

Purification and Partial Characterization of a Raw Starch Digesting Amylase from *Aspergillus tamaraii*

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ABSTRACT

A raw starch digesting amylase (RSDA) was produced from Aspergillus tamaraii using submerged fermentation with soluble starch as carbon source. The enzyme was concentrated from culture supernatants fluid by dialysis and purified by ion exchange chromatography on S- and Q Sepharose (fast flow) respectively and hydrophobic interaction chromatography. The overall yield of the RSDA was 25.47% with a specific activity of 3.63 μ mg-Optimum temperature of the enzyme was 60°C but tolerated a wide temperature range (30-80°C) for 20 minutes at pH 6.0-8.0 in 0.1M citrate phosphate buffer. The activity of the RSDA was stable over a relatively broad pH range (5.0-9.0) suggesting an acid and alkali tolerance when acting on raw starch. The activity of the enzyme was enhanced by Mn^{2+} , Co^{2+} and Fe^{2+} while Ba^{2+} , Ag^+ , and H_g^{2+} had strong inhibiting effects on the activity of the enzyme. The RSDA hydrolysed amylose more substantially relative to pullulan, amylopectin, soluble starch, inulin and dextran. Glucose was the end products of hydrolysis.

Keywords: Aspergillus tamari, starch, amylase and purification.

INTRODUCTION

Enzymes are proteins that are synthesized in living cells which catalyze thermodynamically possible reactions so that the rate of the reaction is compatible with numerous biochemical processes. The amylase family of enzymes is a diverse group of enzymes ubiquitous in microbial world (fungal and bacterial), plant (potato and barley) and animal sources (saliva and pancreas) (Gupta *et al.*, 2003)[1]. However, the majority of the amylases come from microorganisms as they can be grown in large quantity under well controlled conditions to yield amylases that are relatively easy to isolate and purify. Fungal amylases are important in chemical, pharmaceutical and bakery industries[2].

Starch based foods such as cereals (maize, rice, millet and sorghum; and starchy food products such as yams, cassava, potato, coco yams are abundant and widely grown in Nigeria and other developing countries. These products deteriorate early due to poor handling and post -harvest situations .They can therefore be effectively preserved by converting them to other various value-added products through enzymatic processes.

The traditional method of starch hydrolysis to give glucose and other starch derived products is highly energy intensive involving heat liquefaction with thermophilic alpha amylase and subsequent saccharification by fungal amylase (Okolo *et al.*, 2000)[3]. Due to the complexity and high energy requirement involved in saccharification, extensive work has been done within the last few decades on the bioconversion of raw starch substrates by enzymatic digestion without cooking [4].

Enzymatic saccharification of raw starch without cooking has attracted much scientific and industrial interest especially from the point of view of energy saving, minimization of undesirable products, process simplicity and effective biomass utilization. It has been recognized that enzymatic hydrolysis of raw starch without pre-gelatinization has great economic advantage over the traditional method [4],[5].

It is also to be noted that it is more difficult for amylases to act on raw starch granules than on gelatinized starch (Abe *et al.*, 2001)[6]. Generally, cereal starches are more easily digested by amylases than root starches such as potato [7].

Many reports have been published on bacterial and fungal amylases but a few microorganisms seem to possess the capacity to elaborate raw starch digesting enzymes[8].

This research work is aimed at obtaining and partially purifying a raw starch digesting amylase from the culture broth of *Aspergillus tamaris* and followed by evaluation of the enzyme properties.

MATERIALS AND METHODS

The test organism *Aspergillus tamarii* was obtained from the department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka, Nigeria. This organism was maintained on Sabroud Dextrose Agar(SDA) slants at 4°C

ENZYME PRODUCTION

Soluble sugar found to be the best carbon source among other carbohydrate sources tested was used for enzyme production (Onu *et al.*, 2012)[9]. A shake flask fermentation was used for the enzyme production with a view to purifying the raw starch digesting enzyme. Two agar plugs (1.7cm in diameter) of profuse growth of the fungus cultivated on SDA for 96 hr at room temperature were inoculated into four 250ml Erlenmeyer flasks each containing 100ml of sterile fermentation medium which comprised 1% soluble starch, 1%(NH₄)₂HPO₄, 0.1% NaCl, 1%MgSO₄.7H₂O and 1ml of a 5Mm solution of Cobalt Chloride.

Fermentation was carried out in an orbital shaker for four days at 30°C. After fermentation, mycelial cells were separated by filtration through whatman NO. 1 filter paper and cell free supernatant was stored as crude enzyme at 4°C in 0.2M citrated and phosphate buffer pH7.

ENZYME ASSAY

The activity of the crude enzyme extract obtained was assayed. This was done using the method of Bernfield (1995)[7]. One milliliter of 1% (w/v) suspension of the starch was prepared in 0.2M citrate phosphate buffer(w/v) pH7 and 1ml of crude enzyme. The substrate in the buffer was boiled for 10min to gelatinize the starch for enzyme reaction. Incubation of the enzyme reaction mixture was at 40°C for 30min in GFLD-30938 water bath (Germany). The reaction was stopped by adding 2ml of dinitrosalicylic acid reagent (DNS) and heated for 10min. On cooling, 2ml of water was added and absorbance was measured at 540nm in a 1x4.3cm cuvette by using a Jenway 6405 uv/vis spectrophotometer (EU) against a blank prepared by the above procedure but adding DNS before the enzyme. One unit of amylase activity was defined as the amount of enzyme which liberated 1μM of glucose per minute under the assay conditions

PURIFICATION OF RAW STARCH DIGESTING AMYLAASE

The crude enzyme extract (culture filtrate) was concentrated by dialysis against 4M sucrose solution at 4°C overnight. The enzyme activity was determined as described earlier [7].

ION EXCHANGE CHROMATOGRAPHY

An anionic column was prepared by first inserting a glass wool into the separating burette using glass rod. The inserted glass wool was washed by passing citrate phosphate buffer (pH7) through it. Thereafter, S-Sepharose (fast flow) gel already swollen in water was gradually layered and packed into the burette. The column (1.8x12) was washed severally with 0.1M citrate phosphate buffer (pH7). The concentrated crude enzyme (20ml) was carefully applied with the aid of glass pipette and stirring glass rod into the packed column previously equilibrated with 0.1M citrate phosphate at pH7. The sample was eluted first with the same buffer and then a linear gradient of 0.5M sodium chloride solution in 0.2M citrate phosphate buffer. Fractions (10ml) were collected at flow rate of 1ml/min. A total of 45 fractions were collected and fractions 3-17, 24-27, 29 and 33 representing major peak values were pooled and assayed for enzyme activity. The protein absorbance of the pooled fractions was made using Eppendorf Biophotometer at 280nm. The pooled enzyme sample was further concentrated by dialysis at 4°C against 4M solution of sucrose and the enzyme activity and protein content determined as described earlier.

HYDROPHOBIC INTERACTION CHROMATOGRAPHY

To further purify the enzyme, hydrophobic interaction chromatography was applied. The enzyme was applied to a phenyl-sepharose 6 fast flow (high sub) column (1.7 x 4.5) equilibrated with phosphate buffer pH7. Elution was performed with a three - step ammonium sulphate gradient 0.5M to 2M at a flow rate of 1ml/second. Exactly 20 tubes of 10ml each were collected for the 0.5M eluates and six tubes of 10ml each were collected for the 1M and 2M eluates. The activities of the collected fractions were determined in the usual way after which fractions 7-13 and 28-38 were pooled separately, concentrated and their activities determined as described earlier.

PROPERTIES OF THE PURIFIED ENZYME

Phadebas Amylase Test

Into 4ml of 0.25M sodium acetate buffer (pH4.5), 0.2ml of the enzyme was added. 1 tablet of Pharmacia Phadebas reagent (Pharmacia Uppsala, Sweden), shaken vigorously for 10 sec and then incubated at 35°C for 15 min. One millilitre (1ml) of 0.5M NaOH was then added and shaken gently. Development of blue colouration on standing indicated the presence of α amylase. Absorbance was measured at 620nm against a blank without the enzyme.

DETERMINATION OF TEMPERATURE ACTIVITY OF THE PURIFIED AMYLASE

The temperature activity profile of the purified enzyme was determined over the temperature range 30- 90°C. This was done by adding 0.1ml of the purified enzyme to 0.1ml of soluble starch (1% w/v) and incubating at the test temperature for 30 min. The enzyme activity was then determined using DNS reagent as described

DETERMINATION OF THERMOSTABILITY OF THE PURIFIED AMYLASE

The enzyme was incubated at various temperatures (30-90°C for 30 min and promptly chilled in ice. Thereafter, the residual activity was assayed as described earlier

EFFECT OF METAL IONS ON THE AMYLASE

The effects of Ag^+ , Co^{2+} , Zn^{2+} , Mg^{2+} , Ca^{2+} , Mn^{2+} , Ba^{2+} , Cu^{2+} , Pb^{2+} , Fe^{2+} , Hg^{2+} , and Sr^{2+} on the activity of the purified amylase were studied. The reaction mixture of the enzyme (0.2ml), salt of each of the cations (2Mm) and 0.2ml of 1%(w/v) of raw starch in 0.1M citrate phosphate buffer (pH7) was pre-incubated at 60°C (optimum temperature) for 30 min and then the enzyme activity determined thereafter

EFFECT OF INHIBITORS ON THE ACTIVITY OF THE AMYLASE

The effects of the following inhibitors were tested: 2-Mercaptoethanol (2-ME), ethylene diamine tetracetic acid (EDTA), iodoacetic Acid (I.A), N- Bromosuccinic acid, and phenylmethyl sulphonyl fluoride (PSMF). The test procedure was as described for metal ions above.

EFFECT OF SUBSTRATE CONCENTRATION ON THE ACTIVITY OF THE PURIFIED ENZYME

Various concentrations (0mg, 0.2mg, 0.4mg, 0.5mg and 1mg) of carbohydrate substrates (amylose, amylopectin, soluble starch, pullulan, dextran and inulin) in 0.1M citrate phosphate buffer (pH7).were prepared Each of the carbohydrate (0.2ml) was reacted with 0.2ml of the purified enzyme at 60°C and the activity determined using DNS reagent as described earlier. The kinetic constants (K_m and V_m) were estimated by double reciprocal plots of the data according to [10].

RELATIVE RATE OF HYDROLYSIS OF DIFFERENT POLYSACCHARIDES

The relative rate of hydrolysis of various polysaccharides viz. amylose, amylopectin, soluble starch, pullulan, dextrin and inulin were determined. The assay mixture consisted of equal amounts of 0.2ml of 1% (w/v) of each of the various polysaccharides in 0.1M citrate phosphate buffer (pH7 and enzyme. Raw starch digesting amylase activity was assayed at 60°C using DNS as described earlier.

RESULTS AND DISCUSSIONS

The elution pattern from S -sapharose ion exchange chromatography column showed major peaks of enzyme activity occurring between fractions 3-17, 24-27, 29, and 33 (fig.1). The sodium chloride gradient is also indicated. Recovery of those fractions resulted in 27.6% retention of the overall activity and a fold of 1.47 (Table 1). Similarly on hydrophobic interaction on phenyl Sapharose major peaks occurred between fraction 7-13 and 28-38 (Fig 2). Recovery of those fractions led to 25.47% and purification fold of 3.62 (Table 1)

The development of blue colouration in the phadabes arnyiase test showed the presence of amylase as enzyme produced.

The optimum temperature of activity of the purified enzyme is 60°C (Fig.7. This temperature is known to be within the temperature range for alpha amylase to degrade starch to yield fermentable extract (Hough *et al.*, 1971). As much as 70-80% of its activity was retained. Above 80°C the enzyme was denatured. This observation is in agreement with

the findings that alpha amylase is active up to 76°C during mashing (Hough *et al.*, 1971). It is an indication also that the enzyme retained most of temperature resistant characteristics of the organism *Aspergillus tamari* from which it was produced but lost the property at higher temperatures. The enzyme was most stable also at 70°C and also retained 70-80% of its activity at that temperature. It also demonstrated its wide range of thermal activity (30-80°C). The enzyme was therefore substantially resistant to thermal inactivation. These findings partially agree with the suggestion that amylase activity in most cases rise steadily from 0 to an optimum of 60°C and above, after which further increase in temperature will lead to sharp decline in activity (Odibo and Ulbrich-Hofmann, 2001)[2]. They can be related to the decrease in the affinity of the enzyme amylase and other de-polymerizing enzymes that require higher temperatures for optimal efficiency with minimal contamination. Enzyme thermostability is advantageous during hydrolysis (Hayashida, *et al.*, 1986)[1]. Some thermostable raw starch digesting amylase with interesting properties have been reported in fungi and actinomycetes but only few have been reported which can tolerate such broad temperature range[6].

The purified amylase exhibited pH optimum at pH5-6 in citrate phosphate buffer and another activity at pH9 in Tris HCL buffer. This is in agreement with the pH4.5-7.0 for fungal amylase (Onu *et al.*, 2012)[9]. Most of *Aspergillus* glucoamylase shared pH optimal at pH4-6 (Hayashida and Teramoto, 1986)[11]. The amylase was found to be acid - resistant and stable at pH3 that is similar to the acid -stable glucoamylase of most *Aspergillus species* (Taniguchi *et al.*, 2003)[12]. The activity of the purified α - amylase was stimulated by Sr^{++} , Fe^{++} , Cu^{++} , Mg^{++} , Ca^{++} , Zn^{++} and Co^{++} at varying degrees while it was inhibited by Ag^{+} , Ba^{++} , Hg^{++} and Pb^{++} at varying degrees also. The enzyme was stimulated by PMSF and Iodo acetic acid and inhibited by EDTA and 2-Mercaptoethanol. The findings with respect of metal ions and inhibitors are in agreements with the work of [2].

From hydrolytic properties of the enzyme it is shown that the purified enzyme degraded amylose more substantially than other polysaccharides studied. This is in agreement with

the findings on the behavior of novel raw starch digesting amylase from *Aspergillus carbonarius* and that of the findings on another enzyme *Theromomyces lanuginosus* Fl [12].

CONCLUSION

Aspergillus tamaraii produces α -amylase an enzyme that is thermo stable and hydrolyzes raw starch without pre-gelatinization when grown in submerged culture with soluble starch as sole carbon source.

The enzyme produced has several interesting properties for industrial application. It is capable of hydrolyzing various amylaceous polysaccharides especially amylose to produce predominantly glucose, maltose and maltotetraose. In comparison to most microbial amylases, α -amylase from *Aspergillus tamaraii* digests even raw starch thus promising a one step application in starch processing to produce value added products.

Table 2: Effect of Metal Ions on the Activity of the Purified Enzyme

Metal ions A1 (5mM)	Relative activity (%)
No metal	100
Ag²⁺	19.68
Sr²⁺	92.90
Fe²⁺	96.93
Cu²⁺	88.95
Mg²⁺	93.78
Cu²⁺	92.98
Zn²⁺	93.77
Mn²⁺	100
Ba²⁺	22.99
Hg²⁺	2.088
Co²⁺	92.29
Pb²⁺	16.83

Table 3: Effect of Inhibitors on the Activity of the Purified Enzyme

Inhibitors (1 M)	Relative activity (%)
None	100
EDTA	28
Iodo acetic acid	55
PMSF	58
2-Bromosuccinamide	50
2-mercaptoethanol	48

Table 4: Relative Rates of Hydrolysis of Different Polysaccharides

Polysaccharide	Relative rate of hydrolysis (%)
Pullulan	16.81
Amylase	62.61
Amylopeetin	10.68
Soluble starch	6.72
Dextran	9.48
Inulin	11.52

Table 1: Summary of Purification

Steps	Vol.(ml)	Total (μ/ml)	activity	Total protein (mg)	Specific activity (μ/ml)	Yield (%)	purification fold
Supernatant	265	56.68		52.69	1.004	100	1
Dialysis	20	25.35		20.25	1.22	44.7	1.22
Ion exchange chromatography on Q-sepharose	18	15.65		13.09	1.48	27.6	1.47
Hydrophobic interaction chromatography on phenyl sephrose.	10	14.45		3.98	3.63	25.47	3.62

Table 4: Relative Rates of Hydrolysis of different polysaccharides

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Soluble Starch	6.72
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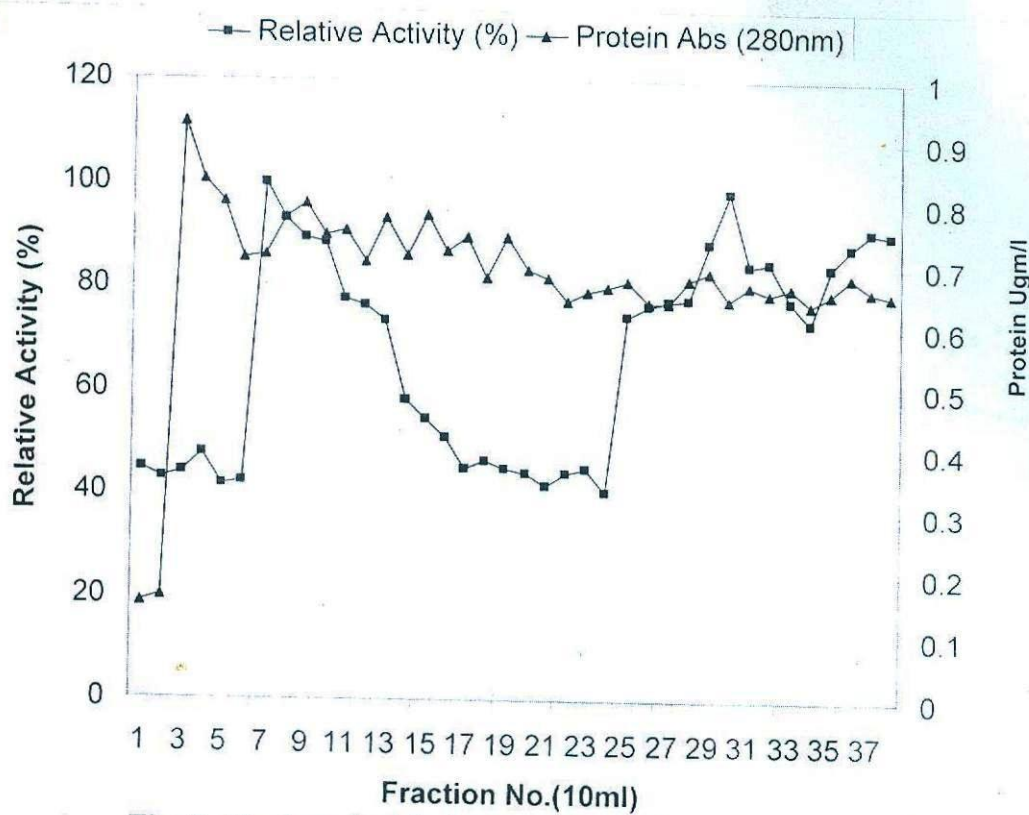


Fig 2. Hydrophobic Interaction Chromatogram Of Fraction A₁

Table 2: : Effect of Metal Ions on the activity of the purified enzyme

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Fe ²⁺	96.93
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Mn ²⁺	100
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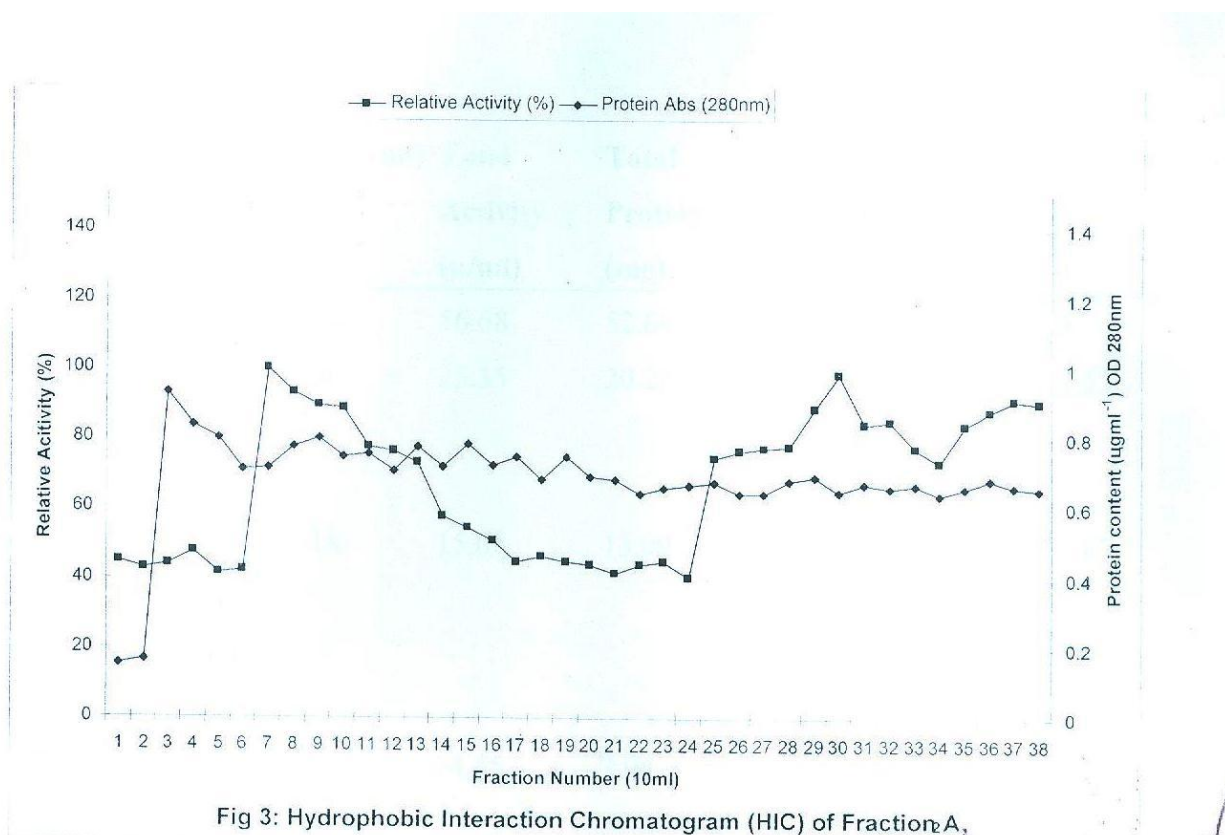


Fig 3: Hydrophobic Interaction Chromatogram (HIC) of Fraction A₂

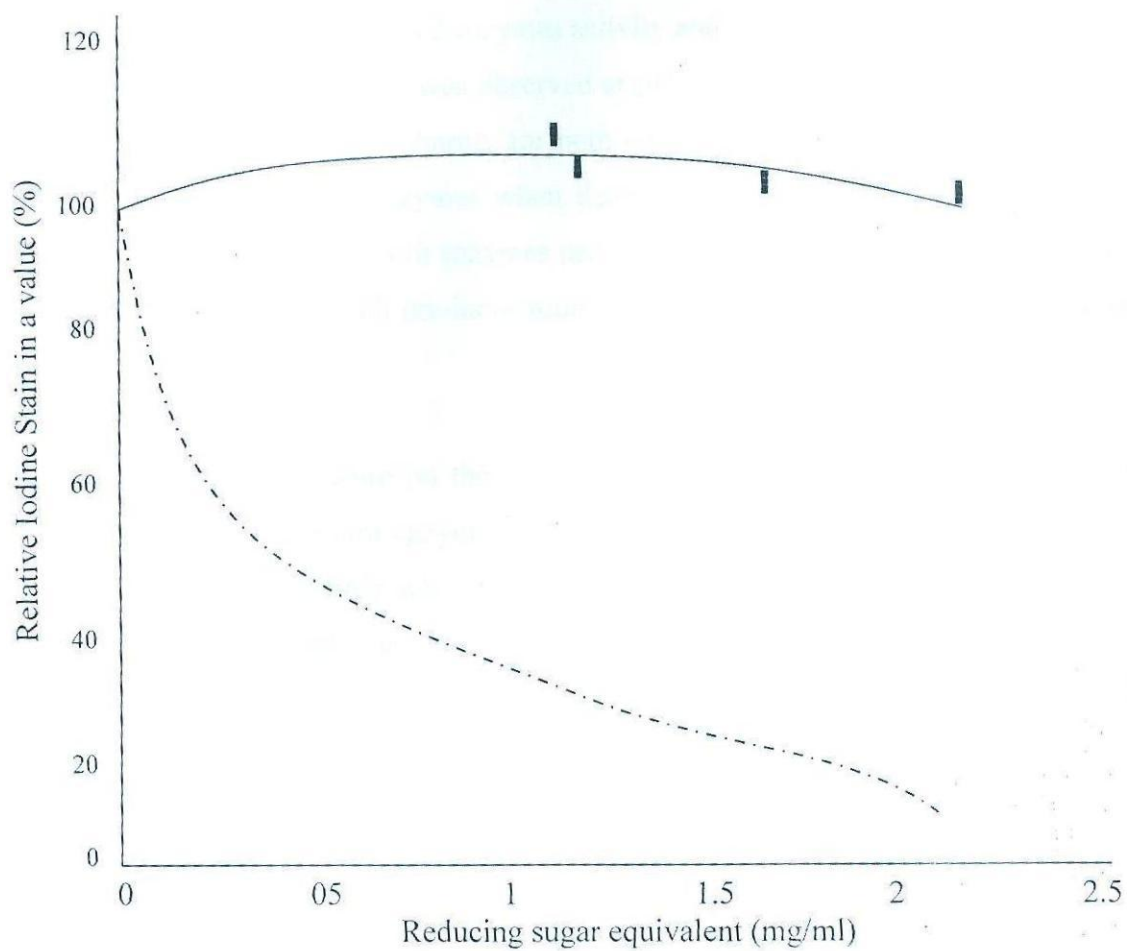


Figure 4. Graph of iodine staining value against reducing equivalent by the α -amylase (Obi and Odibo, 1984).

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