Kinetic Studies During Enzyme Hydrolysis of Potato and Cassava Starches

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The hydrolysis of raw potato and cassava starches by bacterial α-amylase depends on the time of action, temperature and on the specific starch involved. The molecular weight of the trade α-amylase (Termamyl 60L), determined by SDS-PAGE, was found to be 55–65 kDa. The properties of α-amylase such as kinetic parameters, inhibition, stability, and thermostability were studied. The constants K_m and maximum reaction rate V_{max} for α-amylase were fitted to Michaelis-Menten models with these two starches. Differences in response of potato and cassava starches to hydrolysis by Termamyl 60L can explain differences found in K_m and V_{max} values and inhibition properties.

Kinetische Untersuchungen während der Enzymhydrolyse von Kartoffel- und Cassavastärken. Die Hydrolyse von rohen (nativen) Kartoffel- und Cassavastärken durch bakterielle α-Amylase hängt ab von der Einwirkungszeit, der Temperatur und von den verwendeten spezifischen Stärken. Das Molekulargewicht der durch SDS-PAGE bestimmten handelsüblichen α-Amylase (Termamyl 60L) betrug 55–60 KDa. Die Eigenschaften der α-Amylase wie kinetische Parameter, Inhibierung, Stabilität und Thermostabilität wurden untersucht. Die Konstanten K_m und die maximale Reaktionsgeschwindigkeit V_{max} wurden bei den beiden Stärken den *Michaelis-Menten*-Modellen angepaßt. Unterschiede in der Reaktion von Kartoffel- und Cassavastärken in der Hydrolyse durch Termamyl 60L können die bei der K_m und bei der V_{max} sowie bei den Inhibierungseigenschaften gefundenen Unterschiede erklären.

1 Introduction

Potato and cassava *(Manihot esculenta)* represent a popular crop in many countries, including South Africa. Cassava accounts for 57% of the total production of tropical roots and tubers. Cassava flour can be processed into food products, feed and alcoholic drinks such as "Kasiri" and "Kapana" [1–5]. These plants are the main source of starch [4–6]. Potato, sweet potato and cassava starches have been widely used in liquefaction with thermostable endo-amylase [7–12].

Enzyme kinetics of all processes such as mechanism of amylase-inhibitor interaction; enzyme-substrate-inhibition complex; an active site carboxyl group in liquefying α-amylase, purification, characterization and application of enzymes is described in many reports [11, 13–15]. Some preliminary kinetic studies on starch enzymatic hydrolysis were conducted in our previous studies [16, 17]. The raw materials employed vary in their chemical composition and physical properties, therefore all parameters have to be tested. This research included the investigation of the suitability of potato and cassava starches for the production of glucose. Very little has been reported of the effect of bacterial thermostable α-amylase on the kinetic data of potato and cassava starches. This study aims to determine the kinetic and thermodynamic data of potato and cassava starches through their degrees of hydrolysis.

2 Materials and Methods

Medium size potato tubers (*Solanum tuberosum*, cv. Russet Burbank) grown in Alberta and obtained from I & S Produce Ltd. (Edmonton) and cassava roots (*Manihot esculenta*, cultivar C2) which were received from the Experimental Cassava Station (Mtunzini, South Africa) were peeled and sliced. Potato and cassava starches were isolated according to conventional methods [18, 19]. Then potato and cassava starches were washed with distilled water several times to remove the impurities and dried in an oven at 40°C before being used. These pure starches were used for hydrolysis studies.

Termamyl 60L (a thermostable bacterial α-amylase, activity 60 KNU/g, product of Novo-Industri A/S, Denmark) was donated for this investigation. α-Amylase inhibitor (from *Triticum aestibum*, containing approximately 50% protein, activity 1–2 000 units/mg protein) was imported from Sigma Chemical Co.

The moisture, fat and protein contents, swelling power and solubility, water binding capacity, gelatinization temperature and total carbohydrates were performed according to conventional methods of analysis [20].

Amino acid analysis of α-amylase (Termamyl 60L) was performed in a Beckman 120C analyzer after hydrolysis with 6 N HCl at 110°C for 72 h [21]. The carbohydrate content of the enzyme was determined by phenol-sulfuric acid method [22].

Molecular weights (MW) of trade enzyme α-amylase (Termamyl 60L) were determined by the application of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to *Laemmli* [23]. Standard M_r values for protein subunits were obtained from a graph prepared for the following standard subunit analyzed by SDS-PAGE (subunit M_r in parentheses in kDa). Mixture of oligomeric peptides, which have been chemically crosslinked with the following Mr's: (14.3); (42.9); (57.2); (71.5); and (85.0) was produced by Fluka Chemica-Biochemica.

The separation gel contained 10% of acrylamide (PAAG). The running buffer was at pH 6.8. The running time was 130 min, using 18 mA per gel. Mercapthoethanol (ME) and ME with urea were used to provide a reduced condition to the sample preparation. Staining of gels for proteins was performed in 0.25% Coomassie Brilliant Blue 250R (CBBR) [24] and for glycoproteins by a modification of the periodic acid-*Schiff*'s base method [25].

Potato and cassava starch aqueous suspensions were gently boiled until solubilized and then diluted with water to 5%. This ice-cooled solution was treated by dropwise addition of 5 ml 1.5% aqueous sodium borohydrate solution. The temperature of a reaction mixture raised to 25°C and kept at this temperature overnight (stock solution). 10 ml of starch solution were neutralized with 1 N acetic acid and diluted to 1% starch with K-phosphate buffer, pH 6.9 (0.04 M K-phosphate-0.05 M NaCl-1 mM $CaCl_2$).

Stock solution of the enzyme was obtained by dilution of the thermostable bacterial α-amylase (Termamyl 60L) in K-phosphate buffer pH 6.9, containing the enzyme stabilizer (proteolytic enzyme inhibitor). Working solutions of experiments were prepared daily by slowly adding 0.1 ml aliquots of stock solution; 0.1 M sodium hydroxide and by adjusting the solution close to 1 mg preparation per ml with K-phosphate buffer, pH 6.9.

Hydrolysis was done by combining of 1 ml of 1% $NaBH_4$-treated starch solution with1.0 ml of enzyme solution (1 mg liquid enzyme preparation per ml) at 37°C, 60°C, 75°C, and 90°C. The reaction times lasted from 5 up to 90 min. All reagents for this reaction including 3,5-dinitrosalicylic acid and Rochelle salt were prepared according to *Bernfeld* [26]. When reaction was stopped by adding of 2 ml 3,5-dinitrosalicylic acid, the test tubes were heated for 5 min at 100°C. The reaction mixture was diluted by adding 20 ml of distilled water, and absorbance read at 545 nm against the blank in which the enzyme was omitted. A calibration curve was established with glucose (0.2 to 2.0 mg in 2 ml H_2O). Since the hydrolysis of starch by various amylases results in the

formation of maltose, oligosaccharides and glucose, the assay of these enzymes was performed by measuring the appearance of reducing sugars by *Nelson-Somogyi* assay [27–30].

Effect of enzyme concentration on the velocity of hydrolysis was done at pH 6.9, 37°C, using a constant amount of starch samples and varying amounts of enzyme. Rate of inhibition of α-amylase (Termamyl 60L) with an α-amylase inhibitor was determined by measuring their activities. α-Amylase inhibitor solution (0.5 mg/ml) was added to the reaction during inhibition studies [26]. Aliquots of the assay mixture after incubation for 30 min at 37°C were removed at the indicated times into 1% reduced starch in order to determine the amount of α-amylase (Termamyl 60L) activity left. pH dependence of extent of combination of enzyme with inhibitor was done by incubation of both for 30 min at 37°C at varying pHs. The buffers used were 4 mM sodium acetate (pH 4.1–6.3) and 4 mM potassium phosphate (pH 6.25–6.85). The residual α-amylase (Termamyl 60L) activity was measured at pH 6.9 [15].

All parameters affected the velocity of the enzymatic reaction (substrate and enzyme concentrations; inhibition; temperature; and pH) were determined [14, 15]. The substrate as was mentioned above was prepared as sodium borohydrite reduced soluble starch [28]. The initial rates of reaction, kinetic *Michaelis* constants K_m and the maximum reaction rates V_{max} were determined with different substrates under a variety of conditions, including varying concentrations. The kinetic properties of α-amylase were examined by determining the parameters of *Michaelis-Menten*'s kinetic equation of starch hydrolysis at three different temperatures. The numerical value of the kinetic constants were obtained after *Lineweaver-Burk* [31] as well as by other graphical methods [9, 32]. Some data were analyzed for statistical significance by the least significant difference (LSD) test at the 5% level of probability [33].

3 Results and Discussion

The proximate composition of raw potato and cassava starches (fat – 0.06%; nitrogen – 0.02%; carbohydrates – 1.11 g/100 g d. b.; gelatinization temperatures – initial: 60°C and 56°C, mid-point: 63°C and 61°C, and end-point: 66°C and 63°C; swelling power – 2.13 and 2.73; solubility – 0.21% and 1.07%; and water binding capacity – 1.14% and 1.77% fell within the range of values reported in literature [5, 6, 16, 17].

SDS-PAGE and ME-UREA-SDS-PAGE patterns revealed that the α-amylase (Termamyl 60L) consisted of two major subunits with the molecular weight about 55–65 kDa (Fig. 1, lane b). Amino acid analysis showed the enzyme to be low in sulfur-containing amino acids. The enzyme had about 8.2% carbohydrates. This was supported by the glycoprotein presence, confirmed with *Schiff*'s reagent (Fig. 1, lane c). These data correspond with others, who reported for α-amylase from *Bacillus* species about 50–55 kDa [34, 35].

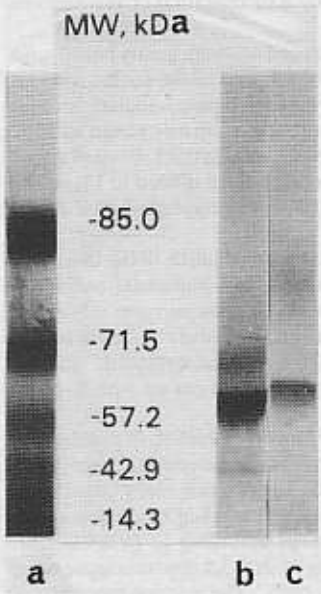

Fig. 1. SDS-PAGE of α-amylase (Termamyl 60L) in 10% PAAG. a. standard protein calibration mixture, stained with CBBR; b. α-amylase (Termamyl 60L) in ME + urea, stained with CBBR; c. α-amylase (Termamyl 60L) in ME + urea, stained with basic fuchsin.

The main factors which determine the initial velocity of enzyme reaction such as effects of enzyme concentration, substrate and its concentration, inhibition and temperature were demonstrated in Figures 2–10.

The pH for α-amylase (Termamyl 60L) was in the range of 5.8–7.0 depending on the type of buffer, temperature and starch concentration. The enzyme concentration varied also between 0.01%–0.1%. The optimal conditions such as constant pH (6.9) and constant enzyme concentration (0.1% v/w) were applied in this study based on several authors [36–43], as well as on our experimental data.

A plot of initial velocity of starch hydrolysis as a function of enzyme is shown on Fig. 2. The velocity is proportional to the enzyme concentration, but the slopes of the straight lines are different for potato and cassava starches. As it was mentioned above that in the enzyme-substrate kinetic study the released glucose was determined colorimetrically. The standard curve used is presented in Fig. 3, position B. Fig. 4 shows two levels of potato starch substrates which provided the expected data. Higher the substrate concentration, higher was the content of glucose in the hydrolysate. The lowest hydrolysis extent was observed at 37°C. At 60°C and 90°C the two concentration levels provided rather close results. The 60°C being preferred by the enzyme. The highest extent of hydrolysis at both concentrations was achieved at 75°C. The hydrolysis rate reached practically a plateau region after 40 min with the exception of the rate recorded at 75°C. Here the hydrolysis is not reaching yet a plateau region even after 80 min (Fig. 4). A plot of initial velocity as a function of potato starch concentration is shown in Fig. 5. The maximum velocity is obtained at 75°C. Starch as a substrate is illustrated at 90°C on Fig. 6 and as well as on Fig. 3, position A. The three concentrations of cassava starch were more readily hydrolyzed than the corresponding levels of potato starch.

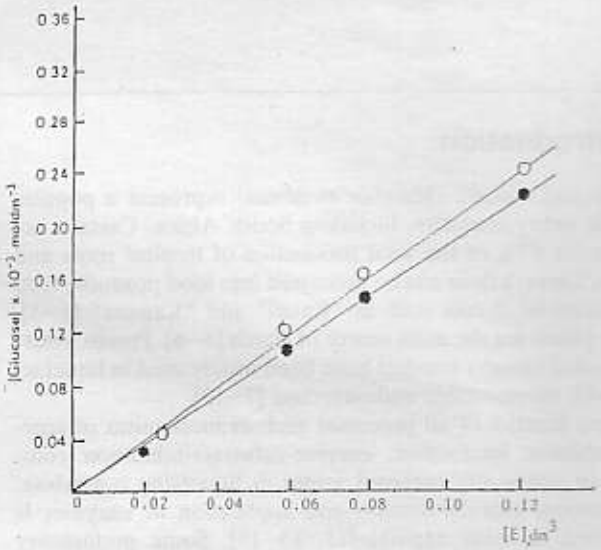

Fig. 2. Effect of α-amylase (Termamyl 60L) concentration on reaction velocity of hydrolysis in potato (—●—) and cassava (—○—) starch samples at 37°C and pH 6.9.

Probably, the samples during enzymatic hydrolysis contained different proportions of low-molecular weight carbohydrates and residual starch, and enzymatic attack was done throughout

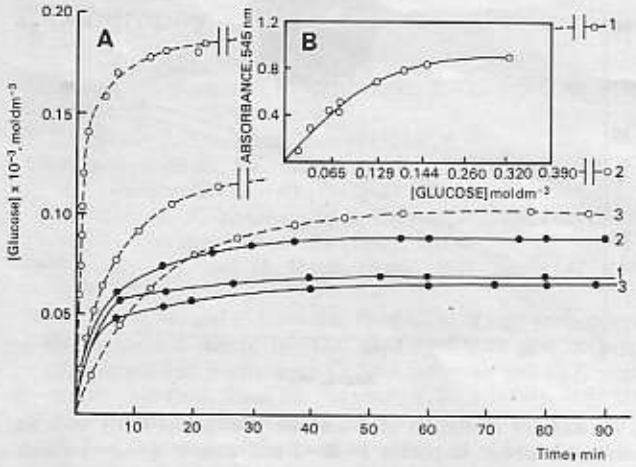

Fig. 3. Effect of substrate material on activity of α-amylase (Termamyl 60L).
(A) The reaction was carried out in the presence of potato (—○—) and cassava (– –○– –) starch samples at 90°C and pH 6.9. 1,2,3-, respectively, 1 ml, 2 ml and 3 ml of substrate. (B) Standard calibration curve.

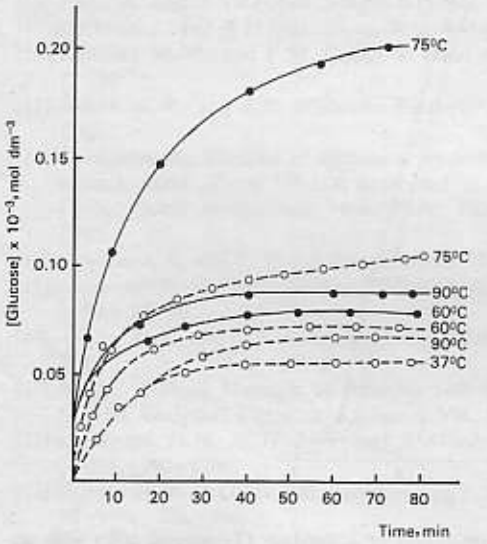

Fig. 4. Effect of substrate material on activity of α-amylase (Termamyl 60L).
The reaction was carried out in the presence of (– –○– –) 1 ml and (—●—) 3 ml of potato starch samples.

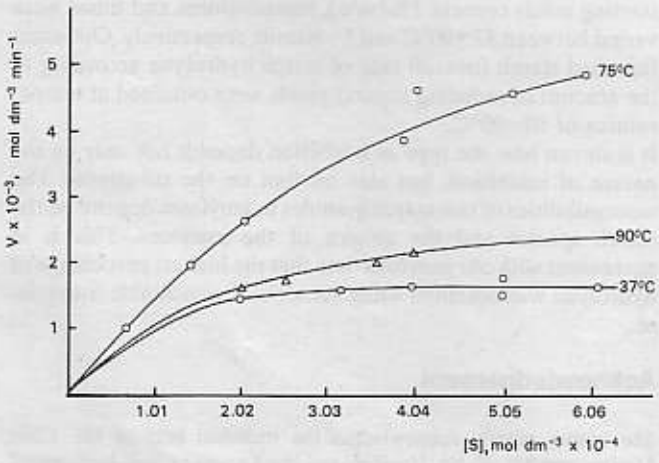

Fig. 5. Effect of substrate concentration on rate of hydrolysis by α-amylase (Termamyl 60L) of potato starch samples.

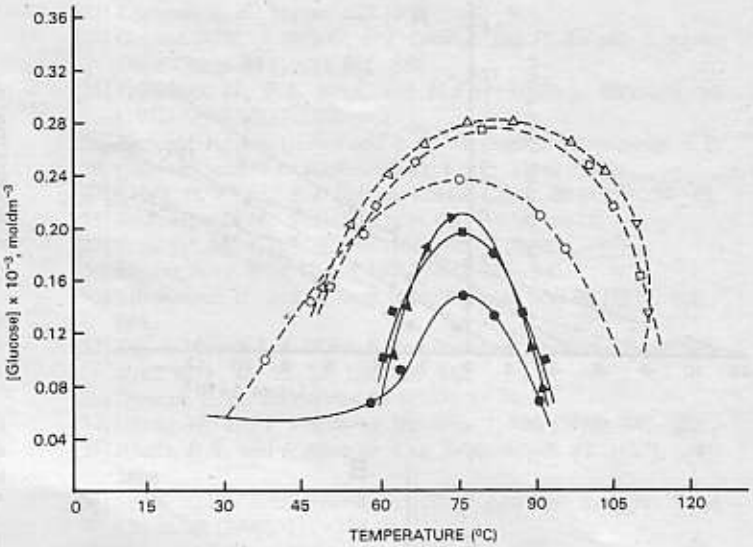

Fig. 6. Temperature stability of α-amylase (Termamyl 60L).
The enzyme was incubated at various temperatures for 80 min in the presence of (—●—) 1 ml; (—■—) 2 ml; and (—▲—) 3 ml of potato starch samples, and (—○—) 1 ml, (—□—) 2 ml, and (—△—) 3 ml of cassava starch samples.

the amorphous granular areas. The cassava was more similar to the cereal starches than to the other root starches. The difference in the digestion of the starches suggests structural differences in the pattern of intermolecular bonding. The intermolecular bonding in the potato seems to quite effectively cover the potential adsorption sites. It was shown that percent of digestion by α-amylase of potato was 4% in comparison with cassava of 34% [43]. This is in agreement with other investigations [36–39].

A double reciprocal of 1/v *versus* 1[S] yielded a straight line from which K_m and V_{max} were calculated by *Lineweaver-Burk* graphical method. The hydrolysis data of substrate concentrations were obtained at 20 and 40 min intervals (Fig. 7, positions A and B). The K_m(mol/dm^3) for potato starch at 37°C, 75°C and 90°C increased from 5.65×10^{-5} to 1.92×10^{-4} at constant time of 20 min. The values obtained at 40 min were nearly in the same range. Cassava starch had a much lower $K_m = 2.25 \times 10^{-3}$ (mol/dm^3) than the potato starch, suggesting a higher affinity of α-amylase to cassava starch. K_m and V_{max} values for potato and cassava starches at 37°C, 75°C and 90°C were within other data [36, 37, 44, 45]. The K_m value which is an indication of the affinity of the enzyme to the substrate is quite close to that of the native α-amylase [37].

The rate of inactivation of α-amylase at pH 6.9 and various temperatures followed a first order rate process. The rate constants were plotted against the reciprocal of absolute T and a straight line relationship is obtained (Fig. 8, position B). The activation energies for denaturation of enzyme and convertion of substrate to product were calculated from the slopes of data plotted according to the *Arrhenius* equation for different temperatures and different substrates (potato and cassava starches). Activation energies (kJ/mol) of the hydrolysis for potato and cassava starches were determined to be 32.75 and 28.65, respectively, which correspond to others [46, 47]. Even a small lowering of the energy of activation results in a significant increase in the rate of this enzymatic reaction compared to the non-enzymatic (non-catalyzed) one.

Rate of inhibition of α-amylase (Termamyl 60L) with an α-amylase inhibitor was performed with variation of time and pH. The inhibition of α-amylase (Termamyl 60L) by α-amylase inhibitor with potato and cassava substrates approaches 40–45% (Fig. 9). The extent of combination of α-amylase inhibitor with

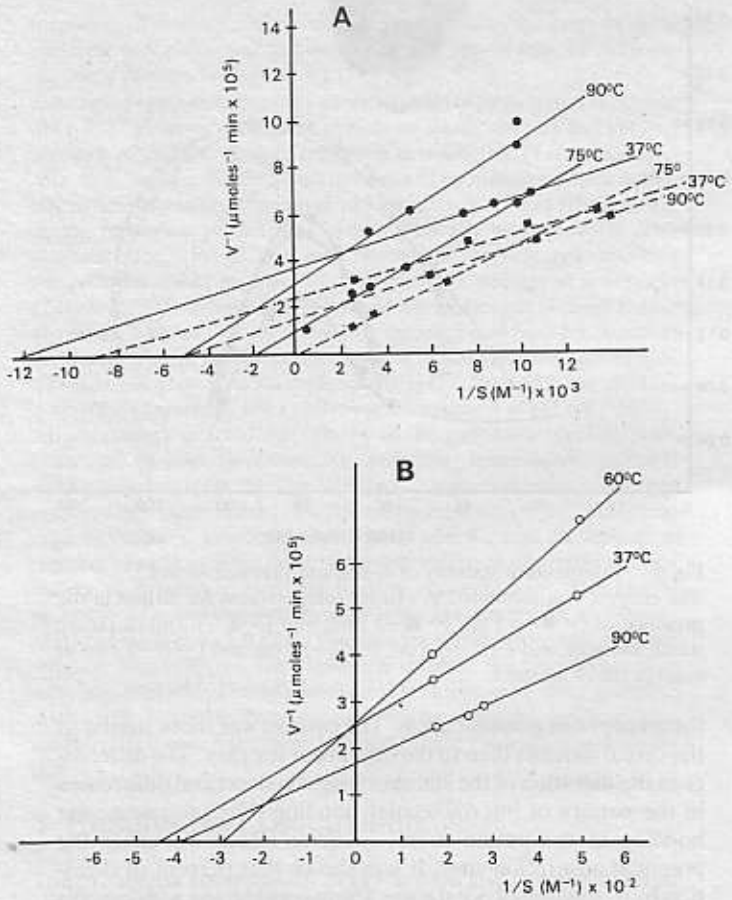

Fig. 7. Effect of substrate concentration of (A) potato and (B) cassava starch samples on rate of hydrolysis by α-amylase (Termamyl 60L). In (A) – reactions were performed (– –■– –) at t = const 20 min; and (—●—) at t = const 40 min. In (B) – at t = const 20 min; The data are plotted by *Lineweaver-Burk* method.

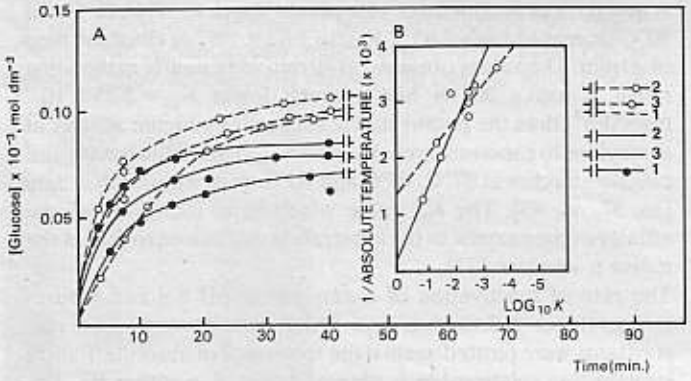

Fig. 8. Effect of substrate material on activity of α-amylase (Termamyl 60L).
(A) The reaction was carried out in the presence of potato (—●—) and cassava (– –○– –) starch samples at 60°C and pH 6.9. 1,2,3- respectively 1 ml, 2 ml and 3 ml of substrate. (B) Temperature profile of α-amylase (Termamyl 60L) plotted according to the *Arrhenius* equation with potato (– –●– –) and cassava (—○—) starch samples.

α-amylase (Termamyl 60L) is dependent on the different pH of incubation with an optimum at approximately pH 5.0–5.2 for potato and cassava starch samples (Fig. 10).

4 Conclusions

Optimum temperature, optimum pH, range of pH stability, maximal activity, initial and relative rates of hydrolysis of

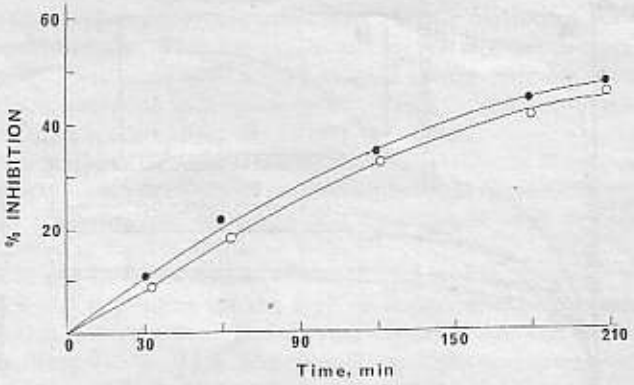

Fig. 9. Rate of inhibition of α-amylase (Termamyl 60L) with an α-amylase inhibitor in potato (—●—) and cassava (—○—) starch samples.

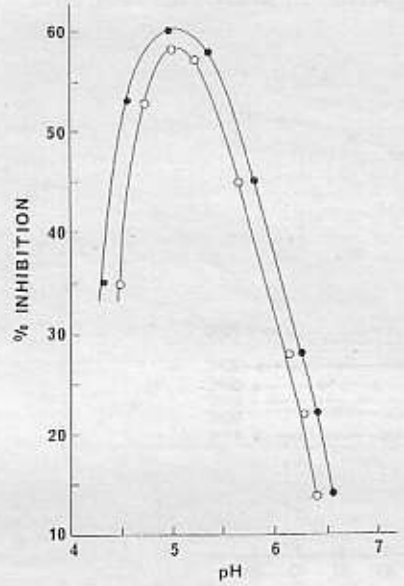

Fig. 10. Rate of inhibition of α-amylase (Termamyl 60L) with an α-amylase inhibitor at different pH in potato (—●—) and cassava (—○—) starch samples.

soluble starch samples, inhibition, activation energy and evaluation of final products of starch hydrolysis were determined. The starting solids content 1%(w/w), temperatures and times were varied between 37–90°C and 5–80 min, respectively. Optimum liquefied starch (overall rate of starch hydrolysis according to the amount of reducing sugars) yields were obtained at temperatures of 80–90°C.
It is shown how the type of inhibition depends not only on the nature of inhibition, but also on that on the substrates. The susceptibilities of raw starch granules to amylases depend on the starch species and the origins of the enzymes. This is in agreement with our previous data that the highest percentage of hydrolysis was obtained using bacterial thermostable α-amylase.

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