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Characterization of Partially Purified α-amylase from Germinating African Breadfruit (*Treculia africana*) Seeds

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Abstract: α-amylase was extracted from the 13th day germinated seeds of breadfruits using 0.2M sodium acetate buffer (pH 5.5). The enzyme was subjected to ammonium sulphate precipitation and dialysis. The partially purified α-amylase showed highest activity at pH 6.0 and temperature 40°C. The Michealis constant (K_m) and the maximum velocity (V_{max}) determined from Lineweaver-Burk plots of initial velocity data at different concentrations of the cassava starch and breadfruit starch were 0.583mg/ml and 1.799µmol/min; 0.332mg/ml 1.65µmol/min, respectively. It is thermally stable between temperatures of 25 - 55°C at pH 6.0. This study indicated that *Treculia africana* seeds which are abundant in Nsukka L.G.A. of Enugu State, Nigeria can be exploited for production of starch and α- amylase for biotechnological applications.

Key words: Characterization · Breadfruit · α-amylase · Starch · Hydrolysis

INTRODUCTION

Breadfruit seed commonly called 'afon' and 'ukwa' by the Yoruba and Igbos of Nigeria is popular as a traditional food item. The seeds of *Treculia africana* (African breadfruit) are widely consumed in West Africa, especially among rural dwellers, thus playing important role in food security, economic empowerment and rural development. African breadfruit seeds have carbohydrate content of 72% [2] with about 40% of the carbohydrate as starch [1]. African breadfruit seeds have been processed into many forms like starch and flour for utilization in food industries [3]. Starch is an energy storing polysaccharides in plants and is mostly made up of a mixture of unbranched amylose and branched amylopectin [4]. Amylases are employed in starch processing industries for the hydrolysis of polysaccharides (starch) into simple sugars. They are classified as α-amylase, β-amylase and γ-amylase. Amylases have been applied in various fields such as brewing, textiles, paper and detergent industries [5]. Various species of bacteria [6 and 7] and plants [8] produce amylases. [9], reported that plants and fungi have formed the basis of important amylase studies in developing countries because of their ubiquitous nature, especially the fungi species (*Rhizopus* species and *Aspergilus niger*). Germinating seeds generally exhibit high amylase and protease activities [10]. This is because these enzymes are synthesized during seed germination to mobilize stored food (starch and protein) for the survival of the young plant until it is capable of making its food by photosynthesis [11]. There is a little information on α-amylases from African Breadfruit. Therefore, the present study is aimed at characterizing α-amylases from germinated *Treculia africana* seeds which can be used to hydrolyze starch for production of glucose which is in high demand for the production of bioethanol, glucose syrup for soft drinks, fermentation and pharmaceutics

MATERIALS AND METHODS

Materials: 3, 5-dinitrosalicylic acid (DNS) was a product of Sigma chemical company, USA. Bovine serum albumin (BSA) (Bio Rad Laboratories, India), Folin-Ciocalteau (E- merk Ltd India). Other chemicals used in this research were of analytical grade. All chemicals were freshly prepared unless otherwise stated.

Collection of Breadfruit Seed: Mature seeds of breadfruits were collected from breadfruit tree located at Eha-Alumona, in Nsukka Local Government Area of Enugu State, Nigeria

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Methods

Extraction of Breadfruit Seeds: The seeds were extracted from the pulpy fruit heads in flowing water with sand, sponge and basket until the slimness of the husk was reduced considerably [12]. The seeds were identified by Mr. Ozioko, Fabian (a taxonomist) of the Department of Botany, University of Nigeria, Nsukka as *Treculia africana.*

Processing of Cassava Starch: Cassava starch was processed using the method described by Corbishley and Miller, (1984) [13] with the following modifications. Freshly harvested cassava tubers were peeled, washed clean and grated. The grated cassava (1.2 kg) was soaked in 4 L of distilled water for an hour after which it was sieved (3 times) with muslin cloth. This was allowed to stand for 4 hrs and the supernatant decanted. The isolated wet starch was sun dried and packaged in plastic air tight container, labeled and kept in a cool, dry place.

Processing of Breadfruit Starch: The breadfruit starch was processed using the method described by Agboola *et al,* [14] with the following modifications. The seeds were dehulled to recover the kernel. The kernels were sun dried and ground to fine flour. 300g of the flour were suspended in 3L of distilled water for 24 hr. The suspended breadfruit seed flour was sieved using muslin cloth. The extracted starch was allowed to sediment for 4 hr at room temperature. The supernatant was decanted off and the starch washed with 3L of distilled water twice and finally allowed to stand for 4 hr. The supernatant was then decanted. The resulting wet starch was sun dried. The dry powder starch was packaged in an air tight container and stored at room temperature.

Germination of Breadfruit Seeds: The viable seeds were imbibed (10 hrs) and aerated (2 hrs). This was followed by another 10 hrs imbibitions. At the end of imbibitions, the seeds were germinated on already prepared germination beds for 20 days. The beds were watered every two days [15].

Extraction of α-amylase: 5, 7, 9, 11, 13, 15, 18 and 20 days germinated seeds as well as un-imbibed seeds (day zero) were decoated separately and the seeds (80g each), were homogenized for 10 min with 160ml of cold 0.2M sodium acetate buffer (pH 5.5) using Philips electric blending machine as described by Afiukwa *et al* (2009) [16]. The homogenate was filtered using Whatman No 1 filter paper and the filtrate centrifuged for 10 min at 5000 rpm. The supernatant was collected and used as the crude extract while the pellets were discarded. Amylase activity was determined for each of the extracts at pH 5.5, 7.5 and 9.0 as in the assay section below.

Purification of Enzyme: The crude α-amylase (300ml) was initially brought to 20% ammonium sulphate $[(NH_4)_2SO_4]$ saturation and allowed to stand for 12 hrs at 4°C. This was centrifuged at 15000 rpm for 10 min. The supernatant was made up to 50% ammonium sulphate saturation and allowed to stand for 12 hrs at 4°C. The precipitate was recovered by centrifugation at 15000 rpm for 10 min. This was dissolved in 0.2M sodium acetate buffer (pH 5.5) to a final volume of 20ml and dialyzed for 12 hrs against the same buffer. The dialysate was used as the partially purified α-amylase.

Assay of Amylase Activity: 1% solution of starch from breadfruit seeds and cassava tubers were prepared by dissolving 1g of starch in 40ml of distilled water and adding it to 50ml of boiling water while stirring. The volume was made up to 100ml after allowing it to cool. α-amylase activity was assayed in duplicate using the method described by Miller (1959). Each of the test tube contained 0.1ml of 1% starch solution, 2.3 ml of 0.2M sodium acetate buffer pH 5.5, 0.1ml of the enzyme and was incubated at room temperature for 30 min after which 0.5ml of DNS (3,5-dinitrosalicylic acid) was added. The test tubes were immersed in boiling water bath for 10 min. The test tubes were allowed to cool at room temperature and absorbance taken against the substrate blank at 540nm using UV- VIS (JENWAY 6405). α-amylase activity was estimated by the analysis of reducing sugar released during hydrolysis of the starch. The amount of glucose produced per unit time was estimated from a standard glucose curve using 5mM glucose solution. One unit of amylase activity (U) is defined as the amount of enzyme that releases 1µmole of reducing sugar as glucose per min under assay conditions. Protein concentration in all enzyme extracts was determined as described by [18].

Optimum pH: This was determined using the following buffer system: 0.2M sodium acetate buffer (pH 3.5 – 5.5), 0.1M sodium phosphate buffer (pH 6 – 7.5) and 0.1M Tris – HCl buffer (pH 8.0 – 10.0) at 0.5 intervals. α-amylase activity was assayed using each of these buffers as in the assay section.

Optimum Temperature: α- Amylase activity was assayed as in the assay section at different temperatures ranging from 25 – 70°C at 5°C intervals in a water bath.

Determination of V_{max} and K_m: Vmax and Km values were determined from Lineweaver-Burk plot of initial velocity data obtained at different concentrations (0 – 1 mg/ml) of starch from breadfruit seeds or cassava tubers separately in 0.1M sodium phosphate buffer (pH 6.0).

RESULTS AND DISCUSSION

The Percentage Starch Yield of Cassava Tubers: The percentage starch yield obtained from the cassava tubers was 34.74%. This fell almost within the 20 – 32% range given for ½ to 1½ year old plants by the International Starch Institute, (2009). Dia *et al,* [19], reported a starch yield of 32% from cassava tubers while [20] reported a percentage yield of 18.32%. The result of this research is higher than 24% reported by [21]. The reason for the differences in the percentage yields might likely be as a result of geographical and breeding influences and possibly age as the age of the plants used were not verified.

The Percentage Starch Yield of Breadfruit Seeds: The percentage starch yield obtained from the breadfruit seeds was 17.14% which is higher than 14.26% reported by [22] and less than 18.05% reported by [23]. This is also higher than 15.4% reported by [24].The moderately high breadfruit starch yield of 17.14% from this study is of significant importance in domestic and industrial food utilization. The differences in yield obtained here might be as a result of difference in processing methods. However, variation in the starch content of breadfruit seeds may depend on the maturity, variety and differences in climatic and agronomic conditions [25].

Variation of Protein and Amylase Activity with Days of Germination: Result showed that germination started 7 days after imbibitions and was completed in 20 days. [26], observed that breadfruit seeds started germinating on the 10th day. The differences in the pattern of germination might be as a result of differences in the method used for seed extraction, climatic condition of the area from where the breadfruit seeds were obtained and the moisture content of the breadfruit seeds. The result of this study showed steady increase in α-amylase activity from day zero through day 5 to the 13th day of germination. Maximum α-amylase activity was detected at 13th day at pH 7.5 (Figure 1). The α-amylase activity started decreasing from day 15 through day 20 after imbibitions. This is possible because α- amylase is expressed and secreted out of cells of aleurone layer and scutellar epithelium in germinated seeds to hydrolyze the endosperm starch to glucose as germination progresses [27]. This result corresponds with the observations made by [28] that during germination, both α- and β- amylase activities increase in the endosperm but the former becomes by far the chief amylolytic enzyme.

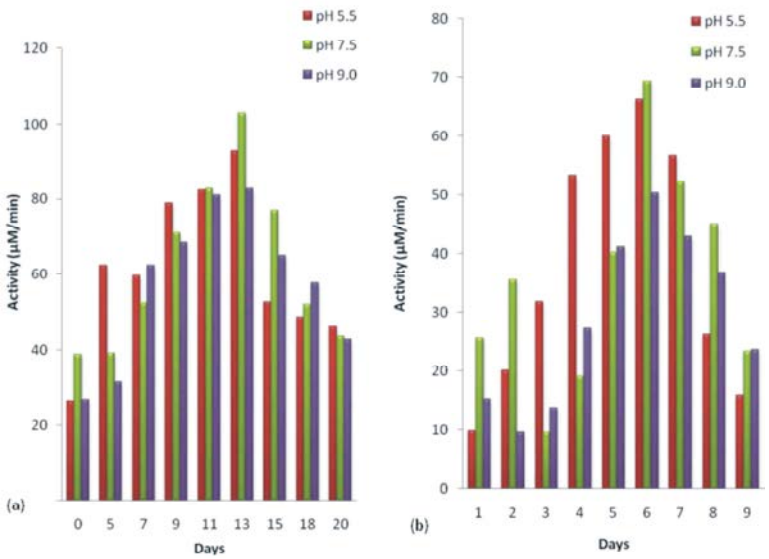

Fig. 1: Determination of day of maximum α-amylase activity after germination of breadfruit seeds (*Treculia africana*) using starch from (a) Breadfruit seeds. (b) Cassava tubers as substrate.

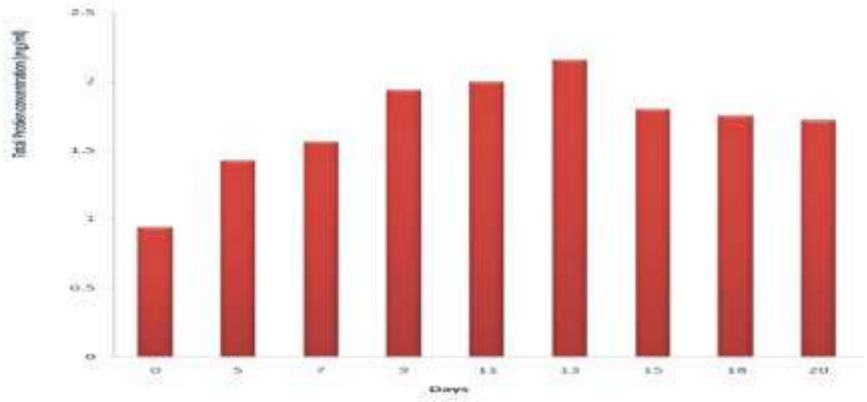

Fig. 2: Determination of day of maximum protein production after germination of breadfruit seeds (*Treculia africana)*

Table 1: Purification Table of the extracted α-amylase

Enzyme sample	Volume	Activity (U/ml)	Total activity	Protein (mg/ml)	Total protein (mg)	Specific activity (U/mg protein)	Yield (%)	Purification factor
Crude	500	93.03	46516.47	2.069	1034.5	0.0899	100	1
Precipitation [$(NH_4)_2SO_4$]	300	223.69	67108.89	2.228	668.4	0.3347	41.59	3.72
Dialysis	20	262.37	5247.47	2.45	49	5.3546	85.26	15.99

Partial Purification of the α-amylase from Breadfruit Seeds: Trial precipitations with solid ammonium sulphate between 10% - 100% were tested to find the proper concentration of ammonium sulphate that will precipitate the enzyme. 50% ammonium sulphate saturation was found to give the best precipitation and was used in subsequent extraction process. The result of the purification profile is shown on Table 1. The result showed that the enzyme was purified 3.72 fold with a specific activity of 0.3347U/mg proteins after ammonium sulphate saturation. The enzyme was then dialyzed and showed 15.99 fold enzyme purification with a specific activity of 5.3546U/mg protein. An extracellular α – amylase produced by *Penicillium chrysogenum* in solid state fermentation was purified with ammonium sulphate and gave 6.2 – fold purification after dialysis [29]. The result of the ammonium sulphate precipitation of α – amylase produced by the strain *Penicillium cameberti* PL21 showed that the enzyme precipitated at 60% ammonium sulphate and when subjected to dialysis resulted in 16.3 – fold purification [30]. [31], reported 60% ammonium sulphate precipitation and a purification fold of 10.9 after dialysis against sucrose. [32], also purified α – amylase using ammonium sulphate precipitation and dialysis against sucrose resulted in 6.20 purification fold. The difference could be because of the source of the enzyme. However, the yield of the enzyme after ammonium sulphate precipitation and dialysis was high (41.59% and 85.26% respectively) (Table 1).

Optimum pH: The optimum pH obtained was 6.0 as seen in Figure 4. This differs slightly from the report given by [33] that optimal pH for the activity of α-amylase from sprouting maize, rice, acha and sorghum with optima at 6.5, 5.5, 6.5 and 5.8, respectively. Similarly, [34] reported optimum pH of 6.0 for amylase from partially germinated mango seeds. [35] reported that α-amylase from *penicillin camemberti* showed optimal activity at pH range of 5.0 – 6.0. Seeds have β-amylase which is present in an inactive form before germination but is activated during germination. The germinating embryo produces gibberellins which stimulate the aleruone layer to produce enzymes including α-amylase, β-amylase and limit-dextrinase during germination [36]. The result suggests that during α-amylase extraction, β-amylase or limit dextrinase may be part of the extracted enzyme since they are present in an active form in the seed during germination and that may account for the two peaks in the pH profile.

Optimum Temperature: The optimum temperature of the enzyme was observed at 40°C as seen in Figure 5. [37] reported an optimum temperature of 60°C for α-amylase extracted from partially germinated mango seeds (*mangifera oraphila)*. Tapan *et al,* (2005) reported that amylase in *Heliodiaptomus viduus* (Gurney) (Crustacean: *Copepod calanoida*) gave optimum activity at 30°C. The observation made by [38] showed that the optimum temperature for α- amylase from sprouting maize and

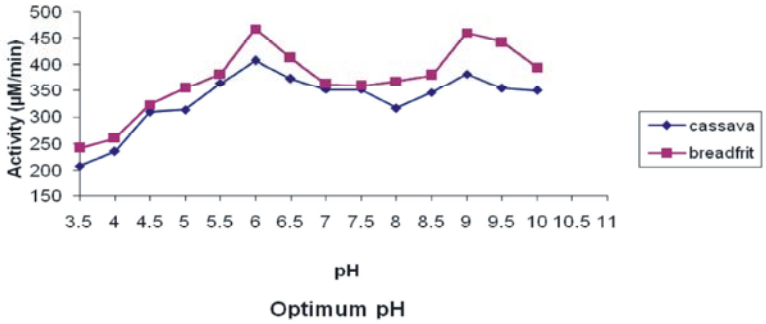

Fig. 3: Effect of pH on α-amylase activity using starch from (a) Breadfruit seeds. (b) Cassava tubers as substrate

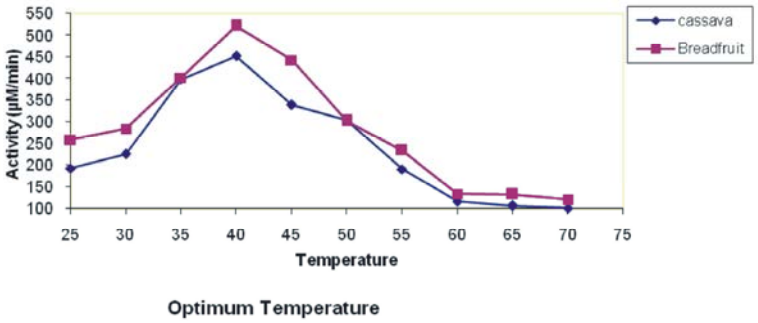

Fig. 4: Effect of temperature on α-amylase activity using starch from (a) Breadfruit seeds.(b) Cassava tubers as substrate

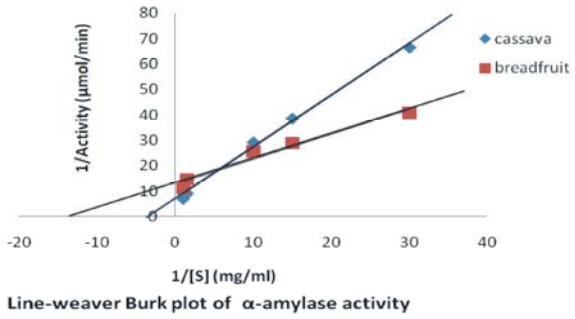

Fig. 5: Line-weaver Burk plot of α-amylase activity

rice was 60°C while that of sprouting acha and sorghum was 70°C. α- Amylase of saccharomyces origin [39] and *penicillium camemberti* PL21 [40] were reported to have optimum activity at 30°C .

Determination of Vmax and Km: The Michealis constant, K_m and the maximum velocity, V_{max} determined from Lineweaver-Burk plots of initial velocity data at different concentrations of the cassava starch and breadfruit starch were 0.583mg/ml and 1.799U; 0.332mg/ml and 1.65U, respectively as seen in Figure 6. [41] gave the Vmax and Km for amylase from sprouting maize, acha, rice and sorghum as 0.09U and 0.23mg/ml; 0.0125U and 0.56mg/ml; 0.07U and 0.29mg/ml and 0.0125U and 0.50mg/ml respectively. [42] reported Km value of 0.6mg/ml for α-amylase from *Bacillus amyloliquifacience* TSWK1-1. [43] reported Km value of 0.68mg/ml from *thermomyces lanuginosus*. [12] as well as [24] observed that the Km of α-amylase from *Bacillus sphaericus* and *penicillium camlemberti* PL21 was 0.92mg/ml each, respectively. [14] reported Vmax of 56.18U and Km of 9.79mg/ml for glucoamylase obtained from *Aspergillus awamori*: *naka zawa* MTCC 6652 using starch as the sole carbon source. Also, [38] observed a Vmax of 0.793U and Km of 0.022mg/ml.

CONCLUSION

The results gotten from this study indicates that the partially purified α-amylase has more affinity for starch from breadfruit seed than that from cassava.

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