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Purification and Interaction of β-amylase from *Dioscorea alata* (Water Yam) with Epicatechin

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Authors' contributions

This is to declare that this is a collaborated project by the authors. Authors OATE and ISA supervised the project, the project analysis was done by authors MAF and AOK. Project carried out by authors MAF and KAO. FMA wrote the manuscript. All authors read and approved the final manuscript

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ABSTRACT

β-amylase is an enzyme that hydrolyzes the α-1,4-glucan bonds from the non-reducing ends of starch and other carbohydrate polymers reducing it to maltose units. Maltose has much application with food processing and pharmaceutical industries**.** The enzyme was purified to apparent homogeneity with a monomeric molecular weight of 30.1 kDa based on SDS-PAGE. The binding Constant (*K*a), K_d Δ*H*, Δ*S* and Δ*G* values were 1.53×10^{3}Lmol^{-1} ,$3.12\text{x}10^{-4}$Lmol^{-1}, 19.35kJmol^{-1}, 56.67Jmol^{-1}K^{-1}, and -18.17kJmol^{-1} respectively. The binding profile of β-amylase with epicatechin was spontaneous with a stoichiometric ratio of 2:1. Hydrophobic bonding played a major role in stabilizing the β-amylase-ligand complex. The mode of reaction was by static quenching. It further dictates that the binding reaction is entropy driven. The inhibitory effect of this plant polyphenols on β-Amylase might contribute to the regulation of β-Amylase activity in plants.

Keywords: β-amylase; purification; epicatechin; fluorescence spectroscopy.

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ABBREVIATIONS

SDS-PAGE : Sodium dodecyl sulphate Poly Acrylamide gel electrop-horesis
DEAE : Diethyl aminoethyl
DNSA : 3,5-Dinitro salicylic acid
µmol : micromole

1. INTRODUCTION

β-amylase (EC 3.2.1.2) is an enzyme that acts on starch with the successive removal of maltose molecules from the non-reducing ends of the glucose polymers [1]. It is widely distributed in most living organisms, plays an important role in starch liquefaction process and starch conversion technology both of biotechnological importance, scientific and industrial interest. Besides being used as a digestive aid and spot remover in dry cleaning, β- amylase is also involved in the production of glucose and fructose syrups, fruit juices, ethanol fuel, sweeteners, and alcoholic beverages, [2-3]. β-amylase is found in higher plants and micro-organisms [4]. The enzyme has been well studied in various plants such as sweet potatoes, [5] and malted barley [6]. Starch amylolytic enzymes are widely distributed in microbes, plants, and animals. They degrade starch and other polysaccharides to yield products characteristic of individual amylolytic enzymes.

Flavonoids have been reported to inhibit toxins, viruses, bacteria and carcinogens. However, molecular mechanisms for these effects, and the role of flavonoid-protein interaction, are yet to be elucidated [7-8]. Data available indicated that molecular size, number and disposition of phenolic nuclei and water solubility affect the strength of flavonoid-protein binding [9]. Among flavanols, monomeric (-)-epicatechin is contained in red wine, tea, cocoa products and many fruits (blackberry, cherries, apple, peach, black grapes). It has been reported to prevent cardiovascular disease, diabetes and some cancers [10-11]. Proteins, especially serum albumin, are possible candidates for its efficient transport in the human body [12].

Yam is a staple food of and nutritional importance in the tropics. It is one of the most cultivated and consumed species. Yam comprises about 75-84% dry weight starch [13] and could serve as a measure of the expression of various enzymes in subcellular locations of the crop responsible for catalyzing starch into its respective products. It could be a good source to meet the high demand for industrial starch hydrolyzing enzymes such as β-amylase. Although, there have been studies on β-amylase from sweet potatoes [14-15], and *Dioscorea dumetorum* tuber [16], there has been a paucity of information on water yam β-amylase. Hence, the study aims to purify β-amylase extracted from *Dioscorea alata* and explore the effect of epicatechin on the enzyme.

2. MATERIALS AND METHODS

2.1 Materials

Water yam was obtained from a local vendor Ketu, Lagos, and identified by a Taxonomist from the University of Lagos, Nigeria.(−)-epicatechin were purchased from Sigma–Aldrich (purity ≥ 98%, M = 290.27 Da), 8-anilino-1-napthalene sulfonic acid (ANS), Trizma base, sodium chloride and spectroscopic grade ethanol were purchased from Sigma-Aldrich Fine Chemicals, St. Louis, MO, USA. BSA standard and Bradford reagent kit were products of Bio-Rad, Palo Alto, CA, USA. All other chemicals were commercial products of reagent/analytical grade. The stock solution of Epicatechin was prepared in ethanol-water mixture and its concentration was confirmed spectrophotometrically using the molar absorptivity at 280 nm (ε280) = 3988 M−1 cm−1 for epicatechin in methanol. The solution was kept in the dark to eliminate light induced photochemical changes.

2.2 Preparation of Crude Extract

Enzyme extraction was carried out according to the method described by [17] with slight modification. Water yam of 600 g was cut into pieces, mixed with an equal volume of cold water, homogenized with a blender, the resulting slurry was filtered with cheesecloth, cold centrifuged at 4000 × *g* for 1 hour to remove the debris and the resulting supernatant was adjusted to pH 5 by adding the same volume of 50 mM acetate buffer pH 5. The obtained solution was mixed with an equal volume of 0.8mM dodecyl trimethyl ammonium bromide and cold centrifuged at 20000 × *g* for 1 hour at 4°C. The supernatant was further used as the crude enzyme solution for subsequent purification steps.

2.3 Acid Treatment

The crude enzyme (1500 ml) was adjusted to pH 3.6 with ice-cold 1 N HCl. This was allowed to stand for 10 minutes to selectively denature α-amylase. The acidified enzyme was readjusted to pH 5 with cold 3 % NH_4OH solution [18].

2.4 Ammonium Sulphate Fractionation

The supernatant was fractionated by ammonium sulfate precipitation (40 - 80% saturation). The precipitate was collected by centrifugation at 10 000 $\times$ *g* for 30 minutes and dissolved in 20 ml of 50 mM acetate buffer, pH 5. The solution was dialyzed overnight extensively against 50 mM acetate buffer, pH 5.

2.5 Ion Exchange Chromatography on DEAE-A_{50}

The dialyzed sample (30ml) was loaded onto a DEAE-Sephadex A_{50} column (2.5 cm $\times$ 20 cm), which was previously equilibrated with over 1000 ml acetate buffer (50 mM, pH 5). The column was washed with the same buffer at 25 ml/hour until the absorbance at 280 nm of the fractions reached zero. The adsorbed proteins were eluted with a linear gradient of 0–1 M NaCl in 50 mM acetate buffer pH 5. Fractions of 5 ml each were collected. The fractions with β-amylase activity were pooled and concentrated with 10 kDa Amicon Membrane.

2.6 β-Amylase Assay Method and Protein Determination

The β-amylase activity was determined by the DNSA method [19]. The reaction mixture contained 100 µL of 1 % soluble starch in 50 mM sodium acetate buffer, pH 5, and the enzyme (100 µl). The reaction was stopped after 3 min of incubation at 25 °C with the addition of 200 µl of DNSA color reagent. The incubated mixture was boiled for 5 minutes, cooled in ice and then diluted with 1000 µl of distilled water. The Absorbance was measured at 540 nm using a UV-visible spectrophotometer against the reagent blank containing no enzyme. The enzyme activity was expressed in micromoles of maltose released per minute per milliliter of the enzyme. The presence of protein was monitored during purification by routinely measuring absorbance at 280 nm.

The protein content was estimated by the Bradford method using bovine serum albumin (BSA) as the standard protein. Measurement of the absorbance was at 595 nm.

2.7 Electrophoresis

SDS-PAGE was performed on a 12 % (w/v) polyacrylamide gel using the Tris-glycine buffer system to determine the homogeneity and the molecular mass of β-amylase. The proteins were stained with 0.05 % Coomassie Blue R-250, and the excess dye was washed out using the destaining solution.

2.8 Equilibrium Titration by Fluorescence Spectroscopy

Equilibrium titration and the fluorescence quenching of β-amylase by the Epicatechin were determined using 5 nm/5 nm slit widths and the excitation wavelength set to 280 nm while the emission was read between 300-500 nm. The Equilibrium titration was performed by continuous addition of 2.5 µM of epicatechin via a mixer to 2ml of 0.230 µM β-amylase in a quartz fluorescence cuvette. Fluorescence quenching constants were analyzed using the Stern-Volmer equation;

$$F_0/F = 1 + k_q\tau_0[Q] = 1 + K_{sv}[Q] \quad (1)$$

Where F_0 and F are fluorescence intensities before and after the addition of the quencher respectively. K_{SV} is the Stern–Volmer quenching constant, [Q] is the concentration of the quencher, k_q is the quenching rate constant of biomolecule and it is equal to K_{sv}/τ_0. τ_0 is the average lifetime of the biomolecule without quencher (τ_0=10^{-8}s)

2.9 Determination of Binding Constant and the Number of Binding Sites

Investigation of the binding constant of the ligand on the protein and the number of binding sites was achieved using the Scatchard analysis:

$$\log\frac{[F_0-F]}{F} = \log K_a + n\log[Q] \quad (2)$$

K_a is the binding/quenching constant of interaction between the quencher and the protein, *n* is the number of binding sites. The value of K_a and *n* were obtained from the plot of log (F_0-*F*)/*F* versus log [*Q*].

The thermodynamic analysis of the binding mode was analyzed from the Van't Hoff equation at different temperature ranges of 15 to 40 ˚C.

$$\ln K_a = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (3)$$

$$\Delta G = \Delta H - T\Delta S = -RT\ln K_a \quad (4)$$

Where R is the gas constant, $T$is the experimental temperature and K_a is the binding constants at corresponding Temperature while G is the Gibbs free energy.

2.10 Determination of Dissociation Constant

The dissociation constant of the ligand on the protein was ascertained by using the Hanes-Woolf plot from the equations below:

$$\Delta F = \frac{\Delta F_{max}[Q]}{K_d + [Q]} \quad (5)$$

$$\frac{[Q]}{\Delta F} = \left(\frac{K_d}{\Delta F_{max}}\right) + \left(\frac{[Q]}{\Delta F_{max}}\right) \quad (6)$$

Where ΔF_{max} is the maximum decrease fluorescence observed when the enzyme is saturated with epicatechin. The ligand concentration was allowed to 25.0μM (see Fig 3). K_d is the dissociation constant and [Q] is the concentration of the epicatechin. The linear regression plot of [Q]/ΔF versus [Q] was used to obtain the values of K_d. The value of K_d derived is shown in Table 2. Epicatechin exhibited a distinct K_d value and this asserted the feasibility of epicatechin dissociation from the enzyme. If binding is too strong as shown by the type of bonding, dissociation may be difficult and consequently the enzyme ligand function impaired

3. RESULTS

3.1 Enzyme Purification

The summary of the purification procedure is presented in Table 1 below. β-amylase from water yam was purified to an apparent homogeneity with 5.53 purification fold and a yield of 24.1 %. The elution profile of the enzyme is shown in Figure 1. A single band after SDS-PAGE shows the homogeneity of the enzyme with a molecular weight of 30.1KDa (Fig. 2). While the apparent relative molecular weight of 31.1 kDa was got by gel filtration on Sephadex G100 (Figure not shown).

3.2 Fluorescence Quenching

The fluorescence spectra of the interaction of β-amylase with epicatechin at different temperatures are shown in Fig. 3. The Fluorescence experiment result revealed that β-amylase quenched through a static quenching process (Table 2). The quenchable fractions of β-amylase fluorophores whose fluorescence intensity was quenched by epicatechin during the fluorescence study can be obtained by the common form of the modified Stern-Volmer equation also called the Lehrer equation.

$$\frac{F_0}{F_0 - F} = \frac{F_0}{\Delta F} = \frac{1}{\alpha} + \frac{1}{\alpha K_{SV}}\frac{1}{[Q]} \quad 7$$

Where α is the quenchable fraction of β-amylase fluorophores. From the intercept of the linear fit, the values of α, at all temperatures studied, were determined and the result is also shown in Table 2. It can be seen that the values of α were less than 1. The total accessible fluorophore fraction was far from unity. The values were between 0.51 and 0.71 suggesting partial accessibility of the epicatechin as quencher to the β-amylase fluorophores. However, the values is in total agreement with earlier submission that the quenchable fraction of fluorophore 'α' is less than 1 ($0 \leq \alpha \leq 1$) provided it obeyed the Stern-Volmer equation and quenching mechanism was not totally static (Fig 4).The temperature dependence of thermodynamic parameters of Epicatechin-β-amylase system at 15-40 ^{0}C expressed by Van't Hoff plot is represented in Fig 5.The values of Ka and n were obtained from the slope and the intercept, respectively. Ka value reflects the ability of epicatechin binding to βamylase. The value of n from the slope was approximately 2 (Table 2). It revealed that two molecules of epicatechin can bind tightly to β-amylase. The Ka value of Epicatechin- β-amylase interaction at 25^{0}C of was 1.53×10^3 Lmol^{-1}. Figure 6 represents the Intensity of fluorophore and its dissociative properties by modified Stern-Volmer plots for the determination of the dissociation constant (Kd) of β-amylase.

4. DISCUSSION

The results showed the presence of β-amylase in water yam. The purification steps employed in this work were adequate for the purification of the β-amylase from water yam to homogeneity. The purification steps brought about low yield and moderate purification fold of the crude extract.

Table 1. Summary of the purification of β-amylase from *D. alata*

	Volume (ml)	Activity (Units/ml)	Protein conc. (mg/ml)	Total activity (Units)	Total Protein (mg)	Specific activity (µmol/min/mg)	Fold	Yield %
Crude	1500	6.74±0.03	4.120±0.03	10115	6180	1.64	1	100
Acid treatment	1500	6.60±0.05	3.520±0.03	9900	5280	1.88	1.14	97.9
40-80 % $(NH_4)_2SO_4$)	30	7.74±0.05	2.100±0.01	232.2	63	3.69	1.96	2.35
Ion exchange on DEAE A_{50}	10	5.59±0.05	0.274±0.01	55.9	2.74	20.40	5.53	24.1

Table 2. Stern–Volmer quenching constants and thermodynamic parameters of the interaction of Epicatechin with β-amylase at six temperatures

T(K)	Ksv (x10^4)	Kq (x10^{12})	Ka (x10^3) L.mol^{-1}	n	K_d (x 10^{-4}) L.mol^{-1}	□□ kJmol^{-1}	ΔS JmolK^{-1}	ΔG kJmol^{-1}	α
288	4.40	4.40	1.84	1.69±*0.06*	2.44			-18.01	0.75
293	2.26	2.26	1.66	1.79±*0.07*	2.93			-18.10	0.56
298	1.72	1.72	1.53	1.78±*0.09*	3.12	19.35	56.67	-18.17	0.63
303	1.55	1.55	1.38	1.62±0.07	3.43			-18.21	0.55
308	1.52	1.52	1.24	1.58±0.05	3.81			-18.24	0.51
313	1.48	1.48	1.13	1.59±*0.08*	4.02			-18.29	0.46

K_q = Quenching rate constant of biomolecules, K_{SV}= Stern–Volmer quenching constant

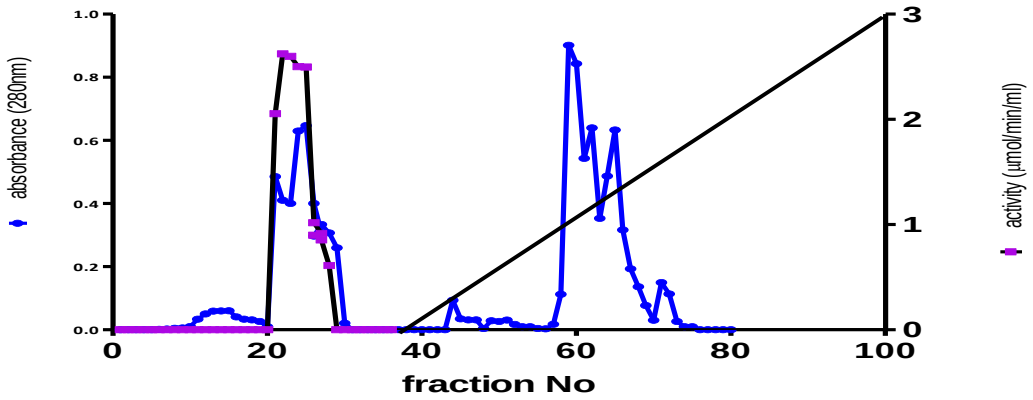

Fig. 1. Elution profile of β-amylase from *D. alata* on DEAE Sephadex A_{50} column.
The protein was eluted with the same buffer containing a linear gradient of NaCl (0-1M)

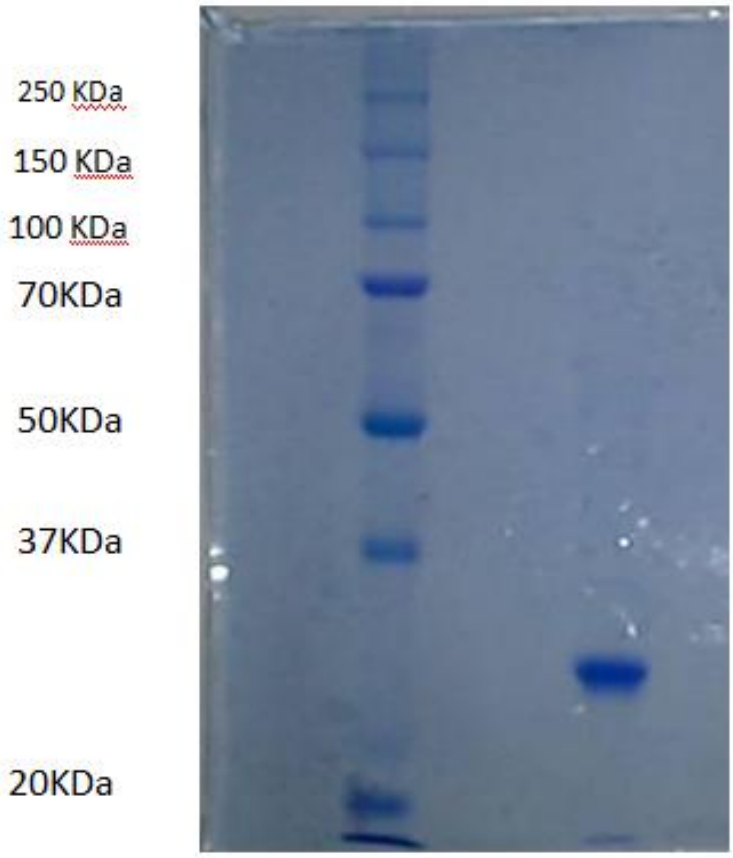

Fig. 2. 10% SDS-PAGE of Electrophoretogram of purified water yam on SDS-PAGE in 10% gel using sodium acetate buffer pH 5
Markers are Myosin (250 KDa), β-Galactoside (150 KDa), Phosphorylase b (100 KDa), Bovine albumin (70 KDa), Ovalbumin (50 KDa), Lactate dehydrogenase (37KDa), and Trypsin inhibitor (20 KDa)

The use of ammonium sulphate must have brought about some loss of activity through inactivation. The purified enzyme was homogenous on SDS-PAGE showing a single band corresponding to a molecular weight of 30.1kDa on SDS-PAGE. This, compared with

the apparent relative molecular weight of 31.1 kDa by gel filtration on Sephadex G100 under non-denaturing condition showed that it is monomeric. Studies on β-amylase from different sources especially cereals have shown the existence of one form of this enzyme; *Bacillus substilis* [14], Cereal [18], Finger millet [20], Chinese yam [17] Soybean [21] and Sweet potato [22]. Although those from sweet potato and bindweed are homotetramers [15,23], existence of five forms of β-amylase in ungerminated and germinated rice seeds had previously been reported [18]. The molecular weight of the enzyme was the same order as values that had been reported for β-amylase from *Bacillus subtilis* Isolated from Kolanut Weevil (39.4 kDa) [24], *Bacillus subtilis* β-amylase isolated from peels of Cassava barley (34 kDa) [23], 24kDa for *Pergularia tomentosa* [25], higher than 6.5kDa reported for sweet potato [26] but lower than that reported from Chinese yam tuber (56kDa) [17], Glycine max seed (57kDa) [25] hedge bindweed (55 kDa) [15] and soybean (57 kDa) [24], millet (58 kDa) [27].

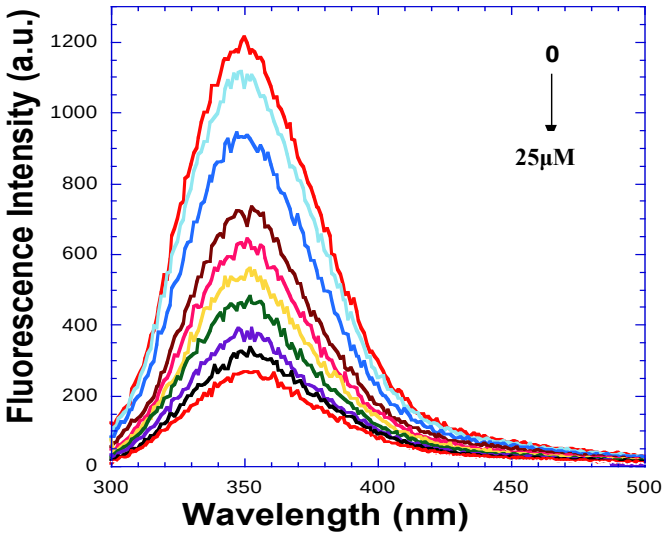

Fig. 3. The fluorescence emission spectra of Epicatechin- β-amylase complexes in the absence and presence of ligand

T = 298 K [β-amylase] was 0.230mM; [Epicatechin] was 0-25 μM at pH 5

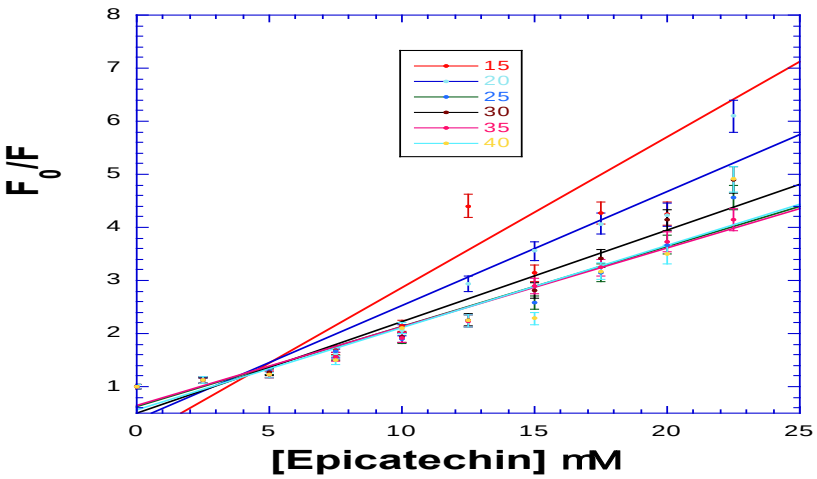

Fig 4. The Classical Stern–Volmer plots of β- amylase by Epicatechin at 288 K-313K to determine the quenching constant (K_{SV} and K_q)

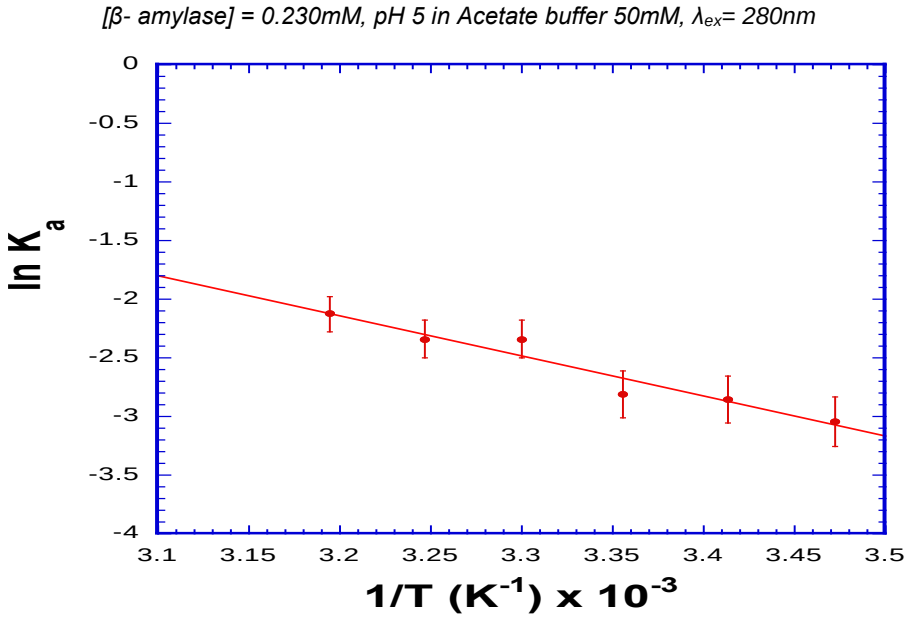

Fig. 5. Van't Hoff plot for the thermodynamic parameters of β- amylase by Epicatechin

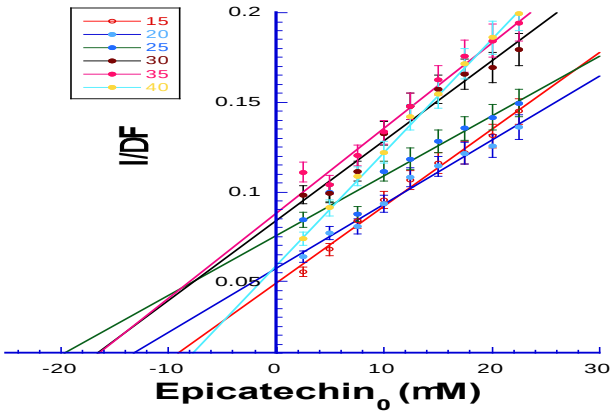

Fig. 6. Intensity of fluorophore and its dissociative properties by
modified Stern-Volmer plots for the determination of the dissociation constant (kd) of β-amylase by Epicatechin at different temperatures 288K-313K

The binding constant (K_a) of the ligand on the protein (β-amylase) and number of binding sites (*n*) was explored and the linear regression plots were shown in Figure 5. As shown in Table 2, at 298K, the binding constant shows a high binding affinity for the enzyme- ligand complex and the number of binding sites for the complex is 2. The thermodynamic parameters according to the Van't Hoff equation show that the reaction was spontaneous and hydrophobic bonding played a major role. The ***K***a value of Epicatechin- β-amylase interaction at 25^0C of 1.53×10^3 $Lmol^{-1}$ was not different from expected values of 10^4 to 10^3 L mol^{-1} of non-specific organic ligands-protein interaction using fluorescence spectroscopic methods [28]. The thermodynamic parameters can be used to predict the binding mode as follows: when (a) $\Delta H > 0$ and $\Delta S > 0$, implies hydrophobic force; (b) $\Delta H < 0$ and $\Delta S < 0$, implies van der Waals force and hydrogen bond; (c) $\Delta H < 0$ and $\Delta S > 0$, indicates electrostatic interactions. The enthalpy change (ΔH) and entropy (ΔS) of reaction, was calculated from the slope of the plot of ln $\boldsymbol{K}_a$

versus 1/*T* (*T*, absolute temperature). Based on Equations (3) and (4), these forces combine with conformational changes and solvent rearrangement account for the signs and magnitude of the protein-ligand binding as well binding stability [29]. The Δ*F* at λex equal to 280 nm with increasing concentrations of epicatechin indicates that the fluorescence of β-amylase due to its tryptophan residues was quenched by epicatechin. The thermodynamic parameters (ΔG) of the binding of β-amylase with epicatechin were slightly affected by a change in temperature. High temperatures result in lower diffusion coefficients. The result shows a high binding affinity for the enzyme-ligand complex and this implies that the rate at which the ligand will bind to the enzyme is higher than the rate of dissociation; this indicates that β-amylase metabolizes Epicatechin.

5. CONCLUSION

The above study has shown the presence of β-amylases in water yam and has demonstrated that it can be purified to apparent homogeneity by a three-step purification process.

The Fluorescence experiments results revealed that the fluorescence of β-amylase was quenched through static quenching process. The values of enthalpy change (ΔH) and entropy change (ΔS) calculated indicated that hydrophobic interactions were the dominant intermolecular forces in stabilizing the complex. The impact on β-amylase by Epicatechin is predicted to result in functional changes which could affect β-amylase normal functions after the bioflavonoid interaction. We hope that further investigations of β-amylases using other approaches, will improve existing knowledge of the enzyme structure, function, and stability.

CONSENT AND ETHICS APPROVAL

Not applicable (the study does not involve hu participant and tissues)

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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