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Purification and kinetic mechanism of alcohol dehydrogenase from coco yam tuber (*Colocasia esculenta*)

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ABSTRACT: Three forms of alcohol dehydrogenase were isolated from coco yam tubers (*Colocasia esculenta*) using ammonium sulphate gradient solubilization and further purified. They showed Michaelis-Menten kinetics and the affinity of the enzymes for their substrates did not influence their rate of reaction. Molecular mass found for F_1, F_2 and F_3 were 80,000±2000; 95,000±3000 and 70,000±1500 respectively. SDS. Polyacrylamide gel electrophoresis indicated a dimmer for F_2 but monomeric enzymes structures for F_1 and F_3. Bisubstrate studies showed double displacement mechanism for F_2 and F_3 in both direction of ethanol oxidation and acetaldehyde reduction, while F_1 indicated sequential reaction mechanism. The high Km values obtained for the enzyme forms with respect to ethanol and acetaldehyde are consistent with high concentrations of these compounds produced by coco yam tubers when subjected to anaerobiosis.

Key words: *Colocasia esculenta*; coco yam; alcohol dehydrogenase; purification; kinetic mechanism.

Introduction

There have been a number of studies on alcohol dehydrogenase (Alcohol: $NAD(P)^+$ Oxidoreductase E.C. 1.1.1.1) from various plant tissues such as maize (1), pea seeds (2), wheat (3), rice (4), pea nut (5), tea leaf (6), soya beans (7), barley grains (8), tomato fruit (9) and potato tubers (10). Some of these studies centred on purification and characterization of the enzymes with respect to multiple forms, substrate specificity and molar mass (1-12).

Recently, various forms of alcohol dehydrogenase (ADH) were isolated from two yam species purified and their kinetic properties studied (13, 14). In spite of these studies on plant ADH, little attention has been ppaid to its kinetic mechanism. This paper reports the isolation, purification and kinetic mechanism of ADH from coco yam tubers.

Materials and Methods

Materials: Coco yam tuber used in this study were obtained from local market in Benin City, Nigeria and kept in a well-aerated place away from direct sunshine. Chemicals and reagents were of analytical grade and were purchased from Sigma Company. Sephadex series were supplied by Pharmacia Fine Chemical.

Isolation and Purification of Enzymes

Separation and purification of ADH from coco yam tubers were carried out as previously described (13).

Analytical Methods

Protein concentration was assayed using protein-dye binding method (15). NH^{+}_{4} ions were determined employing Nesslerization method (16), while sodium was estimated using a flame emission spectrophotometer.

Enzyme assay: Alcohol dehydrogenase activity was assayed in the direction of alcohol oxidation (13). The rate of NADH production was monitored at 34nm, using Pye Unicam SPI800 spectrophotometer fitted with a chart recorder. ADH activity was also assayed in the direction of aldehyde reduction and the decrease in absorbance of NADH at 340nm was monitored.

pH optimum: ADH activity was assayed in pH range of 7.0 to 9.0 using mM Tris/HCL buffer.

Effect of temperature: Enzyme activity was assayed at various temperatures ranging from 20°C to 50°C using assay method.

Molecular mass determination: Molecular mass of each enzyme was determined using disc gel electrophoresis (17), SDS gel electrophoresis (18) and gel filtration (19). The following standard protein markers were used: Ovalbumin (45,000); bovine serum albumin (69,000); hexokinase (96,000); ß-amylase (215,000) and catalase (240,000).

Bisubstrate kinetics: ADH activity was assayed in the direction of alcohol oxidation by varying ethanol concentration at 3 fixed levels of NAD^{+}. Enzyme activity was also assayed at 3 fixed levels of ethanol using NAD^{+} as the variable substrate. In the direction of aldehyde reduction, ADH activity was assayed at fixed levels of NADH with acetaldehyde as the variable substrate. Enzyme activity was also assayed at 3 fixed levels of acetaldehyde using NADH as the variable substrate.

Results and Discussion

Alcohol dehydrogenase was extracted from coco yam tuber and fractionated by $(NH_4)_2SO_4$ gradient solubilization. The isolated enzymes were further purified by gel filtration, ion exchange chromatography and disc gel electrophoresis. Three forms were obtained and designated as F_1, F_2 and F_3. The subscripts 1, 2 and 3 indicate the order of elution from $(NH_4)SO_4$ gradient column.

Fig. 1 shows the elution profile of the enzymes forms from the column F_1 eluted between 66% and 61% $(NH_4)_2SO_4$ saturation, with peak activity at 64% saturation, while F_2 eluted between 58% and 52% $(NH_4)_2SO_4$ saturation, with peak activity at 55% saturation. F_3 eluted between 52% and 48% ammonium sulphate saturation with peak activity at 50% saturation. F_1 was purified 40 fold with specific activity of 250-nkat mg^{-1} protein and a yield of 80%, while 80-fold purification of F_2 yielded 25% and specific activity of 500-nkat mg^{-1} protein. F_1 was purified 28 fold with specific activity of 400-nkat mg^{-1} protein and a yield of 40%. Disc polyacrylamide gel electrophoresis of each enzyme preparation at various gel concentrations, pH values and using different enzymes concentrations showed single band of ADH activity (data not shown).

pH optimum obtained for F_1 and F_2 in the direction of ethanol oxidation was 8.0 but 7.5 for F_3. In the direction of aldehyde reduction, the pH optimum found for F_1 was 8.0 while 7.5 was obtained for F_2 and F_3.

Temperature optimum found for F_1 and F_2 was 38°C while F_3 showed optimum temperature of 40°C.

Fig. 2 and Fig. 3 show the effects of fixed levels of NADH and ADH from coco yam tubers in the direction of aldehyde reduction, when acetaldehyde was varied. The lines of reciprocal plots intersected on 1/S axis for F_1 (Fig. 2). The intercepts and slopes were affected as the NADH concentrations were altered. Symmetrical results were obtained when NADH was varied at 3 fixed levels of acetaldehyde. These results indicate sequential reaction mechanism for the enzyme form. However, the lines of reciprocal plots were parallel for F_2 and F_1 (Fig. 3) with constant slopes. The intercepts were altered by changes in fixed NADH concentrations $1/V_{max}$ intercept replots were linear. Symmetrical results were obtained when NADH concentration was varied at fixed levels of acetaldehyde. These results indicate ping pong reaction mechanism for the two enzymes forms in which the leading substrate binds to the enzyme and is converted to the first product before the second substrate binds and becomes converted to a second product.

$$EH_1 + NADH \leftrightarrow E - NADH \leftrightarrow EH_2\text{-}NAD \leftrightarrow EH_2 + NAD$$

$$EH_2 + B \leftrightarrow EH_2\text{-}B \leftrightarrow E - BH_2 \leftrightarrow E + BH_2$$

Where B = acetaldehyde; BH_2 = ethanol

When the enzyme forms, F_2 and F_3 were assayed in the presence of ethanol with NADH as the variable substrate, the lines of reciprocal plots intersected on $\frac{1}{v}$ axis (Fig. 4A). Similar results were obtained when the activities of the enzyme forms were assayed in the presence of the product, NAD with acetaldehyde as the variable substrate. These results are consistent with non sequential reaction mechanism for F_2 and F_1.

Effects of fixed levels of NAD^+ on enzyme forms in the direction of alcohol oxidation when ethanol was varied are shown in Figs. 5 and 6. The lines of primary plots intersected on 1/s axis for F_1 (Fig. 5). The apparent km values were independent of changes in fixed substrate concentrations. The intercepts and slopes vary as the fixed levels of NAD^+ were altered. The slope and intercepts replots were linear. Symmetrical results were obtained when NAD^+ concentrations were varied at fixed levels of ethanol. These results indicate single displacement mechanism in this direction. However, the lines of reciprocal plots were parallel for F_2 (Fig. 5) and F_3 (Fig. 6). The intercepts changes as the fixed NAD^+ concentrations were altered. The slopes were constant while the intercept replots were linear. Symmetrical results were obtained when NAD^+ was the variable substrate. These results suggest double displacement mechanism for the two enzyme forms in the direction of ethanol oxidation. When the activities of F_2 and F_3 were assayed in the presence of the product, NADH, with ethanol; as the variable substrate, the lines of reciprocal plots intersected on 1/v axis (Fig. 4B). Similar results were obtained when the enzymes were assayed in the presence of the product, acetaldehyde, with NAD^+ as the varied substrate. These results strongly support double displacement reaction mechanism for the two enzyme forms.

The average molecular mass obtained for F_1, F_2 and F_3 using gel filtration and disc gel electrophoresis in non-denaturing buffer system were 80,000±2000, 95,000±3000 and 70,000±1500 respectively. SDS gel electrophoresis showed molecular mass of 49,000 for F_2, 81,000 for F_1 and 69,500 for F_3. These results indicate that F_2 is a dimeric protein with two polypeptide chains, while F_1, F_3 are monomeric enzymes.

Kinetic constants obtained for coco yam ADH in the direction of alcohol oxidation is shown in Table 1A while Table 1B shows the kinetic constants obtained in the direction of aldehyde reduction. In the direction of alcohol oxidation, the Km found for F_1, F_2 and F_3 with respect to ethanol were 6.25 mM, 8.0 mM and 10 mM respectively, while their V_{max} values were 117.6 nkat mg^{-1}, 400 nkat mg^{-1} and 222.2 nkat mg^{-1} protein (Table 1A). The K_m values obtained for the enzymes with respect to NAD^+ were 0.27mM for F_1, 1.66mM for F_2 and 0.71mM for F_3. F_1 showed lowest V_{max} , has a low K_m while F_2 with high Km are about 2 to 3 times more active than F_1.

In the direction of aldehyde reduction (Table 1B), ther Km obtained for F_1, F_2 and F_3 with respect to acetaldehyde were respectively 1.17mM, 13.33mM and 25.0mM, while the V_{max} values were 100.00 nkat mg^{-1}, 111.1 nkat mg^{-1} and 769 nkat mg^{-1} protein. The Km found with NADH as the variable substrate for F_1, F_2 and F_3 were 0.17mM, 0.60mM and 0.9mM respectively. The rate of enzyme reaction increased as the Km values increased. Therefore, these results (Table 1) indicate that the activities of the enzymes are not determined by their affinity for their substrates.

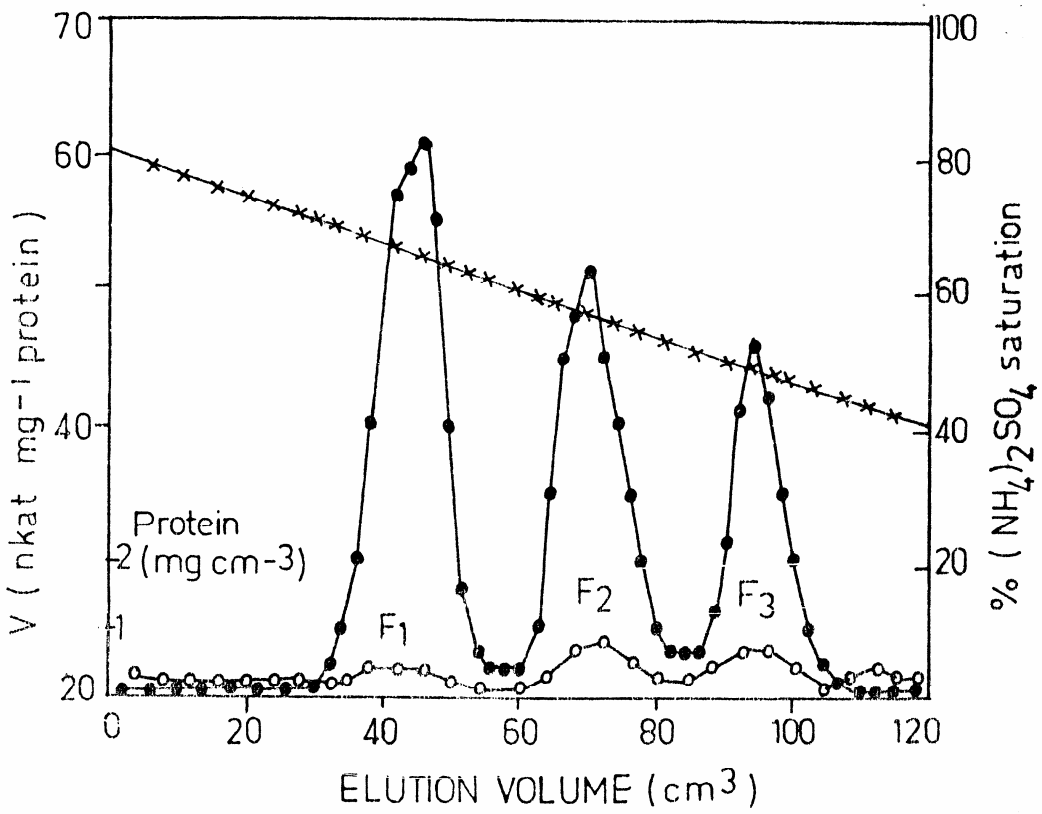

Fig. 1: Separation of ADH fractions from cocoyam tubers using $(NH_4)_2SO_4$ gradient solubilization.
Enzyme activity was assayed in the direction of alcohol oxidation. (●) Enzyme activity; (○) Protein; (x) $(NH_4)_2SO_4$.

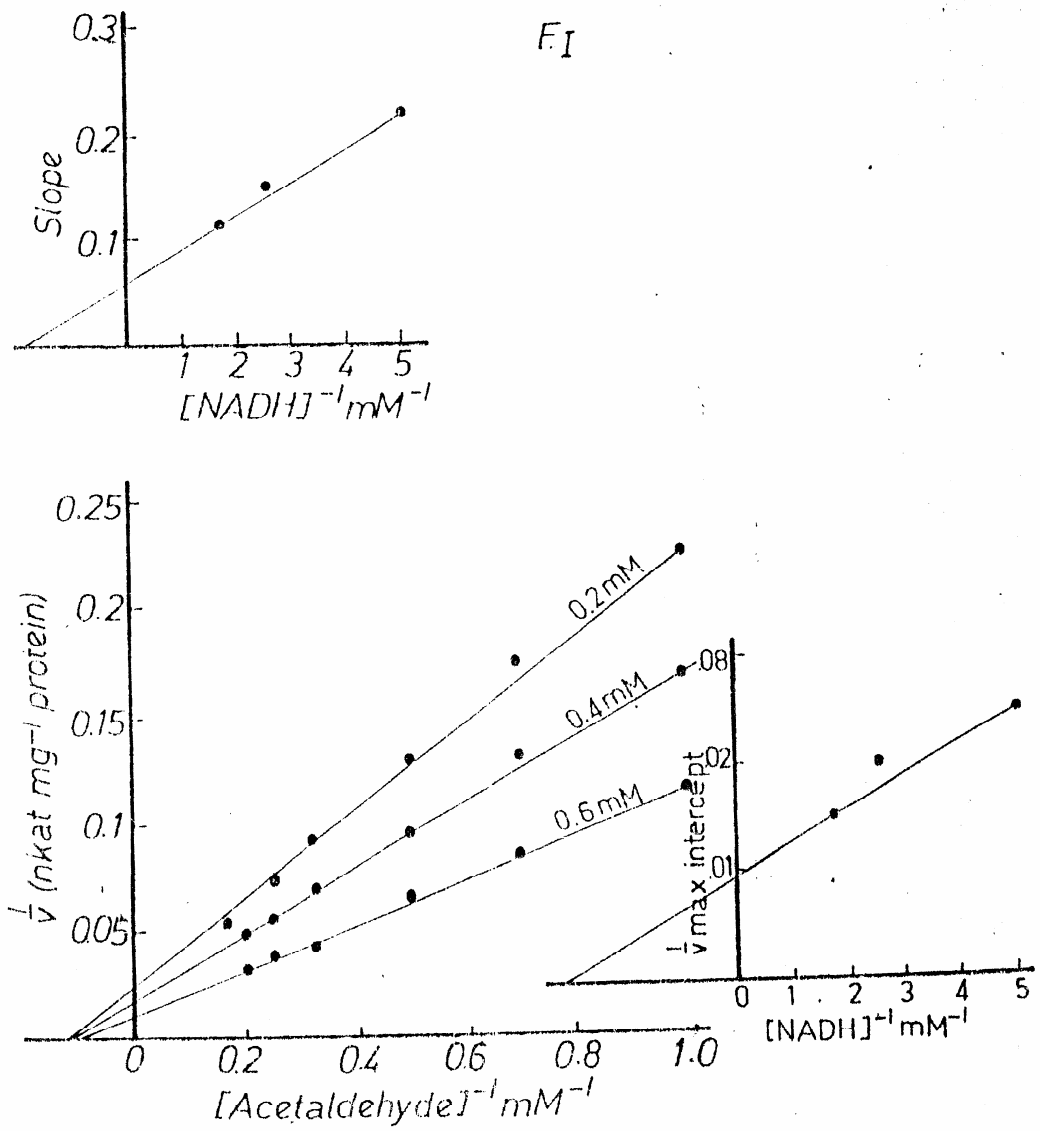

Fig. 2: Effect of fixed levels of NADH concentration on F_1 in the direction of aldehyde reduction when acetaldehyde was varied.

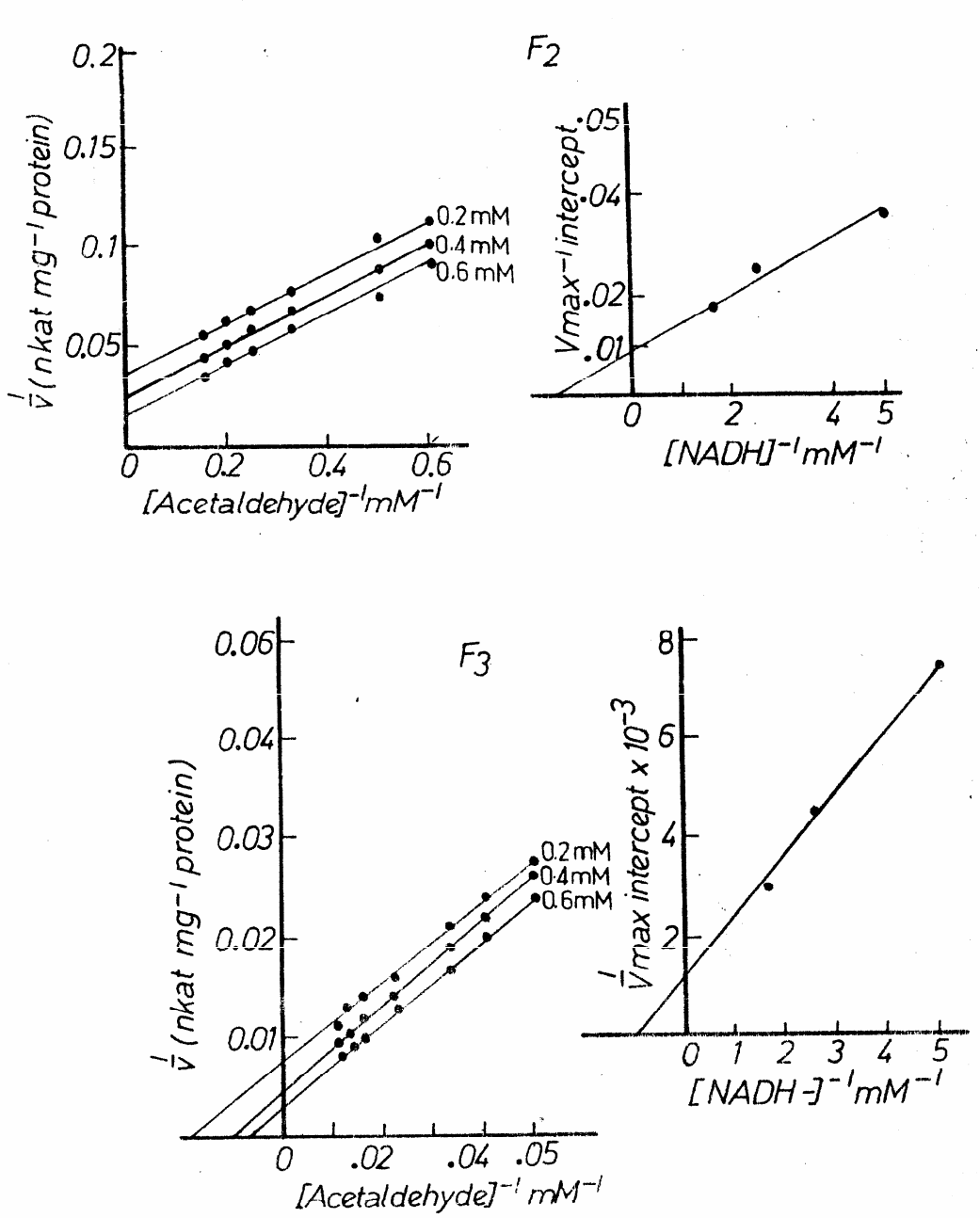

Fig. 3: Effect of fixed levels of NADH concentration on F_2 and F_3 in the direction of aldehyde reduction when acetaldehyde was varied.

However, the Km obtained for these enzymes are high and seem to be non-physiological except for F_1 (Table 1B). This is not surprising as the coco yam tubers produce high level of ethanol and acetaldehyde when subjected to anaerobiosis (20), and therefore ADH from this source does not require low Km to operate. Similar high kM values have been reported for ADH from yam tubers (13, 14) and this was attributed to high ethanol levels produced by yam tuber when subject to anaerobic condition (13).

Table 1: Kinetic constants of alcohol dehydrogenase (ADH) from coco yam tubers.

A. Kinetic constants of ADH forms in direction of alcohol oxidation.

Enzyme form	αK_A*(mM)	K_A (mM)	V_{max} (nkat mg^{-1}	αK_B* (mM)	K_B mM)
F_1	6.25	2.63	117.6	0.27	0.208
F_2	8.0	-	400.00	1.66	-
F_3	10.0	-	222.2	0.71	-

*A = ethanol; B = NAD^+
αK_A = Km for ethanol when NAD^+ is saturating
K_B = Km for NAD^+ when ethanol is saturating.

B. Kinetic constants of ADH forms in direction of aldehyde reduction.

Enzyme form	αK_C^{*+} (mM)	K_C (mM)	V_{max} (nkat mg^{-1}	αK_D* (mM)	K_D (mM)
F_1	1.7	0.7	100.0	0.37	0.55
F_2	13.3	-	111.1	0.66	-
F_3	25.0	-	796	0.90	-

*C = Acetaldehyde; D = NADH
αK_C = Km for acetaldehyde when NADH is saturating.
αK_D = Km for NADH when acetaldehyde is saturating.

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