Full Length Research Paper

Purification and some properties of β-amylase from the nodes of sugar cane, S*accharium offinacium*

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β-amylase (EC 3.2.1.2, α-1,4–D-glucan maltohydrolase) was isolated and purified from the nodes of sugar cane by using ammonium sulphate precipitation, acid-treatment, gel filtration on Sephadex G-75 and ion exchange chromatography on DEAE-Cellulose. Purity was ascertained by the presence of a single band of protein on polyacrylamide gel electrophoresis under non-denaturing conditions. The specific activity was 4.68 $unit.mg^{-1}$ of protein and recovery of 15.67%. The K_m value of the enzyme for starch as substrate was 3.20% while its V_{max} was 1.11$units.min^{-1}.ml^{-1}$. The apparent molecular weight was estimated by gel filtration on a Sephadex G-200 column to be 156,000 Da. The subunit molecular weight was found to be 154,000 Da by sodium dodecyl sulphate polyacrylamide gel electrophoresis. This suggests that the enzyme exists in a monomeric form. The optimum pH for the activity of the enzyme was pH 5.5, while its optimum temperature was 60°C. The hydrolysate of the action of the enzyme showed maltose as the main product of hydrolysis on thin layer chromatography.

Key words: Sugar cane (S*accharium offinacium)*, β-amylase, nodes.

INTRODUCTION

Amylases are enzymes that catalyse the hydrolysis of the 1→4-glycosidic linkages found in polysaccharides, such as amylose, amylopectin, glycogen, or their degradation products. Since the breakdown products of starch are the usual sources of dietary carbon, it is not surprising to find amylases almost universally distributed throughout the animal, plant and microbial kingdoms. Amylases are widely distributed in plant tissues, for example, in storage tissues such as: seeds, nodes and tubers, and in vegetative organs, such as leave (Dunn, 1974).

The major starch hydrolyzing enzyme is believed to be α-amylase, but in leaves and stems, the amylolytic activity of β-amylase has been shown to be substantial (Dreier et al., 1995). It is abundant in sweet potato and in cereals such as: wheat and barley (Okon and Uwaifo, 1984). β-Amylase was first discovered by Caldwell (1931) in the pancreas. It is an exo- enzyme that releases successive maltose units from the non-reducing end of a

polysaccharide chain by hydrolysis of 1,4–glycosidic bonds, leaving a β-limit dextrin when degrading starch and amylopectin, but 1,6- glycosidic links that occur in branched polysaccharides are not hydrolysed (Hyun and Zeikus, 1985). Although α-amylases are endoglyco-sidases, they attack glucans somewhere away from the chain ends at an internal glycosidic bond. The α-amylases are probably the most widely distributed of the amylases; in that they are produced by many different types of bacteria, fungi, animals and some plants, while most of the β-amylases are of plant origin. Another exo-acting amylase is glucoamylase which releases β–D-glucopyranose from the non reducing end of the starch chain. This enzyme differs from β–amylases, in that it does not produce limit dextrins. It catalyses the hydrolysis of both the α–D -(1→4) and α–D-(1→6) linkages, although at widely different rates, and therefore, can completely convert starch to D – glucose. In addition to these amylases, there are also the isoamylases or debranching enzymes that hydrolyse the α–D-(1→6) linkages of amylopectin, for example, pullulanase, an enzyme which was originally observed to hydrolyse theα–D-(1→6) linkages of pullulan, a linear polysaccharide

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composed of maltotriose unit linked by $\alpha-D-(1\rightarrow6)$ bonds (Shannon, 1965).

Sugar cane (*Saccharium offinacium*) is of the family Graminae and it originated from North Guinea and India. It is of high interest majorly from the primary product obtained from it, that is, sucrose which is of commercial interest and domestic needs. The plant part is broadly subdivided into three sectors: the flowering part, stalk and root system. The stalk (which is made up of the nodes, internodes, bud, leaf sheath and leaf blade) is the section of interest in this plant because in addition to performing the biological function as a stem, it also acts as a storage point for sucrose and it houses several enzymes such as invertase, amylases and isomerase that aid in direct and indirect biosynthesis of sucrose and fructans in the plant (Street and Cockburn, 1972).

The biosynthesis of this sucrose takes place via established biochemical pathways occurring in two different sections of the plant. Direct synthesis occurs in the collar, the section that separates the sheath and the blade aspect of the leaves. The collar housed the membraneous ligules and auricle, in which several chloroplasts and cytosols are present and where the synthesis occurs (Salisbury and Rodiskon, 1988). Indirect method of sucrose synthesis occurs in the internode section of the plant via various biochemical hydrolytic actions of several enzymes coupled with the action of the invertase to yields sucrose (Street and Cockburn, 1972). The third system is the secondary system whereby the dissacharide, that is, sucrose is synthesized as a secondary product from the initial plant product such as fats and waxes, which are usually not stored and are biochemically converted to another secondary products (that is, sucrose) by various enzyme present at the older internodes section (Salisbury and Rodiskon, 1988).

In this paper, we demonstrated the presence of β-amylase in the nodes of sugar cane and described some of its properties with the view of exploiting it for industrial purposes, as amylases are important commercial enzymes used in the food processing, brewing, and distilling industries.

MATERIALS AND METHODS

Ammonium sulphate, potassium sodium tartarate (Rochelle salt), sodium hydroxide, potassium iodide, sodium carbonate, sodium bicarbonate, sodium sulphate, copper sulphate, sulphuric acid, ammonium molybdate, sodium arsenate, soluble starch, sodium acetate and mercaptoethanol were products of BDH Chemical Limited, Poole, England. Ninhydrin, sodium dodecyl sulphate, N,N,N[1],N[1]–tetramethylenediamine (TEMED), N[1] N[1]-methylenebisacylamide, ammonium persulphate, Coomasie brillant blue, Molecular weight determination gel filtration kit and SDS-PAGE proteins markers were purchased from Sigma Chemical Company, St Louis, Mo., USA. Glacial acetic acid, n-butanol, maltose, glucose, fructose, sucrose, diethylether, aniline, isopropanol and urea were all product of May and Baker, England. Sephadex G-75 and Sephadex G-200 were bought from Pharmacia Fine Chemical, Upsalla, Sweden. All other chemicals and reagent used were of analytical grade.

Sugar cane was obtained from the sugar cane belt of Ogun State Agricultural Development Project (OGADEP) in Papalanto, Abeokuta, Ogun State, Nigeria.

Enzyme and protein assays

One millilitre (0.1 ml) of the enzyme was added to 0.5 ml of substrate solution (1% starch solution gelatinized with the assay buffer of 0.02 M sodium acetate buffer, pH 4.8). The mixture was then incubated at 37°C for 30 min. The Nelson-Somogyl colorimetric copper method (Nelson, 1944) was used to estimate the reducing equivalent produced and interpolated from a maltose standard curve. A blank was prepared by using 0.1 ml of enzyme with 0.5 ml of the assay buffer. A unit of β-amylase activity was defined as that amount of enzyme which released one micromole of maltose from starch per minute.

Protein concentration determination was by the Biuret method (Gornall et al., 1949) using bovine serum albumin as standard.

Purification of enzyme

Crude enzyme extraction

The nodes of sugar cane were cut, washed with distilled water, peeled and sliced into smaller bits. In a mortar cooled by an ice-bath, the sliced nodes were pressed to extract the juice. The extract was then filtered through a plug of glass wool and the filtrate was subjected to centrifugation at 4,000 rpm for 5 min at 4°C in a refrigerated centrifuge (Sorvail R723) according to Matzui et al. (1977). The supernatant was collected and saved as the crude enzyme.

Ammonium sulphate precipitation

The crude enzyme extract was first subjected to 30% ammonium sulphate precipitation. This was left for 9 h and then brought to 70% saturation. After 18 h, the resulting precipitate was collected by centrifugation at 6,000 rpm for 10 min at 4°C. The precipitate was reconstituted by dialyzing against 0.02 M sodium acetate buffer, pH 4.8.

Acid treatment

The dialyzate was subjected to acid treatment according to Matsui et al. (1977) and Ajele and Oboh (1998) . The solution was gradually made acidic to pH 3.6 by the dropwise addition of ice-cold IN HCl. The precipitate was removed by centrifugation, and then the supernatant was adjusted to pH 4.8 with ice-cold 3% ammonium hydroxide solution. The clear solution was dialyzed against 0.02 M sodium acetate buffer, pH 4.8 for 24 h.

Gel filtration

Seventy grammes of Sephadex G-75 (Super fine) were allowed to swell in 0.02 M sodium acetate buffer, pH 4.8 for 3 days before they were packed into 2.5 x 40 cm column. The packed column was equilibrated with 1 L of the same buffer, after which 20 ml of the enzyme from the previous step was layered into the column. Elution was carried out at a flow rate of 20 ml/h and fractions of 2 ml were collected. The fractions were monitored for protein at 280 nm and assayed for β-amylase activity. Active fractions were pooled and saved.

Ion-exchange chromotography

The combined active fractions from the gel-filtration step were layered on DEAE–Cellulose column (2.5 x 40 cm), which had earlier been equilibrated with 0.02 M acetate buffer, pH 4.8. Elution was carried out with the same buffer at a flow rate of 20 ml/h. The bound proteins were eluted with salt (0.1 M NaCl) in the same buffer. The fractions were monitored for proteins by taking absorbance at 280 nm and assayed for enzyme activity. Active fractions were pooled and saved as 70% ammonium sulphate prepicitate.

Homogeneity test

The homogeneity test was performed by polyacrylamide gel electrophoresis (PAGE) in the absence of sodium dodecyl sulphate (SDS) on a 7% gel according to Pharmacia handbook (Polyacrylamide gel electrophoresis, Laboratory Techniques, Revised Edition, Feb. 1983). The electrophoresis gel buffer was a 0.2 M phosphate buffer, pH 7.2. An aliquot of the purified enzyme in 0.01 M phosphate buffer was mixed with glycerol and tracking dye (0.05% bromophenol blue in 0.01 M phosphate buffer), after which 0.01 ml of this was layered on a rod gel and ran at room temperature at 8 mA per gel. The gels were stained with Coomasie brilliant blue R250 solution (1.25 g of Coomasie brilliant blue in 400 ml of methanol, 70 ml glacial acetic acid, and distilled water to 1 L) followed by destaining in a solution containing 5% methanol and 7.5% glacial acetic.

Determination of subunit molecular weight

The subunit molecular weight of the purified β-amylase was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Weber and Osborn (1975) in a 10% gel, using the phosphate buffer system (pH 7.2). Protein markers used were as contained in the Standard Sigma kits with molecular weight range of 15,000 to 150,000.

Determination of apparent molecular weight

The apparent molecular weight was estimated under a non-denaturing condition by gel filtration on Sephadex G-200 column (2.5 x 70 cm) using the following protein markers: Carbonic anhydrase from bovine erythrocytes (29,000), albumin bovine serum (66,000), alcohol dehydrogenase from yeast (150,000) and β-amylase from sweet potato (200,000), while Blue dextran was used to determine the void volume.

Determination of kinetic parameters

The initial velocity of the reaction was determined at various concentration of soluble starch. Aliquots (0.5 ml) of the desired concentration of the soluble starch solution (previously gelatinized with sodium acetate buffer, pH 4.8) were incubated with 0.1 ml of the purified enzyme. Assay was carried out according to the standard assay procedure earlier described. The values of K_m and V_{max} were estimated from the double reciprocal plot according to Lineweaver and Burk (1934).

Determination of optimum pH

The pH ranged between 3 and 8 using different buffer systems of 0.02 M: Glycine-HCl pH 3, acetate buffer pH 4 and 5, phosphate buffer pH 6 and 7 and Tris-HCl pH 8. Each buffer solution was used to prepare the 1% soluble starch solution used as substrate in assaying the enzyme. The assay was carried out according to the standard assay procedure.

Effect of temperature on enzyme activity

The effect of temperature on the enzyme activity was investigated by assaying the enzyme at temperatures between 0 to 80°C. The substrate was first incubated at the indicated temperature for 10 min before the reaction was initiated by the addition of 0.1 ml of the enzyme that had also been equilibrated at the same temperature.

Effect of temperature on stability

Aliquot (1 ml) of the enzyme solution (which had been previously desalted on a Biogel P-10 column) was incubated at the desired temperature of 40, 50, 60 and 70°C. A 100 μl aliquot was then withdrawn at 10 min interval for 1 h and assayed for residual activity.

Effects of cations

The effect of various metal ions on the activity of β-amylase was examined at 1 and 5 mM final concentration. These salts are barium chloride (BaCl$_2$), calcium chloride (CaCl$_2$), borate chloride (BoCl$_2$), manganese chloride (MnCl$_2$), iron (III) chloride (FeCl$_3$), nickel chloride (NiCl$_2$), magnesium sulphate (MgSO$_4$), copper [II] sulphate (CuSO$_4$), Ethylenediamine tetracetic acid (EDTA) and acetamide.

Tests for hydrolytic activity of the enzyme

The hydrolytic action and hydrolysates were determined according to Ivor and Feinberg (1965) and Matsui et al. (1977). Subsequently, 1 ml of the purified β-amylase enzyme was added to the 5 ml of 1% starch solution (previously gelatinized with sodium acetate buffer, pH 4.8). This was incubated at 37°C for 3 h. The mixture was then centrifuged at 6,000 rpm for 10 min. The concentrated hydrolysate was spotted on a pre-coated thin layer plate. The thin layer chromatography was run for 22 h in a solvent mixture of n-butanol/glacial acetic acid/water (60/15/25 v/v/v), alongside with the sugar markers (maltose, fructose, sucrose and glucose).

The chromatography plate solvent front was marked at the end of the run. The plate was then sun dried, sprayed with locating agent consisting of diphenylamine dissolved in acetone. The plate was then dried in sunlight before it was then used for baking at 105°C for 5 min in an oven. The respective R$_f$ values were determined.

RESULTS

The elution profile on Sephadex G-75 gel filtration chromatography is shown in Figure 1. The result shows one major and three other minor protein peaks. The major protein peak shows β-amylase activity. Figure 2 shows the elution profile on the DEAE-Cellulose column. A summary of the β-amylase purification scheme is presented in Table 1. The enzyme preparation is adjudged pure, since only one protein band was found on the gel after polyacylamide gel electrophoresis in the absence of SDS. The apparent molecular weight of about 153,000
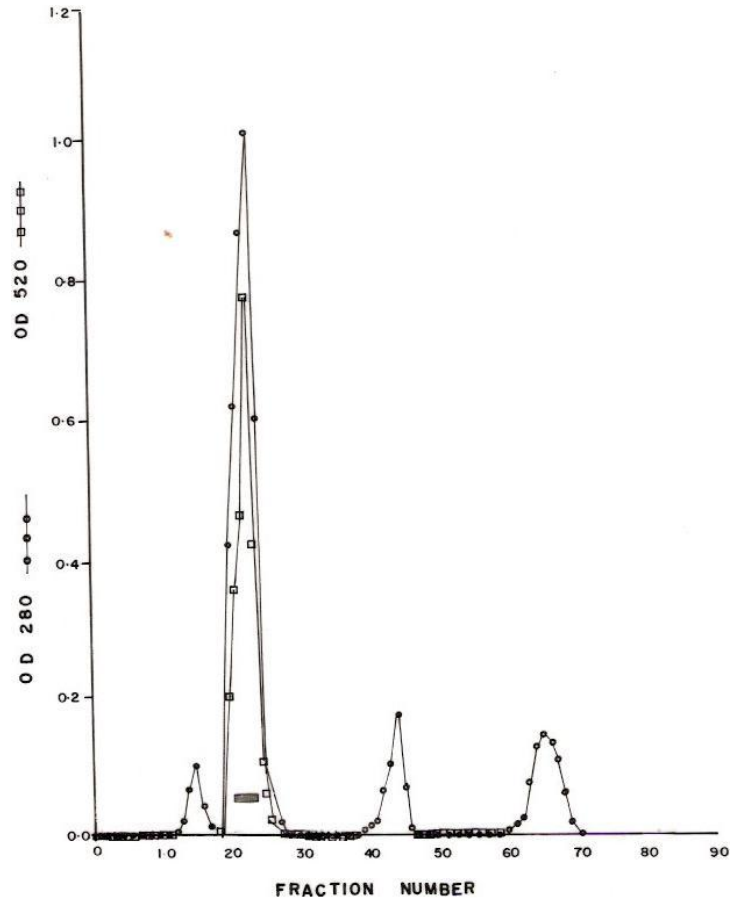
Figure 1. The elution profile on Sephadex G-75 gel filtration chromatography. 20 ml of post acid treated sample was applied on to Sephadex G-75 column (2.5 x 40 cm) previously equilibrated with 0.02 M sodium acetate buffer pH 4.8. Fractions of 2 ml each were collected at a flow rate of 20 ml hr^{-1} (OD$_{280}$), (OD$_{520}$) and (pooled).

was obtained after gel filtration on Sephadex G -200. Two distinct bands were obtained after SDS- PAGE as shown schematically in Figure 3 corresponding to the molecular weight of 88,000 and 68,000 for Bands I and II, respectively. The Lineweaver-Burk plot is shown in Figure 4. The Michaelis-Menten constant K_m for the soluble starch was estimated to be 3.28% starch solution, while its maximum velocity, V_{max}, was 1.11 units.min^{-1}.ml^{-1}. The effect of pH and temperature on the activity of the enzyme resulted in an optimum of 5.5 and 60°C, respectively. The effect of several salts on the activity of the enzyme is presented in Table 2. The activity is expressed as a percentage of the activity in the absence of salts. The hydrolysate of the action of the enzyme on starch showed that maltose was the major product (Figure 5).

DISCUSSION

This research work shows the existence of β-amylase in

the nodes of sugar cane. The presence of amylase in the nodes of sugar cane may be alluded to its role in the indirect method of sucrose synthesis. β-amylase has been isolated and purified from sweet potatoes (Christensen et al., 1997), soya beans (Ajele, 1997), rice (Matsui et al., 1977) and barley (Gestler and Birk, 1985). The result obtained indicates that β-amylase from the nodes of sugar cane is an heterodimer with subunits of 88,000 and 66,000 daltons, while its apparent native molecular weight is 153,000.

Earlier, sweet potatoes β-amylase had been reported to have an apparent molecular weight of 215,000 and con-sisting of four identical subunits of 56,000 (Christensen et al., 1997). Toda and Svensson (2000) reported a mole-cular weight of 206,000 for germinating sweet potatoes β-amylase. However, Gestler and Birk (1985) reported an apparent molecular weight of 176,000 for barley β-amylase. The existence of five forms of β-amylase in ungerminated and germinated rice seeds had also been reported (Matsui et al., 1977). The pH optimum of 5.5 obtained is in good agreement with that reported
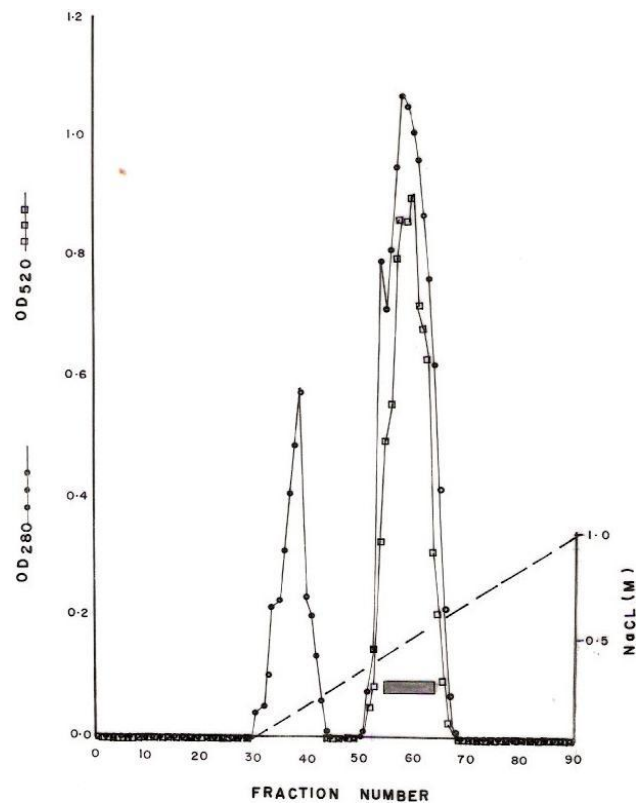
Figure 2. The elution profile on DEAE-Cellulose chromatography. The pooled enzyme from the previous gel filtration step was layered on DEAE-Cellulose column and elution was carried out at a flow rate of 20 ml hr^{-1}, after which 2 ml fractions were collected (OD$_{280}$), (OD$_{520}$) and (pooled).

Table 1. Summary of the purification procedures of sugar cane β-amylase.

| Purification Steps	Volume (ml)	Protein (mg/ml)	Activity (unit/ml)	Total Protein (mg)	Total Activity (units)	Specific Activity (Unit/mg)	Purification fold	Yield (%)
Crude extract	1150	0.25	0.2923	287.5	336.15	1.17	1.00	100
NH$_4$SO$_4$ (70%)	76	2.70	3.80	205.2	288.8	1.41	1.21	85.91
Acid Treatment	58	2.25	3.51	130.5	203.6	1.56	1.33	60.57
Gel Filtration on Sephadex G-75	20	1.75	3.31	35.0	66.2	1.89	1.62	19.69
Ion Exchange on DEAE-Cellulose	15	0.75	3.51	11.25	52.65	4.68	4.00	15.66
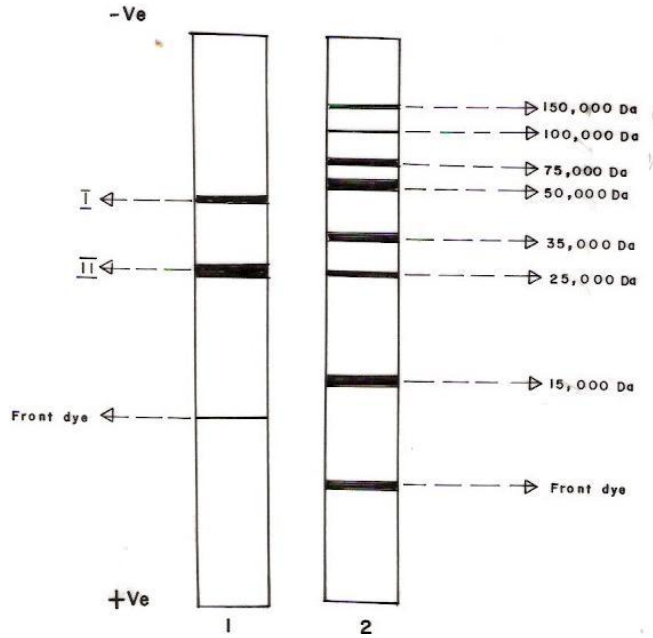
Figure 3. Schematic representation of the result of SDS-PAGE of the purified enzyme. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate for the determination of subunit molecular weight on 10% gel. Two distinct bands were detected (Bands I and II as shown in gel 1). Gel 2 contains the standard protein markers: (150,000Da), (125,000 Da), (75,000 Da), (50,000 Da), (35,000 Da), (25,000 Da) and (15,000 Da).
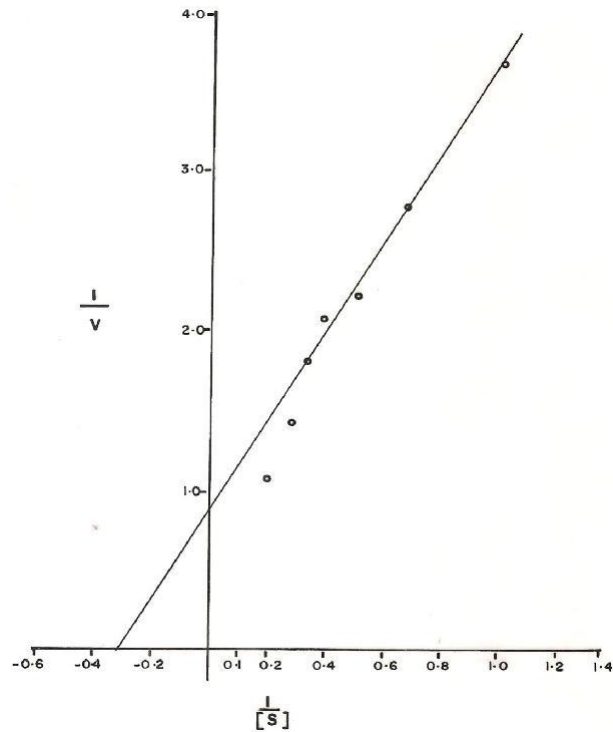

Figure 4. The Lineweaver-Burk plot. Aliquots of 0.5 ml of the desired concentration of soluble starch were incubated with 0.1 ml of β-amylase. The assay was carried out according to the standard assay procedure. The values of K_m and V_{max} were estimated from the double reciprocal plot.

Table 2. The effects of salts and reagent on sugar cane β-amylase.

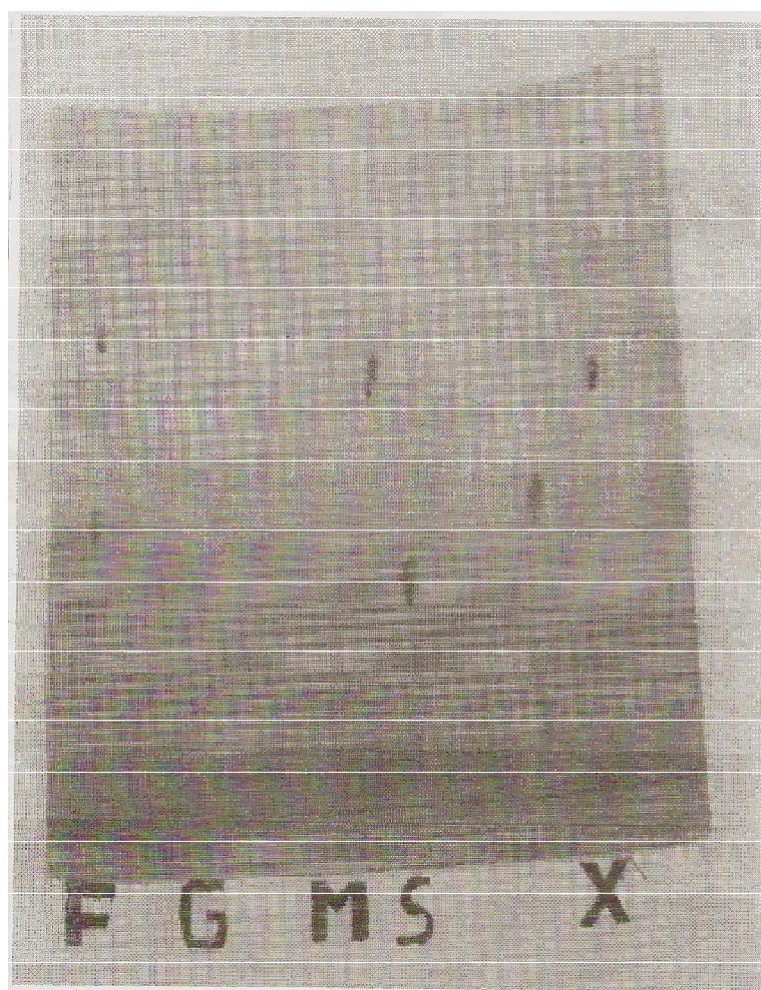| Reagents and Salts	Relative activity (%)	
	1 mM	5 mM
Control (None)	100.0	100.0
$CaCl_2$	72.1	43.0
$NiCl_2$	68.9	26.1
$CdSO_4$	79.0	57.1
$CuSO_4$	108.2	91.8
$MgSO_4$	105.3	192.3
$BaCl_2$	122.0	147.9
MnO_2	116.0	128.1
$SnCl_2$	106.3	87.0
$FeCl_3$	76.0	41.8
$MgCl_2$	102.0	163.0
$ZnSO_4$	117.0	96.4
EDTA	59.2	12.2
Acetamide	41.0	06.2

Figure 5. The chromatogram plate of the sugar makers and the hydrolysate.
F – Fructose; S – Sucrose; G – Glucose; X – Hydrolysate product (sample);
M – Maltose.

for β- amylase from other sources such as sweet potatoes (5.5) (Toda and Svensson, 2000) and barley (5.5) (Gessler and Birch, 1985). Yamamoto et al. (1988) also reported an optimum pH of between 5 and 6 for β-amylase from *Bacillus subtilis*. However, it is worthy to note that the enzyme was active over a wide pH range.

The optimal temperature reported for β -amylase from the pea epicotyl (Lizotte et al., 1990) was 40°C, while a thermostable enzyme reported by Toda and Svensson (2000) on β-amylase from potatoes tuber was 55°C, but this result showed a better thermostable enzyme which has its optimum temperature at 60°C. The enzyme was stable up to 60°C for about 50 min. This agrees very well with what Ajele (1997) reported on β-amylase from soya bean of 60°C. Since this enzyme is thermostable, it could be a target for the production of β-amylase using molecular biology approach.

Like in most other works, maltose was the major detectable product (Matzui et al., 1977; Ivor and Feinberg, 1985). -amylases release high proportion of oligosaccharides along with -D-maltose, whereas -amylases release only -D-maltose as the product (Muralikrishna and Nirmala, 2005).

The attempt to isolate the enzyme from nodes of sugar cane is as a result of showing its presence and underscoring its involvement in the indirect synthesis of sucrose in sugar cane (Street and Cockburn, 1972). Moreover, the occurrence of this enzyme in a thermostable form may be the beginning of its molecular biology and possible exploitation for industrial purposes since this enzyme is mainly present in plants.

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