

Studies on the Amylolytic Enzymes Associated with the Black Mould Deterioration of Eko, a Nigerian Fermented Food

ABSTRACT

Aims: To characterize amylase enzymes responsible for the spoilage of Eko, a Nigerian fermented food and to provide the best way to preserve it.

Study Design: The experiment was carried out to extract the enzyme involved in the deterioration of Eko, it was partially purified using ion-exchange chromatography.

Place and Duration of Study: Department of Microbiology, Obafemi Awolowo University, Ile Ife, between 1994 and 1996.

Methodology: Eko was obtained from the new market Ile Ife and cut into slices, the slices were inoculated with spores of *Aspergillus niger*, on the eleventh day after inoculation, the enzyme was extracted, partially purified using ion-exchange chromatography and characterized.

Results: Within eleven days of inoculation, the Eko slices were extensively covered by the fungal spores, the extracellular enzyme secreted by the organism was harvested and characterized. Its molecular weight was 70,000Da, the optimum pH and temperature were 6 and 35°C respectively, enzyme activity increased with increase in starch concentration with optimum activity at 0.9mg/ml, the apparent K_m for starch hydrolysis was 0.4mg/ml. Cations such as Na and K stimulated amylase activity but was inhibited by EDTA and $HgCl_2$.

Keywords: Amylase, Eko, Fungi, Deterioration

I. INTRODUCTION

Maize (*Zea mays*) belongs to the Division Magnoliophyta, Class Liliopsida, Order Poales, Family Poaceae, Species *mays* and Genus *Zea* [1]. Maize has become a staple food in many parts of the world with the production surpassing that of wheat and rice, it is cultivated throughout the world, and a greater weight of maize is produced each year than any other grain [2]. Maize can be consumed directly by humans or used for corn ethanol, animal feed corn starch and corn syrup [3]. The maize kernel consists of four parts: the pericarp which is the outer covering, the endosperm which contains the starch, the corn's energy storage and proteins for germination, the germ, the living organism in the kernel and the tip cap that attaches the kernel to the cob [4]. Maize is composed of starch 62%, proteins and fiber 19%, water 15%, oil 4%, the B vitamins, niacin, thiamine, pantothenic acid [4], [5]. Fresh green maize is usually roasted or boiled and eaten on the cob. Ripe dry grains are usually cooked with peas and beans together with oil and other condiments and eaten as a meal. The dry grains may be ground instead into flour and mixed with palm oil and other condiments to make delicious refreshment. Most commonly, ripe dry grains are processed and prepared into pap (a paste-like food) or Eko which may be taken as a meal along with other complements [6].

Eko is a thick porridge made from ogi, a fermented cereal food which is commonly consumed in Nigeria along with meat, stew, vegetable soup, steamed bean cake (moin moin), fried bean cake (Akara) [7]. Ogi

is an important food item prepared from maize, sorghum or millet in Southern and Northern Nigeria [8], [9]. The process of manufacture involves fermentation (steeping) of grains in water for 2-3 days followed by wet milling, wet sieving with screen mesh and souring for 12-48 hours. The final product is a white mash which when mixed with water and cooked produces a thin gruel (ogi) which is used as a weaning food for infants and also serves as a major breakfast cereal for adults [10], [11]. Eko (a semi – solid pap from maize) is greatly deteriorated by a number of microorganisms especially fungi. Eko is rich in carbohydrate and starch which is its principal storage carbohydrate is susceptible to attack by amylolytic fungi, the growth of this fungi leads to its spoilage and renders it unfit for human consumption. One of such organisms is *A. niger*, thus for *A. niger* to grow in Eko, it must be able to produce enzymes capable of breaking down starch constituents of Eko.

Starch, the storage carbohydrate and principal constituents of maize, is being degraded by microorganisms. Starch degradation is not limited to maize but also fermented foods such as Eko, it is susceptible to spoilage during storage due to the presence of microorganisms and extracellular enzymes produced, which breakdown the food product into new substances resulting into changes in their organoleptic properties [12]. Factors responsible for the spoilage among others are chance inoculation of spoilage organisms and the humid condition of these foods during storage. *Penicillia* and *Aspergilli* species have been reported as spoilage organisms during storage of a wide range of foods where they may produce a number of mycotoxins [13].

Aspergillus niger belongs to the Class Eurotiomycetes, Order Eurotiales, Family Trichocomaceae and Genus, *Aspergillus*. [14]. *A. niger* is one species of *Aspergillus* responsible for food spoilage during storage, it causes 'black mould' on the outsides of spoilt food on which it grows. The genus, *Aspergillus* comprises one of the most common airborne fungi all over the globe and is known to be a natural and potential pathogen of post-harvest fruit. *A. niger*, a ubiquitous airborne organism has been found to be one of the most important pathogens of harvested fruits [15] [16], and has been shown to cause infection in newly harvested maize grains which were sun dried and also in stored maize grains. Incidence was high in samples dried and/or stored as shelled kernels but low in kernels with intact husks [17].

Amylase is an enzyme which hydrolyzes alpha 1,4-glycosidic bonds of amylose, amylopectin, glycogen and related products [18]. The native starch is susceptible to the action of amylases although their degradation proceeds at a greatly reduced rate compared to gelatinized starch [19]. This study shows preponderance of Amylolytic enzyme in deteriorated Eko and that this enzyme was responsible for the spoilage of Eko since it was broken down into simple sugars. The study also shows the conditions at which the enzyme is active and thus provides information which will help in designing physiological control measures for storage of Eko.

2. MATERIALS AND METHODS

2.1. Organism and Culture Condition

The isolate (IFU.81) of *Aspergillus niger* van Tieghem used in this work was obtained from the culture collection of Professor P. O. Olutiola of the Department of Microbiology, Obafemi Awolowo University, Ile-Ife. It was isolated from deteriorated Eko. The organism was sub-cultured on malt extract agar slants in test tube and 72- hour old cultures were used to prepare the inoculum. Ten milliliters of sterile distilled water were added to each agar slant culture. A sterile inoculating loop was used to dislodge the spores into the sterile water. The spore suspension was filtered through a four layer of sterile muslin and adjusted to approximately 3×10^3 spores/ml.

2.2. Inoculation of Eko (Semi-Solid Pap)

Eko used in this work was obtained from the New Market, Ile-Ife. It was surface sterilized using a solution of 1% sodium hypochlorite for 10 minutes and then rinsed with sterile distilled water. Ten grams of the sterile Eko were carefully weighed out, sliced with sharp sterile scalpel into 20 small pieces to expose more surface area to fungal infection. They were transferred into each 250 ml conical flask containing 10 ml of sterile distilled water. Each flask was aseptically inoculated with 1ml of the spore suspension containing approximately 3×10^3 spores/ml. Uninoculated flask served as control. All flasks were incubated at room temperature for daily observation.

2.3. Extraction of Enzyme

After incubation for 11 days by which time the Eko slices in each experimental flask have become covered extensively with the mycelium and the black spores of *A. niger*, enzyme extraction was carried out. Each flask was filtered through Whatman filter paper No 1. The filtrate from each was tested for amylase activity, control flasks (uninoculated flasks) were treated like the experimental flasks.

2.4. Enzyme Assay

Amylase activity was determined by a modification of the dextrinogenic assay of Pfueller and Elliot [20]. It involved the measurement of dextrinizing power (D. P) which was a direct colorimetric measurement of the changes of the blue color of starch-iodine complexes due to the decrease in the amount of starch. Unless otherwise stated, the reaction mixture contained 2 ml of 0.04 % starch in 0.01 M citrate phosphate buffer (pH 6.0) and 0.5 ml of the enzyme filtrate for the experimental tubes. The control tubes contained only the 2 ml of 0.04 % starch. The experimental and control tubes were incubated inside the water bath at 35 °C for 3 hours. After which the reaction was terminated by adding 2 ml of 1 N HCl to each of experimental and control tubes. To each of the control tubes was added 0.5 ml of the enzyme preparation. Using a clean pipette, 2 ml was transferred into each set of clean test tubes followed by 3 ml of 0.1 N HCl. The content of each tube was mixed thoroughly and 0.1 ml iodine solution was added. Optical density readings were made at 600 nm using a Gallenkamp colorimeter.

One unit of amylase activity was defined in arbitrary units as the amount of enzyme in 1 ml of reaction mixture which produced 0.01 % reduction per min in the intensity of blue color of starch-iodine complex under the conditions of the assay. Specific activity was calculated as enzyme units per mg protein.

Amylase activity was also assayed viscometrically in an Ostward viscometer (Gallenkamp) containing 1 ml of enzyme solution and 9 ml of soluble starch (4 %, w/v) in 0.05 M citrate phosphate buffer (pH 5.0) at 35 °C for 20 min.

2.5. Partial Purification of Enzyme

2.5.1. Concentration of the Crude Enzyme

200 ml of the crude enzyme was concentrated to about one tenth (20 ml) of its original volume in a Rotary Evaporator (Quickfit, Rotavapour-RiBuchi, Switzerland) at 30 °C. Frothing was avoided during this procedure by slow evaporation under low vacuum pressure.

2.5.2. Enzyme Separation On Sephadex G-25 Column

2.5.2.1. Materials and Column Preparation

The Sephadex G-25 (bead size 50-150 micron) was contained in a column with an internal dimension of 2.5 x 40 cm. The column was a vertical glass tube with water jacket and was supplied by Pharmacia Fine Chemicals, Uppsala, Sweden.

The Sephadex was suspended in excess amount of 0.01 M citrate phosphate buffer pH 5.0 containing 5 mM NaN_3 to prevent microbial contamination. The buffer was stirred gently to expel trapped air bubbles. It was allowed to swell for 3 days. The very fine particles remaining were removed by decantation. The gel suspension was deaerated and finally, thin slurry of air-free gel was obtained.

The column was half filled with eluting buffer and the gel slurry was added until the column was almost filled to the brim. The top of the column was connected to reservoir and more buffer was allowed to percolate through it while the outlet was opened until appropriate constant height was obtained for the column. A sample applicator was placed at the top of the column to prevent distortion during sample application and the continuous flow of buffer through it.

2.5.2.2. Application of the enzyme concentrates to Sephadex G-25 column

8ml of the enzyme concentrate was applied to G-25 column and allowed to percolate before adding the eluting buffer [21]. The column eluents were collected as described above for the preparation of the column. Fractions were collected (5 ml per tube) and the optical densities of the fractions were read at 280 nm using spectrophotometer (Cecil). Each of the fractions was then analyzed for amylase activity while its protein content was determined by the method of Lowry *et al.*, [22].

2.5.3. Fractionation by ion-exchange chromatography.

2.5.3.1. Preparation of Sephadex C-50 column

Sephadex C-50 (bead size 40-120 U) contained in a column with internal dimension (2.5 cm x 40 cm) was used. The Sephadex C-50 column was prepared as already described above for Sephadex G-25.

2.5.3.2. Application of enzyme to Sephadex C-50 column

Fractions from Sephadex G-25 column which showed appreciable amylase activity after gel filtration were pooled together and 5ml was applied to the column. The enzyme was eluted in a gradient pattern with 0.01 M citrate phosphate buffer (pH 5.0). Fractions were collected and measured at 280 nm and were examined for amylase activity.

2.5.3.3. Fractionation on Sephadex G-100 Column

2.5.4. Preparation of Sephadex G-100 Column

The Sephadex G-100 was contained in a column with internal dimension of 2.5 cm x 70 cm. The Sephadex G-100 column was prepared as already described above for Sephadex G-25.

2.5.4.1. Calibration of Sephadex G-100 Column.

The column was calibrated with proteins of known molecular weight as has been described by Olutiola and Cole [23].

5mg each of catalase (280,000), glucose oxidase (MW 150,000), human hemoglobin (MW 68,000), egg albumin (MW 45,000), horse myoglobin (MW 17,000) were dissolved in 0.01 M citrate phosphate buffer (pH 5.0) and the solution was applied to the gel column and eluted with 0.01 M citrate phosphate buffer (pH 5.0) [21].

A standard graph was obtained by plotting the elution volume of the reference materials against the logarithm of their molecule weights. The molecular weight of the unknown enzyme proteins were extrapolated from the standard graph.

2.5.4.2. Application of enzyme to Sephadex G-100 Column

The fractions from Sephadex C-50 column which gave maximum enzyme activity were pooled and 5 ml of the mixture was applied to Sephadex G-100 column and eluted with 0.01M citrate phosphate buffer (pH 5.0). Fractions (5 ml per tube) were collected and assayed for amylase activity. The protein content were determined by the method of Lowry *et al.*, [22]

2.6. Characterization of Partially Purified Enzyme

The effects of a number of factors on the activity of the partially purified amylase enzyme were examined. The reaction mixtures and assay methods for the tests were as described under enzyme assay; unless otherwise stated.

2.6.1. Effect of Temperature

The effect of temperature on the partially purified enzyme was examined. The reaction mixtures were incubated at 20, 25, 30, 35, 40 and 45 °C respectively for 3 hours and then analyzed for amylase activity

2.6.2. Effect of Substrate Concentration

Different concentration of starch solutions (0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09 and 0.1 % w/v) served as substrate. Reaction mixtures were incubated at 35 °C for 3 hours and analyzed for amylase activity.

2.6.3. Effect of Chemical Concentration

Different concentrations (0, 5, 10, 15, 20, 25 and 30 mM) of KCl, MgCl₂, NaCl and CaCl₂) respectively prepared in 0.04 % starch solution served as the substrate. Starch solution (0.04 %) containing different concentrations (0, 2, 4, 6, 8 and 10 mM) of mercuric chloride, ethylene diamine tetra acetic acid respectively were also prepared and employed as substrate in enzyme assays. After incubation for 3 hours at 35 °C, amylase activity was determined

2.6.4. Effect of Heat

Samples of the partially purified enzyme were heated at 70 °C for between 0 and 30 minutes. Appropriate quantity (0.5 ml) of the heated enzyme preparation was added to 2 ml of 0.04 % starch solution. The control tubes contained unheated enzyme. After incubation of the reaction mixtures for 3 hours, amylase activity was determined.

2.6.5. Effect of pH

The substrate (starch, 0.04 %) was prepared at different pH values (3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0 respectively). Reaction mixtures were incubated at 35 °C for 3 hours and analyzed for amylase activity.

3. RESULT AND DISCUSSION

3.1. Deterioration of Eko by *Aspergillus niger*

Within four days of inoculating Eko with the spores of *A. niger*, slight growth and sporulation of the organism started to appear. By the 11th day of inoculation, Eko slices in the experimental flasks had become extensively covered with the black fungal spores and mycelium. Eko slices in uninoculated flasks showed no visible signs of fungal growth.

The results of this work showed that amylase enzyme was produced by *Aspergillus niger* during the deterioration of Eko by the organism. *A. niger* and a number of other fungi have been associated with the spoilage of Eko and other fermented foods [24]

3.2. Enzyme Separation on Sephadex G-25

Fractionation of the crude enzyme concentrate on Sephadex G-25 yielded three peaks of absorption designated peaks A, B and C (Fig. 1). Component of peak B exhibited amylase activity while components of peaks A and C lacked amylase activity.

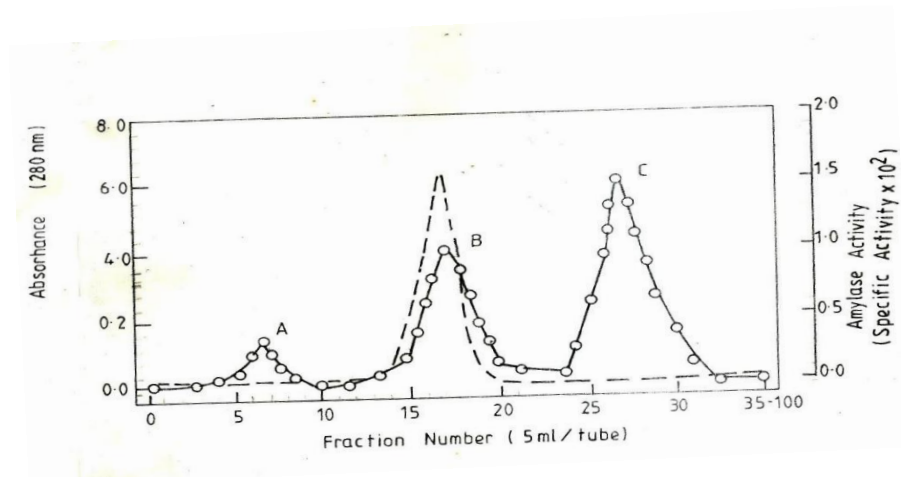


Fig 1: Separation by gel filtration (Sephadex G-25) of proteins in concentrated extracts obtained from Eko (semi solid pap) infected by *A. niger* and enzyme activity of fractions towards starch o-----o, proteins (E_{280}), - - - - - amylase activity,

3.3. Enzyme Separation on Sephadex C-50

Further fractionation of the components of peak B obtained from G-25 column gave two new peaks on C-50 which were designated peaks D and E respectively (Fig.2). Component of peak D exhibited amylase activity. The purification fold of the enzyme was approximately 15. Components of peak E lacked amylase activity.

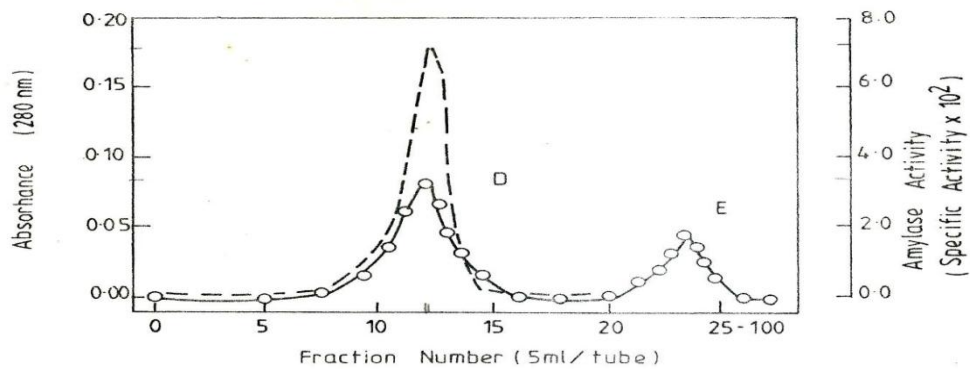


Fig 2: Separation by ion-exchange chromatography (SP Sephadex C-50) of proteins separated by gel filtration, (Fig 1) and enzyme activity of the fractions o-----o, proteins (E_{280}), ----- amylase activity.

3.4. Enzyme Separation on Sephadex G-100

Fractionation of the components of peak D obtained from C-50 column gave two absorption peaks on Sephadex G-100 designated peaks F and G (Fig 3)

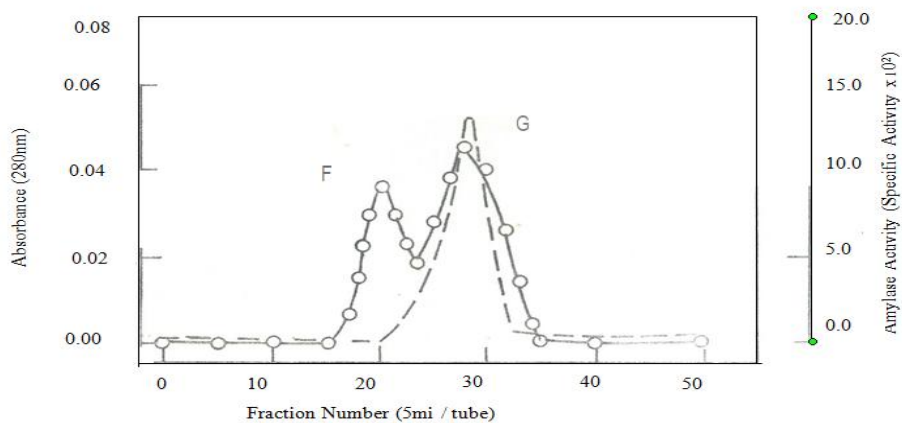


Fig 3: Separation by gel filtration (Sephadex G-100) of proteins (fractions 11 and 12 separated by ion-exchange chromatography (Fig 3) and enzyme activity of fractions o-----o, proteins (E_{280}), ----- amylase activity.

The molecular weight of the components estimated from their elution volumes were approximately 125,000 (Peak F) and 70,000 (Peak G) (Fig. 4). Appreciable enzyme activity occurred in peak G while peak F lacked enzyme activity.

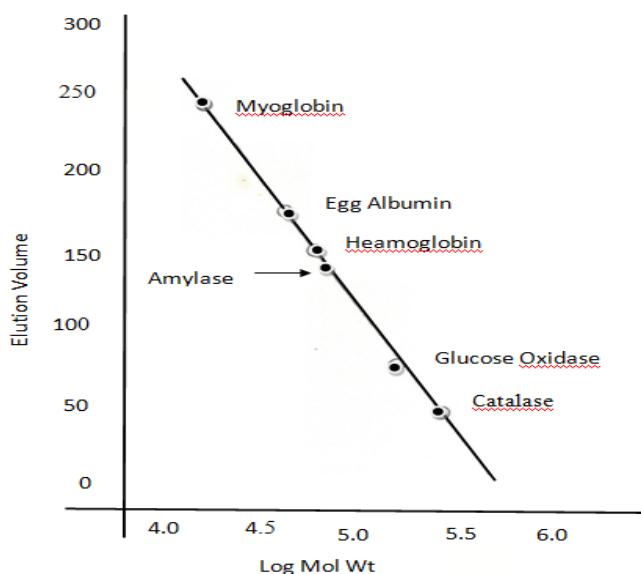


Fig 4: Elution volume against log molecular weight for proteins of known molecular weights separated on a column of Sephadex G-100

The molecular weight of the amylase obtained in this work was approximately 70,000Da. The molecular weight of amylase from different sources varies considerably from 48,000 to 112,000, [25],[26]. Amylase from alkalophilic *Bacillus* sp H-167 was found to have molecular weights ranging from 59,000 to 80,000 [27].

3.5. Properties of partially purified enzyme

The effects of some factors on the activity of the partially purified amylase preparation were examined.

3.5.1. Effect of pH

The activity of the enzyme was affected by the pH of the reaction mixtures (Fig. 5). The activity of the enzyme increased gradually as the pH value increased until an optimum was reached, beyond which the activity started to decrease. The activity of the enzyme was better in acidic conditions (pH 4.0 - 6.0) than in alkaline conditions (pH 7.5 - 8.5). Optimum activity of the enzyme occurred at pH 6.0.

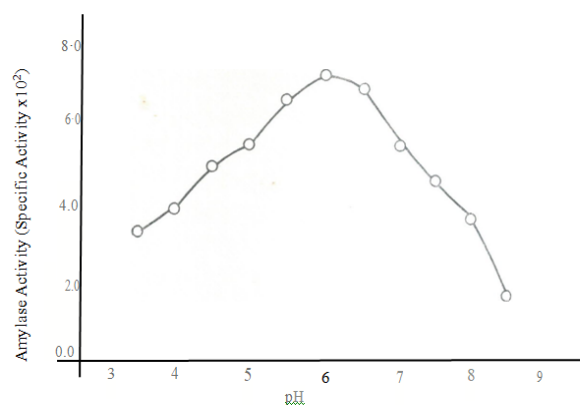


Fig 5: Effect of pH on the activity of partially purified (C-50 fraction) amylase obtained from Eko infected by *A. niger*.

The pH of the reaction mixture had a profound effect on the activity of the enzyme, the optimum activity being at pH 6.0. This result is in agreement with results of Asrat and Girma [28] and Yassien and Asfour [29] who reported an optimum pH of 6.0 for the amylase isolated from *Aspergillus niger* FAB-211 and *Streptomyces clavifer*, Shinde *et al.*, [30] found maximum enzyme activity by *A. niger* and *Bacillus licheniformis* at pH 6 and Ellaiah *et al.*, [31] reported that *A. niger* UO-01 had a preference for amylase production at pH of 6.0

3.5.2. Effect of Temperature

Temperature of the reaction mixture greatly affected the activity of amylase. The activity increased gradually with increase in temperature until an optimum was reached beyond which no further increase in enzyme activity was observed (Fig. 6). Optimum activity of the enzyme occurred at 35 °C

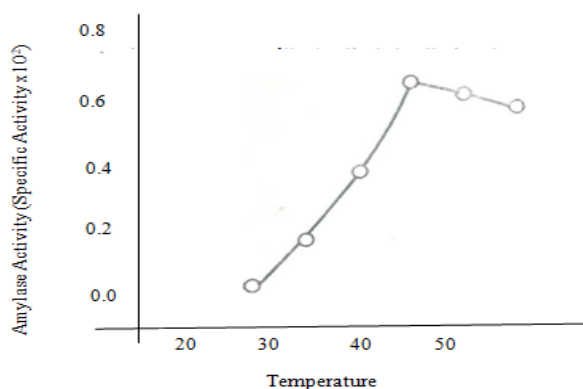


Fig 6: The Effect of Temperature on the activity of partially purified (C-50 fraction) amylase obtained from Eko infected by *A. niger*

The result of this investigation showed that temperature greatly affected the activity of amylase. Optimum activity of the amylase was obtained at 35 °C. This agrees with the report of Adisa [32] who obtained maximum production of amylase and cellulase at 35 - 40 °C from *Aspergillus fumigatus*. Hang and Woodams [33] reported a temperature of 50 °C for amylase from *Byssoschlamys fulva*.

3.5.3. Effect of heat

When the enzyme was heated at 70 °C over a period of 30 minutes, enzyme activity was gradually lost with increase in the length of heating. For instance, when the enzyme was heated for 30 minutes, it lost approximately 100 % activity (Fig. 7).

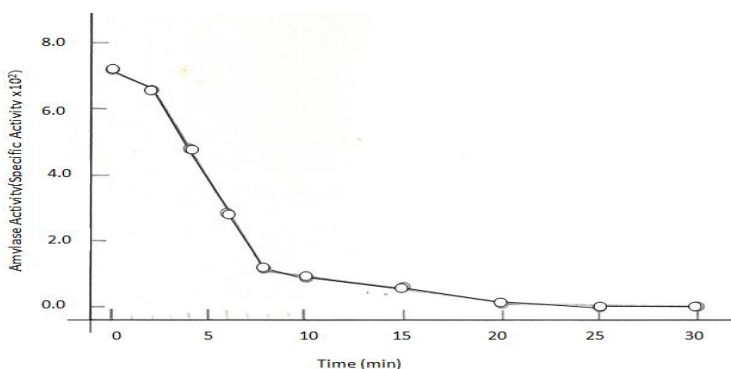


Fig 7: The effect of heating at 70 °C on the activity of partially purified (C-50 fraction) amylase obtained from Eko infected by *A. niger*.

3.5.4. Effect of cations

The effect of some divalent and monovalent cations (Ca^{2+} , Mg^{2+} , Na^+ , K^+) on the activity of the enzyme was examined. The effect obtained was dependent on the type of cations used (Fig. 8). The activity of the enzyme increased as the Ca^{2+} , Mg^{2+} , Na^+ , and K^+ concentrations increased, achieving optimum activities at 25mM concentration for Ca^{2+} and Mg^{2+} , and 30mM for Na^+ , and K^+ respectively. The monovalent cations (Na^+ and K^+) stimulated the enzyme more than the divalent cations (Ca^{2+} and Mg^{2+}).

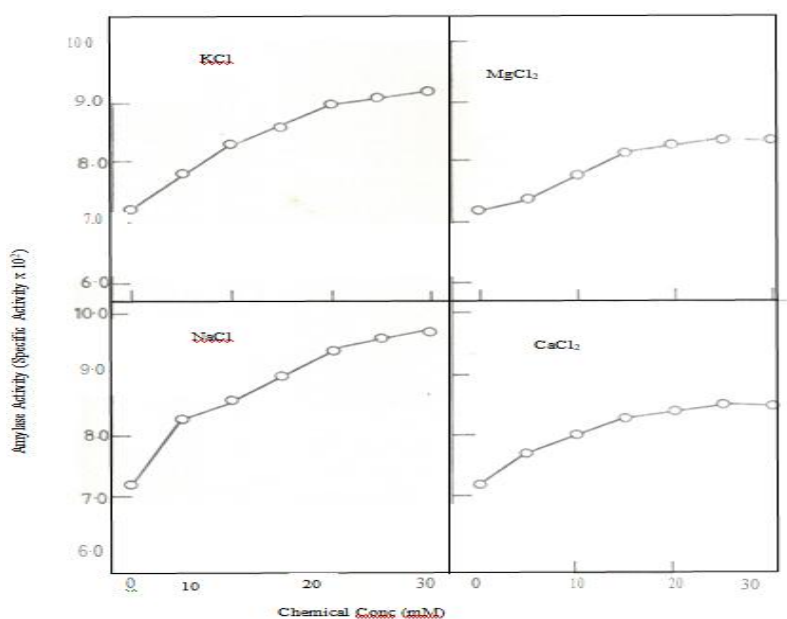


Fig 8: The effect of cations on the activity of partially purified (C-50 fraction) amylase obtained from Eko infected by *A.niger*

The results of this investigation showed that amylase activity was enhanced in the presence of Ca^{2+} and the other cations (Na^+ , K^+ , Mg^{2+}) employed in this work. α -amylase is a metal activated enzyme and has high affinity for Ca^{2+} . In general, Ca^{2+} enhances the activity and thermal stability of most of the α -amylases [34] *Lactobacillus amylophilus* was found to be stimulated by Ca^{2+} [35].

3.5.5. Effect of some chemicals

The effect of two different chemicals, mercuric chloride (HgCl_2), ethylene diamine tetra acetic (EDTA) on the activity of the enzyme was studied. Each of the chemicals inhibited the activity of the enzyme, the level of inhibition being dependent on the type and concentration of the chemical (Fig. 9). Mercuric chloride at 6mM and ethylene diamine tetra acetic at 10 mM brought complete inhibition of the enzyme activity, at 6 mM EDTA, amylase activity was decreased by 87 % while mercuric chloride at the same concentration completely inhibited amylase activity.

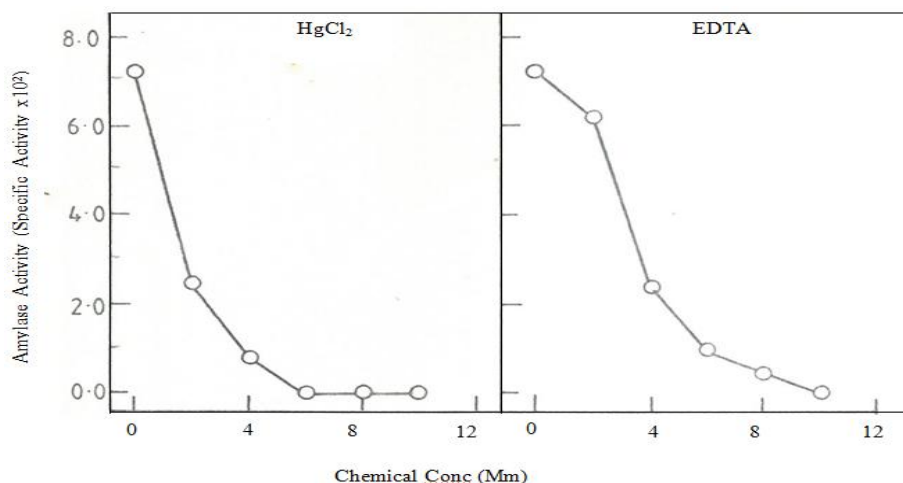


Fig 9: The effect of chemicals on the partially purified (C-50 fraction) amylase obtained froms Eko infected by *A. niger*

Mercuric chloride and ethylene diamine tetra acetic acid (EDTA) were found to inhibit amylase activity. This agrees with the result of Hamilton *et al.*, [36] who reported that Woodward's reagent K, EDTA and EGTA inhibited α -amylase activity.

3.5.6. Effect of substrate concentration

Amylase activity increased with increase in the concentration of starch (Fig 10). Optimum activity occurred at 0.9mg/ml.

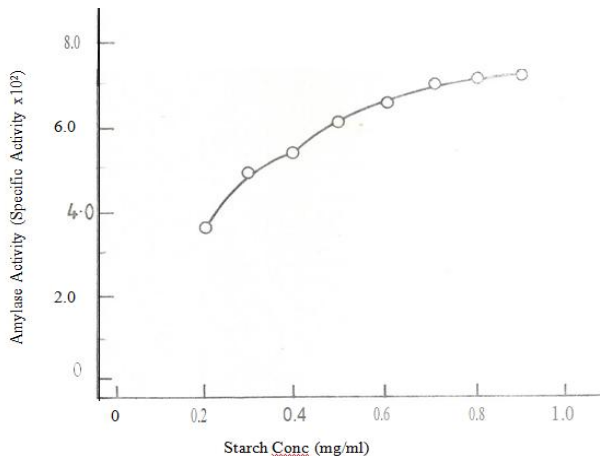


Fig 10: Effect of starch concentration on the partially purified (C-50 fraction) amylase obtained from Eko infected by *A. niger*

From the Line weaver - Burk plot, the apparent km for the hydrolysis of starch was approximately 0.4mg/ml (Fig. 11).

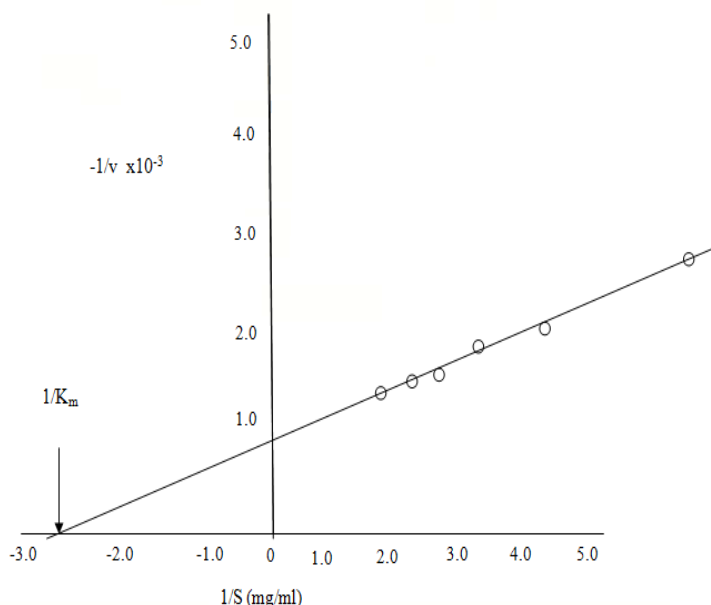


Fig 11: Lineweaver –Burk plot for the hydrolysis of starch by partially purified (C-50 fraction) amylase obtained from Eko infected by *A.niger*

In this work, the activity of the enzyme increased with increase in substrate concentration achieving saturation at 0.9mg/ml. Antranikian *et al.*, [39] reported an increase in amylase activity as a result of increase in starch concentrations.

In this work, it is considered strongly based on the result that the enzyme produced by *Aspergillus niger* in the infected Eko slices is α -amylase and that the enzyme contributed to degradation process. Eko is a starchy food and the physiological significance of the ability of *A. niger* to produce amylase may therefore be that during the infection of Eko slices, the enzyme is able to hydrolyze the complex reserve carbohydrate to the simpler and soluble forms which become absorbed and subsequently metabolized by the pathogen.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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