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KINETICS STUDIES ON NITRATE REDUCTASE FROM MILLET

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

ABSTRACT

Millet seedlings (Ex-Borno, Gero type) were grown and crude cell extract of the leaves was used to assay for the activity of Nitrate reductase (EC 1.6.6.1) spectrophotometrically at 540 nm according to Neyra and Hageman method (1975). Michealis-menten constant, km, and maximum velocity, Vmax, were found to be 106.55 µM and 15.18 µmole min^{-1} $gram^{-1}$ tissue respectively. Optimum temperature was found to be 35°C, while optimum pH was 6.5. The activation energy of the reaction was 38.29 KJ $mole^{-1}$. Glutathione was found to inhibit the enzyme competatively with ki of 2.33 x 10^{-4} µM. Residual activity rate constant at 30°C, 40°C and 50°C were 3x10^{-3} min^{-1}, 6 x 10^{-3} min^{-1} and 9 x 10^{-3} min^{-1} and the biological half life of the corresponding temperatures were 231.0 , 115.5 and 77.0 minutes respectively . Activation energy between 30°C and 40°C was 25.027 KJ mol^{-1} and for 40°C and 50°C it was 292.805 J mol^{-1}. The enthalpy of activation, H was 22.508 KJ mol^{-1} K^{-1} and enthropy S* was -218.27 Jmol^{-1} K^{-1}. The free energy was 88.642 KJ mol^{-1} K^{-1}.*

INTRODUCTION

Nitrate reductase (NADH:Nitrate Oxido-reductase , EC. 1.6.6.1), a substrate inducible enzyme plays a great role in regulating the process of nitrate assimilation in plants and micro-organism (Hewitt *et al.*, 1979). This enzyme is the first in the series of enzymes which reduce nitrate to ammonia and is sensitive to a number of environmental factors. The activity of the enzyme is known to vary under the influence of light intensity, carbon dioxide level, temperature, water availability and nitrate supply (Beevers and Hageman, 1969). However, when other environmental factors are kept constant, nitrate reductase activity (NRA) appears to be inducible by nitrate (Kelpper *et al.*, 1977).

Nitrate reductase belongs to a class of enzymes known as molybdenum-containing multicenter redox enzymes (Hewitt and Notton, 1980). The enzyme from all sources investigated has been shown to be a large (Mw = 200,000 - 500,000) multimeric protein containing flavin, cytochrome b, and molybdenum as prosthetic groups (Hewitt and Notton, 1980). The prosthetic groups serve to transfer reducing equivalents from pyridine nucleotide to nitrate. Nitrate is the principal source of nitrogen for most plants growing under normal field condition

in fertile soil, (Hewitt *et al.*, 1979) which is considered a more favourable source of nitrogen than ammonia for plants(Hewitt *et al.*, 1979).

The major forms of inorganic nitrogen in plants are nitrate, nitrite and ammonia (Hewitt and Cutting, 1979). The concentration of all the three may vary considerably, but in general, nitrite accumulation by plants is rare, while the concentration of ammonia is relatively low between 0.004 and 0.01 M (Hewitt *et al.*, 1979). Nitrate concentration vary enormously (Rawn, 1983). Climate, plant age, developmental stage, nutritional status , management and species are some of the factors that affect nitrate content of plants (Hewitt and Nicholas, 1964). The importance of nitrate reductase in nitrogen metabolism in plants and microorganisms is shown clearly by a highly positive correlation between its activity and protein content in the tissues as reported by Carvin and Atkins (1974).

Of the numerous reports on the use of nitrogen by plants, a few deal with the kinetic studies aimed at delineating the mechanism of nitrate reductase. This communication aims at examining the biochemical characteristics and the factors influencing the activity of the enzyme in millet with a view to determining the kinetic parameters which affect the activity of this enzyme of immense physiological importance.

MATERIALS AND METHODS

Chemicals

Potassium dihydrogen orthophosphate (KH_2PO_4) and dipotassium hydrogen orthophosphate (K_2HPO_4) were G.P.R. grade. Hydrochloric acid (HCl), ethylene diamine tetrachloric acid (EDTA), mercuric chloride ($HgCl_2$) were analar grade. L-cysteine and casein are of L.R. grade. NADH was from sigma, sulphanilamide (Vet. product), potassium nitrate (KNO_3) from May & Baker Chemicals. NaOH (GBD:GPR), Zinc acetate (Canbrian Chemicals), while glutathione and glycine were from BDH Chemical Co., England.

Plant Materials

The millet seeds used for the experiments were a kind gift from Sokoto Agricultural and Rural Development Authority (SARDA). The seeds used were the Ex-Borno (Gero)type.

Growth of Millet Seeds

The millet seeds were soaked in distilled water for one hour, sterilised with 0.1% $HgCl_2$ for 10 minutes and washed several times with distilled water. They were then grown for 12 days in petri dishes layered with Whatman filter paper. The plants were irrigated in the morning and evening with distilled water and exposed to sunlight for 12 hrs. 1 mM KNO_3 was applied to the plants 48 hours prior to harvest and were exposed to sunlight for 8 hours each day. Another set, the control, was supplied with distilled water only untill harvest.

Preparation of Cell Free Extract

When the millet seedlings were 12 days old, 1g of the fresh leaves was mecerated in 10m1 of 0.1 M phosphate buffer (pH 7.0) containing 5 mM EDTA, 10 mM cysteine and 2% casein. The crude homogenate was centrifuged at 5,000 × g for 20 min. The supernatant obtained was then used for the assay of the enzyme activity. Unless

otherwise stated, all crude enzyme preparations were stored at 4°C prior to use.

A. In vitro enzyme assay

Assay of Nitrate reductase activity: Nitrate reductase activity was measured in a 5 ml reaction mixture containing phosphate buffer (0.1M, pH 7.2), nitrate (1 mM) and 0.4 ml of tissue extract. The reaction was initiated by the addition of 0.6 mM of NADH to the test tubes. After 30 minutes of incubation at 30°C, the reaction was stopped by the addition of 0.1 ml of 1 M Zinc acetate. It was followed by the addition of 1.9 ml of 70% ethyl alcohol. The tube contents were centrifuged for 15 minutes at 5,000 × g and a 2 ml aliquot of supernatant solution was assayed colorimetrically for nitrate according to Neyra and Hageman (1975).

B. Kinetic studies

(i) Initial velocity studies: The initial velocity was studied by varying Nitrate concentration between 25 µM and 125 µM.

(ii) Effect of temperature on enzyme activity: In this study, enzyme activity was determined after pre-incubating the enzyme mixture at 30°C, 40 °C and 50°C.

(iii) Effect of temperature on enzyme stability: The effect of temperature on enzyme stability was studied by pre- incubating the enzyme at 50°C, 40°C and 30°C. It differed from the above in that the crude extract was added after the reacting mixture had been incubated in the water bath and the time varied in the range of 5 - 30 minutes.

(iv) Effect of pH on enzyme activity: In this study, instead of the neutral pH, various pH values were used (pH 4,5,6,7,8 and 9).

(v) Effect of inhibitor: The enzyme activity was determined in the presence of 0.1 mM reduced glutathione as inhibitor for 30 minutes. The residual activity was determined according to Neyra and Hageman (1975).

RESULTS AND DISCUSSION

The results of the kinetic studies on nitrate reductase is shown in Figures 1-6. Figure 1. illustrates the effect of varying initial concentration of substrate on the initial rate of the reaction, while Figure 1a shows the Lineweaver-Burk plot (insert) of the same data. The rate is directly proportional to the substrate concentration in a simple Michaelis-Menten fashion. However, it must be borne in mind that nitrate reductase catalyses an essentially bisubstrate reaction, this therefore, does not indicate the operation of a simple Michaelis type mechanism which involves a single substrate and one kinetically relevant enzyme-substrate complex. The apparent km was determined to be 106.55 µM from the linear graph which is slightly lower than the apparent km of 110 µM for Barley as reported by Dalling *et al.*, (1976). However, the relatively low apparent km for nitrate in millet might be due to intensive breeding of the species as reported by Dalling *et al.*, (1976). This discrepancy could be attributable to, among other things, the different levels of the fixed substrate , NADH, used in the different regimes and moreover the enzymes were from different sources. Segel(1975) reported that the km for an enzyme depends not only on the particular substrate but also on the environmental conditions, such as ionic strength, pH and temperature. This may again explain the different apparent Km values observed under a variety of conditions. The apparent V_{max} was found to be 15.18 µmole $min^{-1}g^{-1}$.

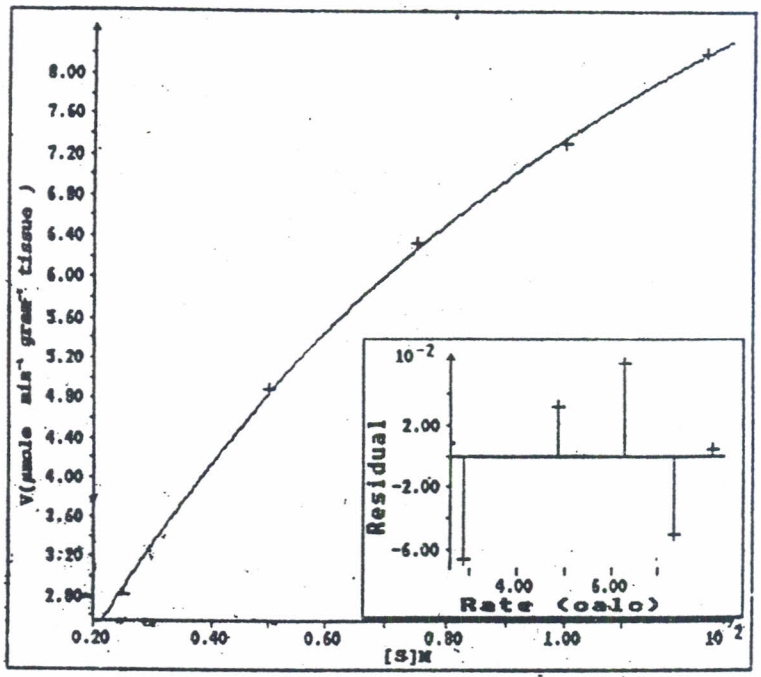

Figure 1. Effect of initial [S] on the rate of reaction [V], with residual shown (insert)

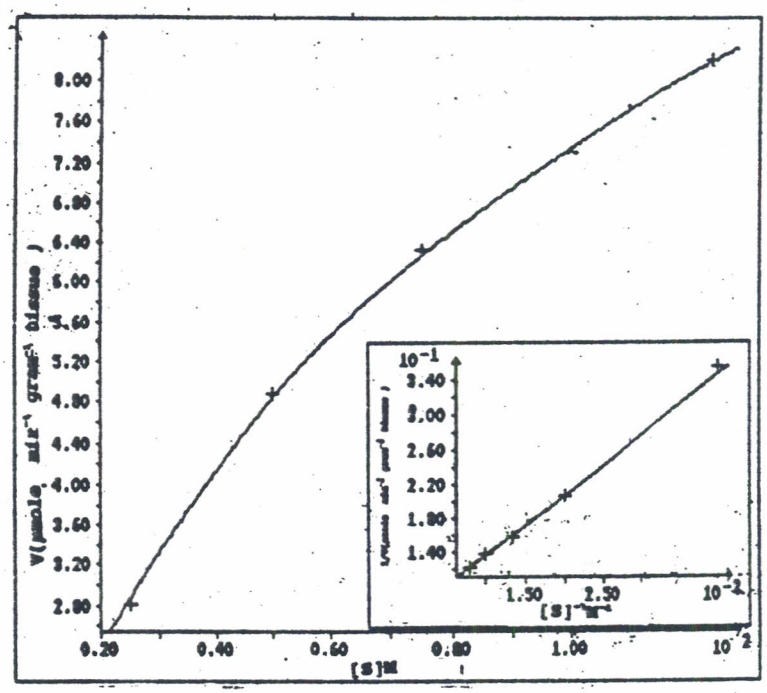

Figure 1a. Effect of initial [S] on the rate of reaction [V], with Lineweaver-Burk plot shown (insert)

The result of the effect of pH on the enzyme activity is shown in Figure 2. The optimum

pH was 6.5. The influence of pH on enzymic reaction may involve several different types of effects which may modify substrate binding and/or catalysis.

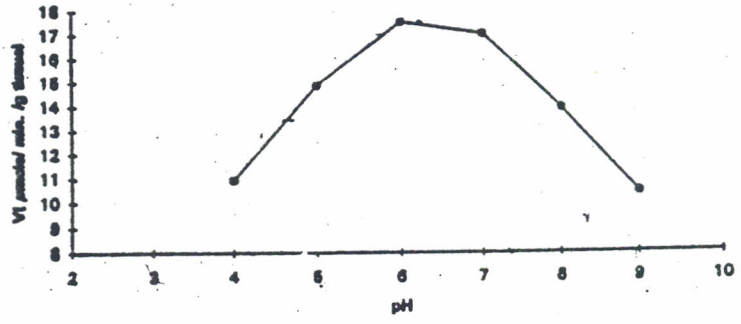

Figure 2. Effect of pH on the activity of nitrate reductase from millet

Figure 3. depicts the effect of temperature on the enzyme activity. The enzyme exhibited a temperature optimum at 35°C. The effect of temperature on enzyme stability is illustrated in Figure 4. Increase in temperature results in progressive decrease in enzyme activity probably due to thermal denaturation of the enzyme as reflected in the rate constants characterising this denaturation at the different temperatures studied. Generally, the rate constant increased as the temperature was increased indicating that the intrinsic stability of the enzyme decreases with increases in temperature; viz:-30°C, 40°C and 50°C which were 3×10^{-3} min^{-1}, 6×10^{-3} min^{-1} and 9×10^{-3} min^{-1}. Thus indicating a first order kinetics of thermal denaturation, while the corresponding half life were 231.0 min, 155.5 min and 77.0 min respectively.

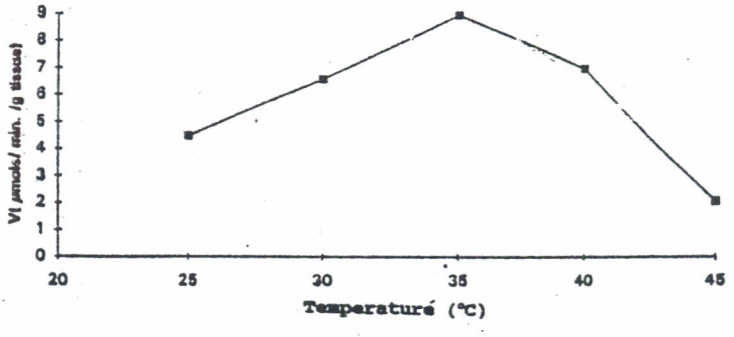

Figure 3. Effect of Temperature on the activity of nitrate reductase from millet

The results indicate that the enzyme is considerably thermolabile. The activation energy of thermal denaturation between 30°C and 40°C was found to be 25.020 KJoules mole^{-1}, while between 40°C and 50°C was 292.805 Joules mole^{-1}. The positive value of the activation energy is to overcome the kinetic barriers to denaturation.

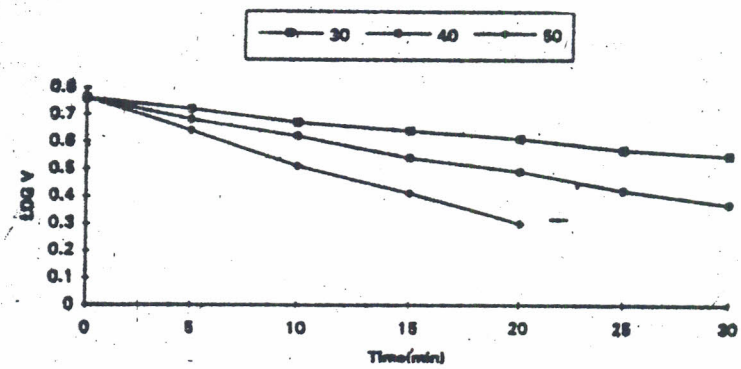

Figure 4. Thermal stability of nitrate reductase (Log V vs Time) from millet

A break in the Arrhenius plot (Figure 5) was observed at 40°C in the semilogarithmic plot of the reaction velocity as a function of reciprocal of absolute temperature. The Arrhenius equation predicts a monotonous, linear and limitless logarithmic response of the rate constant to increase in temperature. However, in enzyme catalysed reaction this is not generally observed. This break in Arrhenius plot is peculiar to enzyme catalysed reactions which is attributable to thermal denaturation. The activation energy was determined from the linear plot to be 38.29 KJ mol^{-1}. This energy is a measure of the energy needed for the conversion of molecules from the ground state to the reactive state.

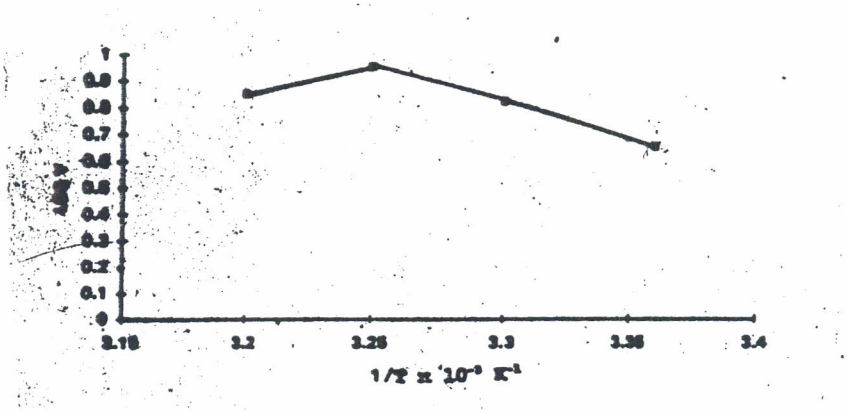

Figure 5. Arrhenius plot for nitrate reductase (Log V vs 1/T) from millet

The enthalpy and entropy of activation at 30°C were found to be 22.508 K Joules mole^{-1} and - 218.27 K Joules mole^{-1} K^{-1} respectively, while the free energy of thermal denaturation was 88.6 4 K Joules mole^{-1} K^{-1}. This is a more acceptable thermodynamic method of determining conformational stability. The positive value of the enthalpy indicates the severance of some bond *en route* the transition state. And the negative value of the entropy of activation of the thermal inactivation of the enzyme indicates a possible decreased randomisation of the protein structure. This interpretation of the negative entropy change is, however, a composite term involving also the solvent effect.

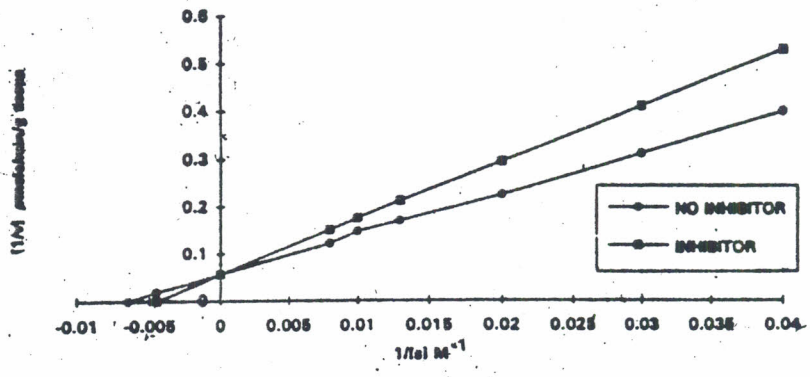

Figure 6. Double reciprocal plot for nitrate reductase from millet in the presence of reduced glutathione

The effect of the inhibitor on the enzyme catalysed reaction is shown in Figure 6. A characteristic competitive inhibition initial velocity pattern was observed. The inhibitor (reduced glutathione) binds reversibly and competes with the substrate for the active site of the enzyme. The site at which the inhibitor binds are those sites that are unavailable for the binding of substrate (Segel, 1975). The actual rate of a competitively inhibited reaction is strictly dependent upon the relative concentration of the substrate and inhibitor at fixed concentration of enzyme (Segel,1975). In this experiment, the km in the presence of the inhibitor was found to be 178.57 µM, that is, there was an increase of km in the presence of inhibitor. This is because at any given concentration of the inhibitor a portion of the enzyme exists in the enzyme-inhibitor form, which has no affinity for the substrate. The dissociation constant for the enzyme-inhibitor complex (ki) was found to be 2.33 x 10^{-4} µM. Conclusively, no any apparent difference was observed between the enzyme from millet and that from rice. However, further studies may be required to ascertain this.

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