Nigerian Journal of Basic and Applied Sciences (1997) 6: 41-50

KINETIC STUDIES ON HEXOKINASE FROM WATER YAM (*DIOSCOREA ALATA*) TUBERS

M.K. ABUBAKAR, L.S. BILBIS and M. LAWAL
Department of Biochemistry, Usmanu Danfodiyo University, Sokoto

ABSTRACT

Hexokinase (E.C. 2.7.11) from Dioscorea alata was isolated and purified 80 - fold using a combination of ammonium sulphate precipitation, gel filtration and ion-exchange chromatography. Electrophoresis of the purified enzyme showed a single sharp protein band which migrated anodically, with a molecular weight of 136,000 daltons as determined using gel filtration.

Initial velocity studies revealed that the enzyme operates via a sequential mechanism with a compulsory formation of a ternary complex of the form enzyme-glucose - $MgATP^{2-}$. The Vmax for the enzyme was determined to be 1.57 Umoles per minute. The Km for glucose and $MgATP^{2-}$ were found to be 3.2×10^{-1} and 8.0×10^{-4}M respectively, corresponding to the total free energy of binding of - 4.33 Kcal per mole. $MgATP^{2-}$ was found to inhibit the enzyme competitively with respect to both glucose and $MgATP^{2-}$ in accord with random order of substrate binding.

The dependence of the rate of reaction on temperature revealed a break in the Arrhenius plot at 40 °C with the activation energy of 11.13 kcal/mole. Temperature stability studies indicated the enzyme is considerably thermolabile with activation energy of thermal denaturation of 31.74 kcal per mole.

INTRODUCTION

The yam tuber under storage epitomises a non-linear, polyphasic and thermodynamically open system that is removed from equilibrium. The weight loss of the tuber in storage is predominantly attributable to respiratory activity (Coursey, 1967; Osuji and Umezurike, 1985). This represents a cascade of reactions characterised by flux and net entropy production (Osuji and Umezurike, 1985). Metabolism in storage therefore connotes an on-going depletion of endogenous negative entropy without a compensatory input of exogenous negative entropy. This predicts inevitable entropic "doom" of the yam tuber whose half-life is calculated to be 1 year by Osuji and Umezurike (1985).

Immediately after harvest, the yam tuber lapses into a period of dormancy characterized by reduced metabolic activity. Dormancy is critical to yam tuber for two

reasons (Ikediobi, 1985), it determines the length of the storage life and reduces the rate of loss of storage carbohydrate. This period corresponds to a phase of minimum entropy production (Passam and Noon, 1977) the depth of which probably represents a critical minimum metabolic flux compatible with the basal level of tuber metabolism. This might be an indication of the lower cut-off point of the depth of dormancy that is compatible with the living state of the tuber. The dormant period is succeeded by a period of enhanced metabolic activity visibly characterized by sprouting, subsequent growth of the resulting seedling and amplication of the activities of some key enzymes of carbohydrate metabolism (Ikediobi and Oti, 1983 and Ugochukwu *et al.*, 1977).

Reasons of academic curiosity apart, kinetic characterisation of enzymes of carbohydrate metabolism whose increase in activities is *sine qua non* to the breakage of dormancy is essential in working out an effective modality whereby net stability may be instilled at the kinetic level by partially inhibiting such enzymes, at about the onset of sprouting, such that their residual activities will be basal. This will complement any genetic or hormonal manipulation that might suppress enzyme amplication in the face of sprouting.

MATERIALS AND METHODS

Materials

The yam tubers used were a kind gift of Professor A.I. Ukoha of the Department of Biochemistry, Federal University of Technology, Owerri, Imo State. The tubers did not show any visible sign of injury or pathological condition.

Nicotinamide Adenine Dinucleotide phosphate (Monosodium salt) and glucose-6-phosphate dehydrogenase were from sigma chemical company, St. Louis, U.S.A. ATP (disodium salt), bovine serum albumin, lactate dehydrogenase, egg albumin and folin-Ciocalteau reagent were from Hopkins Williams, Chadwell Heath, Essex. All other chemicals and reagents were of analytical grades.

Methods

A) Assay of hexokinase activity:

The activity of the enzyme was determined by coupling the glucose-6-phosphate formed in the reaction to glucose-6-phosphate dehydrogenase. The resulting reduced NADP formed was assayed at 340 nm.

(i) *Protein determination*: Total protein was estimated by Lowry method (1951).

B). Enzyme isolation and purification:

(i) *Preparation of crude protein extract:* Cylindrical pieces of the yam tuber were obtained from the "head", "middle" and "tail" regions into separate beakers of distilled water. The pieces were pealed and cut into smaller slices using a sharp knife. 10 g portions from the three regions were pooled and soaked in 30% (w/v) ice cold sodium metabisulphite for 30 minutes. The slices were removed and washed thrice with distilled water and blended in 400 ml of 0.05M Tris/HCl buffer pH 8.0 containing 13.3 mM $MgCl_2$ for 2 minutes. The resulting slurry was centrifuged at 3500 x g for 30 minutes. The resulting supernatant was subjected to ammonium sulphate precipitation.

(ii) *Ammonium sulphate precipitation*: Preliminary experiments have shown that most of the enzyme activity (Hexokinase) was precipitated between 55% and

65% ammonium sulphate saturation.

Enough solid ammonium sulphate was added to 385 ml of the crude extract to bring to 55% saturation. The solution was allowed to remain in ice-bath for an hour with constant stirring and then centrifuged at 10,000 x g for 30 minutes at 4°C. The resultant precipitate was discarded. Solid ammonium sulphate was added to the supernatant to make up to 65% saturation. The solution was again allowed to stand in ice-bath for an hour with constant stirring and then centrifuged at 10,000 x g for 30 minutes. The resulting precipitate was redissolved in minimum amount of 0.05M Tris/HCl buffer, pH 8.0 containing 13.3 mM $MgCl_2$. This is subsequently referred to as ammonium sulphate precipitated enzyme.

(iii) *Dialysis*: 52 ml of the ammonium sulphate precipitated enzyme solution was dialysed for 3 hours against 0.05M Tris/HCl buffer pH 8.0 containing 13.30 mM $MgCl_2$. The system was constantly stirred and the bathing solution changed six (6) times as the dialysis progressed. The resultant dialysed sample was concentrated.

(iv) *Gel-Filtration*: To the fully equilibrated column (2.6 x 80 cm) of sephadex G-150 resin the concentrated dialysed extract was applied and eluted with the equilibration buffer (0.05M Tris HCl pH 8.0 containing 13.30 mM $MgCl_2$) at a flow rate of 30 ml hr^{-1} collecting a 5 ml fraction every 10 minutes. Absorbance of the collected fraction was taken at 280 nm and representative elution profile is shown in Figure 1.

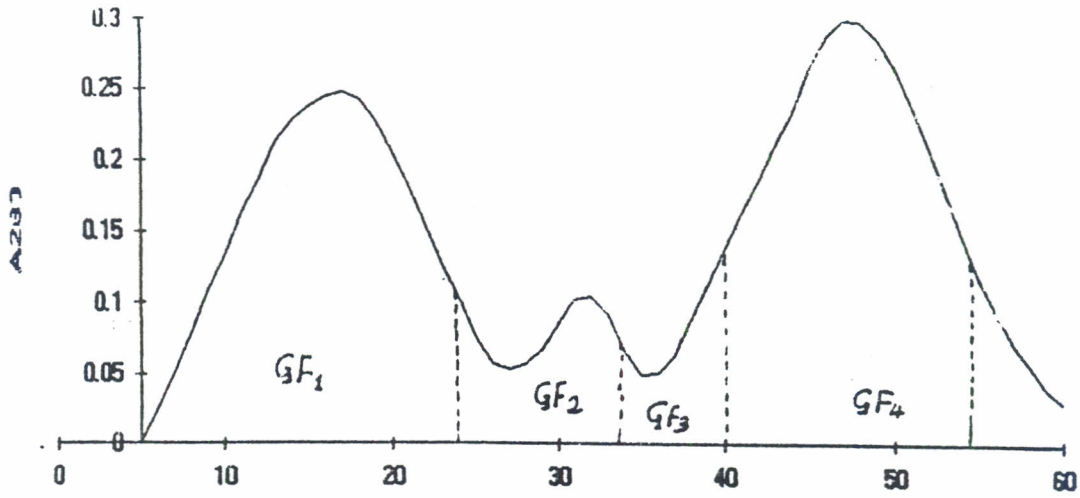

Figure 1. Gel filteration of hexokinase from Dioscorea alata on sephadex G150 column

(v) *Ion-exchange chromatography:* The Hexokinase containing fraction of step (iv) (GF1) was pooled, concentrated and applied to fully equilibrated column (2.6 x 40 cm) of DEAE-sephadex A-50 resin. The column was washed with 20 ml of the equilibration buffer (0.05M Tris/HCl pH 8.0 containing 13.30 mM MgCl2), at a flow rate of 60 ml hr^{-1} collecting 3 ml fraction every 3 minutes.

The bound proteins were eluted with a linear salt gradient consisting of 150 ml of 0.05M Tris/HCl buffer containing 13.30 mM $MgCl_2$ as the starting buffer and 150 ml of the starting buffer containing 0.5M NaCl as the finishing buffer. The absorption profile at A_{280} ηm is shown in Figure 2.

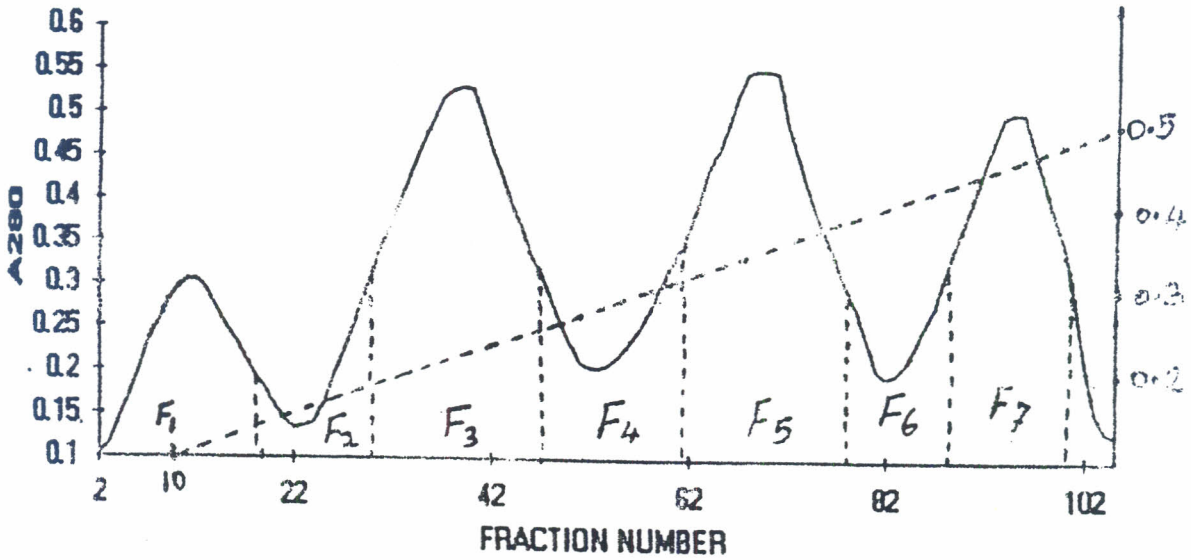

Figure 2: Ion-exchange chromatography profile of hexokinase on DEAE sephadex A50

(vi) *Polyacrylamide gel electrophoresis (PAGE)*: The PAGE was carried out as described by Ornstein and Davis (1964) in the absence of sodium duodecylsulphate (SDS).

(vii) *Molecular weight determination*: Molecular weight of the purified enzyme was estimated by gel filtration according to the method described by Andrews (1978) using lactate dehydrogenase, bovine serum albumin, haemoglobin and egg albumin as standard.

C) **Kinetic Studies:**

(i) *Initial velocity studies*: The initial velocity was studied by varying glucose concentration between 30mM and 150mM at changing fixed concentrations of $MgATP^{-2}$ (0.3 mM, 0.78 mM and 1.48 mM). The experiment was repeated using $MgATP^{-2}$ as the variable substrate at four fixed concentrations of glucose (data not shown).

(ii) *Product inhibition studies*: This was carried out using both glucose and $MgATP^{-2}$ as variable substrates in the concentration range stated in B (I) at different fixed concentrations of $MgATP^{-2}$ (0.63 mM, 1.26 mM, 1.89 mM and 2.5 mM).

(iii) *Effect of temperature on enzyme activity*: In this study, enzyme activity was determined after pre-incubating the hexokinase assay mixture at 26°C, 30°, 40° and 50°C. After temperature equilibrium for 5 minutes, 2.61 ml of the mixture was pipetted into the cuvette and reaction started by the addition of 0.4 ml of the purified enzyme solution.

(iv) *Effect of temperature on enzyme stability*: The effect of temperature on enzyme stability was studied by pre-incubating the enzyme at 30°C and 40°C. Aliquots were withdrawn after five minutes, allowed to cool and 0.4 ml was used to determine the activity at 25°C.

RESULTS AND DISCUSSION

A summary of the purification of *Dioscorea alata* Hexokinase is shown in Table 1. The purity of the final preparation was checked by PAGE and the preparation was found to be electrophoretically homogenous as no detectable contaminant was seen.

Table 1. Purification of *Dioscorea alata* Hexokinase

Step	Total Protein (Mg)	Specific activity (units/mg protein)	Total act. (units)	Yield (%)	Purification Fold
Crude extract	3193.75	12.57	40145.44	100.00	1.00
$(NH_4)_2SO_4$ Precipitation	141.81	212.66	30159.04	75.00	16.92
Gel Filtration	19.74	718.10	14175.29	35.31	57.13
Ion-exchange Chromatography	8.47	1006.97	8529.00	21.24	80.10

Molecular weight

Molecular weight of the preparation was estimated by gel filtration. The molecular weight was found to be 136,000 which is in agreement with the literature value of 100,000 reported for Hexokinase from other sources (Colowick, 1973). The small discrepancy may be due to different methods used in the determination of the molecular weight in the two cases.

Kinetic Studies

The results of the initial velocity studies is shown in Figure 3. Varying the glucose concentration at changing fixed concentration of $MgATP^{-2}$ yielded a family of reciprocal plots that intersect on 1/[Glucose] axis, i.e $MgATP^{-2}$ behaved as if it were a non-competitive inhibitor of hexokinase with respect to glucose. This is a kinetic pattern expected for a sequential mechanism with a compulsory formation of a ternary complex of the form enzyme-glucose-$MgATP^{-2}$ (Cleland, 1970 and Segel, 1975). This finding is in accord with the report of others for hexokinase from a variety of sources (Colowick, 1973). The results of product inhibition using $MgADP^{2-}$ is shown in Figures 4 and 5 with both glucose and $MgATP^{2-}$ as variable substrates, the enzyme was found to be inhibited competitively by $MgADP^{2-}$ suggesting a random order of the binding of glucose and $MgATP^{2-}$ on the condition that an abortive ternary complex is not formed.

The kinetic constants V_{max} for the enzyme and K_m for both glucose and $MgATP^{2-}$ were

determined from the analysis of the double reciprocal plot using the equation of a rapid equilibrium random bireactant enzyme-catalysed reaction as derived by Segel (1975). The V_{max} was found to be 1.57 µmoles min^{-1}. This is 2.4 times greater than that of erythrocyte hexokinase (Rijksen and Staal, 1976). The K_m for glucose and $MgATP^{2-}$ was 0.32M and 8.0 x 10^{-4}M respectively. The K_m value for glucose is greater than that of human iso-enzyme type IV (Colowick, 1973). The high K_m value for glucose compared to that of $MgATP^{2-}$ is not unexpected for a tissue whose bulk of storage food material is carbohydrate. Glucose is expectedly present at a higher concentration in the yam tuber than $MgATP^{2-}$, in order for the reaction to occur *in vivo* at an appreciable rate the enzyme should have higher affinity for $MgATP^{2-}$ and hence lower K_m value. This difference in affinity is also reflected in the standard free energy of binding at pH 8.0, as determined from the K_m value under rapid equilibrium. The free energy for glucose and $MgATP^{2-}$ binding are -0.57 and -3.65 Kcal $mole^{-1}$ respectively.

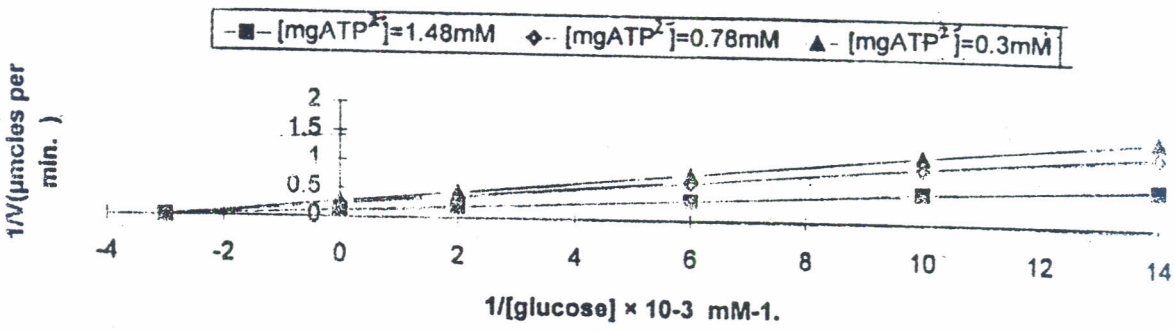

Figure 3: Double reciprocal plot of the enzyme activity using glucose as a variable substrate and mgATP2- as a changing fixed substrate

This shows the binding is spontaneous under standard conditions in each case but more spontaneous in respect of $MgATP^{2-}$

The results of the effect of temperature on the enzyme reaction is shown in Figure 6. A break in the Arrhenius plot was observed at 40°C. This is peculiar to enzyme catalysed reactions which is attributable to thermal denaturation. At temperatures between 26°C and 40°C, the rate of reaction is rate limiting, but above 40°C, a different step, enzyme denaturation become rate limiting.

The activation energy was found to be 11.13 Kcal $mole^{-1}$, this is rather small but compares favourably with the value of 14.7 Kcal $mole^{-1}$ for erythrocyte hexokinase

(Rijksen and Staal, 1976). Both the activation energy and the breaking point of the Arrhenius plot were reported to be dependent on the ratio of Mg^{++} to ATP (Rijksen and Staal, 1976).

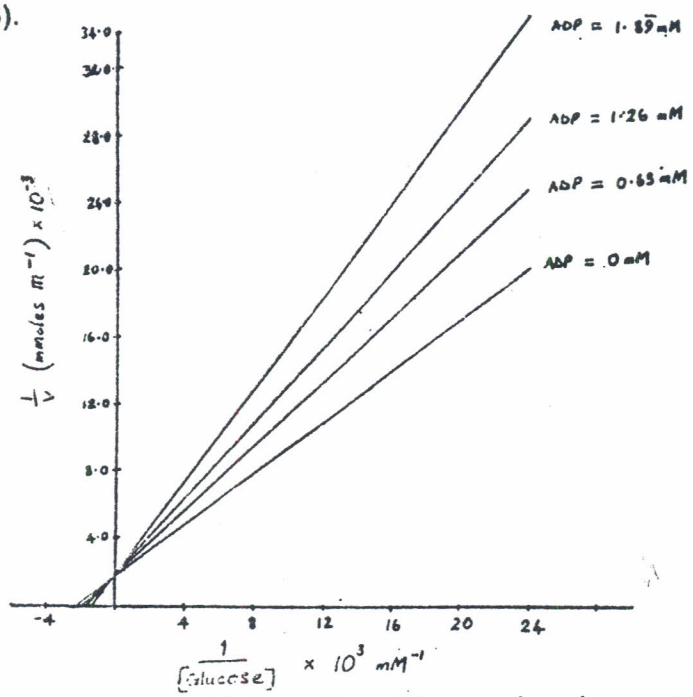

Figure 4: Product inhibition of hexokinase using glucose as a variable substrate and $MgADP^{2-}$ as a product inhibitor

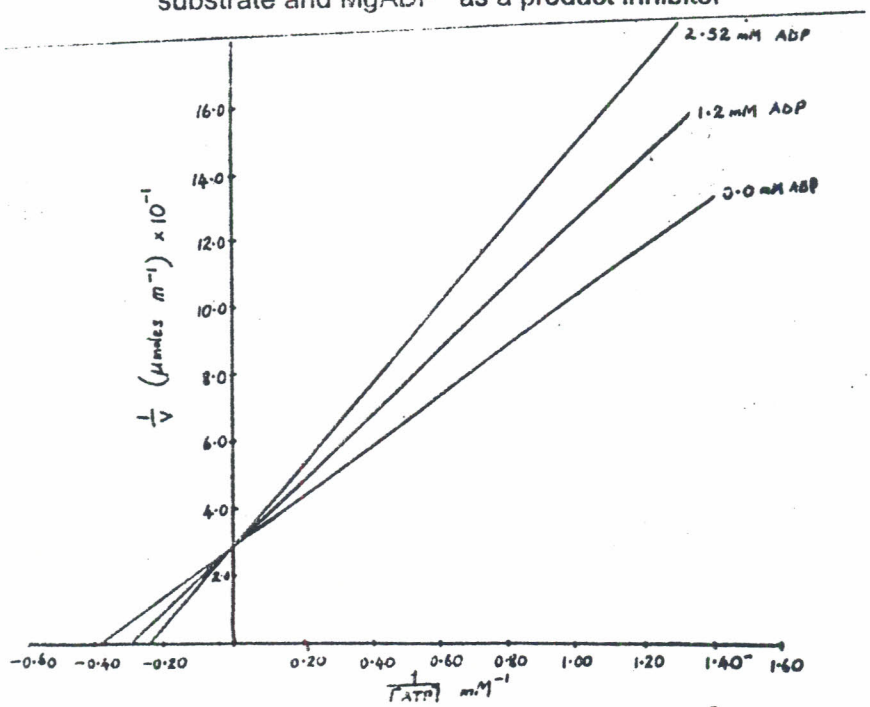

Figure 5: Product inhibition of hexokinase using $MgATP^{2-}$ as a variable substrate and $MgADP^{2-}$ as a product inhibitor

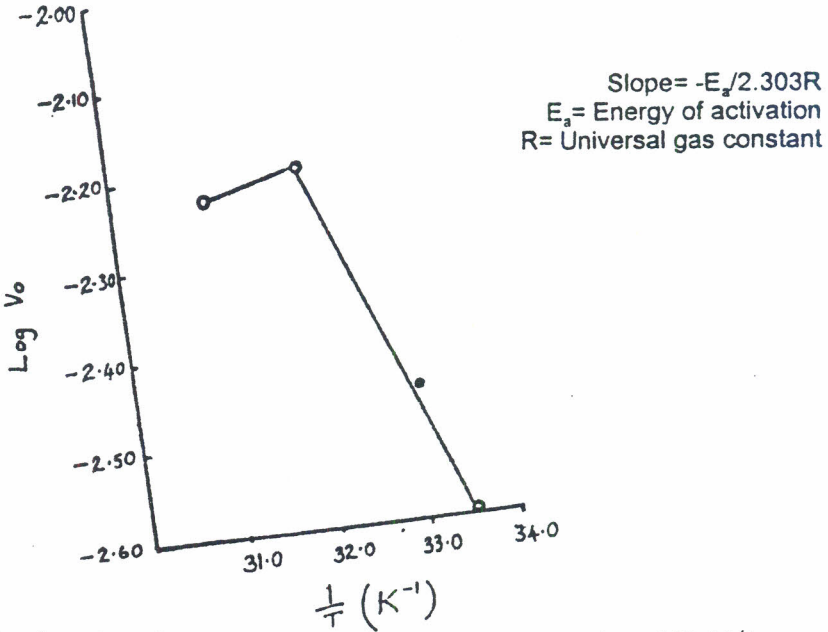

Figure 6: Arrhenius plot of the logarithm of initial velocity against 1/T (K^{-1})

The results for the effect of the pre-incubation of the enzyme at 30°C and 40°C is shown in Figure 7. The semi-logarithmic plots are linear. This suggest that the kinetic of thermal denaturation is first order. The activation energy of thermal denaturation between 30°C and 40°C was found to be 31.74 Kcal $mole^{-1}$. The enthalpy and entropy of activation at 30°C were found to be 31.14 Kcal $mole^{-1}$ and 23.64 Kcal $mole^{-1}$ respectively. The positive value of the activation energy, is to be overcome the kinetic barrier to denaturation. The half lives of the enzyme at 30°C and 40°C were found to be 5.8 hrs^{-1} and 1.2 hrs^{-1} respectively. This indicate that the enzyme is considerably thermolabile. The positive value of entropy of activation of the thermal inactivation of the enzyme indicates an increased randomisation of the protein structure while the positive value of the enthalpy indicates the severance of some bonds enroute the transition state. The values of entropy and enthalpy of denaturation as determined experimentally fall within the range for most proteins (Segel, 1975).

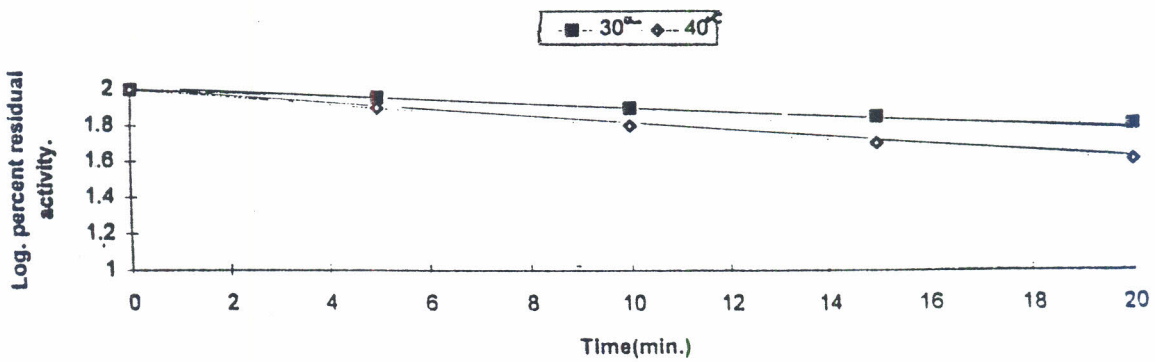

Figure 7: Semi-logarithmic plot of the percent residual activity of the incubated enzyme against time (min.) at 30° and 40°C

It is noteworthy that the activation energy of denaturation is higher than the activation energy of the reaction. This suggests that at relatively low temperature, the rate of catalytic reaction supersedes the rate of thermal denaturation. The reverse is the case at relatively high temperature. This naturally explains for the break in Arrhenius plot at relatively high temperatures.

As hexokinase is one of the rate limiting enzyme in glycolysis, one should be prompted to investigate the possibility of designing a selective inhibitor and evaluate its potential as sprout suppressant. This may help toward prolonging the shelf-life of yam tuber.

Ugochukwu *et al.* (1977) have reported a 2-fold increase in hexokinase activity in physiologically old *D. rotundata* tubers. This suggests that the activity of hexokinase and that of other glycolytic enzymes increases at the onset of sprouting (Ikediobi and [illegible]). Hexokinase reaction is therefore a likely reaction that can be exploited to prolong the storage life time.

REFERENCES

Andrews, P. (1978). Estimation of Molecular size and Molecular weights of Biological compounds by Gel-Filtration. *Methods of Biochemical Analysis*. 18:1-51.

Coursey, D.G. (1967). **Yams**. Tropical Agriculture Series, Longmans Green, London 230pp.

Clelard, W. (1970). Enzyme Mechanism. In: Boyer, P.D. (Ed.) **Enzyme**. 3rd edition, Academic Press, New York and London.

Colowick, P.S. (1973). The Hexokinase. In: Boyer, P.D. (Ed.) **Enzyme**. 3rd edition, Academic Press, New York and London. pp. 1-46.

Ikediobi, C.O. and E. Oti (1983). **Some Biological changes associated with post-harvest storage of white yam (*D. rotundata*) tubers.** *J. Sci. Fed. Agric.* 34:1123-1130.

Ikediobi, C.O. (1985). Biochemistry and physiology of yam storage. In: Osuji, G.O. (Ed.) **Advances in Yam Research**. Biochemical Society of Nigeria. 109 pp.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randali (1951). Protein measurement with folin-phenol reagent. *J. Biol. Chem.* 193:265-275.

Ornstein, L. and B.J. Davies (1964). Disccelectrophoresis-1 Background and Theory. *Annal of New York Academy of Science*. 121: 321-349.

Osuji, G.O and G.M. Umezurike (1988). The Biochemistry of yam tuber deterioration. In: Osuji, G.O. (Ed.) **Advances in Yam Research**. Biochemical Society of Nigeria. pp. 261-276.

Passam, H.C. and R.A. Noon (1977). Deterioration of Yam and Cassava during storage. *Ann. Appl. Biol.* 85: 436-440.

Rijksen, G. and G.E.J. Staal (1976). Kinetics of human erythrocytes hexokinase influence of temperature, ATP and Magnesium ions. *Bioch. Biophys. Acta* 453: 382-391.

Segel, I.H. (1985). **Enzyme Kinetics. Behaviour and Analysis of rapid equilibrium and steady state enzyme system**. Wiley Interscience. New York. pp. 273-327.

Ugochukwu, E.N., E.O. Anosike and S.E.O. Agogbua (1977). Changes in enzyme activity of white yam tubers after prolong storage. *Phytochrome*. 16: 1159-1162.