



Full Length Research Paper

Effects of Temperature, pH and Substrate Concentration on the Kinetics of Salivary Alpha-Amylase Activity among Cigarette Smokers in Awka, Anambra State, Nigeria.

*¹Enemchukwu, B.N., ²Ubaaji, K.I., ²Igwilo, I.O., ²Udedi, S.C.

¹Department of Science Laboratory Technology, Akanu Ibiam Federal Polytechnic, Unwana, P.M.B.1007, Afikpo, Ebonyi State, Nigeria.

²Department of Applied Biochemistry, Nnamdi Azikiwe University, Awka, Nigeria.

*Correspondence author's E-mail: achylesma@yahoo.com

ABSTRACT

Alpha- amylase enzyme was characterized from the saliva samples collected from one hundred healthy adult smokers and fifty non smokers (control) in order to determine the effects of temperature, pH and substrate concentration on the kinetics of the enzyme. The analysis of the saliva samples revealed specific activities ranging from 1.25 to 2.20U/mg for smokers and 1.23 to 2.10U/mg for non smokers, with mean values of 1.70 ± 0.33 U/mg and 1.72 ± 0.30 U/mg respectively. The Michaelis constant (K_m) values of 3.30×10^{-2} mg/ml and 3.37×10^{-2} mg/ml observed for smokers and non smokers were obtained from the double reciprocal plot of initial velocity($1/V_0$) and substrate concentration($1/[S]$). Maximum activities were obtained at an optimum temperature of 40°C and an apparent pH of 7.0 for both subjects. Therefore, this prospective study shows that cigarette smoking has no effects on the kinetics of salivary alpha-amylase activity.

Keywords: Velocity, Michaelis constant, temperature, enzyme kinetics.

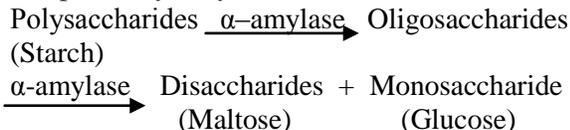
INTRODUCTION

Alpha-amylase (α -1,4-glucan 4-glucanohydrolase EC 3:2:1:1) is an enzyme found in human saliva, pancreatic juice, human breast milk, serum and certain tissues such as the liver which is responsible for the breaking down of starch into maltose and glucose by hydrolyzing the α (1-4) linkages in starch. Salivary alpha amylase functions as the first main step of the process of digestion. The process of digestion begins with the chewing of food in the presence of salivary alpha amylase in the mouth to convert the starch in food into sugar (Maureen, 2000). Foods that are high in starch, like potatoes or bread, will taste slightly sweet when chewed; this is because of the action of amylase. The enzyme amylase is also secreted by the pancreas, where it is called pancreatic amylase; its presence in the gastrointestinal tract aids in the breakdown of food molecules for the body to store and use (Rosenblum *et al.*, 1998).

Starch which is the substrate for ptyalin(alpha amylase) and their products (short chains of glucose) are able to partially protect it against inactivation by gastric acid as the food goes down the stomach in acid medium of about pH 3.3. At this acid medium, ptyalin added to buffer at pH 3.0 underwent complete inactivation in 120 minutes; however addition of starch to 0.1% level resulted in about 10% of the activity remaining, and similar addition of starch to a 1.0% level resulted in about 40% of the activity remaining at 120 minutes (Rosenblum *et al.*, 1998).

In vitro, α -amylase is also able to hydrolyze α -(1 4) linkages in glycogen but has no activity on α -(1 6) linkages responsible for the more highly branched structure of glycogen. These branched structures also reduce the activity of the α -amylase towards glycogen by limiting the accessibility of the target α -amylase bonds, requiring the action of an enzyme that acts on

these branched products to accomplish this compound hydrolysis (Maureen, 2000).



Apart from its function as digestive enzyme, amylase is also useful outside the human body. In bread making, amylase is useful in breaking down starch in flour into simpler forms of sugar, which yeast can then feed on, causing the bread to rise and also imparting flavor. Another form of amylase, "termamyl" sourced from *Bacillus licheniformis*, is also used in some detergents to dissolve starch from stains and dishes when used in dish washer detergents (Robyt and Ackerman, 1973).

Characterization of salivary α -amylase activity showed that the enzyme was stable for at least six months at 25°C (Sampson *et al.*, 1981). It was also observed that this enzyme requires a neutral pH (7.0) or slightly alkaline medium of between pH 7.0 to pH 7.4 for activity and the body temperature is optimal for its best action (Henry and Chiamori, 1960).

Salivary α -amylase has received attention over the past years as a marker for Sympathetic Nervous System (SNS) activity (Rohleder *et al.*, 2004). Evidence has been accumulating showing that salivary α -amylase responds to acute psychosocial stress in a pattern suggesting association with the SNS; and stress-induced increases have been shown to be associated with increasing SNS parameters (Nater *et al.*, 2007). Furthermore, salivary α -amylase increases can be elicited by SNS activating drugs (yohimbine) and prevented by beta blockade, (propranolol) (Ehlert *et al.*, 2006).

More recent work has described a reliable diurnal pattern of salivary α -amylase activity and association of daily amylase secretion with relevant psychological states have been reported (Nagler *et al.*, 2001; Wingefeld *et al.*, 2010). Daily amylase secretion for example changes in response to the chronic stress of caring for a family member with brain cancer; daily secretion is furthermore altered in individuals

with Post Traumatic Stress Disorder (PTSD) in people with higher self-rated depression, and is associated with chronic stress in children with asthma and low socioeconomic status (Granger *et al.*, 2007).

MATERIALS AND METHODS

SAMPLE COLLECTION

About 1.0ml of the saliva samples were collected from each of the one hundred healthy adult smokers and fifty non smokers (control) using specimen sample bottles and diluted to 1:100 with physiological saline (0.85% NaCl) using the technique proposed by Jose and Marriana (2001). The subjects were selected randomly from Awka, Anambra State, Nigeria. The specimens were stored at 4°C to 8°C until analysis which kept the specimens stable for at least twelve months (Lorentz, 1998). The starch substrate which was used in this study was washed in order to reduce the viscosity using dilute NaOH (0.25%) and then allowed to dry at room temperature as recommended by Somogyi (1960). This process of washing the substrate produced gel that was less viscous and sufficiently fluid to permit accurate pipetting and was completely free of the reducing matter (Somogyi, 1960). The starch was then prepared by suspending about 15g of the washed dried starch in 100ml of the buffered solution and with the addition of another 900ml of the buffered solution that was heated to boiling point, thereby forming a gel with pH of 7.0.

Then glucose standard curve was calibrated using a glucose oxidase Kit (GOD/PAP Kit) containing a working glucose standard and a buffered glucose oxidase reagent. The amount of the reducing sugar (glucose) in the buffered starch substrate was determined with the glucose standard curve. Also protein standard curve was calibrated using a standard Bovine Serum Albumin (BSA) in which 0.5g of BSA was weighed and dissolved in one litre of distilled water giving a standard BSA concentration of 500 μ g/ml. This curve was then used to estimate protein concentration of the saliva samples (Lowry *et al.*, 1951; Sengupta and Chattopadhyay, 1993).

ALPHA-AMYLASE ENZYME ASSAY

In order to determine the effects of temperature, pH and substrate concentration on the kinetics of salivary alpha-amylase activity among cigarette smokers as well as non smokers, routine analysis of the saliva samples was carried out with the buffered starch substrate using the enzymatic method for the α -amylase assay for human biological fluids (Scharpe, 1972; Kondo *et al.*, 1988; Henry and Chiamori, 1960) releasing glucose whose concentration for each subject was determined using the glucose standard curve.

The enzyme specific activities for smokers and non smoker subjects were determined over a range of substrate concentration; 15g/L, 12g/L, 8g/L, 6g/L, and 4g/L in a Potassium Dihydrogen Phosphate-Disodium Hydrogen Phosphate (KH₂PO₄-Na₂HPO₄) buffer system (pH 7.0) with the same enzyme concentration. The Line-Weaver Burk plot of the reciprocal of the substrate concentration 1/[S] against the reciprocal of the initial velocity 1/V₀ (the specific activity) was drawn from where the Michaelis constant (K_m) and the maximum velocity (V_{max}) were determined as shown in Figure(1a) and Figure(1b) respectively for both cigarette smokers and non cigarette smokers. The same assay protocols were carried out for smokers and non smoker subjects at varying incubating temperature (20°C, 30°C, 40°C, 50°C and 60°C) with the same enzyme concentration, and then at different pH of the buffered starch substrate solution from pH 6.0, 6.5, 7.0, 7.5 and 8.0 at 40°C respectively.

RESULTS AND DISCUSSION

This prospective study which was undertaken to assess the effects of temperature, pH and substrate concentration on the activities and the kinetics of salivary α -amylase on cigarette smokers as well as non smokers revealed specific activities ranging from 1.25 to 2.20 U/mg for smokers and 1.23 to 2.10 U/mg for non smokers. Mean values of 1.70 ± 0.33 U/mg and 1.72 ± 0.30 U/mg were obtained for smokers and non smokers (control) respectively. These mean values were subjected to a statistical analysis for differences in mean using the

students' t-tests which revealed no significant difference at 95% level of confidence interval.

The Michaelis constant (K_m) and the maximum velocity (V_{max}) values obtained in this study by the double reciprocal plot or the Line-Weaver Burk plot (Figure.1a and Figure. 1b) gave values of 3.30×10^{-2} mg/ml and 10.0 U/mg for apparent K_m and V_{max} respectively for smokers and 3.37×10^{-2} mg/ml and 10.10 U/mg for apparent K_m and V_{max} respectively for non smokers.

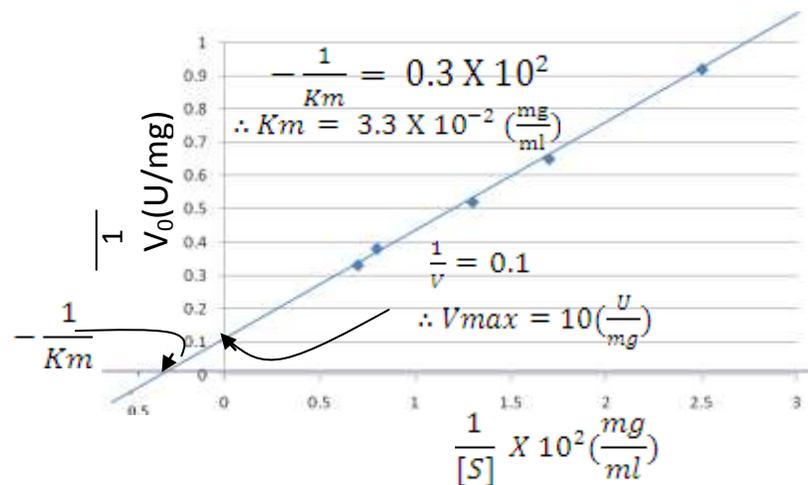
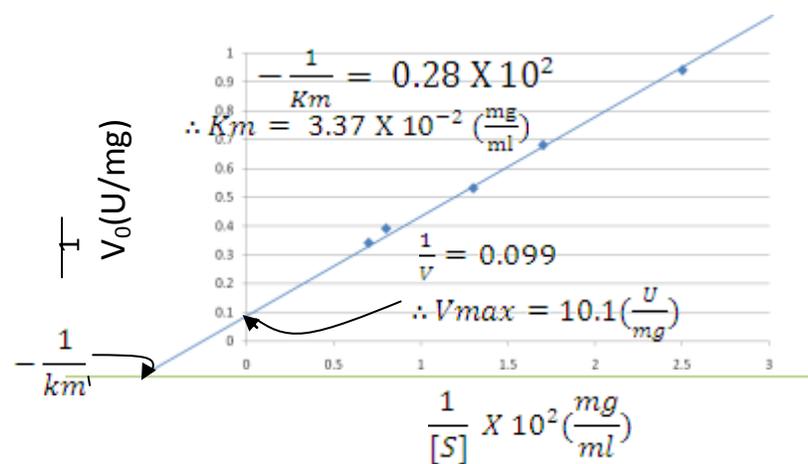


Fig 1(a): K_m and V_{max} determination for smokers.



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These values were in line with the values of 2.9×10^{-2} mg/ml and 10.8 U/mg for a research work carried out by Henry and Chiamori (1960) on urine and serum amylases. These apparent K_m and V_{max} values obtained for smokers and non smoker subjects also revealed no statistical difference at 95% level of confidence interval.

The result from the analysis of the temperature effects on salivary α -amylase activity showed that the enzyme had maximum specific activities of 2.20 U/mg and 2.10 U/mg at 40°C for smokers and non smokers respectively as shown in Figure 2(a) and Figure 2(b). This temperature optimum of 40°C was a little bit different from the optimum temperature of 42°C obtained by Matthew *et al.* (1973) on a research work carried out on urine and serum amylases. The difference may be due to the different population as well as the physiological conditions and other parameters under which the research was carried out.

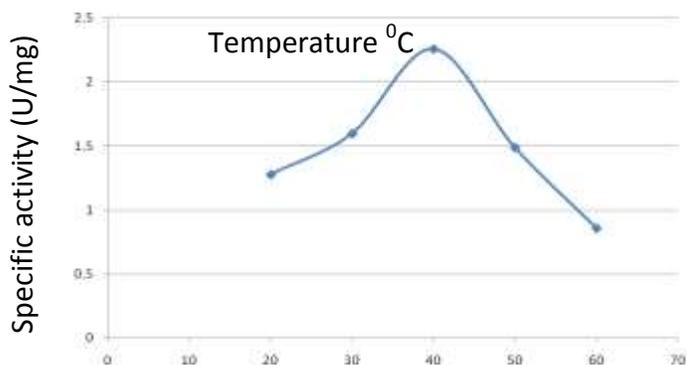


Fig. 2(a): Optimum temperature determination for smokers

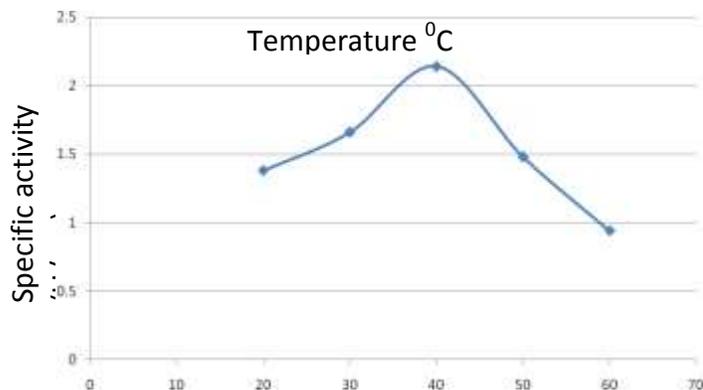


Fig. 2(b): Optimum temperature determination for non smokers

The assay result for optimum pH determination for smokers and non smokers showed an apparent pH optimum of 7.0 for both subjects corresponding to specific activities of 2.62 U/mg and 2.50 U/mg respectively as could be observed from the graphs (Figure 3a and Figure 3b). And from the specific activities profile, activities increased to 76.99% from pH 6.0 to pH 7.0 for smokers and 82.1% from pH 6.0 to pH 7.0 for non smokers which is in agreement with the observation of Matthew *et al.* (1973) who recorded an increased activities of 77% from pH 6.0 to pH 7.0 for both serum and urinary amylases.

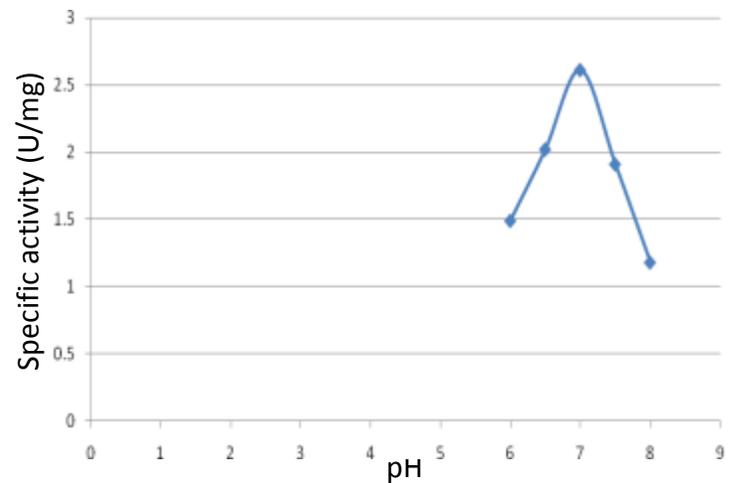


Fig. 3(a): Optimum pH determination for smokers

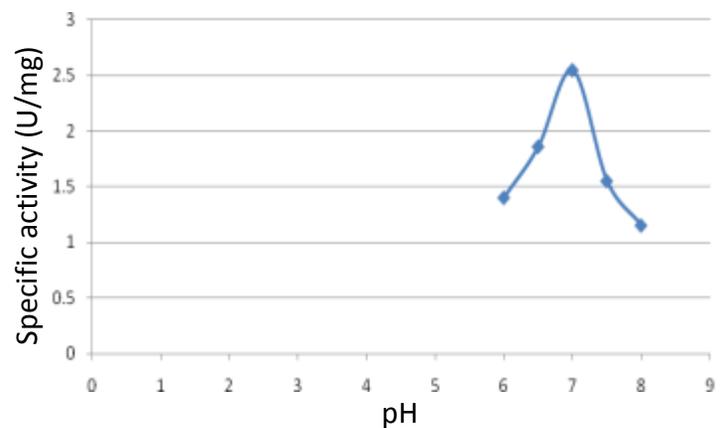


Fig. 3(b): Optimum pH determination for non- smokers

Salivary alpha-amylase activities and protein levels were observed to be high for those cigarette smokers who also indulge in hemp (marihuana) smoking. Kola nuts were also observed to exhibit the same effect of elevated salivary α -amylase activities and protein levels among cigarette smokers as hemp (marihuana). In other words, hemp (marihuana) and kola nuts were observed to enhance activities and protein levels in cigarette smokers. The active ingredients contained in these substances that were responsible for this enhanced activities and protein levels are yet to be determined. This of course will give room for further scientific investigation. On the other hand, kola nuts were observed to show no marked effect among non-smoker subjects.

Furthermore, both subjects exposed to fluoride ions through toothpaste and mouth rinses showed decreased salivary α -amylase activities and protein levels which also give room for further scientific research.

CONCLUSION

From the findings, therefore, cigarette smoking does not have any temperature, pH and substrate concentration effects on the kinetics of salivary α -amylase digestion of starch.

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