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An Alpha Amylase Like Protein from Plantains

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Authors' contributions

This work was carried out in collaboration between all authors. Author IKA designed the study and performed the experiments. The three authors reviewed the design of the study and performed analysis of the data. Author IKA wrote the protocol and the first draft of the manuscript. Authors IKA, AAI and BAB wrote, read and approved the final manuscript.

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Short Research Article

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ABSTRACT

Carbohydrates and lignocellulose biomass are the major feedstock for the bioethanol production. The processing of starch to bioethanol is a challenging process that requires several agents and varying conditions. The starch liquefaction and saccharification are key processing steps in the bioethanol industry. The rate-limiting α-amylase plays an important role due to its endo-glycosidic activity. α-amylase endo-glycosidic action on long glucan chains may be rate limiting in starch. The purpose of this study is to purify an alpha amylase from plantains. Water soluble proteins were extracted from fully ripened plantains, and α-amylase activities were measured. It was observed that there is a strong variation in α-amylase activities among individual plantains, although the protein concentration was generally low. The proteins were fractionated using ammonium sulphate, the α-amylase precipitated at 30% of the salt. This is characteristically low and desirable since most proteins precipitate at higher ammonium sulphate concentration. It suggested that majority of contaminating proteins and other molecules may be removed in the single step; dialysis was used to remove the salt. Consequently, significant enrichment in α-amylase activity was recovered after dialysis. Subsequent purification of the protein was attempted using ion exchange chromatography.

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The protein binds to Q-sepharose at neutral pH but this was not successful at acidic pH. Therefore, the result revealed that the protein could be negatively charged at that condition. Hence, a new alpha amylase like protein was purified from plantains.

Keywords: Hydrolases; alpha amylase; starch; glucose; proteins; ion exchange chromatography.

1. INTRODUCTION

Glucose in plants is stored in the form of starch thus giving the structure its compactness. Starch is a feedstock for a variety of industrial processes; food and beverages, gums and thickeners as well as the energy industry [1-4]. It is a polymer which compose of amylose and amylopectin. Amylose is a linear glucose polymer with the sugars linked by α-1,4 glycosidic bonds. In contrasts, amylopectin is branched in nature and at the branched points the sugars are linked by α-1,6 glycosidic bonds [5-9].

Due to its physiological importance, starch is found nearly all classes of crops. Cereals such as maize, rice and millet; fruits such as apple, banana; tubers such as yam, cassava and potato; legumes among others [10-13]. In addition, starch can be in transitory and storage forms. Transitory as the name implies is synthesized in leaves during the day and degraded at night; while the reserves that are stored in seeds and tubers or other vegetative tissues represent the second form [10-13].

In plants, the chloroplasts or amyloplasts are the sites of starch hydrolysis; a process that requires a variety of enzymes [14-19]. These enzymes include hydrolases; endoamylases, exoamylases, debranching enzymes, and transferases. The endoamylase α-amylase hydrolyses α-1,4 glycosidic bonds in internal positions of amylose and amylopectin structures leading to the generation of smaller water soluble glucans [17,20,21]. Exoamylases such as β- and gluco-amylases cleave both α-1,4 and α-1,6 glycosidic bonds at the external part of starch molecule. Debranching enzymes, example isoamylase and pullulanase hydrolyse the α-1,6 glycosidic bonds while the transferase cuts α-1,4 glycosidic bond of a donor and transfers it to an acceptor molecule [22]. Of all these enzymes, α-amylase is considered as a key enzyme for industrial starch hydrolysis due to its endo-amylase activity [23,24].

Alpha amylase (1,4-α-D-glucan-4-glucanohydrolase, EC 3.2.1.1) is a member of the glucosyl hydrolase class-13 [20,25]. The enzyme is found in humans, animals, microbes, plants and the archaea [26,27]. High activity of α-amylase has been reported during seed germination, indicating its role in starch mobilization in germinating seeds where starch reserves are used for energy [13,20].

Plant α-amylases are grouped into three families; one, two and three. Family one α-amylase are secreted proteins with a signal peptide for entry into the endoplasmic reticulum. It is found in cereals and seeds of dicot plants [28,29]. In plants such as barley, α-amylase may be produced and secreted by the aleurone cells or scutellum or both of seeds [30-32]. The second family do not have any targeting peptide; thus are localised to the cytoplasm. The proteins are found in leaves of monocots and dicot plants as well as gymnosperms and degrade cytosolic α-glucan or heteroglycan [28,29]. Family three α-amylases are almost twice the sizes of amylases of families one and two and have a chloroplast transit peptide in the N-terminal domain [28,29].

Starch is semi-crystalline in nature; thus some amylases have additional features for effective binding to the starch molecule. It is referred to as a carbohydrate binding module (CBM) or starch binding domain (SBD) [33-35]. They are structural motifs that facilitate effective binding of the amylase with starch leading to hydrolysis [36-38]. Previous research efforts were mostly focused on identifying heat stable alpha amylase due to industrial processing at high temperature [39,40].

Plantain is a climacteric fruit that consists of high amount of crystalline and resistant starch when unripe. However, ripening take place in eight stages during which the starch is progressively converted into soluble sugars [41-43]. This conversion is thought to be mediated by different hydrolases. One of these enzymes is α-amylase which has an endo-glycosidic activity to attach an intact starch granule [40,44,45]. Similarly, ripening fruits are able to hydrolyse starch to soluble sugars at ambient temperatures; hence hydrolases may be expressed that utilise crystalline starch as their substrate [41,46,47]. In view of the above, such plants may be considered as good models for the discovery of α-amylase with unique properties.

2. METHODS

2.1 Protein Extraction

Fully ripened plantain were identified and collected from local market in Kano, Nigeria. Water soluble proteins were extracted from the plantain as followed; the plantain was peeled, the pulp was cut and measured. Alpha amylase extraction buffer in the ratio of 1:2 was added to the pulp and ground using a domestic blender. The homogenate was spun at maximum using the refrigerated centrifuge, the supernatant was recovered and pellet discarded. Total protein concentrations were measured by Bradford method [48] using the Bio-rad reagent. The alpha amylase activity was measured as described below.

2.2 α-amylase Assays

The amylase activities were measured according to the procedure described on the Megazyme kit. The amylase extraction buffer (50 mM malic acid, 50 mM NaCl, 2 µM $CaCl_2$, 0.02% sodium azide and 0.02% BSA). The α-amylase substrate contained blocked P-nitrophenyl maltoheptaosides (54.5 mg), glucoamylase (100 U, pH 5.2), α-glucosidase (100U, pH 5.2) and dissolved in 10 ml of distilled water according to manufacturer's instructions. The assay was performed as followed: 30 µl of the substrate was added to 30 µl of sample, and the mix was incubated at 45ºC; the reaction was stopped by adding 150 µl of 1% (w/v) Tris pH 11. 200 µl of the solution was then added into the well of microtitre plate and the optical density was read at 405 nm. Values greater than O.D of 1 were considered out of scale; and the respective samples were diluted. The amylase activity was calculated as the change in optical density divided by the volume of extract used (µl) and incubation times and multiplied by 1000 to convert to ml. The assays were repeated either twice or thrice and the average was computed.

2.3 Ammonium Sulphate Precipitation and Dialysis

The protein was fractionated ammonium sulphate ($(NH_4)_2SO_4$) precipitation at 30%. Ammonium sulphate fractionations were performed repeatedly for plantain and barley extracts. Dialysis membrane CelluSep® (T2, MWCO 6000 – 8000) was used according to the manufacturer's instruction. The ammonium sulphate fractions were dialysed against distilled water overnight at 4ºC. The alpha amylase activity was measured using the Ceralpha Method from Megazyme according to manufacturer's instructions [49,50]. Total protein concentrations were measured by Bradford method [48] using the Bio-rad reagent.

2.4 Column Purification

Alpha-amylase purification was attempted using the GE Healthcare HiTrap column for anion exchange chromatography (Q-IEX) and cation exchange chromatography (SP-IEX) with Hepes buffer at pH 7.5 and elution by application of a salt gradient (0-1 M). A further purification of alpha-amylase using Q-HiTrap column was performed with alpha amylase buffer at pH 5.5 instead. The binding of alpha amylase to sepharose was tested in sepharose binding assay; 200 µl of sepharose slurry (initially washed with HEPES buffer) was added to 600 µl of HEPES buffer and 200µl of dialysed amylase extract were mixed and used.

3. RESULTS

3.1 Extraction of Water Soluble Proteins from Plantains

The protein was extracted in by mixing and blending the tissues with the amylase buffer. It was then centrifuged, and the supernatant was recovered. Water soluble extracts from fully ripened plantains were tested for α-amylase activity using blocked para-nitrophenyl maltoheptaoside. The oligosaccharide substrate mimics starch; a compound is released upon cleavage of the substrate by α-amylase. Plantain ripening is a long process from a green plantain to an almost liquid fruit. Generally, higher activities and protein concentration were observed at later stages of ripening. However, analysis of ripe plantains still revealed enormous variation in enzyme activity that could not be correlated to a specific property. Fig. 1 shows the variability in α-amylase activity of different plantains. The extract obtained using the standard low-pH alpha-amylase extraction buffer showed a greater enzyme activity.

3.2 Ammonium Sulphate Fractionation of Alpha Amylases Leads to Strong Enrichment of the Activity

In order to enrich the extract for α-amylase and to separate proteins from soluble cell wall polysaccharides and other contaminants; proteins were precipitated using ammonium

sulphate. The results showed that that maximum α-amylase activity was recovered when precipitation was carried out at 30% ammonium sulphate (Fig. 2). Further increase in ammonium sulphate did not increase the recovered activity. This is good since most proteins precipitate at higher ammonium sulphate concentration, a 30% precipitate may lead to a strong enrichment of the desired protein in a single step.

3.3 Dialysis of the Fractionated Fractions

The remaining salt in the pellet was removed by subsequent dialysis of the re-suspended protein pellet against distilled water overnight. This will ensure stability of the protein. Alpha amylase activity and protein concentration were measured in the starting material, the re-suspended precipitate before and after dialysis. A remarkable recovery and enrichment of enzyme activity after dialysis was observed as shown in Table 1. A 20-fold enrichment of the enzyme relative to the total proteins was obtained. On the other hand, there was a significant loss of protein during dialysis, which could be due to the overall low protein concentration of the plantain extracts.

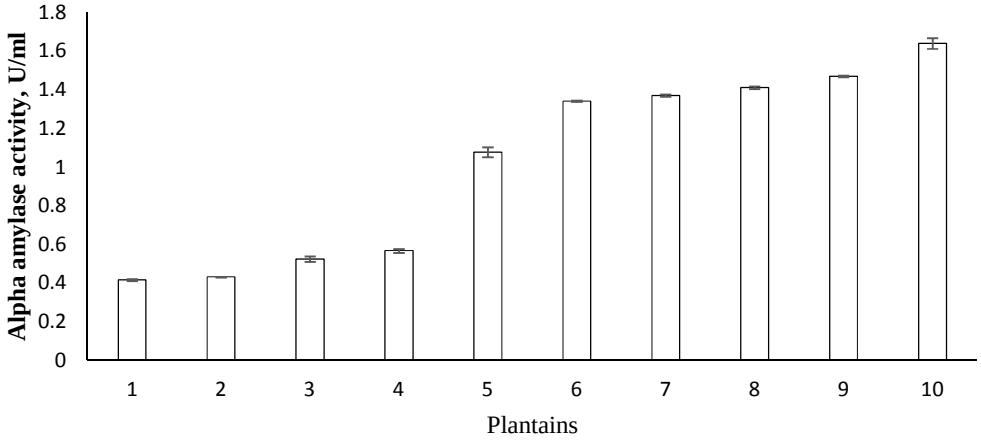

Fig. 1. Plantains α-amylase activity profile. Soluble proteins were extracted from different ripened commercially available plantains that were fully ripened. Error bars indicate repetitions of the assays for individual plantains

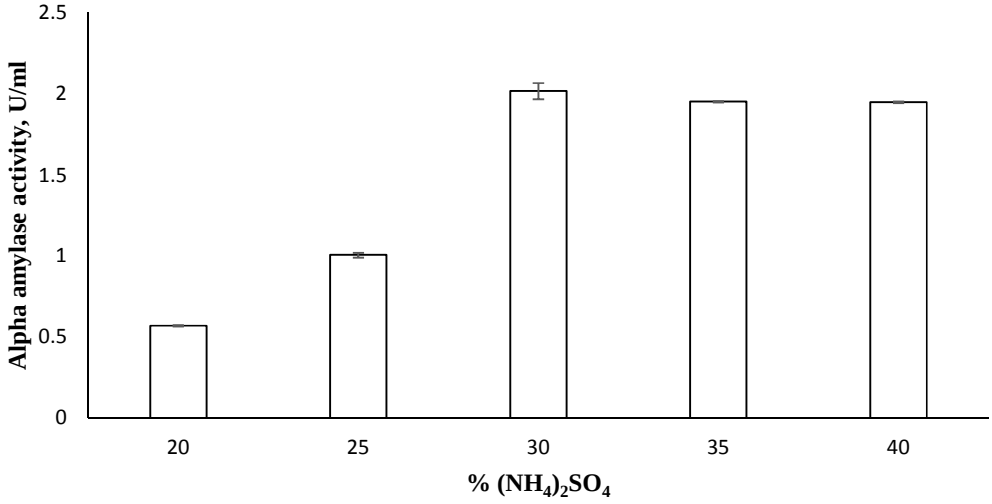

Fig. 2. Enzyme activity of the precipitates, the pattern of enzyme activity on fractionation of amylase at different percentages of ammonium sulphate are shown. It shows the α-amylase activity obtained on fractionation of plantain α-amylase. Error bars indicate standard deviations

Table 1. Enrichment of amylase activity

	Starting	Re-suspended precipitate	Dialysed
α-Amylase activity (U/ml)	3.50	9.53	8.44
Protein concentration (mg/ml)	1.12	0.30	0.14
Specific activity	3.12	31.54	59.45

Table 2. Activities of the eluted fractions

Fractions	1	2	3	4	5	6	7	8
Amylase activity (U/ml)	0.037	1.722	3.502	1.536	1.685	2.603	1.423	0.487
Protein concentration	0.022	0.085	0.173	0.072	0.071	0.099	0.043	0.023
Specific activity	1.672	20.270	20.299	21.410	23.498	26.389	32.914	20.772

3.4 Purification of Alpha Amylase

The next step was to purify the protein using an ion exchange column. The experiments revealed that at neutral pH, the measured α-amylase Eactivity remained bound to Q-sepharose, suggesting that the enzyme is negatively charged under these conditions. Other conditions of pH and S-sepharose were attempted, but did not give a defined result. In one experiment, a defined α-amylase activity peak was eluted (Table 2), in subsequent repetitions the protein bound to the column but failed to elute.

4. DISCUSSION

4.1 Plantains Exhibit Varlability in α-amylase Activity

Significant α-amylase activities were measured from fully ripened plantains, however there is strong variation among the extracts. High activity of α-amylase which is one of the principal enzymes in starch degradation has been reported in ripening fruits [10,41,46,51,52]. Higher α-amylase activity in plantains at later stages of ripening may suggest the role of the enzyme in starch hydrolysis during ripening [42,47].

4.2 An Alpha Amylase-like Protein was Purified from Plantains

The pattern showed by plantain α-amylase is in close agreement to the precipitation of α-amylase *Eisenia foetida* of 35% $(NH_4)_2SO_4$ [53]. Ammonium sulphate fractionations of α-amylases have been achieved at 35-65% concentration for α-amylase from cowpea, millet, soybean, safflower and azuki bean with a high recovery of enzyme activity has also been reported [20,54,55]. These differences can be attributed to lots of factors, among which are the size of the proteins, stability at high salt concentration and isoelectric point (pI) [39,56,57]. Since most proteins need more than 30% ammonium sulphate to precipitate, fractionation of α-amylase at this concentration leads to higher specific activity thus enrichment of enzyme activity.

In the present work, attempted purifications of the amylase extract on ion exchange columns after dialysis produced results that were inconsistent and not reproducible. Initial experiments showed that at neutral pH, the measured α-amylase activity remained bound to Q-sepharose, suggesting that the enzyme is negatively charged. The α-amylase failed to bind to either Q-sepharose or S-sepharose at pH 5.5, this may indicate that the protein was not charged coupled with loss of proteins on the column which leads to a reduced amylase activity. A challenge to the purification is the fact that plantain extracts exhibit extremely low protein concentrations even upon optimisation. In addition, is the high viscosity exhibited by the extract which may be due to polysaccharides. The polysaccharide molecules may compete with the protein for the column, thereby interfering with the binding of the protein to column. Consequently, these have overall negative effect on purification of the amylase by chromatography.

5. CONCLUSION

Amylases including alpha-amylase, beta-amylase and glucoamylase are important hydrolases for the different processing and conversion of starch. The alpha amylase is known to play roles in liquefaction and saccharification of starch. These enzymes are ubiquitous, and can be found and isolated from various sources including bacteria, fungi and

plants. An alpha-amylase like protein was identified from plantains, however this protein showed some uncommon characteristics such as precipitation at low salt concentration which is typical of proteins due to their dynamism.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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