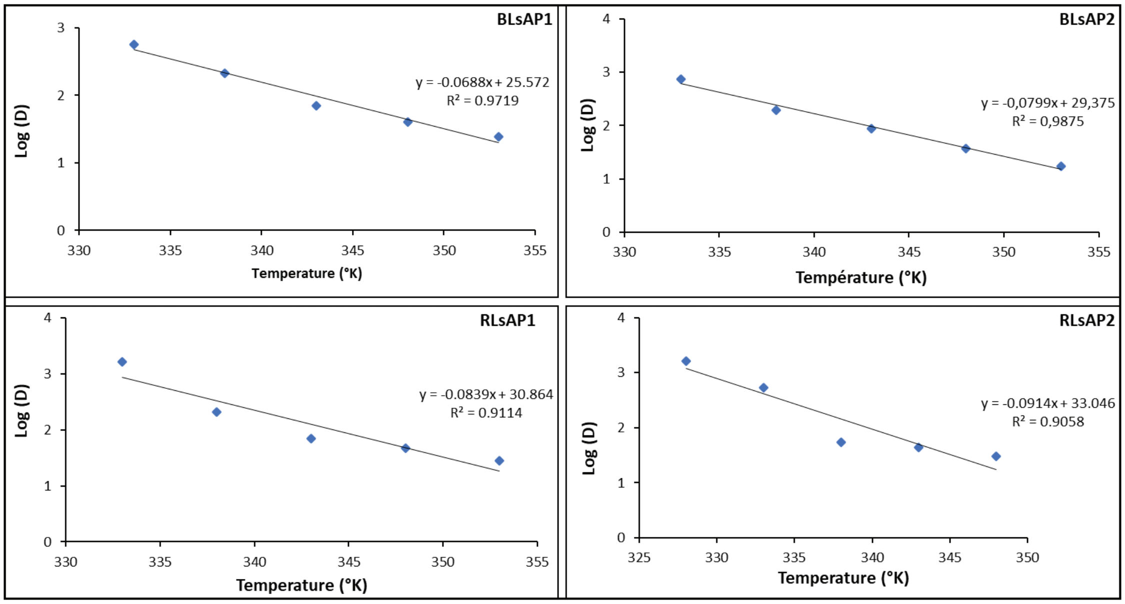

Figure 2: Effect of temperature on *D* values for inactivation of acid phosphatases B*Ls*AP1, B*Ls*AP2, R*Ls*AP1, and R*Ls*AP2 isolated from *L. siceraria* seeds.

is the proof that the inactivation time of the studied enzymatic molecules is shortened rapidly at high temperatures. As shown in Table 2, at their optimum temperature, *D* values obtained were of 561.71, 742.90, 1645.00, and 1645.03 minutes for acid phosphatases B*Ls*AP1, B*Ls*AP2, R*Ls*AP1, and R*Ls*AP2, respectively. It is noteworthy that at 60°C, the *D* value for R*Ls*AP1 inactivation was, respectively, 2.93 and 2.21 times higher than the corresponding value for inactivation of B*Ls*AP1 and B*Ls*AP2. This indicates probably the higher thermal stability of R*Ls*AP1. Otherwise, for this acid phosphatase, the decimal time is reduced about 8-fold when the optimal temperature increases by 5°C (from 1,645.00 to 209.36 minutes). *D* values (ranged from 258.7 ± 11.4 minutes to 0.41 ± 0.05 minutes in buffer solution) reported for alkaline phosphatase (EC 3.1.3.1) from calf intestine were lower than those obtained in this study [35].

The temperature resistance values (temperature increase needed for a 10-fold decrease of *D*) were calculated and results are shown in Table 2. The corresponding *z* values were 14.53°C, 12.52°C, 11.92°C, and 10.94°C for the inactivation of acid phosphatases B*Ls*AP1, B*Ls*AP2, R*Ls*AP1, and R*Ls*AP2, respectively. These results are consistent with values obtained for alkaline phosphatases in raw milk (11.8°C) by Fadiloğlu et al. [36] and in whole milk (12.27°C) by Stănciuc et al. [35]. Previously, Blel et al. [37], Claeys [38], Levieux et al. [39], and Marchand et al. [40] reported *z* values ranging from 5°C to 8°C (for thermal inactivation of alkaline phosphatases) lower than *z* values obtained in this study.

The slopes of Arrhenius plots in Figure 3 allow us to calculate the activation energy (*Ea*) of each enzyme. Activation energy is a minimum amount of energy required for the native enzyme in order to initiate the inactivation or denaturation process. This activation energy can be seen as the energy barrier that molecules need to cross to be able to react [41]. Beyond the activation energy, the enzyme is denatured and cannot refold to the native form as reported by Siddiqui et al. [28]. The *Ea* values of acid phosphatases from *L. siceraria* seeds were ranged from 155.08

(for B*Ls*AP1) to 200.55 kJ/mol (for R*Ls*AP2) (Table 2). For example, it means that for acid phosphatase B*Ls*AP1, it was necessary to absorb 155.08 kJ from the external medium to start its inactivation at temperatures between 60°C and 80°C. These values were higher than those found (92.4 and 81.243 kJ/mol) for α-amylases from sprouted pearl millet [32]. The relative high values obtained for activation energies suggest that important energy amounts must be supplied to the studied enzyme in order to initiate the denaturation process. It means that these acid phosphatases are relatively thermostable.

The activation energy value enabled the determination of enthalpy, entropy, and free energy of activation of the four enzymes as shown in Table 3. Enthalpy ($\Delta H^{\#}$) is the total amount of energy required to bring the enzyme from the ground state to the activated intermediate. In this study, enthalpy values ($\Delta H^{\#}$) decreased as the temperature increased (Table 3). The obtained $\Delta H^{\#}$ average values were 152.23, 177.04, 186.82, and 197.74 kJ.mol^{-1} for B*Ls*AP1, B*Ls*AP2, R*Ls*AP1, and R*Ls*AP2, respectively. According to Lai and Tabatabai [42], a greater amount of stretching, squeezing, or breaking of chemical bonds required to reach the transition state corresponds to a larger value of $\Delta H^{\#}$. Among the four studied acid phosphatases, it appears that R*Ls*AP2 (which shows the much higher $\Delta H^{\#}$) would undergo the most conformational change during thermal inactivation as reported by Marin et al. [43]. Being well known that $\Delta H^{\#}$ and *Ea* are directly related, the high enthalpy ($\Delta H^{\#}$) change for R*Ls*AP1 and R*Ls*AP2 indicates that these enzymes need greater energy for their thermal denaturation [44]. So, the positive value of the enthalpy change ($\Delta H^{\#}$) would suggest the endothermic nature of these enzymatic reactions.

Table 2: *D*, *Z* and *Ea* values for thermal inactivation of phosphatases B*Ls*AP1, B*Ls*AP2, R*Ls*AP1, and R*Ls*AP2 from *L. siceraria* seeds.

***D* values (minute)**	**Phosphatases**			
	B*Ls*AP1	**B*Ls*AP2**	**R*Ls*AP1**	**R*Ls*AP2**
D_{55}	nd	nd	nd	1,645.03
D_{60}	561.71	742.90	1,645.00	535.58
D_{65}	211.28	196.84	209.36	54.44
D_{70}	70.86	87.57	70.00	43.70
D_{75}	40.12	37.33	47.29	29.91
D_{80}	24.60	5.18	27.71	nd
z values (°C)	14.53	12.52	11.92	10.94
Ea (kJ/mol)	155.08	179.89	189.67	200.55

nd: not determined.

The positive values of the obtained entropy ($\Delta S^{\#}$) and free energy ($\Delta G^{\#}$) corroborate the fact that the studied acid phosphatases underwent a large change in conformation [35]. Activation entropy ($\Delta S^{\#}$) is defined as the degree of disorder in the transition complex, while Gibbs activation free energy ($\Delta G^{\#}$) represents the spontaneity of a reaction and the energy required for the enzyme-substrate complex to breakdown into a catalytic reaction product [29]. the obtained $\Delta S^{\#}$ values of acid phosphatases from *L. siceraria* seeds were ranged from 175.34 (for B*Ls*AP1) to 313.35 J.mol^{-1}.K^{-1} (for R*Ls*AP2) (Table 3). The positive values obtained for entropy changes ($\Delta S^{\#}$) would translate a high disorder at the transition state, which is not the case in the native state the native state. Also, it would indicate that aggregation processes are not significant.

The Gibbs free energy of activation ($\Delta G^{\#}$) represents a good index to measure stability of biocatalyst [45]. The reaction is said spontaneous when the Gibbs free energy is low or negative. In this study, positive $\Delta G^{\#}$ values obtained imply that thermal inactivation reactions of acid phosphatases from *L. siceraria* were not spontaneous. So, the decrease of $\Delta G^{\#}$ for all studied acid phosphatases with increasing temperature suggests lower stability at high temperature [46,47].

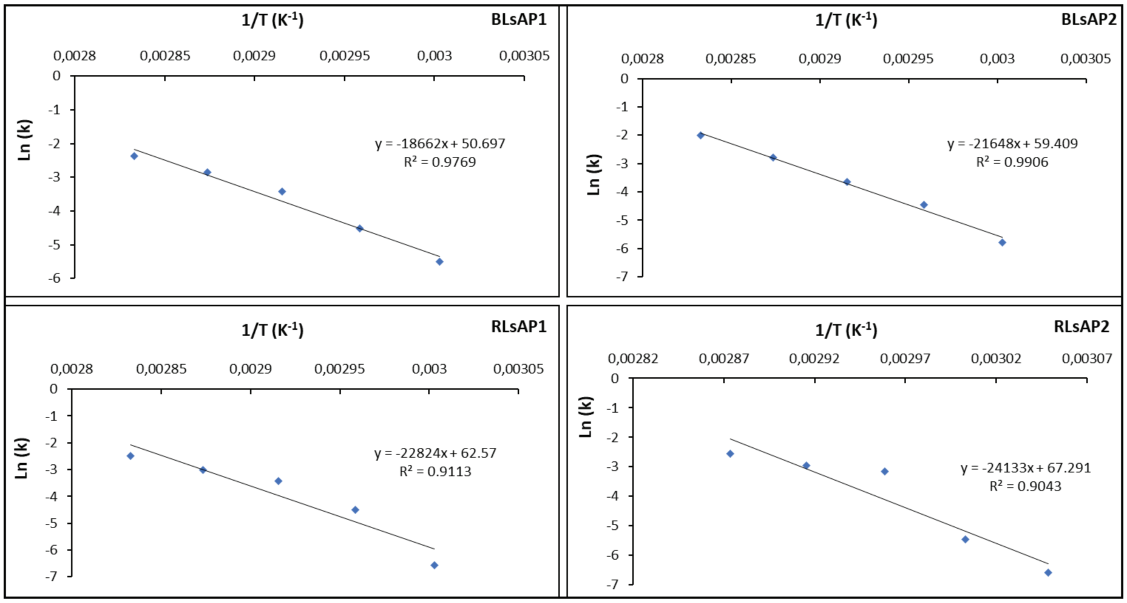

Figure 3: Arrhenius plot for thermal inactivation of acid phosphatases B*Ls*AP1, B*Ls*AP2, R*Ls*AP1, and R*Ls*AP2 isolated from *L. siceraria* seeds.

Table 3: Thermodynamic parameters of acid phosphatases B*Ls*AP1, B*Ls*AP2, R*Ls*AP1, and R*Ls*AP2 from *L. siceraria* seeds.

Thermodynamic parameters	Phosphatases	Temperature (°C)						Means
		55	60	65	70	75	80	
$\Delta H^{\#}$ (kJ.mol^{-1})	B*Ls*AP1	nd	152.31	152.27	152.23	152.20	152.15	152.23
	B*Ls*AP2	nd	177.13	177.09	177.05	177.00	176.96	177.04
	R*Ls*AP1	nd	186.90	186.86	186.82	186.78	186.73	186.82
	R*Ls*AP2	197.82	197.78	197.74	197.69	197.65	nd	197.74
$\Delta S^{\#}$ (J.mol^{-1}.K^{-1})	B*Ls*AP1	nd	175.58	175.46	175.33	175.21	175.10	175.34
	B*Ls*AP2	nd	247.98	247.85	247.73	247.61	247.49	247.73
	R*Ls*AP1	nd	274.25	274.12	274.00	273.88	273.76	274.00
	R*Ls*AP2	313.60	313.48	313.35	313.23	313.11	nd	313.35
$\Delta G^{\#}$ (kJ.mol^{-1})	B*Ls*AP1	nd	93.85	92.97	92.09	91.21	90.34	92.09
	B*Ls*AP2	nd	94.55	93.31	92.07	90.83	89.60	92.07
	R*Ls*AP1	nd	95.58	94.21	92.84	91.47	90.10	92.84
	R*Ls*AP2	94.96	93.39	91.82	90.26	88.69	nd	91.82

nd: not determined.

4. CONCLUSION

Investigation on thermal inactivation of acid phosphatases from *L. siceraria* seeds indicated a first-order kinetics for the four enzymes. They showed high activation energies (*Ea*) and change in enthalpy ($\Delta H^{\#}$) suggesting that these acid phosphatases are relatively thermostable since they need important energy amounts before triggering the inactivation process. The comparison of the rate constants, half-lives, and *D* values revealed a better thermostability of the two acid phosphatases (R*Ls*AP1 and R*Ls*AP2) isolated from the round-fruited cultivar of *L. siceraria*.

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