Journal of Advances in Biology & Biotechnology

24(6): 46-62, 2021; Article no.JABB.72147
ISSN: 2394-1081

Evaluation of Inactivation Kinetics and Thermodynamic Properties of Peroxidase from *Cucumis sativus* for Biotechnological and Industrial Applications

Iweha Christian Chukwunyere[1], Ugwoke Faith Ifeanyi[1,2], Ogara Lydia Amaechi[3], Nweze Justus Amuche[3], Ogbonna Kenneth Ejike[3], Chinedum Patricia Eluka[1], Akor Joseph[3*], Odu Maureen Nwamaka[4] and Joshua Parker Elijah[1]

[1]Department of Biochemistry, Faculty of Biological Sciences, University of Nigeria, Nsukka, Enugu State, Nigeria.
[2]Uburu Bioresources Development Center, National Biotechnology Development Agency, Abuja, Nigeria.
[3]Department of Science Laboratory Technology, Faculty of Physical Sciences, University of Nigeria, Nsukka, Enugu State, Nigeria.
[4]Department of Biochemistry, School of Biological Sciences, Federal University of Technology, Minna, Niger State, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JABB/2021/v24i630221
Editor(s):
(1) Prof. C.J. Chiang, China Medical University, Taiwan.
Reviewers:
(1) Giorgio Sonnino, Universite' Libre de Bruxelles, Belgium.
(2) Rinku Chakrabarty, Alipurduar University, India.
Complete Peer review History: https://www.sdiarticle4.com/review-history/72147

Original Research Article

Received 14 May 2021
Accepted 27 July 2021
Published 28 July 2021

ABSTRACT

The unpurified enzyme gave specific activity of 51.36 µ/mg and thereafter it was subjected to two phases of purification process of salt precipitation and gel filtration. The precipitating agent was ammonium sulphate while Sephadex-G100 served as purification matrix. The purification fold achieved after precipitation and filtration was 3.3 and with corresponding specific activities of 34.22

**Corresponding author: E-mail: Joseph.akor@unn.edu.ng;*

µ/mg and 116.31 µ/mg. The substrate used for the assay was o-dianisidine. Within 40-80°C of temperature, the kinetics of the peroxidase inactivation was evaluated. The results from assays showed that cucumber peroxidase conformed to the hypothesis of Michealis-Menten Theory. From the Lineweaver-Burk plot, Michaelis Constant (Km) and maximum velocity (Vmax) were evaluated and obtained 5.02mg/ml and 11.57µmol/min respectively. The heat induced inactivation gave biphasic curves, where initial rise in temperature was rapidly succeeded with much slower decrease. A first-order kinetic behaviour was observed for cucumber peroxidase heat inactivation. The *k* values of between 3.49×10^{-2} to 8.38×10^{-2} min^{-1} was obtained while the *Z* value was found to be 22.3°C. Decrease in *k* values with rise in temperature suggests that cucumber peroxidase was rapidly inactivated at elevated temperature. The slope of Arrhenius plot gave the activation energy of 127.99KJMol^{-1}K^{-1}. Also evaluated were thermodynamic constants (ΔH, G Δ, ΔS) for inactivation of peroxidase at variable temperatures. Cucumber peroxidase activity was observed to be pH sensitive and stable within pH range of 5.6-8. Further decrease or increase from this range resulted to decrease in peroxidase stability.

Keywords: Concentration; enzyme; peroxidase; cucumber; homogenization; industrial; bioremediation.

1. INTRODUCTION

Peroxidases are biocatalyst that are sourced from varieties of living organisms such as plants, fungi and bacteria. They are haem proteins that speed up the rate of biochemical reactions [1]. This involves the oxidation of various substrates capable of donating electrons such as phenols, aromatic amines why hydrogen peroxide receive electrons to be converted to water [2]. Peroxidases are ubiquitary and participate in various metabolic functions in living organisms such as fruits maturation [3] and strengthening of the cell wall architecture in bacteria [4]. There is increasing interest in peroxidase not only in reference to their physiological roles, but there ever increasing usefulness in industrial, biotechnological and analytical applications. This is because they are operationally stable to the prevailing extreme conditions associated with industrial processes and also possess the ability to amplifying weak signals when they are incorporated in biosensors for analytical applications [2]. Biotechnologically, peroxidases have gained prominence as they are important in the production of various items such as detergents and fructose corn syrup.

Peroxidase has widespread applications. It is employed in the green process of waste water treatment as they have the ability to remove deleterious phenolic compounds that usually accompanies industrial effluents which are emptied in water ways. When untreated, they constitutes environmental and water toxins leading to the death of aquatic organisms [5]. Through this bioremediation, phenolic compounds which causes health challenges such as cancer and teratogenic effects are polymerized via redox reaction [6]. Peroxidase is used for Immuno labelling during research and in medical diagnosis [1] as secondary antibody. It is also useful in food processing as it serves as biological signal for the formation of harmful reactive oxygen species [7] and as catalysts for breaking down of lignocellulosic components into its fibrous units during paper pulp [8,9]. Peroxidase applications are also seen in area of recovery of polluted soils when it is being immobilize for bioremediation purposes (8). Although peroxidases are found from various sources such as fungi, plants are the major target if it is for commercial scale applications. This is because of inherent advantages such as being cheap in terms of production cost, high sensitivity and stability during application.

Cucumber is fruit that is widely cultivated which belong to the gourd family of Cucurbitaceae with botanical name *Cucumis sativus*, with its associated features of broaden leaves and usually long green fruit that diminishes in size at one end [10]. Cucumber are nutritious fruits when eaten raw or in cooked form and because of its nutrition benefits are cultivated in various regions of world with varieties of species.

High effeciency in the industrial application of peroxidases are based on operational modalities being employed and chemical properties of such enzyme. Some of these properties that needed to be evaluated to ascertain the applicability of such enzyme include but not limited to its thermostability, solubility, catalytic efficiency, substrate affinity, optimum pH and temperature all for enhanced Biotechnological and industrial

applications. This research is geared towards evaluation of kinetics and thermodynamics of peroxidase isolated from cucumber and ascertain the viability of its usefulness in industrial and biotechnological applications.

2. MATERIALS AND METHODS

2.1 Sample Collection

Cucumber fruits were purchased from Ikpa Early Morning Commodity Market in Nsukka with GPS coordinate (6° 50' 34.5912" N and 7° 22' 23.7576" E.), in Nsukka Central Development Center Enugu State, Southeast Nigeria.

2.2 Peroxidase Extraction

Crude peroxidase from cucumber fruits was extracted, following the method of Khali-Ur-Rehaman [11] modified by Eze [12]. The fruits of cucumber were thoroughly washed with tap water and 600 g was weighed and blended for homogenization in presence of 1000 ml of ice cold phosphate buffer of 0.05 M. The homogeneous mixture passed through rigorous stirring and the solution filtered into a conical flask. The filtrate was centrifuged at 4000 rpm for 30 min. The supernatant was measured and found to be 2000 ml. Increase in volume after homogenization is as a result of the water content of the fresh cucumber. The enzyme extract was stored at temperature of 4ºC.

2.3 Assay and Protein Determination

Mclellan and Robinson [13] method guided activity assay of the peroxidase. The standard assay vial had 0.3 ml of 1% o-dianisidine, 0.2 ml of hydrogen peroxide, 2.4 ml of sodium phosphate buffer of pH 6.5 and 0.1 ml of cucumber peroxidase as its constituents. The change in absorbance at 460 nm is as a result of the reduction of hydrogen peroxide in the presence of o-dianisidine. For every 30 sec at time interval of 5 min the readings were taken. Using Bovine Serum Albumin as standard the concentration of protein present in cucumber peroxidase was evaluated according to the Lowry method [14].

2.4 Peroxidase Purification

Peroxidases were precipitated with gentle stirring at 20-90% saturation of solid ammonium sulphate. Eight test samples were obtained in all. Ammonium sulphate-crude enzyme solutions were kept at cold temperature of about 4^{o}C for 30 hours till the supernatant could be gently decanted off. The test tubes were centrifuged at 4000 rpm, for 20 mins. Precipitates from the individual percentage ammonium sulphate saturations were re-dissolved, respectively, in equal volume of 2.4 ml of the working buffer solution. Peroxidase activities of the precipitates were assayed to determine the percentage ammonium sulphate saturation that precipitated enzyme with highest activity.

A known volume (1.5 litres) of unpurified enzyme filtrate was precipitated. From the studies, seventy percent saturation gave the highest activity and hence, suitable for mass precipitation of the enzyme. Precipitation at seventy percent saturation was done by gently dissolution of 654 g of the precipitant (salt) in the filtrate and then carefully stirred until the precipitant was thoroughly dissolved. To preserve the precipitate for further studies, it was dissolved again in 40 ml phosphate buffer and thereafter freezed.

Sephadex G-100 was used for the gel purification of the enzyme. The G-100 gel has the dual ability of removing other ions that can interfere with the enzyme activities and removing other lower molecular weight proteins. The gel was carefully loaded in a chromatographic column of dimension 50cm by 2.5cm and was equilibrated using 0.05M of the working buffer of pH 6.5. 30ml of precipitated enzyme was delivered into the column and fractions were taken at a volume-interval ratio of 5ml/min. The protein concentration of each fraction was monitored using a spectrophotometer at 280nm. The peroxidase activity of each fraction was also monitored spectrophotometrically at 460 nm. The active fractions were pooled into two (2) fractions (A and B) and stored at temperature of 4ºC.

2.5 Characterization of Enzyme

2.5.1 Determination of optimum pH

With the buffering medium of Tris – HCl buffer (0.05M, pH 8– 9.5), Sodium-acetate buffer (0.05M, pH3.5 - 4.5) and sodium phosphate buffer (0.05 M, pH 5.0 –7.5), within the pH range of (3.5-9.5) the activity of the peroxidase was assessed at intervals of pH 0.5. The assay was done by incubating 1 M of each of the buffer (2.4 ml), hydrogen peroxide (0.2ml), enzyme (0.1ml) and 1% o-dianisidine (0.3 ml). The reaction

mixture was read spectrophotometrically at 460 nm for 5 min at 30 sec intervals.

2.5.2 Temperature

The determination of the optimal temperature for the peroxidase activity was conducted by incubating 0.1 ml of the enzyme and other constituents of the assay vial (2.4ml of 0.05M sodium phosphate buffer pH 6.5, 0.2ml of hydrogen peroxide and 0.3 ml of 1% o-dinanisindine for 20min). For 5 mins at every 30s, the absorbance was taken at 460nm.

2.6 Substrate Concentration

For the evaluation of substrate concentration effect on enzyme activity, the enzyme was incubated in different concentrations of the substrates by incubating various concentrations of the substrates (o-dianisidine) in equal volume of the enzyme at 50ºC. Double reciprocal plot of initial velocity data at variable concentrations of the substratehe enabled us to calculate kinetic parameters; V_{max} and K_m values of cucumber peroxidase.

2.7 Thermal Stability

Heat inactivation experiment at pH 6.0 was carried out in the same manner described by Elwahab [15]. Isothermal inactivation experiments for different time span in a temperature-regulated water bath enabled us to determine the kinetic and thermodynamic constants from the thermal inactivation of peroxidase from cucumber fruits. Incubating the enzyme in water bath at the temperature range of 40-90^{o}C at interval of 10O^{o}C for 10 minutes was the basis for the thermal stability study of peroxidase. The assay protocol is composed of 2.4 ml of 0.05 M phosphate buffer pH 6.0; 0.1 ml of enzyme, 0.2 ml of H_2O_2, and 0.3 ml of 1% concentrations of the substrate (o-dianisidine). Aliquots (0.2ml) of the enzyme solution were pipetted into glass test tubes of the same diameters and covered with cotton. The test tubes containing enzyme solution were transferred to a thermostatic water bath, which was previously set at a variable temperature of 40°, 50°, 60°, 70°, 80° and 90° C. The test tubes were withdrawn immediately from water bath and cooled in an ice-cold water and stored at -18°C until time for assaying at a variable time interval of 2, 4, 6, 8 and 10 mins. The residual enzyme activity was then determined as described in the peroxidase assay section. The enzyme stability was calculated as percentage residual enzyme activity. A plot of time against change in optical density (OD) per minute was done which gave rise to a biphasic first-order inactivation curve.

2.8 Thermal Inactivation

The enzyme activity was assayed un-preheated. The assay protocol contained 0.05 M phosphate buffer pH 6.0, 0.3ml of 1% concentration of o-dianisidine and 0.1 ml of the enzyme, 0.2 ml of H_2O_2, for 30 mins at optimum conditions to determine A_0, which is the heat untreated sample. The test sample was incubated for 1 hour at different temperature of 40º, 50º, 60º, 70º and 80^{o}C and an aliquot of 0.5 ml was taken from enzyme extracts at every 10 min interval, cooled at 4ºC for 20 mins to ensure that the secondary and tertiary subunits of the enzyme assume it's initial state of stability after the thermal inactivation period and eventually reverse to its native structure [15]. This was followed by assaying the activity of cucumber peroxidase as was done for unheated enzyme and the activity obtained from the samples was denoted as $A_{t.}$

2.9 Percentage Residual Activity

The enzyme fraction percentage residual activity was calculated using equation 1 below

$$A_t/A_o x\ 100\% \quad (1)$$

Where A_t = Activity of the enzyme at time t
A_o = Initial enzyme activity.

2.10 Denaturation Constant

Enzyme denaturation constant was evaluated by utilising the plot of lnA_o/A_t against t (mins) as shown below.

$$A_t/A_o = \exp(-kdt) \quad (2)$$

$\ln(A_t/A_o) = -kdt$ first order kinetics

Where,

kd = Denaturation constant
t = time.

The reaction depicting first-order kinetics suggest that it occurs at one inactivation rate (k-value) in a single step.

Half life of Cucumber Peroxidase $t_{1/2}$:

Equation 3 guided the calculation of the $t_{1/2}$ of the enzyme.

$$t_{1/2} = \ln 2/kd \quad (3)$$

Activation energy of inactivation:

The Ea(inact) was deduced from the Arrhenius equation:

$$\ln k = Ae - EaR/T \quad (4)$$

where,

k = Rate constant value
A = Arrhenius constant
Ea = Activation energy
R = Gas constant
T = Temperature.

The slope of the graph of equation 4 above helped us to evaluate the type of reaction rate involved and rate constant (kd) of thermal inactivation of the cucumber peroxidase.

2.11 D-values of Peroxidase

D-value is the time required at a given temperature to reduce the initial enzyme activity (A_o) by 90%.

$$D = \ln 10Kd \quad (5)$$

2.12 Z-Values of the Enzyme Fractions

The Z-value (°C) is the temperature increament required to induce a 10-fold reduction in D-value.

$$Slope = (1/z) \quad (6)$$

2.13 Evaluation of Thermodynamics Properties

The enthalpy of activation of thermal denaturation ΔH(inact), Gibbs free energy of activation of the thermal denaturation (ΔG(inact)) and the entropy of activation of the denaturation (ΔS(inact)) were all evaluated from the values of activation energy and Arrhenius constant [16,17] as shown in the equations below.

$$\Delta H(inact) = Ea(inact) - RT \quad (7)$$

Where;

ΔH(inact) = Inactivation enthalpy
Ea(inact) = Activation energy of inactivation
R = Gas constant
T = Time

$$\Delta G(inact) = -RT \times \ln(Kd \times h/Kb \times T) \quad (8)$$

Where;

ΔG(inact) = Free energy change of activation
h = Plank's Constant
Kb = Bolztman constat

$$\Delta S(inact) = \Delta H(inact) - \Delta G(inact)/T \quad (9)$$

ΔS= change in entropy of inactivation

2.14 pH Stability

pH stability was evaluated by incubating a known volume of the enzyme in different buffers of various pHs within the range of 3.5 and 9.5 for 5 min at room temperature and subsequently, the enzyme residual activity was monitored as described above.

2.15 Statistical Analysis

To obtain a more reliable statistical data, all evaluation reported in this study were done in triplicate. Results were expressed as means ± standard deviation.

3. RESULTS AND DISCUSSION

Peroxidase is very essential in plants metabolism as it is involve in various physiological processes including but not limited to plant growth, differentiation and development. Crude peroxidase was isolated from cucumber fruits using sodium phosphate buffer as extraction medium. The pilot studies for purification protocol revealed that 70% ammonium sulphate saturation was suitable to precipitate protein as it gave the result with highest activity. This could be attributed to the nature and types of the hydrophobic amino acid directly involve in the reaction. A purification fold of 0.65 and 33.3, and specific activity of 34.22μ/mg and 116.31μ/mg were achieved following precipitation and filtration purification steps respectively. Desalting of the enzyme after gel filtration led to increment in purification fold. This also showed that the enzyme has a high level of reduced protein contaminants in comparison to crude and precipitated enzymes. Lettuce stems peroxidase yielded a purification fold of 17.92 when it was

subjected to the purification steps of ammonium sulphate precipitation, sephadex G-100 and affinity chromatography [18]. On the other hand, Turkish black radish peroxidase gave a purification fold of 9.7 [19]. This was after a thorough purification process comprising ammonium sulphate precipitation, dialysis and CM Sephadex ion exchange chromatography. Small purification fold achieved as shown by the result could be attributed to only two phases of purification levels, unlike results from other researchers who engaged in more than two purification steps for peroxidase from other sources. The activity of the crude is the highest when compared to that of ammonium sulphate precipitate and gel filtration (Table 1). This could be as a result of other proteins that were complexed with crude. Removal of more contaminated proteins after gel filtration led to the observed rise in specific activity [16].

Enzyme activity is largely influence by degree of alkalinity or acidity of the reacting medium as it alters ionization states of parcipating reactants [17]. pH 5.5 gave the maximum enzyme activity as seen from Fig. 3 and there was a fall in activity when the pH was either increased or decreased and the drop was more prominent in the alkaline compare to the acidic region. This indicates that pH optimal for peroxidase isolated from *Cucumis sativus* is 5.5 and suggests that the active site of the enzyme might be constituted with acidic amino acids like aspartic and glutamic acids. The result as shown are in line and is in consistent with the findings from the body of literature. For instance, studies revealed that optimum pH from various sources of peroxidases as follows; *Allium sativum* (5.5), *Ipomoea batatas* (6), *Raphanus sativus* (5) and *Sorghum bicolor* (4) [20]. *Also* Khali-Ur-Rehaman previously reported optimum pH range of 4.5-6.5 on peroxidases isolated from vegetables of different kinds [11]. pH optimum of 8.5 and 7.2 were obtained from horseradish peroxidase and beans cell peroxidase respectively [21]. The nature of the soil where these fruits were planted, the kind of substrate involved in the assay and type of amino acids found in the active site also may have given rise to the slight variation in the pH [21]. It is also worthy to note that pH of peroxidase isolated from cucumber is close to pH neutrality which is preferable for biotechnological applications. Such pH is usually recommended in other to mimic physiological environment or medium where metabolic activity occurs naturally.

For enzyme characterization, the effects of substrate concentration and temperature of the reacting medium were understudied. The investigation revealed that 50^{o}C was an optimum temperature of peroxidase from Cucumber (Fig. 2) and lowest activity occurred at temperature of 80^{o}C, an indication of inactivation of the enzyme at extreme temperature. Optimum temperature of 50^{o}C for peroxidase from Ipomoea batatas [22], Allium sativum, Sorghum bicolor, Ipomoea batatas [20] and 55^{o}C for peroxidase from Dioscorea esculenta L. tubers are consistent with the findings from this result [23]. The little variation seen in the optimum temperature is largely caused by isoenzymes from different species of plants and type of reducing substrates that was used for the assay. Industrially, peroxidase applications occur at temperature range of 25 - 55^{o}C or even above, based on this, peroxidase from *Cucumis sativus* will be a veritable tool in the industry since it's optimum performance is at 50^{o}C which is within the application range. The Lineweaver Burk's plot, enabled us to deduce Km and Vmax values and was found to be 5.44mg/ml and 12.57µmol/min respectively based on studies on effect of substrate concentration. A low Km indicates that the cucumber peroxidase exhibit high affinity for the substrate. The values are in consistent with peroxidases from other sources [24] as seen in Streptomyces sp. MSC702 whose Km and Vmax values gave 2.407mg/ml and 21853µmol/min respectively [25]. The variation could be attributed to difference in the type of substrates used by the researchers.

Naturally, enzymes have distinctive structural level of organizations that is made up of primary, secondary and tertiary subunits based on its amino acid components in the polypeptide chain. Higher temperature via heating which is common in industrial setting destroys enzyme motifs (folding) which defines it's functionality and consequently renders them inactive.

The ability of an enzyme to withstand or resist unfolding when heated is best describe as it's thermostability. Thermal denaturation of enzyme occurs when the enzyme passes through two physical state of configurations from its native form (N) to an unfolded enzyme (U) which could be reversibly restored back to (N) form upon cooling and lastly to the inactivated enzyme form I which occur after extensive heating. Degree of thermal inactivation of Peroxidase from Cucumber was calculated under a temperature range of 40-80°C. Both time of heating and

temperature under evaluation significantly affected the inactivation of peroxidase, although, absolute inactivation of peroxidase was not possible. According to Deepa and Arumughan [26] cucumber peroxidase had shown high stability under intense heating due to extra conformational stability confered on it by glycosylated proteins and also the presence of many cysteine amino acids in the protein. Over the years and based on proven theories, enzyme thermal stability have been evaluated by calculating kinetics and thermodynamics parameters such as: half-life, minimum amount of energy required for the enzyme to be denatured called activation energy of denaturation, change in entropy arising from thermal denaturation, the enthalpy of denaturation and the change in Gibbs free energy during the period [27].

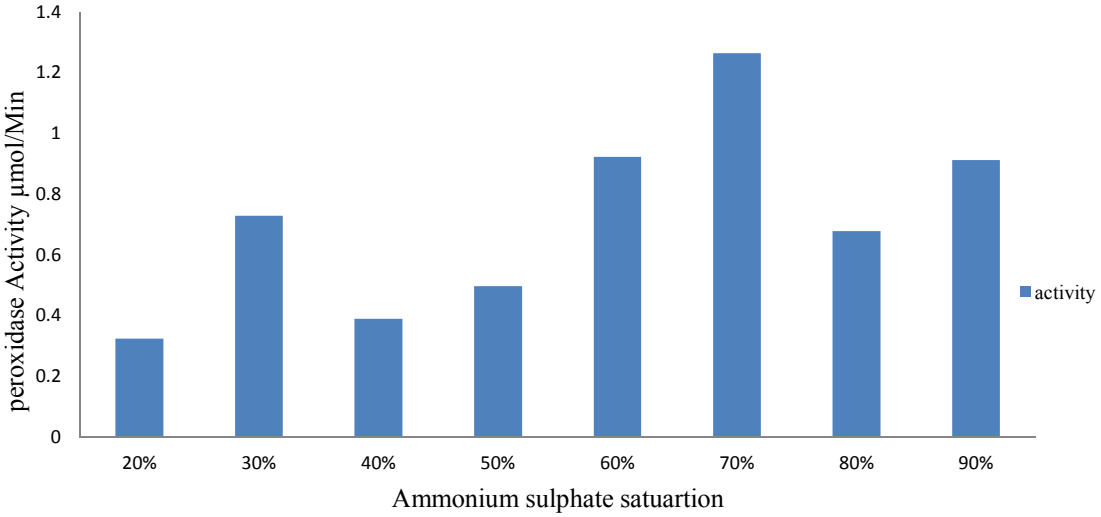

Fig. 1. Ammonium sulphate precipitation profile for peroxidase isolated from *Cucumis sativus*

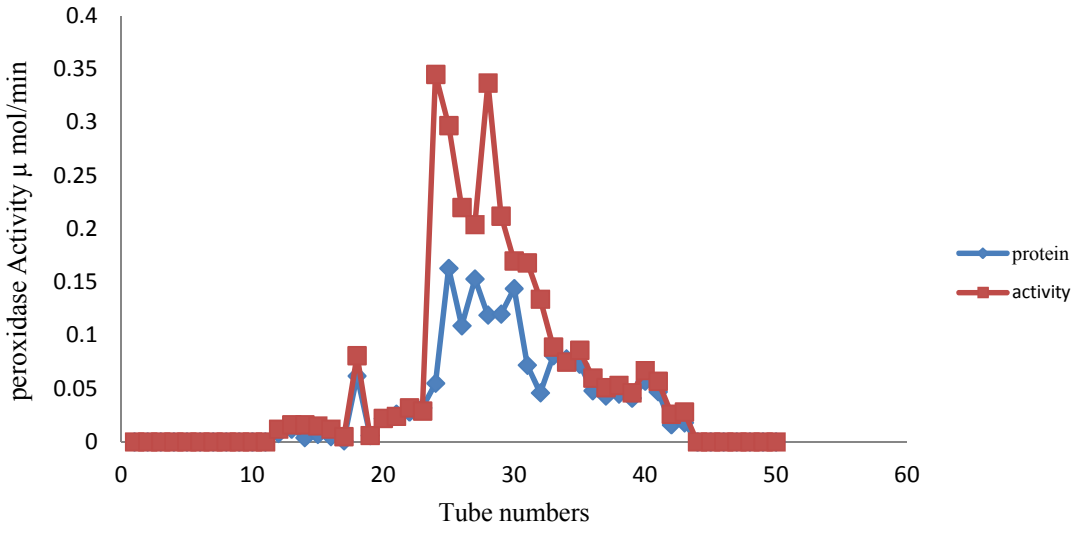

Fig. 2. Elution profile of gel filtration for peroxidase isolated from *Cucumis sativus*

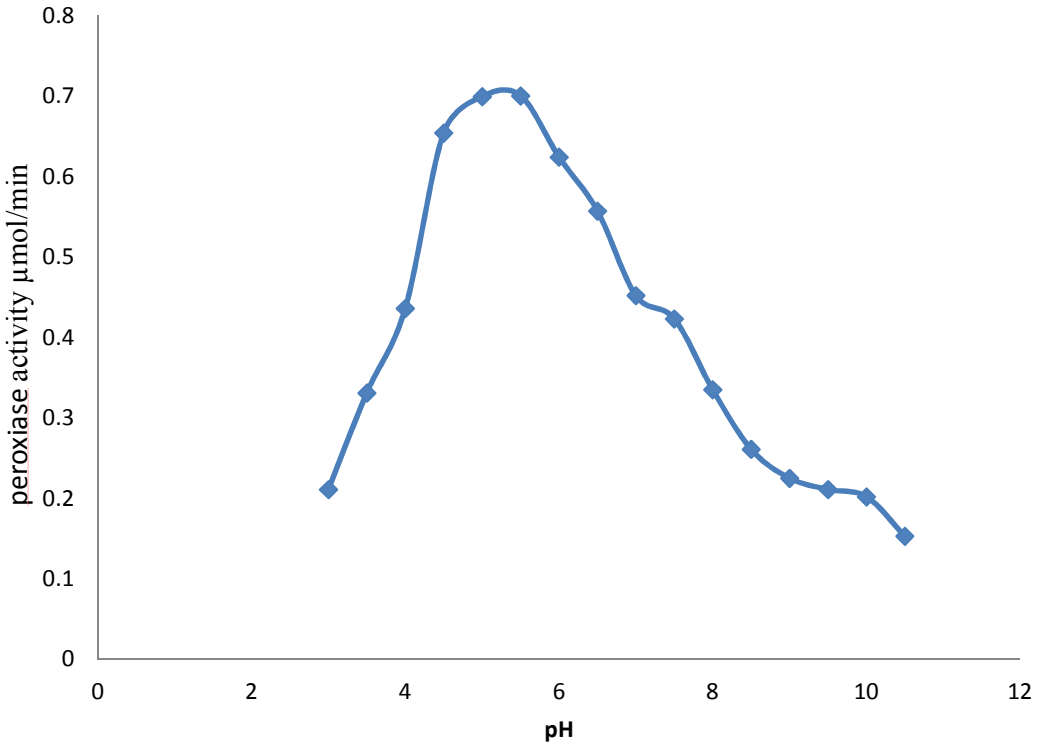

Fig. 3. Effects of pH on perxoidase activity

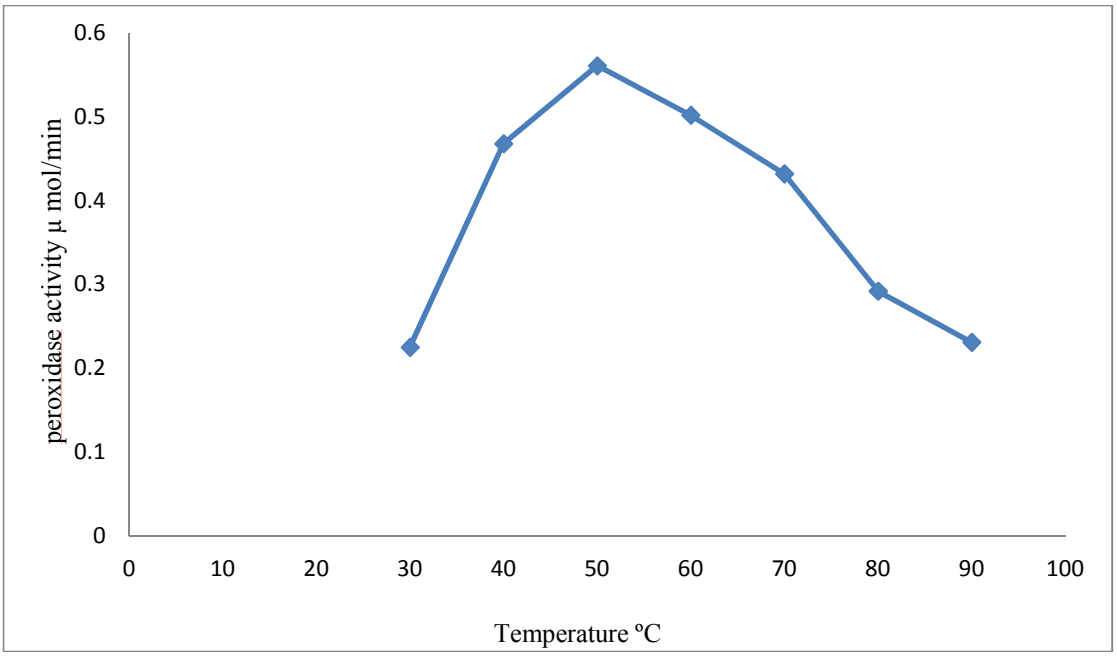

Fig. 4. Effects of temperature on peroxidase activity

Table 1. Purification Table of peroxidase extracted from *Cucumis sativus*

Enzyme	Volume (ml)	Protein (mg/ml)	Total Protein	Activity (µmol/min)	Total Activity	Specific activity (µ/mg)	Purification fold	Percentage yield
Crude	500	1.775	887.5	86.323	49211.5	51.36	1	100
$(NH_4)_2S04$	60	0.583	34.98	20.33	1257	34.22	0.65	2.55
Gel filteration	30	0.163	4.89	18.417	584.91	116.31	3.33	46.53

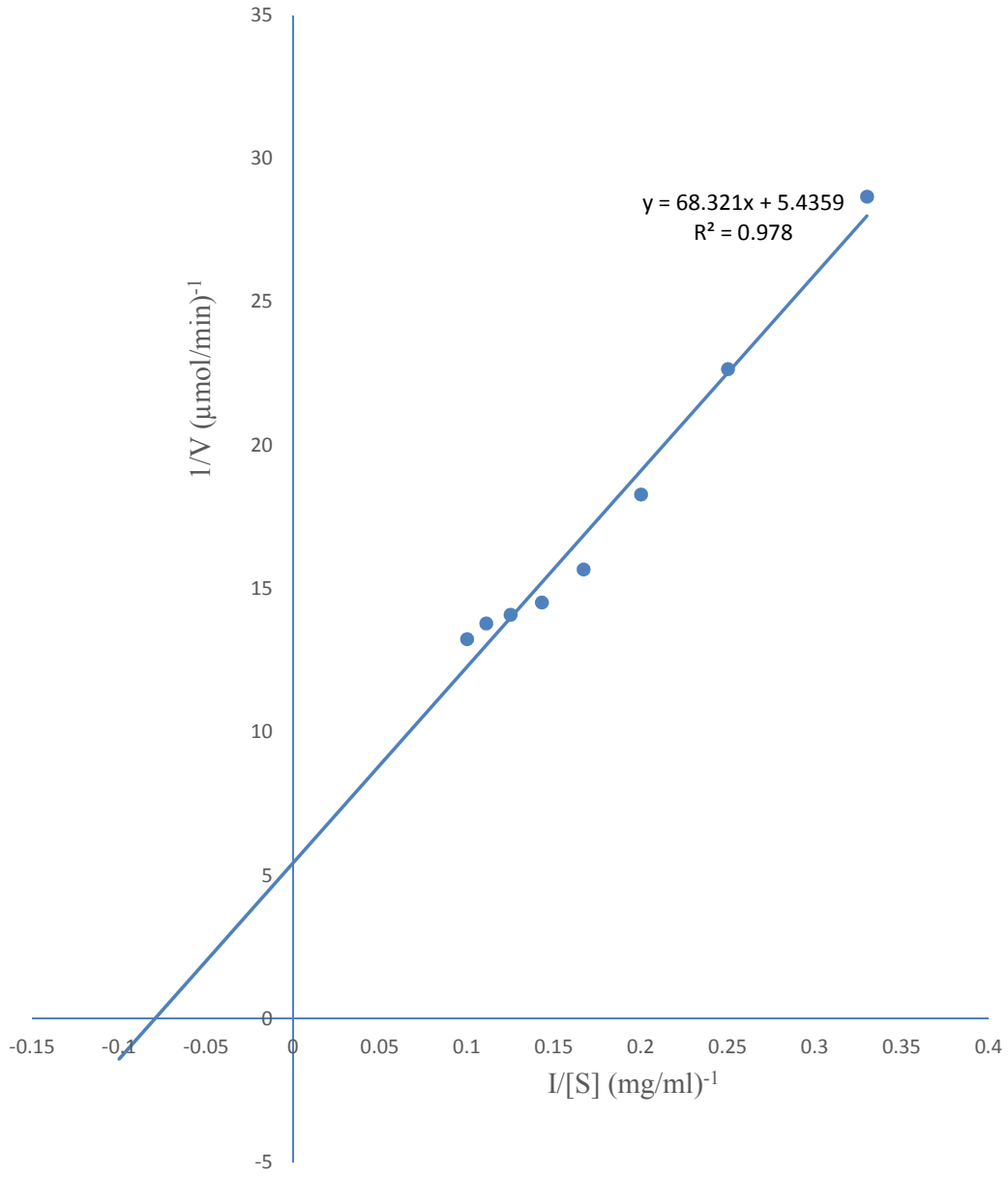

Fig. 5. Line Weaver-Burk plot of peroxidase isolated from *Cucumis sativus* using different concentrations of o-dianisidine as substrate

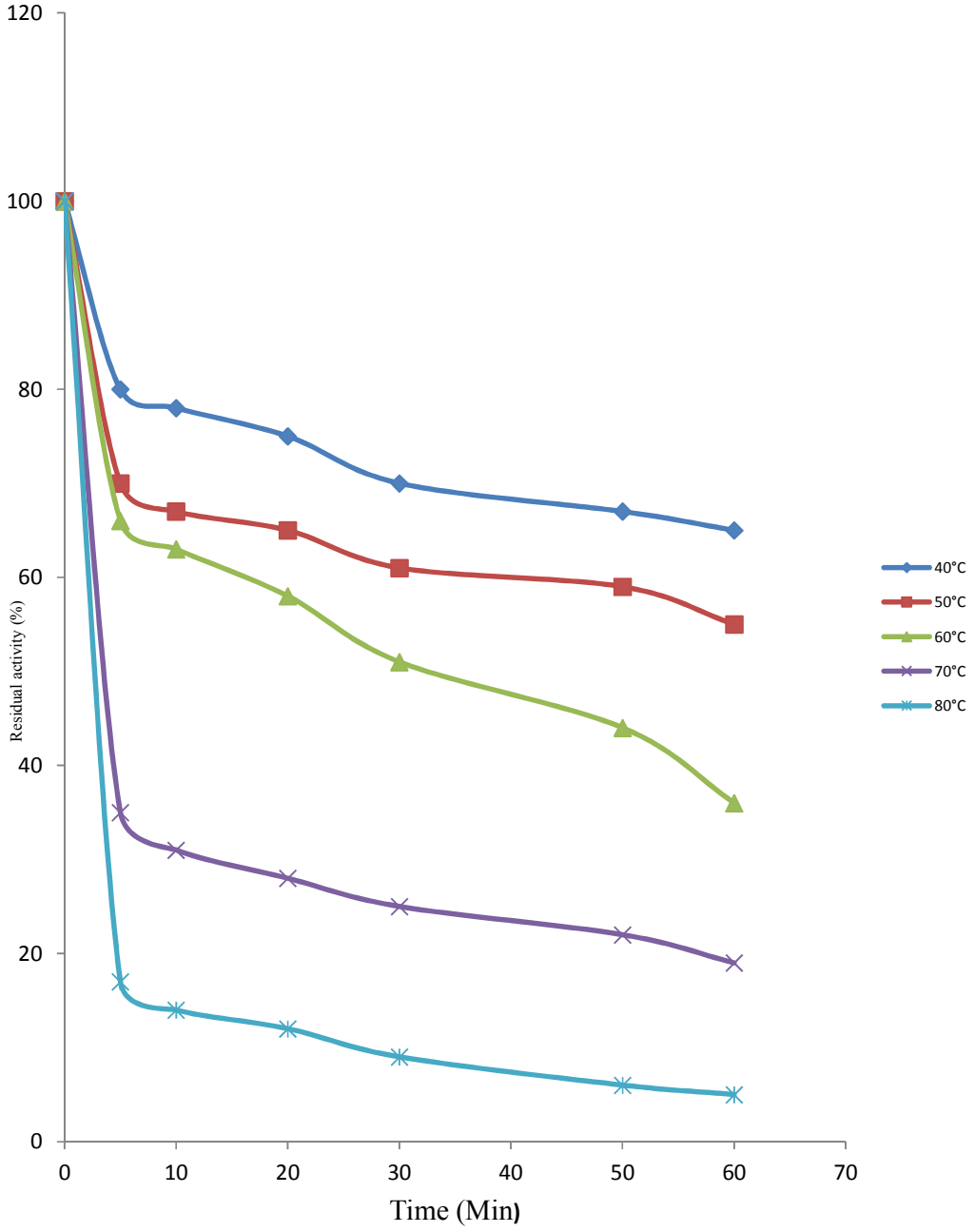

Fig. 6. Percentage residual activities against time for peroxidase isolated from *Cucumis sativus*

Table 2. Characterisation of Peroxidase Obtained from *Cucumis sativus*

Properties	*Cucumis sativus*
pH	5.5
Temperature (°C)	50
Km (mg/ml)	5.02
Vmax (µmol/min)	11.57

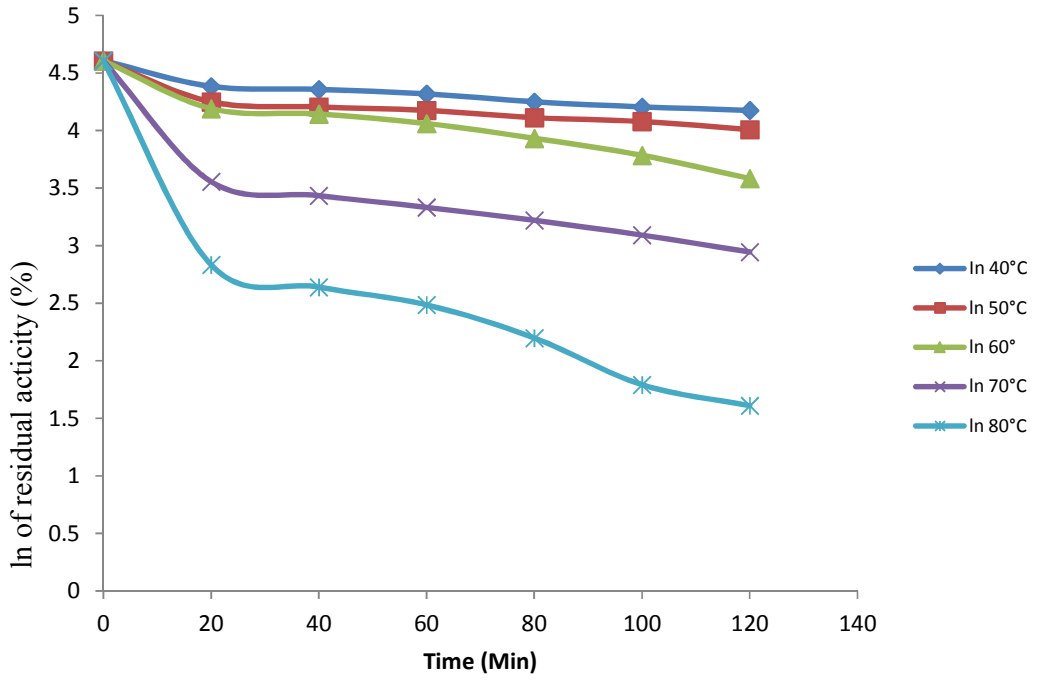

Fig. 7. Percentage residual activities against time for peroxidase isolated from *Cucumis sativus*

On half-life ($T_{1/2}$) of the enzyme, a prolonged half-life was recorded at 40ºC and 50ºC, signifying that cucumber peroxidase has higher thermal stablity at those temperatures under evaluation. The half-life of the peroxidase isolated from Cucumber at the optimum temperature of 50ºC is 15.05 min. The half-life of 11.82 and 8.5 mins were observed for peroxidases sourced from mangosteen pericarp [28] and strawberry at 50ºC respectively [29]. Meta analysis based on available data had shown that enzyme studied herein is more stable than those in body of literature and hence should be a target to source peroxidase needed in many industries were thermostable peroxidase is needed.

The D value of Cucumber peroxidase was 383.7642 min at optimum temperature of 50°C. It was calculated from the reciprocal of the slope of the inactivation curves. *D* value of Cucumber peroxidase decrease with increase in temperature suggesting that it is thermostable. The D-value obtained in this study was more than that obtained from mongosteen pericap (152.2 min), strawberry (141.4 min) and matured coconut water (102.22 min) at the same 50°C of temperature inactivation [30].

Table 3. Summary of the thermoinactivation and thermodynamics of the enzyme denaturation of peroxidase isolated from *Cucumis sativus*

Thermal Stabilty and Thermodynamics of Peroxidase Denaturation Table

Temperature (ºC)	*T1/2 (min)*	Kd (*min*−1)	D-values (mins)	ΔHº(D) (KJ/mol)	ΔGº(D) (KJ/mol)	ΔGº(D) (KJ/mol)
40	21.12	0.035	575.6463	30.321	103.53	-121.34
50	15.05	0.028	383.7642	30.271	104.31	-123.01
60	11.7	0.022	209.3259	29.123	107.29	-126.61
70	9.3	0.019	127.9214	28.040	111.32	-127.31
80	6.51	0.014	65.78815	26.78	112.71	-128.45
Ea	127.99KJMol^{-1}K					
Z-value	22.3ºC					

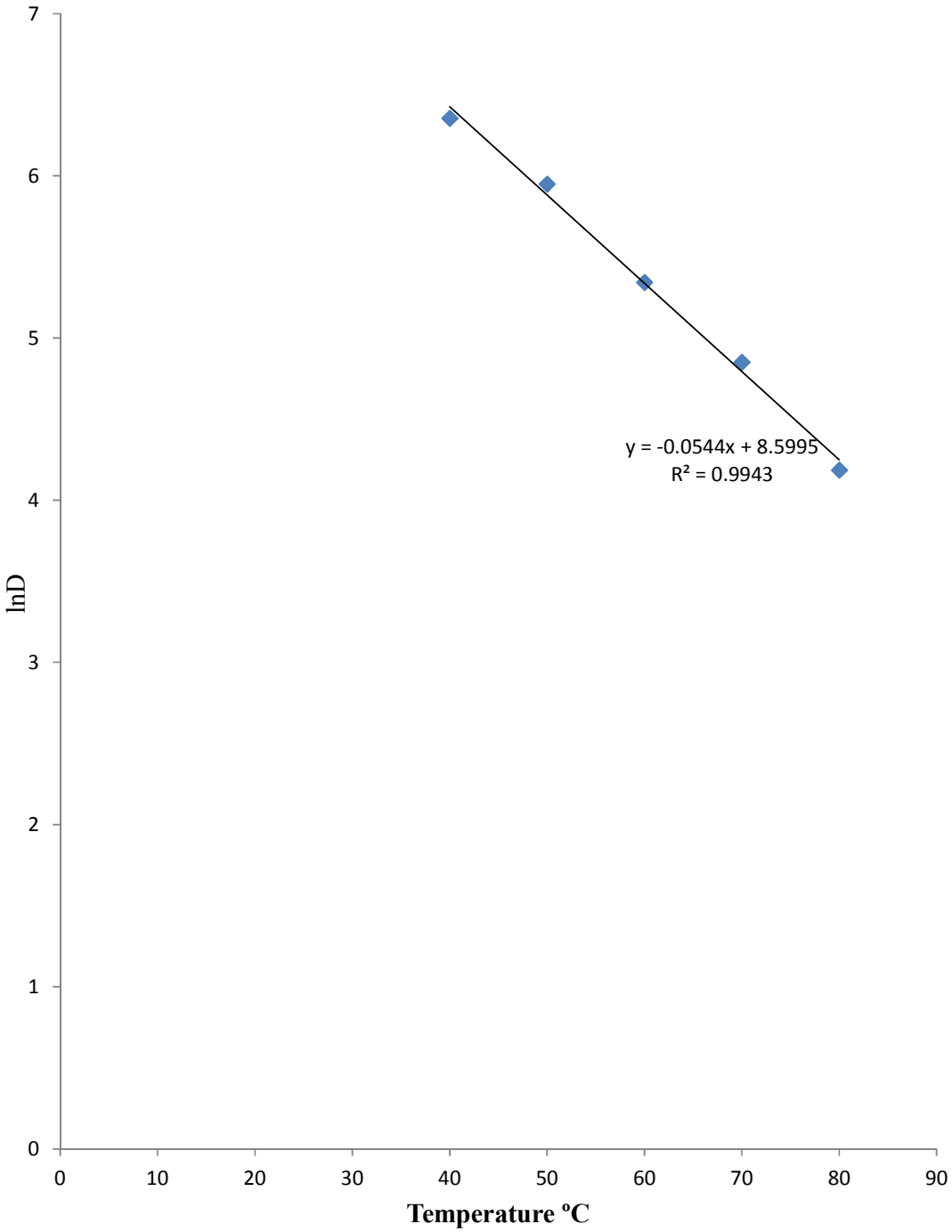

Fig. 8. Calculation of Z-value for peroxidase isolated from *Cucumis sativus*

Calculated Z values for Cucumber peroxidase was 22.3°C (r=0.970). The z value of 22.3°C for peroxidase inactivation was in agreement with the z value of peroxidase from other sources. Apricot yielded 20.5°C for peroxidase and polyphenol oxidase complex [31]. It was reported from the existing literature that heat stable isoenzyme of carrot peroxidase gave Z value of 20.1°C [32]. These results are in consistent with the findings of this research.

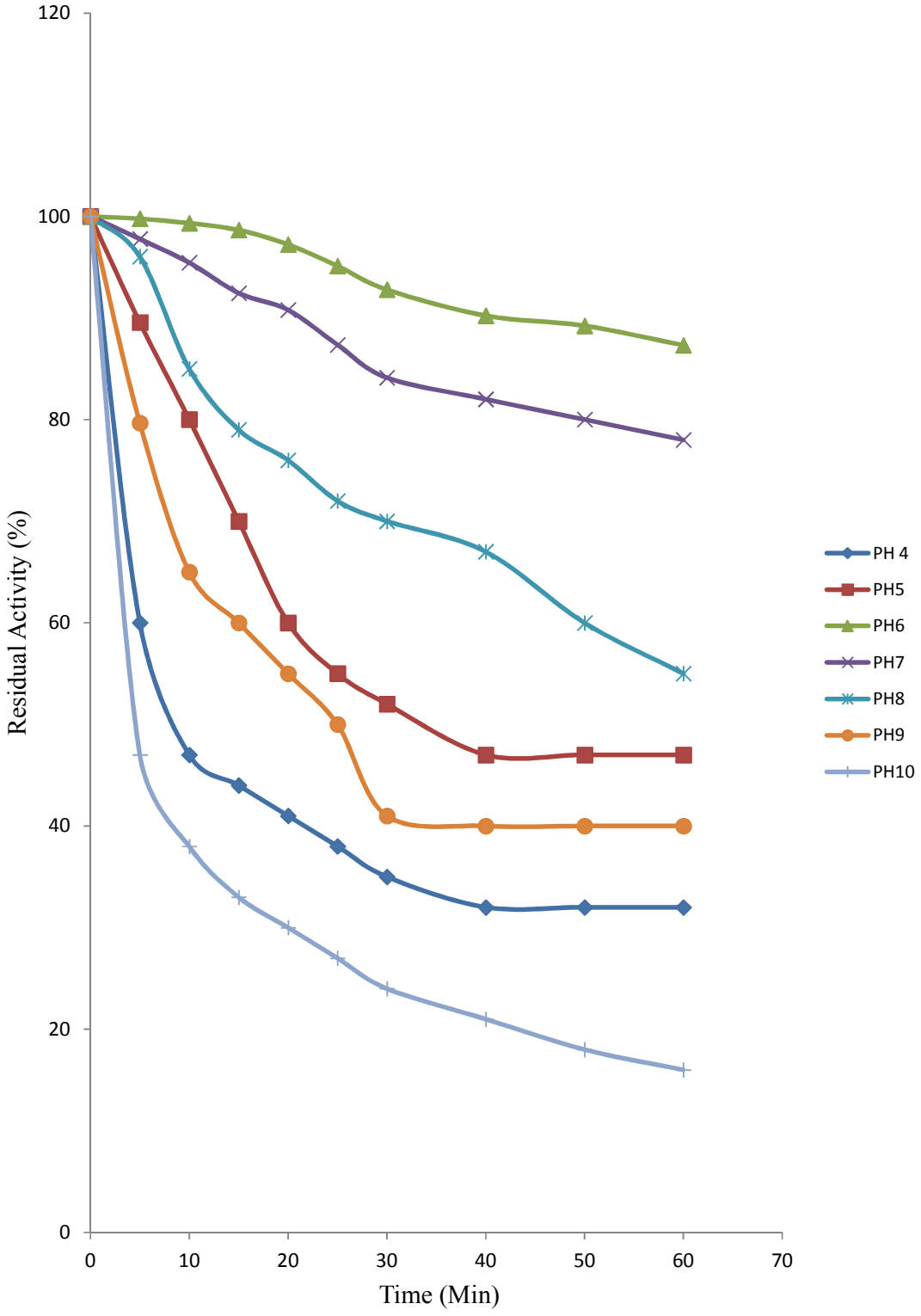

Fig. 9. pH stability plot of residual activity of peroxidase from Cucumis sativus

Table 4. Parameters for pH stability studies

pH	$T_{1/2}$ (Min)	Kd (Min $^{-1}$)
4	0.57	0.006
5	2.34	0.04
6	8.12	0.001
7	5.23	0.002
8	4.12	0.003
9	1.99	0.005
10	0.17	0.007

For successful denaturation of an enzymes by heating, there is a minimum amount of energy needed to be introduced to the reacting medium. This energy is called activation energy of denaturation (Ea(D) which causes disorientation of the native conformational structure of the enzyme and consequently makes the enzyme inactive. Before the enzyme is finally denatured, it passess through an intermediate state (U) which is highly unstable [33]. For this intermediate form of enzyme to be finally denatured, the energy supplied must be greater than activation energy of denaturation Ea(D). Peradventure the energy supplied is lesser to that of Ea(D), the intermediate (U) which is unstable can revert to its native form (N) when it has cooled off [34]. Biomolecules naturally survive instability by the help of this energy barrier. Once the Ea(D) is less than the energy supplied, the enzyme is permanently denatured (I) and will never reassume its native conformation with its attendant loss in function. This process is irreversible thermal denaturation of enzyme. The estimated Ea(D) which was 127.99KJmol^{-}1K^{-}1 was deduced from the Arrhenius plot. This is a plot of rate of thermal denaturation (K_d) against temperature. The Ea(D) of 127.99KJmol-1K-1 was small in value compared to that found for peroxidase obtained from fresh pinto beans, 186.2 KJmol-1K-1, Elsanta strawberry 196.2 KJmol^{-}1K^{-}1 and melon 186.3 KJmol^{-}1K^{-}1 [35]. Inactivation rate constants was observed to be dependent of temperature as it fitted into Arrhenius Equation. This linearity suggests that the inactivation of cucumber peroxidase was a function of reaction condition(s) and pricesely in this case, temperature, resulting to protein unfolding [36].

It is not ideal to depend only on the result of half-life to characterize enzyme thermodynamically [28]. To do thorough investigation, other thermodynamic parameters are paramount as they give further insight into its degree of stability in reacting medium. Ea(D) is directly proportional to ΔH(D). Findings from this research revealed that ΔH(D) values decreased with increase in temperature. High values ΔH(D) as obtained here suggests that the native conformation of this enzyme would undergo denaturation-induced modification [37]. Inverse relationship between ΔH values with temperature increment shows that less energy is needed for the denaturation of enzyme at elevated temperature [38]. The same trend in ΔH values was calculated for white yam though with variable degree and in most other research findings [12].

Despite that a high enthalpy of activation energy of denaturation (ΔH(D)) is an indication that an enzyme is thermally stable, it is also essential that other thermodynamic parameters such ΔG(D) and ΔS(D) are evaluated. Theoretical and research evidence have proven that Gibbs free energy of activation of denaturation ΔG(D) gives a reliable indicator for stability [27]. A negative or a very small value of change in Gibbs free energy suggests that the reaction can occur spontaneously. Cucumber peroxidase has an elevated ΔG(D) suggesting that it is thermally stable at a temperature of 50^{o}C where it gave ΔG(D) of 104.31 KJmol^{-}1 and is in consistent with the finding from cauliflower peroxidase [29]. The value of ΔG(D) which is an evaluation of the spontaneity of the reaction is higher when compare to the value of ΔH(D). This is as a result of negative entropic conditions associated with the reaction medium [39]. A negative ΔG(D) was reported for white yam peroxidase, an indication that the thermostability of the enzyme is high and not at par with that of cucumber peroxidase [12]. The variation could be linked to various factors such as the nature of amino acids in the protein polypeptide chain and the kind of solvation layer (depending on the stabilizing forces) existing in the reaction media where the two enzymes were involved.

Thermal denaturation of enzymes does contribute to rise in entropy. A positive entropy of activation of denaturation, ΔS (D) signifies disorderliness that is associated with transformation of the enzyme from its native (N) state to the unstable intermediate state (U) which ultimately decreases the stability of enzyme while a negative value suggest high stability and molecular orderliness. Peroxidase from *Cucumis sativus* had a negative entropy which shows an ordered molecular state of the enzyme in reaction medium. The orderliness of molecules occured as a result of protein compactness, hydrophobic interactions of the amino acid residues and reaggregation of the proteins

before overcoming the activation energy of denaturation [40,41].

Peroxidase from *Cucumis sativus* showed maximum stability at pHs of 5.5, 6, 7 and 8 when preincubated for an hour as the enzyme maintained above 50% residual activity up to an hour. Similar results were obtained using peroxidases from grape, banana and pineapple [42]. The loss in activity in low and high pHs typified the pH dependent of enzyme active site and was so because of the release of heme group which occurs at such pH range [43]. On the other hand, the active site of peroxidase is known to be constituted of ionic groups which must be in the right ionic state and in that regards, sustain the native orientation of the enzyme active site for effective reaction catalysis [44].

4. CONCLUSIONS

Present study revealed that cucumber fruits produces substantial amount of peroxidase. The partially purified peroxidase recorded maximum activity at temperature of 50°C. The study showed that the enzyme was still active even at temperature of 70 degrees centigrade although it lasted for a very shot period of time which shows that the enzyme could be exploited for industrial applications which usually occur at high temperature without easily being denatured. The pH studies reported that this enzyme perform maximally at pH close to neutrality which is good for biotechnological applications usually carried out in a manner that mimicks physiological environment. The widespread application of peroxidase due to its ever growing need in analytical and biotechnological applications has necessitated it's exploration. Since the chemical and kinetic parameters (K_m and V_{max}) of peroxidase from cucumber fruits as seen from this stiudy are suitable for industrial and biotechnological applications, then it becomes imperative to source peroxidase from cucumber fruits to boost its commercial availability which is currently in short supply. Thermal inactivation of cucumber peroxidase was evaluated and it fitted into a first-order kinetic model. The thermodynamic parameters such as D, Z and K values and high values of activation energy and change in enthalpy suggest that a high amount of energy is needed to induce denaturation on cucumber peroxidase, indicating that it is highly stable which is of one of the features required of enzyme usable for industrial applications. Also, the procedure enumerated in this study to obtain this enzyme and to evaluate its kinetic and thermodynamics parameters is effective and suitable for analytical applications.

ACKNOWLEDGEMENT

The authors wish to appreciate the staff of department of Biochemistry University of Nigeria Nsukka for their support and encouragement in the course of this research.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Abdel-Aty AM, Hamed MB, Gad AM, El-Hakim AE, Mohamed SA. Ficus sycomorus latex: An efficient alternative Egyptian source for horseradish peroxidase in labeling with antibodies for immunodiagnostic kits. Vet World. 2018;11:1364–1370.
2. Pandey VP, Manika A, Swati S, Sameeksha T, Dwivedi UN. A comprehensive review on function and application of plant peroxidases. Biochem Anal Biochem. 2017;6:308-325.
3. Sahin ES and Soya C. Properties of Tomato. Journal of Advancement in Plant Science. 2018; 206:1-6.
4. Chen LC, Chung YC, Chang CT. Characterisation of an acidic peroxidase from papaya (*Carica papaya* L. cv Tainung no. 2) latex and its application in the determination of micromolar hydrogen peroxide in milk. Food Chem. 2012;135:2529–2535.
5. Mirazizi F, Bahrami A, Haghbeen K, Zahiri HS, Bakavoli M, Legge RL. Rapid and direct spectrophotometric method for kinetics studies and routine assay of peroxidase based on aniline diazo substrates. J Enzyme Inhib Med Chem. 2016; 31:1162–1169.
6. Elena-Florentina G, Gabriela C, Renato F. Development of horseradish peroxidase/ layered double hydroxide hybrid catalysis for phenol degradation. Res Chem Intermediat. 2018;44(12):7731–7752.
7. Kondeti RS, Korivi M, Kesireddy N, Hua KC, Kesireddy SR. Protective effect of dietary ginger on antioxidant enzymes and oxidative damage in experimental diabetic

rat tissues. Food Chem, 2011;124:1436–1442.
8. Moubasher H, Mostafa FA, Wahsh S, Haroun O. Purification and characterization of lignin peroxidase isozymes from *Humicola grisea* (Traaen) and its application in bioremediation of textile dyes. Egypt J Bot. 2017;57:335–343.
9. Mohamed SA, El-Badry MO, Drees EA, Fahmy AS. Properties of a cationic peroxidase from Citrus Jambhiri cv. Adalia. Appl Biochem Biotechnol. 2018;150:127–137
10. Abiodun OA. Comparative studies on nutritional composition of four melon seeds varieties. Pakistan Journal of Nutrition. 2010;9:905-908.
11. Khali-Ur-Rehaman M, Yaqub M.A, Sheikh S, Arshad S. Extraction and evalution of peroxidase from various vegetable sources. International Journal of Agriculture and Biology. 1999;3:170-173.
12. Eze SOO, Chilaka FC, Nwanguma BC. Studies on thermodynamics and kinetics of thermo-inactivation of some quality-related enzyme in white yam (*Dioscorea rotunda).* Journal of Thermodynamic Catalysis. 2010;1:104.
13. McLellan KM. and Robinson DS. Purification and heat stability of *Raphanus sativus* peroxidase isoenzymes. Food Chemistry, 2009;23:305-319.
14. Lowry HO, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry. 1951;193:265-275.
15. Griffin H, Dintzis FR, Krull L, L. Baker LFL. A microfibril generating factor from the enzyme complex of Trichoderma reesei. Biotechnology and Bioengeneering. 1984; 26:269–300.
16. Lukong B, Awah F, Nwuke C, Fobellah H. Protein isolation, purification and estimation: In: Introduction to Protein Science, Osprey Publication Center Owerri, Nigeria. 2007;55-56.
17. Gawlik-Dziki U, Zlotek U, Swieca M. Characterization of polyphenol oxidase from butter lettuce (*Lactuca sativa*). Journal of Food Chemistry. 2008;107:129-135.
18. Yihong H, Juan W., Ping L, Yiwei M. Purification and partial characterization of peroxidase from lettuce stems. African Journal of Biotechnology. 2012;11:2752-2756.
19. Melda S, Ihami G, Murat C, Ali M, Habibe BK, Hasan O. Purification and characterization of peroxidase from Turkish black radish (*Raphanus sativus* L.). Journal of Medicinal Plants Research. 2010;4:1187-1196.
20. Mamounata D, Oumou HK, Nafissetou O, Romaric GB, Imael HNB, Mamoudo HD. Comparison of peroxidase activity from Allium sativum, Ipomoea batatas, *Raphanus sativus* and *Sorghum bicolor* grown in Burkina Faso. African Journal of Biochemistry Research. 2011;5:124-128.
21. Blee KA, Bolwell GP, Bindschedler VL, Butt V, Davies DR, Gardner SL, Gerrish C, Minibayeva F. The apoplastic oxidative burst in response to biotic stress in plant: A three component system. Journal of Experimental Botany. 2003;53:1367-1376.
22. Civello PM, Martinez AG, Chaves AR, Anon MC. Peroxidase from strawberry fruit: Partially purification and determination of some properties. Journal of Agriculture and Food Chemistry, 2010;43:2596-2601.
23. Omidiji O, Okpuzor JE, Otubu O. The contribution of an ionic peroxidase isozyme to enzyme-mediated browning in *Dioscorea esculenta* L. Tubers. Pakistan Journal of Nutrition. 2006;5(5): 478–480.
24. Ganjloo A, Rahman RA, Osman A, Baka J, Bimakr M. Kinetics of crude peroxidase inactivation and color changes of thermally treated seedless guava. Food and Bioprocess Technology. 2009;4(8):1442-1449.
25. Gutteridge JM. Iron promoters of the fenton reaction and lipid lipid peroxidation can be released from haemoglobin by peroxides. FEBS Lett. 1986;201:291–5.
26. Deepa, SS, Arumughan C. Oil palm fruit peroxidase: purification and characterization. Journal of Food Science and Technology, 2002; 39.8–13.
27. Maragoni AG, Jackman RL, Stanley WD. Chilling-associated softening of tomato fruit is related to increased pectinmehylesterase activity. Journal of Food Science. 1995; 60.1277–1281.
28. Kouadio EL, Konan HK, Dabonne S, Due DE, Kouame LP. Study of thermal stability of α-Amylase sourced from digestive tract of the tropical house cricket, *Gryllodes sigillatus*: Kinetic and thermodynamic analysis. Journal of Novel Applied Sciences. 2013;2(3):74-82.

29. Kumar SR. Structure, Function and Stability of Alpha-Amylase from Jowar (*Sorghum bicolar*). Ph.D. Thesis, University of Myrose. 2008;99.
30. Chisari M, Barbagallo RN, Spagna G. Characterization of polyphenol oxidase and peroxidase and influence on browning of cold stored strawberry fruit. Journal of Agricultural and Food Chemistry. 2008;55(9):3469-3476.
31. Arnnok P, Ruangviriyachai C, Mahachai R, Techawongstien S, Chanthai S. Optimization and determination of polyphenol oxidase and peroxidase activities in apricots. International Food Research Journal. 2010;17(2):385-392.
32. Goncalves EM, Pinheiro J, Abreu M, Brandao TRS, Silva CLM. Carrot peroxidase inactivation, phenolic content and physical changes kinetics due to blanching. Journal of Food Engineering, 2013;97(4):574-581.
33. Siddiqui KS, Azhar MJ, Rashid MH, Rajoka MI. Stability and identification of active-site residues of carboxymethylcellulases from *Aspergillus niger* and *Cellulomonas biazotea*. Folia Microbiology. 1997;42(4): 312-318.
34. Saqib AN, Hassan M, Khan FN, Baig S. Thermostability of crude endoglucanase from *Aspergillus fumigatus* grown under solid state fermentation (SSF) and submerged fermentation (SmF). Process Biochemistry. 2010;45:641-646.
35. Yemenicioglu A, Ozkan M, Cemeroglu B. Thermal stabilities of peroxidases from fresh pinto beans. Journal of Food Science. 1998;63(6):987-990.
36. Griffin H, Dintzis, FR, Krull L, Baker FL. A microfibril generating factor from the enzyme complex of Trichoderma reesei. Biotechnology and Bioengeneering. 1984; 26:269–300.
37. Marin E, Sanchez L, Perez MD, Puyol P and Calvo M. Effect of heat treatment on bovine lactoperoxidase activity in skim milk: Kinetic and thermodynamic analysis. Journal of Food Sciences. 2003;68(1):89-93.
38. Bhatti HN, Madeeha M, Asgher M, Batool N. Purification and thermodynamic characterization of glucose oxidase from a newly isolated strain of Aspergillus niger. Canadian Journal Microbiology. 2006;52: 519-524.
39. Riaz M, Perveen R, Javed MR, Nadeem H, Rashi MH. Kinetics and Thermodynamic Properties of noval glucoamylase from *Humicola* sp. Enzyme and Microbial Technology. 2007;41:558-564.
40. Marin E, Sanchez L, Perez MD, Puyol P, Calvo M. Effect of heat treatment on bovine lactoperoxidase activity in skim milk: kinetic and thermodynamic analysis. Journal of Food Sciences. 2003;68(1):89-93.
41. Gummadi SN. What is the Role of Thermodynamics on Protein Stability? Biotechnology and Bioprocess Engineering. 2003;8:9-18.
42. Smith JA, Hammerschmidt R. Comparative study of acidic paroxidases associated with induced resistance in cucumber, muskmelon and watermelon. Physiological and Molecular Plant Pathology. 1998;33: 255-261.
43. Farrell RL, Murtagh KE, Tien M, Mozuch, MD, Kirk, TK. Physical and enzymatic properties of lignin-peroxidase isoenzymes from Phanerochaete chrysosporium. Enzyme Microbiology and Technology. 2007;11:322-328.
44. Whitaker JR, Lee CY. Recent advances in chemistry of enzymatic browning. Phytochemistry. 2007;29:1051-1056.

Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle4.com/review-history/72147