

## Purification and some properties of *Dioscorea alata* (water yam) $\beta$ -amylase

### ABSTRACT

$\beta$ -amylase is an enzyme that hydrolyzes the  $\alpha$ -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 ( $K_a$ ),  $K_d$ ,  $\Delta H$ ,  $\Delta S$  and  $\Delta G$  values were  $1.18 \times 10^3$ ,  $178.8 \mu\text{M}$ ,  $-12.81 \text{ kJmol}^{-1}$ ,  $15.91 \text{ Jmol}^{-1}\text{K}^{-1}$ , and  $-17.55 \text{ kJmol}^{-1}$  respectively. The binding profile of  $\beta$ -amylase with epicatechin was spontaneous with a stoichiometric ratio of 1:1. Hydrophobic bonding played a major role in stabilizing the  $\beta$ -amylase-ligand complex. The mode of reaction was by static quenching. It further dictates that the binding reaction is enthalpy and entropy driven. The inhibitory effect of this plant polyphenols on  $\beta$ -Amylase might contribute to the regulation of **an *in vitro* glycemic activity of the enzyme for whatever reason.**

**KEY WORDS:**  $\beta$ -amylase, Purification, Epicatechin, Fluorescence Spectroscopy.

### 1.0 INTRODUCTION

$\beta$ -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. Amylases are one of the most important industrial enzymes with applications in various industrial fields such as food, textile, paper, detergent, and baking industries. Besides being used as a digestive aid and spot remover in dry cleaning,  $\beta$ -amylase is also involved in the production of glucose and fructose syrups, fruit juices, ethanol fuel, sweeteners, and alcoholic beverages [2, 3].  $\beta$ -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 amyolytic enzymes are widely distributed in microbes, plants, and animals. They degrade starch and other polysaccharides to yield products characteristic of individual amyolytic enzymes.

The inhibition of enzyme activity is one of the major regulatory devices of living cells and one of the most important diagnostic procedures used by the enzymologist. It provides valuable information about the specificity of an enzyme, the physical and the chemical architecture of the active site, the kinetic mechanism of the reaction, various aspects of enzymatic catalysis, and metabolic pathways.

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 [7] 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  $\beta$ -amylase. Although, there have been studies on  $\beta$ -amylase from sweet potatoes [8,9], and *Dioscorea dumetorum* tuber [10], there has been a paucity of information on water yam  $\beta$ -amylase. Hence, the study aims to purify  $\beta$ -amylase extracted from *Dioscorea alata* and explore the effect of epicatechin on the enzyme.

### 2.0 MATERIALS AND METHODS

Water yam was obtained from Ketu, Lagos, and identified by a Taxonomist from the University of Lagos, Nigeria, and the research work was carried out between January 2017 and December 2019.

#### 2.1 Preparation of crude extract

Enzyme extraction was carried out according to the method described by [11] 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  $4000 \times g$  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  $20000 \times g$  for 1 hour at  $4^\circ\text{C}$ . The supernatant was further used as the crude enzyme solution for subsequent purification steps.

#### 2.2 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

93 10 minutes to selectively denature  $\alpha$ -amylase. The 153  
94 acidified enzyme was readjusted to pH 5 with cold 3 % 154  
95  $\text{NH}_4\text{OH}$  solution [12]. 155  
96 156  
97 **2.3 Ammonium Sulphate Fractionation** 157  
98 158  
99 The supernatant was fractionated by ammonium sulfate 159  
100 precipitation (40 - 80% saturation). The precipitate was 160  
101 collected by centrifugation at 10 000  $\times$  g for 30 minutes 161  
102 and dissolved in 20 ml of 50 mM acetate buffer, pH 5. 162  
103 The solution was dialyzed overnight extensively against 163  
104 50 mM acetate buffer, pH 5. 164  
105 165  
106 **2.4 Ion Exchange Chromatography on DEAE-A<sub>50</sub>** 166  
107 The dialyzed sample (30ml) was loaded onto a DEAE- 167  
108 Sephadex A<sub>50</sub> column (2.5 cm  $\times$  20 cm), which was 168  
109 previously equilibrated with over 1000 ml acetate 169  
110 buffer (50 mM, pH 5). The column was washed with 170  
111 the same buffer at 25 ml/hour until the absorbance at 171  
112 280 nm of the fractions reached zero. The adsorbed 172  
113 proteins were eluted with a linear gradient of 0–1 M 173  
114 NaCl in 50 mM acetate buffer pH 5. Fractions of 5 ml 174  
115 each were collected. The fractions with  $\beta$ -amylase 175  
116 activity were pooled and concentrated with 10 kDa 176  
117 Amicon Membrane. 177  
118 178  
119 **2.5  $\beta$ -Amylase Assay Method and Protein** 179  
120 **Determination** 180  
121 The  $\beta$ -amylase activity was determined by the DNSA 181  
122 method [13]. The reaction mixture contained 100  $\mu\text{L}$  of 182  
123 1 % soluble starch in 50 mM sodium acetate buffer, pH 183  
124 5, and the enzyme (100  $\mu\text{L}$ ). The reaction was stopped 184  
125 after 3 min of incubation at 25  $^\circ\text{C}$  with the addition of 185  
126 200  $\mu\text{L}$  of DNSA color reagent. The incubated mixture 186  
127 was boiled for 5 minutes, cooled in ice and then diluted 187  
128 with 1000  $\mu\text{L}$  of distilled water. The Absorbance was 188  
129 measured at 540 nm using a UV-visible 189  
130 spectrophotometer against the reagent blank containing 190  
131 no enzyme. The enzyme activity was expressed in 191  
132 micromoles of maltose released per minute per 192  
133 milliliter of the enzyme. The presence of protein was 193  
134 monitored during purification by routinely measuring 194  
135 absorbance at 280 nm. 195  
136 The protein content was estimated by the Bradford 196  
137 method using bovine serum albumin (BSA) as the 197  
138 standard protein. Measurement of the absorbance was at 198  
139 595 nm. 199  
140 200  
141 **2.6 Electrophoresis** 201  
142 SDS-PAGE was performed on a 12 % (w/v) 202  
143 polyacrylamide gel using the Tris-glycine buffer system 203  
144 to determine the homogeneity and the molecular mass 204  
145 of  $\beta$ -amylase. The proteins were stained with 0.05 % 205  
146 Coomassie Blue R-250, and the excess dye was washed 206  
147 out using the destaining solution. 207  
148 208  
149 **2.7 Equilibrium Titration by Fluorescence** 209  
150 **spectroscopy** 210  
151 Equilibrium titration and the fluorescence quenching of 211  
152  $\beta$ -amylase by the Epicatechin were determined using 5 212

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 12.5  $\mu\text{M}$  of epicatechin via a mixer to 2ml of 0.230  $\mu\text{M}$   $\beta$ -amylase in a quartz fluorescence cuvette. Fluorescence quenching constants were analyzed using the Stern-Volmer equation

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

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  $k_{sv}/\tau_0$ .  $\tau_0$  is the average lifetime of the biomolecule without quencher ( $\tau_0=10^{-8}\text{s}$ ).

**2.8 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:

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

$K_a$  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  $^\circ\text{C}$ .

$$\Delta G = -RT \ln K_a \quad (3)$$

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

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.

**2.9 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:

$$EI \rightleftharpoons E + I \quad (5)$$

Where  $E$ ,  $I$ , and  $EI$  represent the enzyme, inhibitor, and the enzyme – inhibitor complex respectively. In conditions where the enzyme concentration  $[E]$  is negligible compare to the inhibitor  $[I]$  concentration, it can be written as:

$$[EI] = [E]_t I / (K_d + [I]) \quad (6)$$

Where  $K_d$  is the dissociation constant of the  $EI$  complex and  $[E]_t$  is the total enzyme concentration.

209 Dissociation constant  $K_d$  can then be calculated from  
 210 the Equation below

$$I/\Delta F = K_d/\Delta F_{max} + I/\Delta F_{max} \quad (8)$$

$$\Delta F = \frac{\Delta F_{max}}{K_d + [I]} [I]$$

212 By considering that  $\Delta F$  is proportional to  $E$ , it can be re  
 213 written in a linear form

216  
 217 Where  $\Delta F_{max}$  is the maximum decrease in fluorescence  
 observed when the enzyme is saturated by epicatechin.

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### 222 3.0 RESULTS

#### 225 3.1 Enzyme Purification

226 The summary of the purification procedure is  
 227 presented in Table 1 below.  $\beta$ -amylase from water yan  
 228 was purified to an apparent homogeneity with 5.53  
 229 purification fold and a yield of 24.1 %. The elution  
 230 profile of the enzyme is shown in Figure 1. A single  
 231 band after SDS-PAGE shows the homogeneity of the  
 232 enzyme with a molecular mass of 30.1KDa (Fig. 2).  
 233 While the apparent relative molecular weight of 31.1  
 234 kDa was got by gel filtration on Sephadex G100 (Figur  
 235 not shown).

241 Fluorescence experiment result revealed that  $\beta$ -  
 242 amylase quenched through a static quenching process  
 243 (Table 2; Figure 4). The temperature dependence of  
 244 thermodynamic parameters of Epicatechin- $\beta$ -amylase  
 245 system at 15-40 °C expressed by Van't Hoff plot is  
 246 represented in Table 3. At 298 K, the binding constant  
 247 shows a high binding affinity for the enzyme-ligand  
 248 complex and the number of binding sites for the  
 249 complex is approximately 1 (Table 4). The  $K_a$  value of  
 250 Epicatechin-  $\beta$ -amylase interaction at 25°C of was  
 251  $1.18 \times 10^3 \text{ Lmol}^{-1}$ . Figure 5 represents the Intensity of  
 252 fluorophore and its dissociative properties by  
 253 modified Stern-Volmer plots for the determination of  
 254 the dissociation constant ( $K_d$ ) of  $\beta$ -amylase.

#### 237 3.2 Fluorescence quenching

238 The fluorescence spectra of the interaction of  $\beta$ -  
 239 amylase with epicatechin at the different  
 240 temperatures are shown in Figure 3. The

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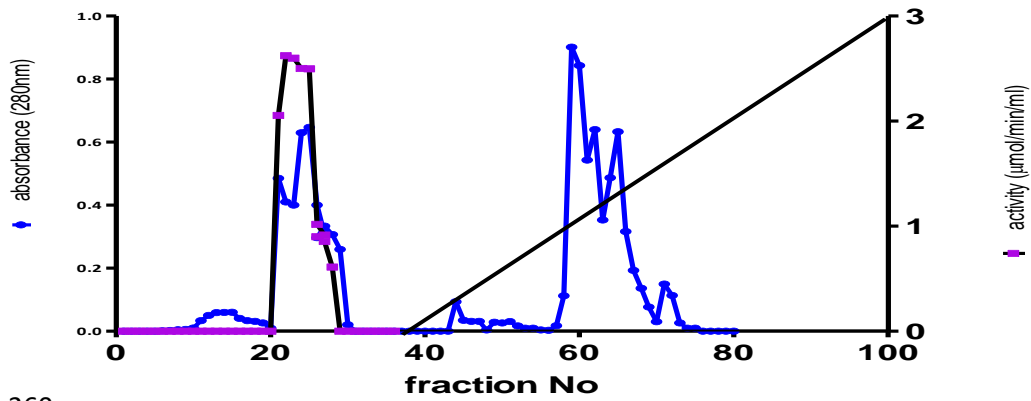
256 **Table 1: Summary of the purification of  $\beta$ -amylase from *D. alata***

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	Volume (ml)	Activity (Units/ml)	Protein conc. (mg/ml)	Total activity (Units)	Total Protein(mg)	Specific activity ( $\mu\text{mol}/\text{min}/\text{mg}$ )	Fold	Yield %
Crude	1500	6.74 $\pm$ 0.03	4.120 $\pm$ 0.03	10115	6180	1.64	1	100
Acid treatment	1500	6.60 $\pm$ 0.05	3.520 $\pm$ 0.03	9900	5280	1.88	1.14	97.9
40-80 % (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	30	7.74 $\pm$ 0.05	2.100 $\pm$ 0.01	232.2	63	3.69	1.96	2.35
Ion exchange on DEAE A <sub>50</sub>	10	5.59 $\pm$ 0.05	0.274 $\pm$ 0.01	55.9	2.74	20.40	5.53	24.1

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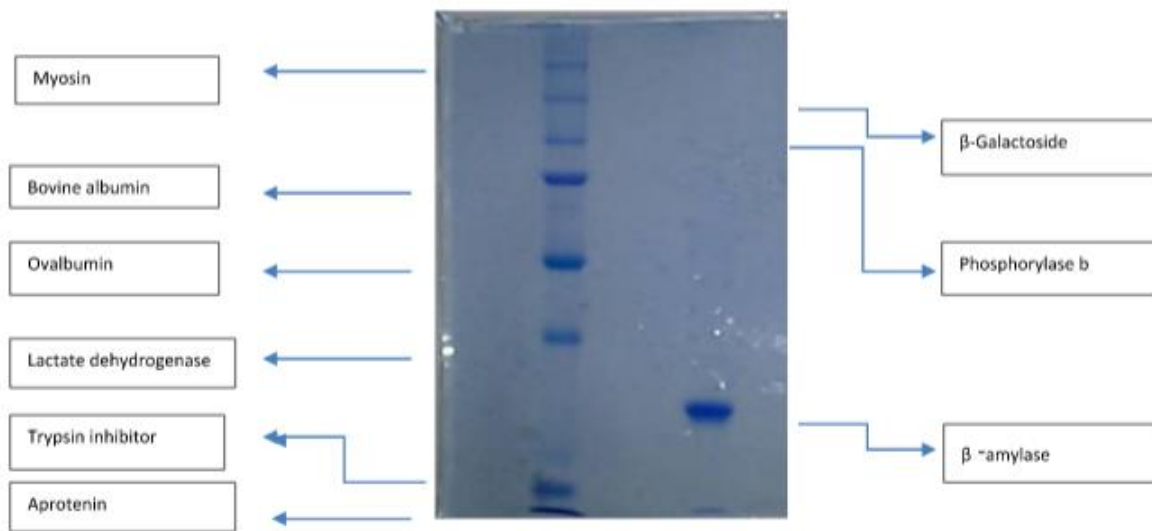
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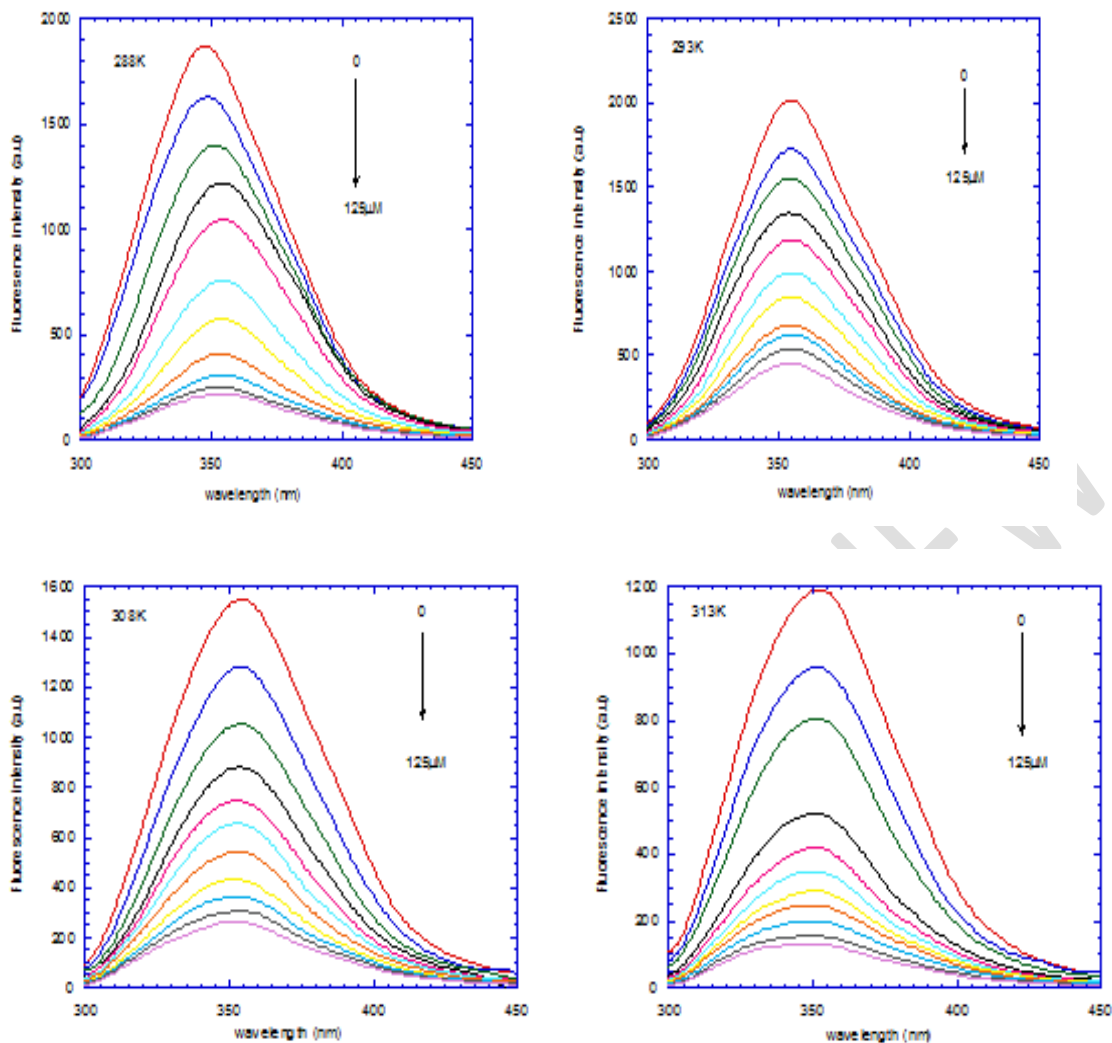
261 **Fig.1: Elution profile of  $\beta$ -amylase from *D. alata* on DEAE Sephadex A<sub>50</sub> column. The protein was eluted with**  
 262 **the same buffer containing a linear gradient of NaCl (0-1M)**

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273 **Fig. 2: Electrophoretogram of purified water yam on SDS-PAGE in 10% gel using sodium acetate buffer, pH 5.**  
 274 **Markers are Myosin(250 kDa),  $\beta$ - Galactoside (150 kDa), Phosphorylase b (100 kDa), Bovine albumin (70 kDa),**  
 275 **Ovalbumin (50 kDa), Lactate dehydrogenase (37kDa), Trypsin inhibitor (20 kDa), and Aprotinin (10 kDa).**



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283 Fig. 3: The fluorescence emission spectra of epicatechin-  $\beta$ -amylase complexes in the absence and presence  
 284 of ligand (From [0] to [125  $\mu$ M]).  $T = 288\text{K}-313\text{ K}$ . [ $\beta$ -amylase] was 0.230 mM; [Epicatechin] was 12.5, 25,  
 285 37.5, 50, 62.5, 75, 87.5, 100, 112.5 and 125  $\mu$ M at pH 5.

286

287 Table 2: Stern–Volmer quenching constant ( $K_{sv}$ ) and ( $K_q$ ) of the interaction of Epicatechin with  $\beta$ -amylase at six  
 288 temperatures.

Temperature (K)	$K_{sv} (\times 10^4 \text{M}^{-1})$	$K_q \times 10^{12} \text{MS}^{-1}$
288	7.62	7.62
293	4.40	4.40
298	3.57	3.57
303	2.92	2.92
308	2.80	2.80
313	2.64	2.64

290  $K_q$  = Quenching rate constant of biomolecules

291  $K_{sv}$  = Stern–Volmer quenching constant

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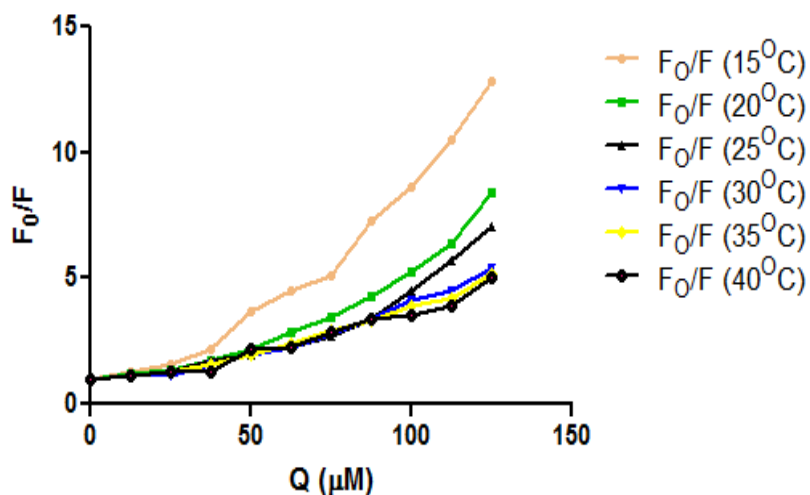
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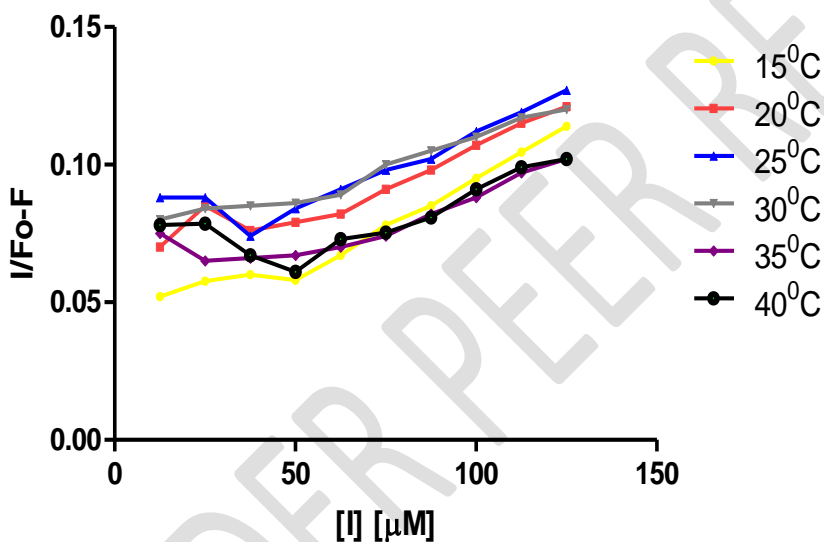
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305 Fig 4: The Classical Stern–Volmer plots of  $\beta$ - amylase exposed to Epicatechin at 288 K-313K to determine the  
 306 quenching constant ( $K_{sv}$  and  $K_q$ ). [ $\beta$ - amylase] = 0.230mM, pH 5 in Acetate buffer 50mM,  $\lambda_{ex}$ = 280nm  
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309 Fig 5: Intensity of fluorophore and its dissociative properties by modified Stern-Volmer plots for the  
 310 determination of the dissociation constant ( $k_d$ ) of  $\beta$ -amylase exposed to Epicatechin at different temperatures  
 311 288K-313K.  
 312  
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316 Table 3: The temperature dependence on thermodynamic parameters of Epicatechin-  $\beta$ -amylase system at  
 317 15-40°C by Van't Hoff plot  
 318  
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Temperature (K)	$\Delta H$ (KJ/mol)	$\Delta S$ (J/mol/k)	$\Delta G$ (KJ/mol)
288			-17.39
293			-17.47
298	-12.81	15.91	-17.55
303			-17.63
308			-17.71
313			-17.79

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328 **Table 4: Binding Parameters of  $\beta$ -amylase**  
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Temperature (K)	Binding constant $K_A \times 10^3$ ( $\mu\text{M}$ )	n	$K_d \times 10^{-6}$
288	1.48	$172 \pm 0.07$	69.34
293	1.24	$1.66 \pm 0.08$	145.4
298	1.18	$1.61 \pm 0.10$	178.8
303	1.11	$1.61 \pm 0.04$	188.0
308	1.06	$1.61 \pm 0.04$	190.2
313	0.92	$1.64 \pm 0.05$	238.9

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331 **4.0 DISCUSSION**

332 The results showed the presence of  $\beta$ -amylase in water  
 333 yam. The purification steps employed in this work were  
 334 adequate for the purification of the  $\beta$ -amylase from  
 335 water yam to homogeneity. The purification steps  
 336 brought about low yield and moderate purification fold  
 337 of the crude extract. The use of ammonium sulphate  
 338 must have brought about some loss of activity through  
 339 inactivation. The purified enzyme was homogenous on  
 340 SDS-PAGE showing a single band corresponding to a  
 341 molecular mass of 30.1kDa on SDS-PAGE. This,  
 342 compared with the apparent relative molecular weight  
 343 of 31.1 kDa by gel filtration on Sephadex G100 under  
 344 non-denaturing condition showed that it is monomeric.  
 345 Studies on  $\beta$ -amylase from different sources especially  
 346 cereals have shown the existence of one form of this  
 347 enzyme; *Bacillus subtilis* (8), Cereal (12), Finger millet  
 348 (14), Chinese yam (11) Soybean (17) and Sweet potato  
 349 (18). Although those from sweet potato and bindweed  
 350 are homotetramers (9,19), existence of five forms of  
 351 amylase in ungerminated and germinated rice seeds  
 352 had previously been reported (12). The molecular  
 353 weight of the enzyme was the same order as values that  
 354 had been reported for  $\beta$ -amylase from *Bacillus subtilis*  
 355 isolated from Kolanut Weevil (39.4 kDa) (16), *Bacillus*  
 356 *subtilis*  $\beta$ -amylase isolated from peels of Cassava barley  
 357 (34.67 kDa) (17), 24kDa for *Pergularia tomentosa* (20),  
 358 higher than 6.5kDa reported for sweet potato(23) but  
 359 lower than that reported from Chinese yam tuber  
 360 (56kDa) (11), Glycine max seed (57kDa) (18) hedge  
 361 bindweed (55 kDa) (9) and soybean (57 kDa)(18), millet  
 362 (58 kDa) (15).  
 363 The binding constant ( $K_A$ ) of the ligand on the protein  
 364 ( $\beta$ -amylase) and number of binding sites ( $n$ ) was  
 365 explored and the linear regression plots were shown in  
 366 Figure 5. As shown in Table 4, at 298K, the binding  
 367 constant shows a high binding affinity for the enzyme-  
 368 ligand complex and the number of binding sites for the

369 complex is 1. The thermodynamic parameters  
 370 according to the Van't Hoff equation show that the  
 371 reaction was exothermic, spontaneous and  
 372 hydrophobic bonding played a major role. The  $K_a$  value  
 373 of Epicatechin-  $\beta$ -amylase interaction at 25°C of  
 374  $1.18 \times 10^3 \text{ L mol}^{-1}$  was not different from expected  
 375 values of  $10^4$  to  $10^3 \text{ L mol}^{-1}$  of non-specific organic  
 376 ligands-protein interaction using fluorescence  
 377 spectroscopic methods [21]. The thermodynamic  
 378 parameters can be used to predict the binding mode as  
 379 follows: when  
 380 (a)  $\Delta H > 0$  and  $\Delta S > 0$ , implies hydrophobic force;  
 381 (b)  $\Delta H < 0$  and  $\Delta S < 0$ , implies van der Waals force  
 382 and hydrogen bond; (c)  $\Delta H < 0$  and  $\Delta S > 0$ ,  
 383 indicates electrostatic interactions. The enthalpy  
 384 change ( $\Delta H$ ) and entropy ( $\Delta S$ ) of reaction, was  
 385 calculated from the slope of the plot of  $\ln K_a$  versus  
 386  $1/T$  ( $T$ , absolute temperature). Based on Equations  
 387 (3) and (4), these forces combine with  
 388 conformational changes and solvent  
 389 rearrangement account for the signs and  
 390 magnitude of the protein-ligand binding as well  
 391 binding stability [22]. The  $\Delta F$  at  $\lambda_{\text{ex}}$  equal to 280  
 392 nm with increasing concentrations of epicatechin  
 393 indicates that the fluorescence of  $\beta$ -amylase due  
 394 to its tryptophan residues was quenched by  
 395 epicatechin. The thermodynamic parameters ( $\Delta G$ )  
 396 of the binding of  $\beta$ -amylase with epicatechin were  
 397 slightly affected by a change in temperature. High  
 398 temperatures result in lower diffusion coefficients.  
 399 The result shows a high binding affinity for the  
 400 enzyme-ligand complex and this implies that the  
 401 rate at which the ligand will bind to the enzyme is  
 402 higher than the rate of dissociation; this indicates  
 403 that  $\beta$ -amylase metabolizes Epicatechin.

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409 **CONCLUSION**

410 The above study has shown the presence of  $\beta$ - 419 dominant intermolecular forces in stabilizing the  
411 amylases in water yam and has demonstrated that 420 complex. The impact on  $\beta$ -amylase by Epicatechin is  
412 it can be purified to apparent homogeneity by a 421 predicted to result in functional changes which could  
413 three-step purification process. 422 affect  $\beta$ -amylase normal functions after the  
414 The Fluorescence experiments results revealed that 423 bioflavonoid interaction. We hope that further  
415 the fluorescence of  $\beta$ -amylase was quenched through 424 investigations of  $\beta$ -amylases using other approaches,  
416 static quenching process. The values of enthalpy 425 will improve existing knowledge of the enzyme  
417 change ( $\Delta H$ ) and entropy change ( $\Delta S$ ) calculated 426 structure, function, and stability.  
418 indicated that hydrophobic interactions were the

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428 **LIST OF ABBREVIATIONS:**

429 **SDS-PAGE:** Sodium dodecyl sulphate Poly Acrylamide gel electrophoresis

430 **DEAE:** Diethyl aminoethyl

431 **DNSA:** 3,5-Dinitro salicylic acid

432  **$\mu$ mol:** micromole

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434

**Declarations**

435 **Ethics approval and consent to participate:** Not applicable (the study does not involve human participant and tissues)

436 **Consent for publication:** The authors approve the consent for publishing the manuscript

437

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