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DETERMINATION OF CYANOGENIC POTENTIAL PARTIAL PURIFICATION AND HYDROLYTIC ACTIVITY OF ß- GLUCOSIDASE FROM TWO CULTIVARS OF CASSAVA- 505 AND 419

NWOKO NNAEMEKA SLADEN

Department of Biochemistry, Michael Okpara University of Agriculture Umudike, Abia State, Nigeria

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ABSTRACT

ß-glycosidase was partially purified from plant source (Cassava-505 and 419 Cultivars) by extraction with acetate buffer. Separate fractional precipitation was carried out using ammonium sulphate and acetone, followed by heat treatment. The Content of partially purified enzyme from each cultivar gave 0.46mg/ml and 0.069mg/ml respectively. Mathematical treatment of the data from the degradation of linamarin by the partially purified enzyme generated HCNValues that were used to construct line weaver Burk plot. This gave apparent Km and Vmax value of 5.75mm and 3.2×10⁻³mmol HCN /min/ml for 505 cultivar. 7.80mm and 3.7×10⁻³mmol HCN /min/ml for 419 cultivar. The Temperature and PH optima obtained for both the crude and partially purified enzyme showed high degree of hydrolysis towards standard linamarin and cyanogenic glucosides of cassava.

Keywords: ß -glycosidase, Cyanogenic glycosides, Enzyme, Cyanide.

INTRODUCTION

Cassava (Manihot esculenta crantz or Manihot utilissima phol) is widespread in the tropical world. Its primary attraction is that in its tuberous root, it isthe highest yielding starchy staple where yield as high as 50 to 80 metric tons per hectare have been recorded. Cassava roots are an important staple food for about 200 to 300 million people in tropical areas (nestel, 1973).

Cassava contains the cyanogenic glucosides Linamarin and Lotaustralin, which may be hydrolyzed to hydrogen cyanide by endogenous Linamarase on tissue damage (conn, 1969).

ß – glucosidase is the enzyme that degrades cyanogenic glucosides through exogenious and endogenious means to librate hydrogen cyanide (HCN) contained in foods (Okolie,et al,1988, Okafor et el, 2003).

People living in the tropics are faced with chronic and acute toxicity on cassava diet due to high cyanide content. These arouse the curiosity on how to reduce high content of cyanogenic glucoside using both exogenious and endogenious enzymes. Therefore, the potential toxicity of these widely consumed staple food crops requires a reliable, fast and cheap analytical method to measure the cyanogenic content.

The separate quantification of the three categories of cyanogens is a prerequisite for studies of the breakdown and removal of cyanogens during processing of cassava and for monitoring safety of product. This work therefore aimed at the determination of the cyanogenic potential of cassava, its partial purification and hydrolytic activity of Beta glucosidase.

Taking cognizance of the fact that the use of exogenous *ß*-glucosidase in the hydrolysis of cyanogenic glucosides is inevitable, the ß-glucosidase of some plants have been commercialized. The prominent among them is linamarase

Which is *ß*-glucosidase of cassava (cook,et al 1978). However, the high cost Of this commercial ß-glucosidase as well as the unavailability of equipment Required for their production makes it necessary for the production of readily available and inexpensive *ß*-glucosidase.

As a means of producing readily available form of this enzyme, *ß*- Glucosidase extracted from cassava was used for some kinetic studies. The Kinetic property of the enzyme was determined from the Line Weaver-Burk Plot.

MATERIALS AND METHODS

APPARATUS AND EQUIPMENTS

Cap fit test tubes, automatic micropipette, pH meter, water bath, spectrophotometer, beaker, reagent bottles, wash bottles, kitchen blender, centrifugation machine, glass pipettes, stop watch, magnetic stirrer, analytical balance, kitchen knife, measuring cylinders, funnels, whatman's filter paper.

REAGENTS

0.2M sodium hydroxide, 0.5% chloramin T, 0.1m orthphosphoric acid, 0.1 phosphate buffer pH 6.0, colour reagents, acetate buffer pH 5.5, linamarin standard, potassium cyanide standard, acetone, cyanohydrin standard.

REPARATION OF REAGENTS

0.2M SODIUM HYDROXIDE

4.0g of NaoH pellets were weighed out and dissolved in 1 liter of distilled water.

0.5% CHLORAMIN T

0.5g of chloramin T were weighed out and dissolved in 100ml of distilled water. This was made fresh every day.

0.1M ORTHOPHSPHORIC ACID

6.74ml of Orthophosphoric acid were measured and mixed with 1 liter of distilled water.

0.1M PHOSPHATE BUFFER pH 6.0

7.30g of Na_2HPO_4 and 10.04g NaH_2PO_4 of were weighed and dissolved in 200ml and 400ml of distilled water respectively. 4.92ml of Na_2HPO_4 and 350.8ml of NaH_2PO_4 were mixed together and the solution made up to 800ml with distilled water. The solution was adjusted to pH 6.0 using 5M NaOH in a pH meter.

ACETATE BUFFER pH 5.5

5.8ml of glacial acetic acid were mixed with 1 liter of distilled water. The solution was adjusted to pH 5.5 using 5M NaOH.

COLOUR DEVELOPER

3.7g NaOH, 7.0g 1,3-dimethyl barbituric acid and 5.7g isonicotinic acid were weighed and dissolved in 200ml of distilled water. The pH was adjusted between 7 and 8 with HCL or NaOH.

POTASSIUM CYANIDE STANDARD PREPARATION

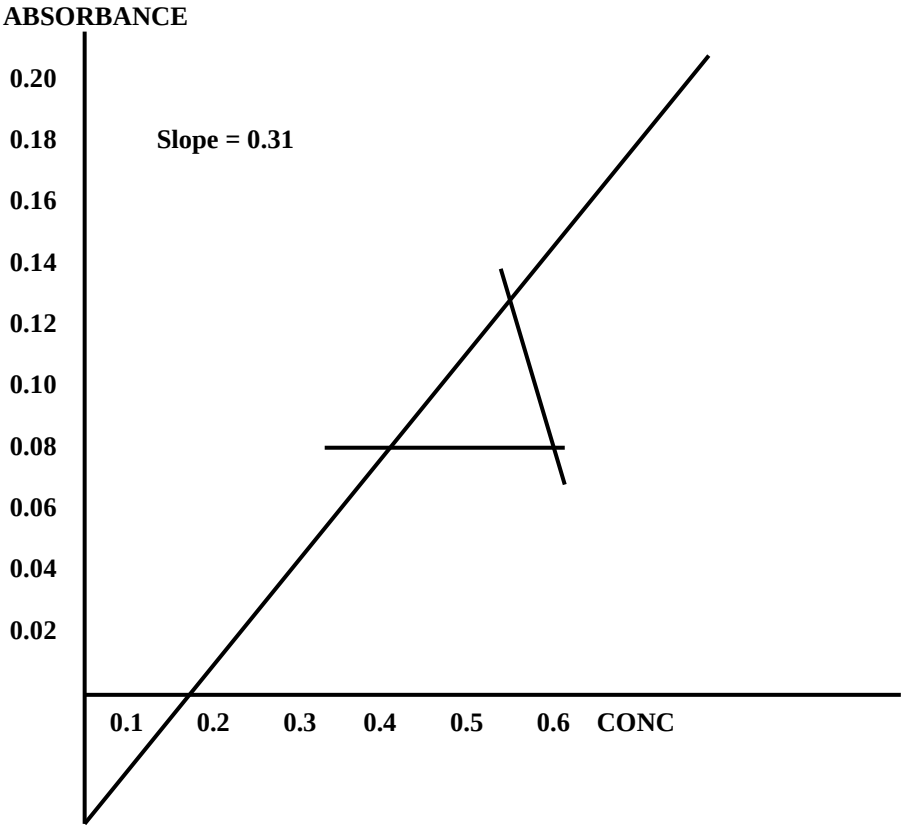
0.125g potassium cyanide was weighed and dissolved in 100ml of distilled Water. This gave a concentration of 1.25mg/ml. 1ml of the above solution was added to 100mg of distilled water. This mixture gave a solution of 12.5mg/ml. 0.5ml of the above solution was made up to 1ml by adding 0.5ml of distilled water. This gave a stock solution of 2.5**µ**gCN/ml. Serial dilutions were made by pipetting 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6ml of the above solution into test tubes and made up to 1ml by pipetting 0.9, 0.8, 0.7, 0.6, 0.5 and 0.4ml of distilled water. 2.5ml of 0.1M phosphate buffer PH 6.0 was added across. This gave cyanide stock volume of 0.25, 0.50, 0.75, 1.00, 1.25 and 1.50mg/ml respectively. Chloramin T was added across followed by colour development and spectrophotometric determinations at wavelength of 605nm.

The table of the above is given below.

TABLE 1: POTASSIUM CYANIDE STANDARD

VOL. OF STOCK	CONCENTRATION (Mg/ 100g)	CONCENTRATION (µg / ml)	ABSORBANCE (605NM)
0.00	0.00	0.00	0.000
0.25	0.01	0.10	0.031
0.50	0.02	0.20	0.063
0.75	0.03	0.30	0.094
1.00	0.04	0.40	0.123
1.25	0.05	0.50	0.162
1.50	0.06	0.60	1.183

FIGURE 1: POTASSIUM CYANIDE STANDARD CURVE



SOURCES OF THE CASSAVA

The cassava used for this research work was gotten from National Root And Crops Research Institute, Umudike, in Ikwuano Local Government Area of Abia State, Nigeria.

EXTRACTION AND PURIFICATION OF *β* – GLUCOSIDASE

100g of cassava peels (505 and 419 cultivars) were weighed and Homogenized with 250ml of acetate buffer (0.1M) pH 5.5 using Kitchen blender. The homogenate was filtered using whatman's filter Paper to obtain the filtrate. 100ml of the filtrate for each sample was collected, and 60g of Ammonium Sulphate $(NH_4)_2SO_4$ salt was added and this was kept overnight in order to obtain precipitate.

The precipitation formed was collected and centrifuged at 10,000G for an hour. The precipitate obtained after centrifugation was dissolved in 50ml of 0.1 phosphate buffer pH 6.0 and dialyzed in the same buffer with 3 changes of the buffer every 8 hours (cooke et al 1978).

NB: The partially purified enzyme from each cultivar was used for Enzyme activity and kinetic studies.

PROTEIN DETERMINATION

The kjedahl method of Nitrogen estimation was used to determine the protein content of partially purified enzyme. The nitrogen Content was determined and multiplied with the factor 6.25 used for Plant protein.

PROCEDURE

0.1ml of the partially purified enzyme for each of the sample 419 and 505 respectively were measured into microkjeldahl digestion Flask. 0.5g anhydrous sodium sulfate, 0.1g copper sulfate and 0.4g Selenium catalyst, followed by 3ml conc. H_2SO_4 was added.

The mixture was digested in the fume cupboard by heating. The digest was cooled with 100ml of water. 10ml of the digest were mixed with Equal volume of 45% NaOH solution in a microkjeldahl distillation Apparatus. The mixture was each distilled into $10cm^3$ of 4% boric acid solution containing 2 drop of Zug Zaga indicator. 50ml distillate were collected and titrated against 0.2 HCL using an automatic micro burette to pink end- point. The same process was applied to blank. (kjedahl 1983).

DERERMINATION OF *ß* - GLUCOSDASE ACTIVITY (LINAMARASE)

ß-glucosidase activity was determined by the amount of cyanide produced by the enzymatic average of linarmarin. Standard linamarin solution was pipetted into stopped test tubes at 0.5, 1.0, 1.5, 2.0 and 2.5ml. The test tubes were incubated at 30^{o}C.

The reaction started by the addition of 0.2ml of the enzyme in the test tubes and allowed to stand for 15minutes. The reaction was quenched by the addition of 0.2ml of 0.2M NAOH. Colour development followed immediately by the addition of phosphate buffer pH 6.0, 0.2ml chloramin T and colour developer. The cyanide released was measure using a spectrophotometer at λ 605nm. (essers, et, al, 1993).

PREPARATION FOR CYANIDE ANALYSIS

CASSAVA PEEL

50g each of the two cassava cultivars (505 and 419) peel were Collected and homogenized in 160ml cold orthophosphorc acid Buffer medium in a kitchen blander. The homogenate was kept in the refrigerator for further analysis.

CASSAVA PULP

50g of each of the two cassava cultivars pulps were homogenized in 160ml 0.1M orthophosphoric acid buffer medium in a kitchen blender. The homogenate were filtered and the filtrate was used for analysis.

CASSAVA LEAVES

10g of each of the two cultivars of cassava leaves were homogenized in 50ml 0.1M orthophosphoric acid medium using a kitchen blender.

The filtrate was used for further analysis.

CYANIDE DETERMINATION

ASSAY FOR TOTAL CYANIDE

One enzyme tablet was used to make a solution of the enzyme. 1ml of each aliquot was pipetted into duplicate test tubes. 0.1ml of Linamerase enzyme was added to each duplicate. This was incubated in a water bath at 40^{o}C for 20 minutes. They were allowed to cool for 15 minutes. 0.1ml of NAOH was added followed by the addition of 2.8ml of phosphate buffer, colour developer and spectrophotometric reading at 505nm.

ASSAY FOR FREE CYANIDE

0.2ml of each aliquot was pipetted into duplicate test tubes. 0.1ml of linamerase enzyme was added, and this was left to stand for about 30miniutes without incubation. 2.8ml Phosphate buffer were added followed by colour development and spectrophotometric determination at 605nm.

RESULTS

TABLE 2: RESULT FROM NITROGEN ESTIMATION TO DETERMINE PROTEIN CONTENT

CULTIVARS USED	OPTICAL DENSITY	CONC.(GLDL)
419	0.090	0.197
505	0.050	0.131
STD	0.273	6.0g/dl

PROTEIN DETERMINATION

% protein = % N× F

Where F= conversion factor = 6.25

$$\% \text{ Nitrogen} = \frac{(Vt - Vb) \times Na \times gN \times \text{digest obtained} \times 100}{\text{Digest distilled} \times 10}$$

Where V_t = volume in ml of standard HCL used in titration
V_b = volume in ml standard HCL used in blank titration
N_a = Normality of molarity used
W = weight in ml of the sample digest

FOR 419 CULTIVAR

$$\% \text{ Nitrogen} = 0.197 \times 0.02 \times 0.014 \frac{\times 100 \times 100}{5 \quad 10}$$

$$= 0.01104\%$$

% Protein = % Nitrogen x 6.25

= 0.01104 x 6.25

= 0.069mg/ml

FOR 505 CULTIVAR

$$\%\text{Nitrogen} = 0.131 \times 0.02 \times 0.014 \frac{\times 100 \times 100}{5 \quad 10}$$

= 0.00734 %

% Protein = % Nitrogen x 6.25

= 0.00734 x 6.25

= 0.046mg/ml

RESULT FROM DETERMINATION OF FREE AND TOTAL CYANIDE CONTENT IN FRESH CASSAVA PEEL, PULP AND LEAVE.

TABLE 3: FOR TROPICAL MANIHOT SELECTION (505 CULTIVAR)

CASSAVA CULTIVAR TMS/98/505	CONC OF FREE CYANIDE (µG/ML)	CONC OF TOTAL CYANIDE (µG/ ML)
Peel	2.45 ± 0.01	2.66 ± 0.01
Pulp	0.84 ± 0.01	1.55 ± 0.03
Leaves	0.52 ± 0.02	1.55 ± 0.03

TABLE 4: TROPICAL MANIHOT ESCULENTA (419 CULTIVAR)

CASSAVA CULTIVAR TME419	CONC OF FREE CYANIDE (µG / ML)	CONC OF TOTAL CYANIDE µg/ ml
Peel	1.80 ± 0.04	3.12 ± 0.02
Pulp	0.62 ± 0.02	1.30 ± 0.03
Leaves	0.53 ± 0.01	0.89 ± 0.02

RESULT OF DEGRADATION OF LINAMARIN BY THE PARTIALLY PURIFIED *ß* – GLUCOSIDASE AND THE CYANIDE PRODUCTION VALUES.

TABLE 5: FOR 419 CULTIVARS

Linamarin conc. (s) in incubation Medium	Absorbance	CN^- Production (µmol/ mm)	CN^-/60sec. (V)	1/(S)	1/(V)
5	0.276	7.62×10^{-1}	12.2×10^{-3}	0.200	81.97
10	0.297	9.42×10^{-1}	18.4×10^{-3}	0.100	54.35
15	0.306	11.38×10^{-1}	21.6×10^{-3}	0.067	46.30
20	0.308	14.41×10^{-1}	24.2×10^{-3}	0.050	41.32
25	0.334	15.91×10^{-1}	27.3×10^{-3}	0.040	36.65

FIGURE 2: A LINEWEAVER- BURK PLOT OF CYANIDE RELEASED DURING DEGRADATION OF CYANOGENIC GLYCOSIDES OF 505 CASSAVA CULTIVAR BY PARTIALLY PURIFIED *ß*- GLUCOSIDASE OF CASSAVA.

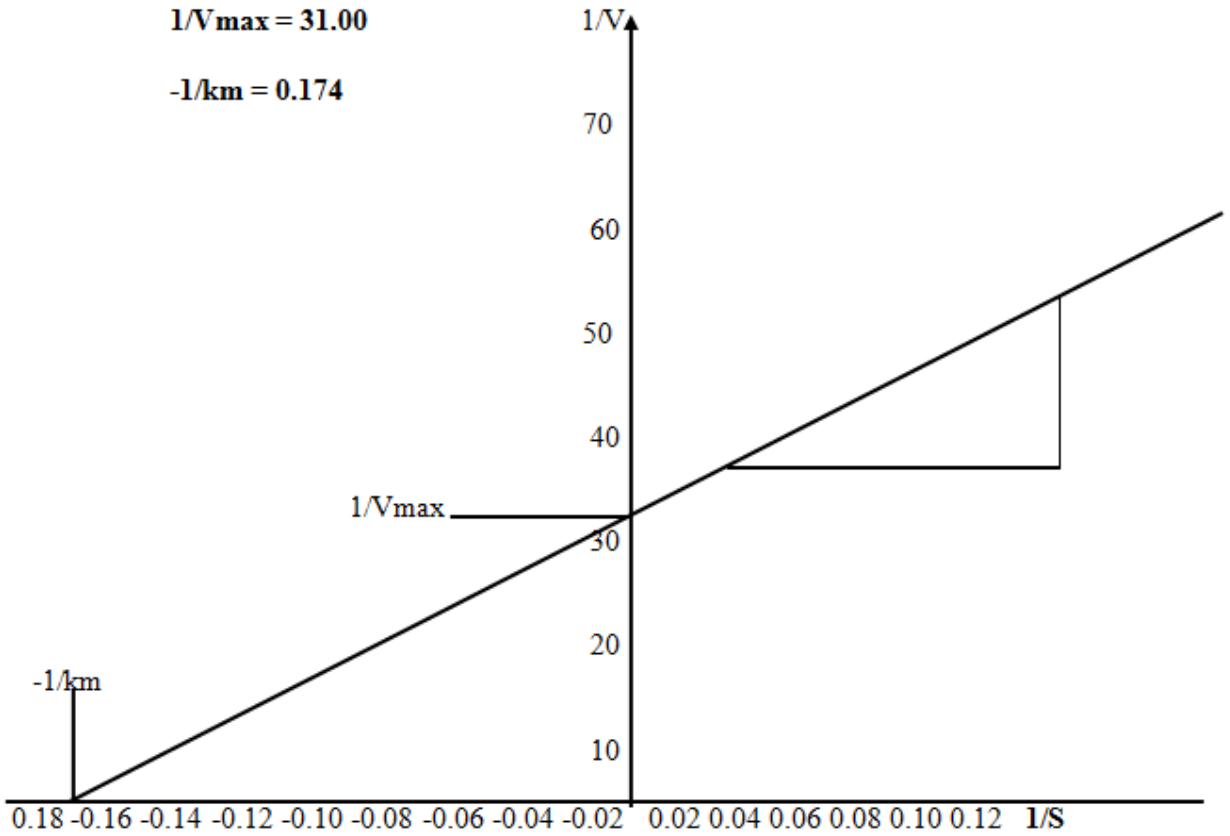


FIGURE 3: A LINEWEAVER – BURK PLOT OF CYANIDE RELEASED DURING DEGRADATION OF CYANOGENIC GLYCOSIDES OF 419 CASSAVA CULTIVAR BY PARTIALLY PURIFIED *β* – GLUCOSIDASE OF CASSAVA.

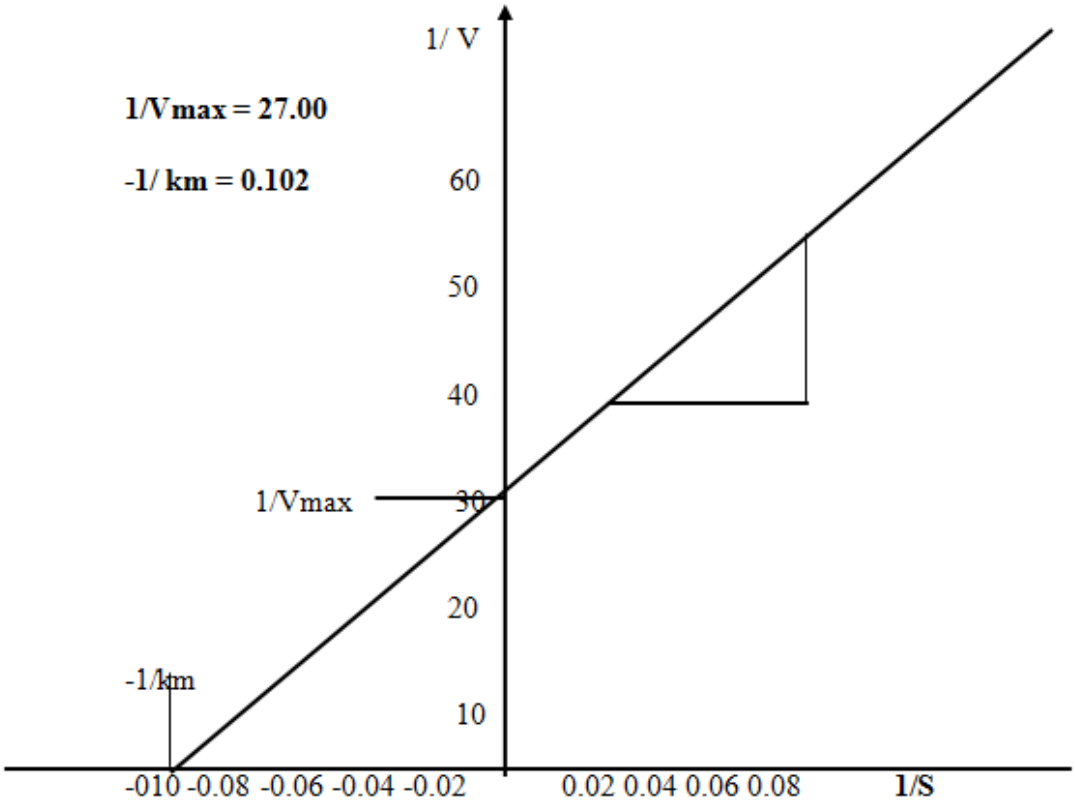


TABLE 6: FOR 505 CULTIVARS

Linamarin conc (s) in incubation Medium	Absorbance	CN^- Production (µmol/mm)	CN^-/60sec (V)	1/(S)	1/(V)
5	0.360	8.24×10^{-1}	16.1×10^{-3}	0.200	62.11
10	0.390	11.48×10^{-1}	21.4×10^{-3}	0.100	46.73
15	0.413	13.31×10^{-1}	23.2×10^{-3}	0.067	43.10
20	0.420	16.23×10^{-1}	25.4×10^{-3}	0.050	39.37
25	0.460	18.21×10^{-1}	28.6×10^{-3}	0.040	34.97

KINETIC PROPERTIES

The kinetic properties (Km and Vmax) were calculated from Line weaver – Burk plot of cyanide production from the Degradation of standard linamarin by the partially purified *β* – glucosidase. The cyanide (CN^-) production and the rate (V) used in plotting the curve are show on figure 2 and 3 respectively for 419 cultivars and505 cultivars.

FOR 419 CULTIVARS

(A) Maximum velocity (V max) was calculated from the graph as follows:

1/Vmax = 27.00
1 = 27.00 Vmax
Vmax = 1/27.00
Vmax = 3.7×10^{-2} µmol/min

(B) Maximum velocity (Vmax) for 505 cultivars was calculated from the graph.

1/Vmax =31.00
1 = 31.00 Vmax
Vmax = 1/31.00
Vmax = 3.2×10^{-2}µmol/min

(C) The Michaelis Menten consant (km) from the graph of 419 cultivars are given as follows:

-1/km = -0.102
1 =0.102km
Km = 1/0.102
Km =9.80

(D) The Michaelis Menten constant (km) from the graph of 505 cultivars is given as follows

1/km = -0.174
1= 0.174km
Km = 1/0.174
Km = 5.75

DISCUSSION

KINETIC PROPERTIES OF THE PARTIALLY PURIFIED ENZYME

Mathematical treatment of the data from the degradation of linamarin by the β – glucosidase, gave values that were used to plot the Line Weaver – Burk plot which gives Km and Vmax values. For 419 cultivars, Km and Vmax gave 9.80 and 3.7 x 10^{-2}µmol/mins respectively. While 505 cultivars gave Km and Vmax values of 5.75 and 3.2 x 10^{-2} µmol/mins respectively. 419 cultivars have both a higher Km and Vmax values than 505 cultivars. The higher Km indicates a low affinity of the enzyme for its substrate.

DETERMINATION OF FREE AND TOTAL CYANIDE CONTENT IN FRESH CASSAVA PEEL, PULP AND LEAVES

For 505 cultivars, concentration of free cyanide in the peel, pulp and leaves are 2.45± 0.01, 0.84± 0.01, 0.52 ± 0.02 respectively. While the concentration of total cyanide in the peel, pulp and leaves are 2.66 ± 0.01, 1.55 ± 0.03, and 1.55± 0.03 respectively. The result shows that the peel of the 505 cassava cultivars has the highest concentration of free cyanide and total cyanide, followed by the pulp and then the leaves. For the 419 cassava cultivars, concentration of free cyanide in the peel, pulp and leaves are 1.80 ± 0.04, 0.62 ± 0.02, 0.53 ± 0.01 respectively.

While concentration for total cyanide is 3.12 ± 0.02, 1.30 ± 0.03, 0.89± 0.02, respectively. The result shows that the peel has the highest concentration of free cyanide and total cyanide. The pulp and leaves follows respectively.

TOTAL PROTEIN CONTENT OF THE TWO CULTIVARS OF CASSAVA.

The total proteins content of the partially purified enzyme from the two Cassava cultivars (419 and 505) are 0.069mg/ml and 0.046mg/ml respectively.

This indicates that 419- cassava cultivars have higher protein content than 505 Cultivars. The protein content of the partially purified enzyme from A. achatina was reported by Okafor and Ezeronye (2003) to be 0.076mg/ml.

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REFERENCES

[1] E.E,Conn, *Journal of Agriculture. F.d Chem*, **1973**, 17.519.
[2] E.E, Conn, The biosynthesis of cyanogenic glucosides and other simple nitrogen compound perspectives in photochemistry, Academic press London, **1969**, Chapter 2, Page 47-74.
[3] R.D, Blake, G. Blake, J.M, Bethershill, Purification of cassava Linamarase, Academic press London, **1978**, 17, 381-383.
[4] Delange, R, Ahluwalia, Proceedings of a workshop on cassava and toxicity, 31 May-2 June **1982**, Ottawa, Canada, 7-146.
[5] A.J.A, Essers, M. Bosveld, R.M, Vandergrift, *Journal of Science of Food and Agriculture*. **1993**, 65, 287-296.

[5] B.L, Nestel, Chronic cassava toxicity. International Development Research center, Ottawa, Canada, **1973**, 6-12.
[6] L, Nicholis. Tropical Nutrition and Dietetics. Baillier London, **1949**, 106-150.
[7] P.N, Okafor, O.U, Ezeronye, Partial purification and some properties of ß- glucosidase from the gut of the giant African snail, Achatina ,achatina PhD thesis, Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria, **2003**.