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Isolation, Purification, Characterization and the Possible Involvement of Histidine and Cysteine in the Catalytic Mechanism of Beta-amylase Sourced from Cassava (*Manihot esculenta* Crantz) Peel

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Abstract: Beta-amylase is a starch hydrolyzing enzyme which is fondly used in both foods, pharmaceutical and brewing industries to convert starch into maltose. Hence, this study was carried out to isolate, purify, characterize and determine the possible involvement of histidine and cysteine in the catalytic mechanism of beta-amylase sourced from cassava (*Manihot esculenta*, Crantz) peels. Beta Amylase was obtained from cassava peels and purified by gel filtration and ion exchange chromatography. The homogeneity of the enzyme was established by polyacrylamide gel electrophoresis and its molecular weight by Sodium Dodecyl Sulphate Polyacrylamide Electrophoresis (SDS-PAGE) on 10% gel. The Michealis-Menten constant, Km, maximum velocity, Vmax and Kcat were obtained from Line-Weaver Bulk plot. From the plot of logVmax/km Vs pH, the apparent pk values of 5.21 and 6.58 were obtained. Effects of temperature, pH, salts concentration and temperature on stability of beta amylase activity at pH ranging from 5-8 were determined. The polyacrylamide gel electrophoresis in the presence and absence of SDS produced a single bond. The enzyme was found to have an optimum activity at pH 5 and 60°C. The current work confirmed the presence of beta-amylase in cassava peels and was also found to be thermostable and thermoactive, good enough for some industrial applications.

Key words: Beta-amylase, *Manihot esculenta* Crantz, isolation, enzyme activity purifications, characterization, catalytic mechanism

INTRODUCTION

Beta-amylases (EC 3.2.1.2) are exo-hydrolases which release beta-maltose from the non-reducing ends of alpha-1, 4 linked poly and oligoglucans until the first alpha-1, 6 branching point along the substrate molecule is encountered (Sakai, 1988).

It is a starch hydrolyzing enzyme which is fondly used in both foods, pharmaceutical and brewing industries to convert starch into maltose (Aiyer, 2005). Starch is a carbohydrate food reserve in plants and major energy source for non-photosynthetic organisms. It is abundant in nature and is composed of two distinct polysaccharides, amylose and amylopectin (Lizotte *et al*., 1990). The main changes in its structure, which involve the depolymerization of these two forms, can be accomplished by mechanical hydrothermal and enzymatic treatments (Grime and Briggs, 1995). Beta-amylase occurs widely in higher plants (Aunstrup, 1978; Evans *et al*., 1997) and also in micro-organisms (Nanmori, 1988). It has been well studied in a number of plants such as sweet potatoes (Cochrane *et al*., 1991) malted barley (Evans *et al*., 1997). Not-withstanding most of the microbial beta-amylases produced, however have been found not to be active and thermostable enough to replace the indigenous plant enzymes from agarian sources such as barley, soyabean, cassava (Fossi *et al*., 2005). Starch degrading enzymes are distributed widely in the microbes, plants and animals. They degrade starch and related polymers to yield products characteristic of individual amylolytic enzymes.

Fig. 1: Heap of cassava peels

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High value is placed on the thermostability and thermoactivity of amylase used in the bio-processing of starch into various maltose syrups (Saha and Zeikus, 1989). However, some reports show that beta-Amylase from higher plants and from micro-organisms are neither active nor stable at temperature above 60°C (Fogarty and Kelly, 1990). Hence current study focus on providing beta-Amylase that can withstand the stress of operating conditions either through genetic manipulation to improve beta-Amylase from a known source or searching for a new source with the potentials of producing amylase of desirable physicochemical properties.

MATERIALS AND METHODS

Raw materials and enzyme extraction: Samples of cassava peels were obtained from domestic sources. Cassava peels were dried and ground into fine powder. The powder (50 g) was suspended in 300 ml of 0.02 M sodium acetate buffer pH 5.0. The mixture was stirred overnight and filtered using cheesecloth. The filtrate was centrifuged at 4000 rpm for 10 min with Automatic super speed centrifuge at 4°C. The filtrate served as the crude extract.

Purification: Crude extract was applied to a column packed with sephadex G - 100 (2.5 x 68 cm) that had previously been equilibrated with 0.02 M sodium acetate buffer, pH 5.

Fractions of 5.0 ml were collected at flow rate of 20 ml/hour. The pooled unbound beta-amylase was further purified on a column packed with CM cellulose (2.5 x 27 cm) that had previously been equilibrated with 0.02 M sodium acetate buffer pH 5. The unabsorbed proteins were eluted with the same buffer. Fractions of 5.0 ml at a flow rate of 20 ml/hour were collected. The bound proteins were eluted with linear gradient of 0-1.0 M NaCl in the same eluting buffer. Unless otherwise stated all purification was carried out at 4°C in the cold laboratory.

Enzyme assay: beta-Amylase activity was measured as described by Oboh and Ajele (1998) using 3,5-Dinitriosalicylic Acid (DNSA) colour reagent. The appropriately diluted enzyme solution (0.5 ml) was added to 0.5 ml of 1% (w/v) soluble starch dissolved in the appropriate buffer solution. Control consisted of 0.5 ml buffer solution plus 0.5 ml soluble starch solution. Reaction tubes were incubated in a water bath at 100°C for 5 min. The tubes were cooled on ice, followed addition of 10 ml distilled water and the absorbance was taken at 540 nm. The unit of beta-amylase activity was defined as that amount of enzyme which released one micro mole of maltose from starch in a minute at 25°C and pH 5.0. Protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Homogeneity test: Homogeneity test was performed by Polyacrylamide Gel Electrophoresis (PAGE) in the absence of Sodium Dodecyl Sulphate (SDS).

Electrophoresis in the absence of SDS was performed on 10% gel according to the method of Weber and Osborn (1969). The electrophoresis buffer was 0.2 M phosphate buffer pH 7.2. A known volume of the purified beta-amylase sample in 0.01 M phosphate buffer with glycerol and tracking dye (0.05% bromophenol blue in distilled water) was electrophoresed at room temperature (25°C) at 8 mA per gel. The gels were stained after electrophoresis with 0.25% Coomasie brilliant blue R250 solution. (10.25 g dissolved in 22.7 ml methanol 4.6 ml glacial acetic acid and distilled water to 100 ml) These gels were destined in the destining solution (50 ml methanol, 7.5 ml glacial acetic acid plus distilled water to 100 ml).

Determination of subunit molecular weight: The subunit molecular weight of the purified beta-amylase was determined by Sodium Dodecyl Sulphate Polyacrylamide Electrophoresis (SDS-PAGE) according to the method of Weber and Osborn (1969) in 10% gel using the SDS Tris-HCl buffer system pH 8.0. Protein markers used include; bovine serum albumin (66,000), chicken ovalbumin (45,000), porcine stomach mucosa (34,700), bovine pepsin trypsinogen (24,000) bovine lactoglobulin (20, 120), egg white lysozyme (45,000). A volume of 0.01 ml of a solution of the electrophoresis low molecular weight calibration kit protein standards in sample buffer was boiled for 2 minutes in a boiling water bath. A solution of the protein (20 µl) was laid on the gel and electrophoresis was run for 5 h at 4 mA/gel during stacking and 8 mA/gel during separation. After electrophoresis, the lengths of the gel and the distance migrated by the tracking dye were measured before the gels were stained with 0.25%. Coomasie blue R-250, they were subsequently destined with the destining solution. After destining, the lengths of the gel and distances migrated by the various protein bands were measured, The relative mobility (Rm) of each of protein band was obtained according to the method of Weber and Osborn (1969).

Determination of apparent molecular weight: The apparent molecular weight of beta-amylase was estimated under non denaturing condition by gel filtration on a Sephadex G-100 column (2.5 x 100 cm) using the following protein markers BSA (Mr 66,000; 3 mg/ml), ovalbumin (Mr 45,000; 3 mg/ml), cytochrome c (12,400), alpha-globulin (150,000) and 3.0 ml of each standard were applied to the column and run individually using 10mM Tris Hcl buffer, pH 8.0 as eluent at a flow rate of 8.4 ml/hour. Fractions (2 ml) were collected and

elution was monitored for each protein at 280 nm. The void volume (V_0) of the column was determined with blue dextran, its elution being monitored by measuring absorbance at 620 nm.

Determination of kinetic parameters: The apparent kinetic parameters; Michaelis-Menten constant, Km and the maximum velocity, Vmax, were determined for the purified beta-amylase enzyme by determining initial velocities of the reaction at varying concentrations of 1% soluble starch (0.5 mg/ml). Aliquots of 0.5 ml of the desired concentration of soluble starch were incubated with 0.5 ml of the enzyme. The assay was carried out according to the standard assay procedure. The values of Km and Vmax were estimated from the double reciprocal plot of 1/V against 1/S.

Determination of amino acids participating in catalysis: Effect of pH on enzyme kinetic parameters Michaelis-menten constant, km and the maximum velocity, Vmax and the catalytic constant (kcat) were determined for the purified beta-amylase by determining initial velocity of the reaction at varying concentrations of soluble starch. (0-20 mg/ml) buffer of different pH ranging from pH 4-10 at a concentration of 0.01 M were prepared using different buffer system: acetate buffer pH 4 and pH 5; phosphate buffer pH 6 and pH 7 and Tris-HCl pH 8, 9, 10. Each buffer solution was used to prepare the 1% soluble starch solution used as substrate in assaying the enzyme. Aliquots of 0.5 ml of the desired concentrated with 0.5 ml of the enzyme at 27°C (room temperature). The assay was carried out according to the standard assay procedure. The values of km and Vmax were estimated from the Eadie-Hofstee plot.

Physicochemical properties

Effect of pH on enzyme activity: Buffers (0.1 M) each within the pH range 3 to 9 were prepared using different buffer systems, acetate buffer (pH 4 and 5), phosphate buffer (pH 6 and 7), Tris/HCL (pH 8 and 9). Each buffer solution was used to prepare the 1% soluble starch solution used as substrate in assaying the enzyme activity in different buffer system. The assay was carried out according to the standard assay procedure.

Effect of pH on the stability of the enzyme: Aliquots of 1 ml enzyme solution were mixed with 2 ml of the buffer at room temperature for six hours. At one hour interval, aliquot of 0.5 ml from the mixture was assayed for residual activity under standard assay conditions. The procedure was repeated for various pH ranging between pH 4 and pH 8.

Effect of temperature on enzyme activity: A mixture of 0.5 ml of 1% soluble starch and 0.5 ml of the enzyme solution was incubated at the temperature of interest for 3 min in regulated Gallen Kamp water bath. 1 ml of colour reagent (3, 5-dinitrosalicyclic acid) was added to the mixture after incubation. The mixture was placed in boiling water bath for 5 min. After cooling on ice, 10 ml of distilled water was added and the absorbance of the mixture was measured at 540 nm.

Effect of temperature on the stability of beta-amylase: The thermal stability of the enzyme was determined by incubating 4 ml of the pooled enzyme at various temperatures between 50°C to 90°C without the substrate for 1 h. At intervals of 10 min, aliquots of 0.5 ml of the incubated enzyme were assayed for residual activity.

Effect of some salts on the activity of beta-amylase: A stock solution of 0.02 M of each of the salts was prepared. An assay mixture containing a final concentration of 20 mM of each salt was then prepared from the stock solution. The assay was carried out according to the standard assay procedures. The blank consist of soluble starch and distilled water without the salt and the control consisted of 0.5 ml soluble plus 0.5 ml enzyme solution without salt.

RESULTS AND DISCUSSION

Enzyme purification: The enzyme was purified to an apparent homogeneity with a specific activity of 0.65 mg/min/ml.

Crude extract (29.4 mg) was applied to a Sephadex G-100 (2.5 x 70 cm) previously equilibrated with 0.02 M sodium acetate buffer pH 5.0. The flow rate was 20 ml/hour and 0.02 M acetate buffer, pH 5 was used to elute the protein.

Homogeneity test and molecular weight determination: A polyacrylamide gel electrophoresis in the absence and presence of SDS produced a single band as represented in Fig. 4 and 5, respectively. The sub-unit molecular weight of the purified enzyme was estimated to be 60,700 Daltons by SDS-PAGE, while the apparent molecular weight by gel filtration gave 58,500 Daltons.

Effect of amino acids participating in catalysis: From the plot of log Vmax/Km important apparent pK values of 5.21 (general base) and 6.58 (general acid) were obtained. The values are suggestive of the presence of imidazole group of histidine at the active site of cassava (*Manihot esculenta* crantz) peels beta-amylase that was needed for the catalysis.

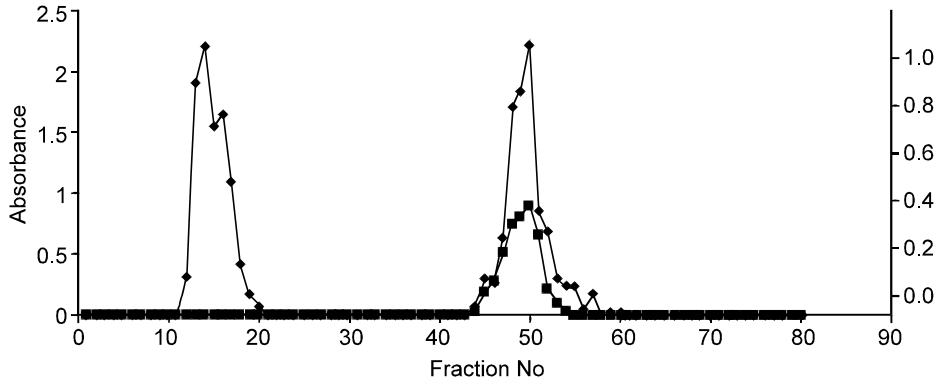

Fig. 2: Elution profile of crude beta-amylase on sephadex G-100

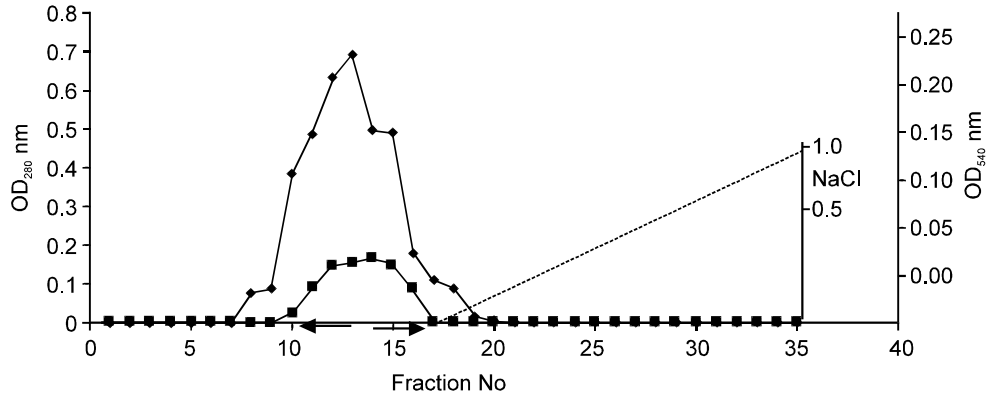

Fig. 3: Elution profile of pooled beta-amylase fractions from Sephadex G-100 on CM-cellulose. A pooled fraction (35.46 mg) from Sephadex G-100 was applied to CM cellulose column (2.5 x 35 m) after equilibration with 0.02 M acetate buffer, pH 5. Fractions (5 ml) were collected at a flow rate of 20 ml/h. The adsorbed proteins were further eluted with a linear gradient 0.1 M NaCl elution buffer

Table 1: A summary of the results of the purification of the beta-amylase from cassava peel

Purification step	Vol. (ml)	Protein (mg/ml)	Activity (Units/ml)	Total protein	Total activity (Units)	Specific activity (Units)	Purification fold	Yield (%)
Crude extract	14	2.10	4.06	29.40	56.84	1.93	1.00	100
Gel filtration on sephadex G 100	40	0.95	1.63	38.00	65.20	1.72	0.89	40
Ion exchange on CM cellulose	40	0.92	1.60	36.80	76.80	1.74	0.90	39

The beta-amylase from cassava peels was purified to homogeneity by a combination of ion exchange chromatography and gel filtration chromatography on Sephadex G-100. The presence of a single band clearly suggests that the beta-amylase from cassava peels exists in one form. Studies on beta-amylase from different sources; soya-bean (Gertler and Birk, 1965) and sweet potato (Babu *et al*., 1996) have shown the existence of one form of this enzyme. However, existence of five forms of beta-amylase in germinated rice seeds had previously been reported by Matsui *et al.* (1977).

The fact that the molecular weight obtained from gel filtration chromatography (58,500 Daltons) is almost equal to that obtained by SDS-PAGE (60,707 Dalton) indicated that the beta-amylase from cassava peel was monomeric. Tanaka *et al.* (2001) reported the existence of four identical subunits in sweet potato beta-amylase. Various molecular weights have been reported for beta-amylase from other sources, for example, 50,000 daltons was reported for beta-amylase from sweet potato (Babu *et al*., 1996); 53,000 daltons was reported for *Bacillus polymyxa* 26-1 (Sohn *et al*., 1996) 209,000 daltons and 239,000 daltons for two different species of sweet potato beta-amylase (Chang *et al*., 1996) and 105,000 daltons for the beta-amylase from *Bacillus megaterium* B_6 (Ray, 2000).

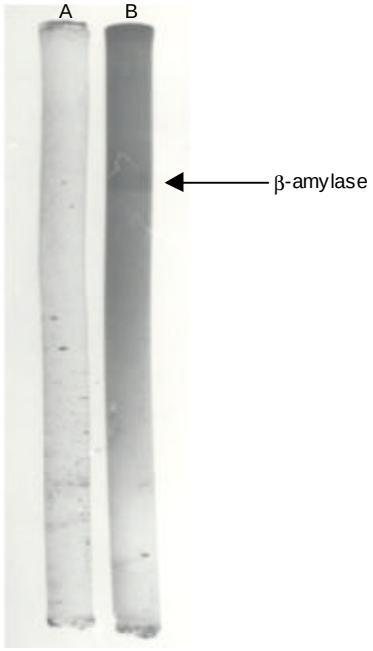

Fig. 4: Photograph of polyacrylamide gel in the absence of Sodium dodecyl sulphate. Lane A showed the gel without protein while lane B represented the single band of ß-amylase

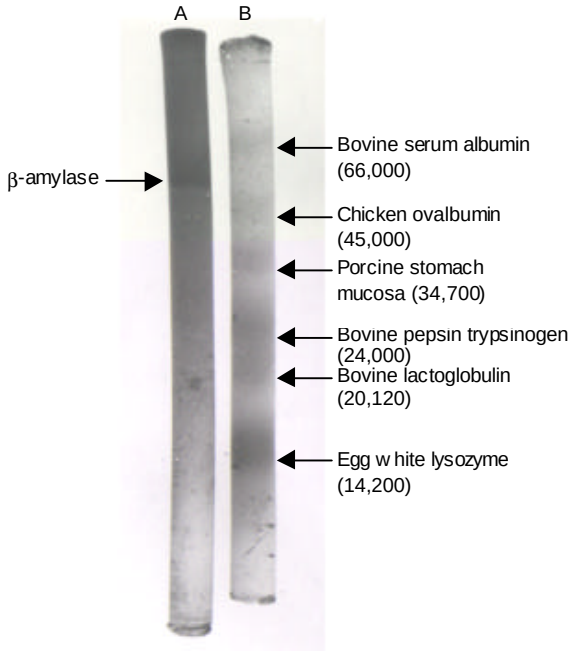

Fig. 5: Photograph of polyacrylamide gel electrophoresis of the pooled fractions from ion exchange chromatography in the presence of SDS. The molecular weight of beta amylase was estimated to be 60,707 daltons

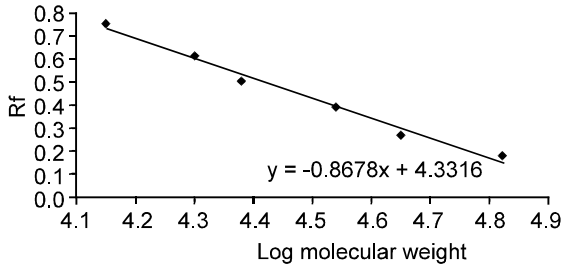

Fig. 6a: Graphical illustration of logarithm of molecular weight of standard protein markers against their relative mobilities on 10% gel

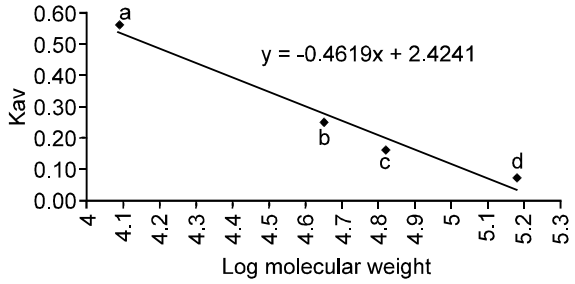

Fig. 6b: Graphical illustration of the plot of logarithms of the molecular weights of the standard protein markers against the Kav under non denaturing condition by gel filtration on Sephadex G-100 column (2.5 x 100 cm) using the following protein markers: cytochrome c (a), ovalbumin (b), bovine serum albumin (c), chicken alpha-globulin (d)

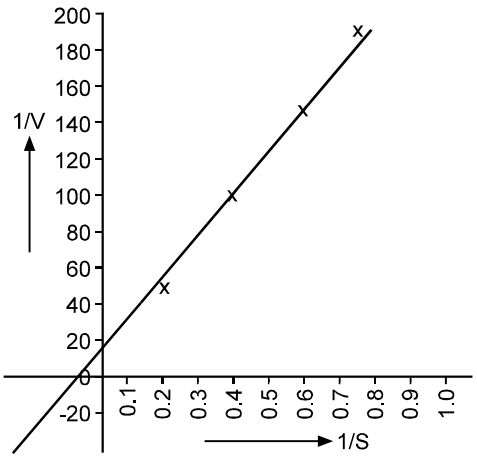

Fig. 7: Line weaver-burk double reciprocal plot. Aliquots of 0.5 ml of the desired concentration of soluble starch was incubated with 0.5 ml of the beta-amylase. The assay was carried out according to the standard assay procedure. The values of Km and Vmax were estimated from the double reciprocal plot

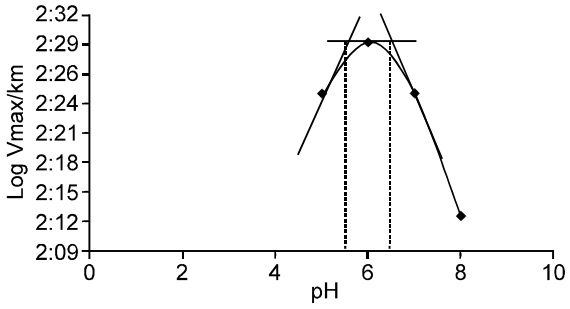

Fig. 8: Graphical illustration of logarithm of Vmax/Km vs pH

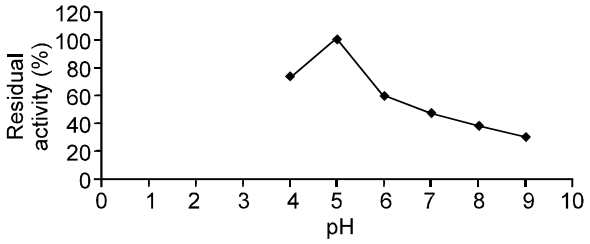

Fig. 9: The enzyme activity was measured at various pH. Aliquots of 0.5 ml of enzyme solution were added to 0.5 ml of 1% soluble starch prepared by dissolving in buffer of the pH of interest. The assay was carried out as stated in the procedure for enzyme assay. The activity was expressed relative to the maximum activity obtained at pH 5, which was taken as 100%

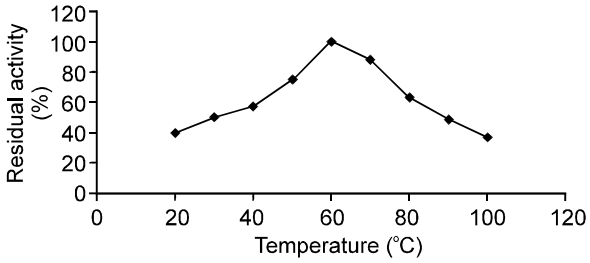

Fig. 10: The enzyme activities were measured at various temperatures, 0.5 ml of the enzyme solution was added to 0.5 ml of 1% soluble starch. The mixture was incubated at a temperature of interest for 3 min. The assay was carried out according to the standard assay procedure. The relative activity at each temperature was expressed as a percentage of the activity obtained at 60°C which was taken is 100%

The Michaelis-Menten Constant, Km value obtained in this report was similar to the value (3 mg/ml) reported by Matsui *et al.* (1977) for rice. Apparent pK values of 5.21 (general base) and 6.58 (general acid) obtained. These results were suggestive of the presence of imidazole

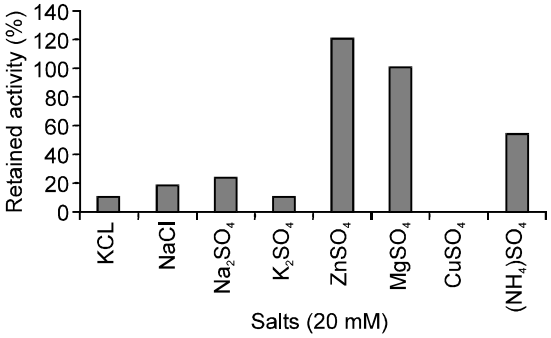

Fig. 11: The effect of salts on the activity of beta-amylase. The salts; $CuSO_4$, $(NH_4)_2SO_4$, KCl, NaCl, K_2SO_4 and Na_2SO_4 had inhibitory effect. $CuSO_4$ is the most potent inhibitor at concentration of 20 mM. $ZnSO_4$ has activating effect while $MgSO_4$ showed little or no effect on the enzyme

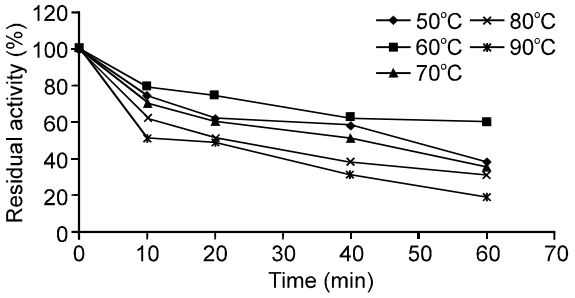

Fig. 12: The enzyme solution (4 ml) was incubated at of 50°C for 60 min. Aliquot (0.5 ml) was withdrawn at an interval of 10 min after which it was allowed to cool at room temperature and assay was carried out according to standard assay procedure. The procedure was repeated for 60°C, 70°C, 80°C and 90°C

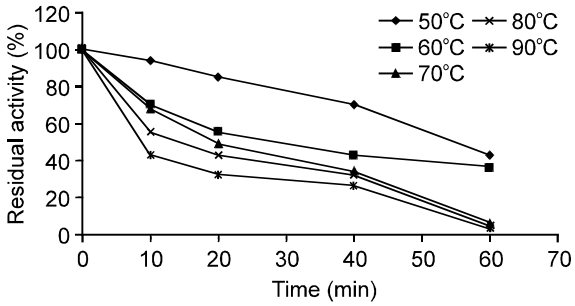

Fig. 13: The enzyme solution (4 ml) was incubated at of 50°C for 60 min. Aliquot (0.5 ml) was withdrawn at an interval of 10 min after which it was allowed to cool at room temperature and assay was carried out according to standard assay procedure. The procedure was repeated for 60°C, 70°C, 80°C and 90°C

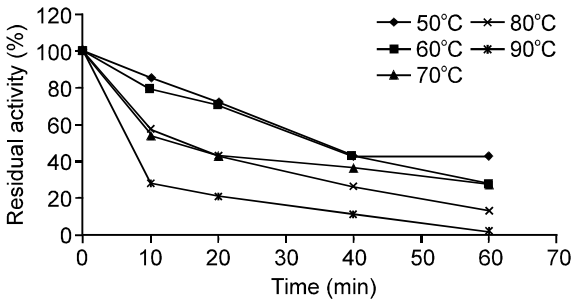

Fig. 14: The enzyme solution (4 ml) was incubated at of 50°C for 60 min. Aliquot (0.5 ml) was withdrawn at an interval of 10 min after which it was allowed to cool at room temperature and assay was carried out according to standard assay procedure. The procedure was repeated for 60°C, 70°C, 80°C and 90°C

side chain of histidine residue and the thiol group of cysteine residue as being involved in the catalytic mechanism of the beta-amylase. The X-ray crystallographic work by Mikami *et al.* (1994) on soyabean beta-amylase confirmed Glu 186 and Glu 380 as catalytic residue. It was also reported that the non-reducing end (Glc I) is hydrogen bonded to four amino acid residues of Asp 101, Asp 53, His 93 and Arg 420 in the catalytic site. It was further suggested that Asp 101 might play a key role in the catalytic mechanism of soyabean beta-amylase. These amino acid residues may be distinct from the amino acids of the cassava peel beta-amylase. It could be speculated that the role of imidazole group of histidine was to electrostatically stabilize the positively charged oxonium ion intermediate formed during catalysis.

The optimum temperature 60°C suggested that the enzyme can be employed in food and beverage industries to convert starch into maltose where high value has been placed on the thermostability and thermoactivity of the enzyme because of high temperature operating conditions (Swamy *et al.*, 1994). The optimal temperature for beta-amylase activity from potato leaf was 40°C. This value was also reported by Bartholomew *et al.* (2000) for a raw starch-digesting enzyme. An optimum temperature of 50°C was reported by Martinez *et al.* (2002), whereas, the pea epicotyl beta-amylase was unstable above 40°C (Lizotte *et al.*, 1990). Hyun and Zeikus (1985) reported that most of the beta-amylase earlier isolated showed optimum activity at low temperature and were not thermostable. Microbial beta-amylases such as *Bacillus polymyxa* 26-I beta-amylase has an optimum of 45°C. The optimum temperature for *Clostridium thermocellum* S58 beta-amylase was 60°C (Swamy *et al.*, 1994). *Bacillus circulan* beta-amylase was also reported by Ray *et al.* (1994) to have an optimum temperature of 60°C.

The pH optimum reported for cassava peel beta-amylase is quite similar to those of beta-amylases from other sources. Ajele (1997) reported pH 4.5 for Soyabean beta-amylase, Babu *et al.* (1977) obtained an optimum pH of 5.5-6.5 for rice beta-amylase. An optimum pH of 5.5 was reported by Martinez *et al.* (2002) for raw potatoes while the optimum pH of 6-9 was reported by Bartholomew *et al.* (2000) for raw potatoes beta-amylase. The fact that the beta-amylase from cassava peel showed improved stability to temperature in weakly acidic medium as being reported by the work of some researchers. Hyun and Zeikus (1985) reported that *Clostridium thermosulfurogenes* beta-amylase was very stable at the acid region after 1 h of incubation. Gertler and Birk (1965) also observed no change in activity of beta-amylase from soyabean after two hours of incubation in the acidic region (pH 3-pH 6) while there was a decrease in the activity at the alkaline medium. However amylase of *Bacillus mesentericus* retained a very high activity at weakly alkaline medium for up to 2 h while there was a rapid inactivation of the enzyme after 1 h at the acidic region. The Previous studies by Oboh and Ajele (1998) has shown that metallic chlorides are usually potent activators of amylases. However this was not the case in the present study because NaCl and KCl inhibited the enzyme from the cassava peel very strongly. Marked activation of beta- amylase from cassava peel by $ZnSO_4$ was consistent with previous findings that divalent ions enhanced amylase activity (Matsui *et al.*, 1977). The present study confirmed the presence of beta-amylase in cassava (*Manihot esculenta* Crantz) peels. The beta-amylase was found to be thermostable and thermoactive, good enough for some industrial applications and also established the possible involvement of histidine and cysteine in its catalytic mechanism.

Conclusion: In conclusion, this study, has shown that cassava could serve as an alternative and a very cheap source of plant beta-Amylase with desirable physicochemical properties. In addition, it will be a sort of an environmental control measure, whereby the cassava peels which form a nuisance to the community may be converted to a very useful raw material in the industries.

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