SCREENING FOR LINAMARASE IN SOME YEAST STRAINS, AND CHARACTERIZATION OF LINAMARASE FROM DRIED CASSAVA (MANIHOT ESCULENTA) CORTEX.

BY

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(B.A., M.SC, BIOCHEMISTRY)

A thesis submitted to the Postgraduate School, Ahmadu Bello University, Zaria, in Partial fulfillment of the requirement for the degree of:

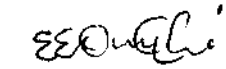
DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY

DEPARTMENT OF BIOCHEMISTRY
FACULTY OF SCIENCE
AHMADU BELLO UNIVERSITY
ZARIA, NIGERIA

JANUARY, 1996

DECLARATION

I, Elewechi Onyike hereby declare that this thesis is a record of my own research work and has not been submitted for award of a degree of any other university. Information and excerpts from published and unpublished works of others have been acknowledged in the text.



Elewechi Onyike

January 1996

CERTIFICATION

This thesis, titled, screening for linamarase in some yeast strains, and characterization of linamarase from dried cassava (Manihot esculenta) cortex, by Elewechi Onyike meets the regulations governing the award of a degree of Doctor of Philosophy of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literal presentation.

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DEDICATION

TO THE ALMIGHTY GOD,

who made cassava and allowed linamarin/ lotaustralin and linamarase to co-exist.

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ACKNOWLEDGEMENT

I express my profound gratitude to my supervisors, Professor A.I Ukoha, and Professor C.O. Ikediobi, for advice, suggestions and assistance during the entire period of this work.

I am also grateful to my head of Department Professor G.H. Ogbadu, for allowing me to use the facilities in the Department, and also to the teaching and technical staff of the Biochemistry Department, Ahmadu Bello University, Zaria, Nigeria, especially Mr. Owoshaba.

My thanks go to the programme leader NAPRI, Zaria Unit, Dr. Eduvie for allowing me to use their refrigerated centrifuge; and to Dr. Ameh of the Department of Microbiology for providing yeast strains and isolates. I am grateful to my family for their love and support.

I also appreciate the care and company I received from Mr. Enoch G. Dzingina. I extend by gratitude to Wilson Maikano, Michael Ogo, Kofo Ajala, Josephine Adejoh, Martina Amakwe, Mr and Mrs Alex Lakoju, Mr and Mrs Samson Alonge, Dr and Mrs T. Iyaniwura, Rahila Musa and all the Brethren for their love and prayers.

I am eternally grateful to the Almighty God whose love, protection and provision encouraged my heart and finally anchored my hope. May His name be praised for ever-Amen.

ABSTRACT

Dried Cassava (Manihot esculenta) cortex contains 1inamarase with high activity. The 1inamarase was purified 97 fold by ammonium sulphate fractionation, and gel filtration on Sephadex G-150. The enzyme has Km values of 0.75 and 2.22mM with respect to the artificial substrate, p-nitropheny 1-B-D-glucoside, and its natural substrate linamarin, respectively. D-gluconolactone is a strong competitive inhibitor of the enzyme with Ki of 0.22mM when p-nitopheny 1-B-D-glucoside is the substrate. The pH optimum of the enzyme is 6.5. Analysis of the effects of pH and temperature on 1inamarase activity and kinetic constants indicates that the carboxyl group of aspartate or glutamate and the imidazole of histidine may be involved in the enzyme catalysis. Linamarase from cassava was significantly inhibited by potassium cyanide and citric acid. The enzyme was found to be stable up to 48°C and the optimum temperature of the linamarase catalyzed hydrolysis was 36°C. The energy of activation was found to be about 3.92 Kcal/mole.

The isolated enzyme was found to be very efficient in the hydrolysis of bound cyanide in any of the cassava products, ' gari' and ' lafun' . A substantial quantity of cyanide, was found in sour ' gari' which is generally considered as having little or no cyanide in it.

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CHAPTER 1

INTRODUCTION

On a world-wide basis, the tropical root crop, cassava (Manihot exculenta Crantz) provides 42 million tons of dry matter which is equivalent in energy terms to 40-50 million tons of grain (Cock, 1985).

In nutritional terms cassava is considered primarily as a source of energy. The use of cassava as energy source is limited by the presence of cyanogenic glycosides, linamarin (2-(β-D-glucopyranosyloxy) Isobutyronitrille and lotaustralin, methyl (2-(β-D-glucopyranosyloxy) methyl butyronitrille). These cyanogenic glycosides are hydrolyzed to hydrogen cyanide by endogenous linamarase (EC. 3.2.1.21, linamarin β-D-glycoside glucohydrolase) on tissue damage (Conn, 1969).

The traditional food preparation techniques used for processing cassava reduce the cyanide content in cassava, but there are often substantial residual quantities which may be sufficient to produce chronic symptoms and occasionally, even acute poisoning.

Reports in the literature have associated tropical ataxic neuropathy (a disease of the nerves), and endemic goiter, to chronic consumption of cassava products (Osuntokun 1968; Ekpechi 1967).

Osuntokun (1968) hypothesized that tropical ataxic

neuropathy in Nigeria and West African, is associated with high consumption of cassava derivatives. Ekpechi (1967) has also shown that cassava consumption has adverse action on the function of the tyroid gland.

Two major international agricultural research centres: International Centre for Tropical Agriculture (CIAT) in Colombia, and International Institute of Tropical Agriculture (IITA) in Ibadan, Nigeria, have programs aimed at improvement of cassava in both quality and quantity. In IITA the program involves eradication of diseases that affect cassava, bacterial diseases and insect born diseases and also selection of cassava lines for acyanogenesis. These selections are based on lines that lack the glucoside, glucosidases or both glucoside and glucosidases. The cassava line will also retain its resistance to insects and diseases in the cyanogenic cassava plant. This has not been achieved yet. The CIAT has undertaken a full scale program to develop cassava as an efficient producer of calories for human and animal feed, and for industrial starch markets. Their research concentrates on increasing yields, lowering production cost, and developing simple storage and processing methods. They are also involved with the screening of large germ plasm collections for a cultivar with a zero or very low cyanide level, but have not found any yet.

Accurate determination of bound cyanide (Linamarin and lotaustralin) as well as free cyanide (HCN) has been a major problem until the introduction of linamarase-based method in

the assay protocol (Cooke, 1978; Ikediobi et al, 1980). It has also been demonstrated that exogenously added linamarase preparation is effective in detoxifying grated fermenting cassava during manufacture of gari, a popular cassava product (Ikediobi and Onyike, 1982a). Cassava, especially the cortex, is a major source of linamarase (De Bruiyn, 1973; Wood, 1966). Some fungi and bacteria have also been shown to produce linamarase in large quantities (Okafor and Ejiofor, 1985; Ikediobi and Ogundu, 1985). Fusarium equiseti and Aspergillus sydowi have specifically been shown to be excellent candidates for commercial production of linamarase via aerobic fermentation (Ikediobi and Ogundu, 1985). But the linamarase from Fusarium equiseti is capable of hydrolyzing the substrate analogue, P-nitrophenyl -β-D- glucoside only, but not capable of hydrolyzing the substrate linamarin/lotaustralin (Ogundu, 1988). Sacharomyces cerevisae, Yeast strain foam have been shown to produce β-glucosidase (Duerkson et al, 1958) and have also been induced to produce β-Glucosidase which have been purified and characterized. Other yeast strains can be screened and induced to produce β-glucosidase and specifically linamarase activity.

The proposed research involves screening, isolation and characterization of linamarase from dried cassava cortex, which is normally a waste product in cassava processing industries (e.g starch and gari manufacture) and screening of local yeast strains for linamarase production.

The objectives are:

1. Developing a means of producing pure linamarase in large quantities to be use for,
    a. Quality control in food processing industries.
    b. Diagnostic laboratories.
2. To provide basic information on properties of linamarase from cassava (cortex)
3. To provide an insight into the nature of the groups at the active site of the enzyme and thus understand the enzyme's machanism of action. Also, information will be derived about the nature of the activation process, from the ionization of the enzyme-substrate complex. This will confirm the group suggested as being present at the active site.
4. To obtain other inexpensive and safe sources of linamarase, for cassava, food and feed processing industries.

CHAPTER 2

LITERATURE REVIEW

2.1 Cyanogenic glucosides

In a review by Conn (1969), it was observed that 1000 plant species representing about 90 families and at least 250 genera have been reported to be cyanogenic. Several dozen species have been studied in greater detail, in some instances because of their economic significance. Table 2.1 (Conn, 1969), is a collection of cyanogenic glucosides of known structure, their sugar, aglycone, and plant species where they are found.

TABLE 2.1 CYANOGENIC GLYCOSIDES OF KNOWN STRUCTURE

(Conn, 1969)

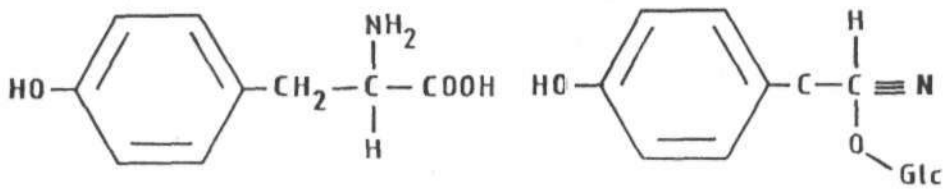
Glycoside	Sugar	Aglycone	Occurrence
Amygdalin	Gentiobiose	D-Mandelonitrile	Prunus Sp.
Prunasin	D-Glucose	D-Mandelonitrile	Prunus Sp., Many Rosaceae Eucalyptus Sp.
Sambunigrin	D-Glucose	L-Mandelonitrile	Sambucus nigra Acacia Sp.
Prulaurasin Vicianin	D-Glucose Vicianose	DL-Mandelonitrile D-Mandelonitrile	Vicia angustifolia L. and other vicia
Dhurrin	D-Glucose	L-p-Hydroxymandelonitrile	Sorghum Sp.
Taxiphyllin	D-Glucose	D-p-Hydroxymandelonitrile	Taxus Sp.
Zierin	D-Glucose	m-Hydroxymandelonitrile	Ziera laevigate Sm.
Linamarin	D-Glucose	α-Hydroxylobutryronitrile	Linum Usitatissimum, L.;Tri Phaeolus lunatus L.; Trifolium repena. L. Lotus Sp., Manihot Sp., Dimorphotheca Sp.
Lotaustralin	D-Glucose	α-Hydroxy α-Methyl Butyronitrile	(See Linamarin)
Acacipatalin	D-Glucose	β-Dimethyl-α-Hydroxyacrylonitrile	Acacia Sp. (South African)
Gynecardin	D-Glucose	Gynocardinonitrile	Gyanocardia odorata R. Br. Pangium edule, Reinw.

2.2 Biosynthesis of Cyangenic glucosides

Research in several Laboratories has shown that four of the known cyanogenic glucosides are derived from closely related amino acids (Conn and Butler, 1969) as shown in Fig. 2.1.

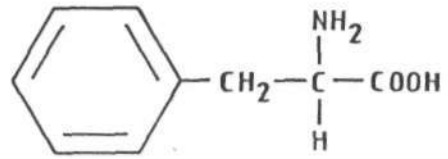
Tyrosine is known to be effectively converted into dhurrin in the sorghum plant (Conn and Akazawa, 1958; Gander, 1958), and into taxiphyllum in shoots of the yew three (Bleichart et al, 1966). Phenylanine has been shown to be converted to prunasin in cherry laurel leaves (Mentzer and Farre-Bonvin, 1961). Butler and Butler (1960) first showed the conversion of valine to linamarin in new zealand clover as well as the conversion of isoleucine to lotaustralin. Conversion of valine and isoleucine to linamarin and lotaustralin have also been observed in flax seedlings (Butler and Conn, 1964).

In each of these biosyntheses, there is a loss of the carboxyl carbon and oxidation of the amino carbon and nitrogen atoms to the level of a nitrile (Conn, 1969). Uribe and Conn, (1966) have shown that the nitrile nitrogen atom of dhurrin, is directly obtained from the α-amino group of tyrosine. They showed that the biosynthetic route involves the modification of the amino acid in a manner such that the C6-C2 Nitrogen unit remains intact.

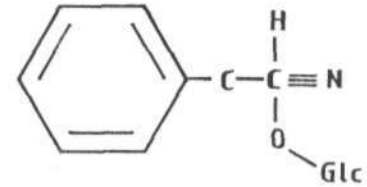


L-TYROSINE

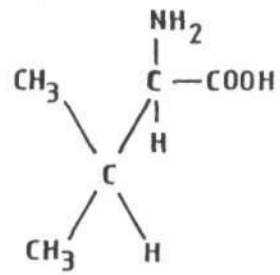
DHURRIN



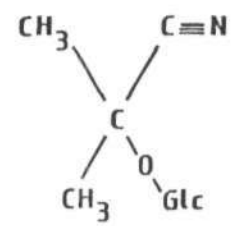
L-PHENYLALANINE



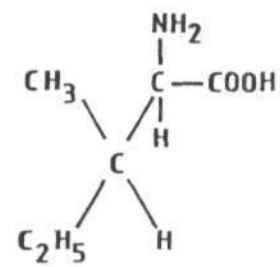
PRUNASIN



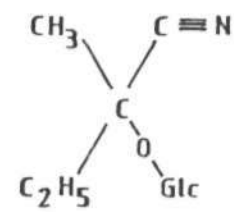
L-VALINE



LINAMARIN



L-ISOLEUCINE



LOTAUSTRALIN

Fig. 2.1. The precursor - product relationship between certain amino acids and certain cyanogenic glucosides (Conn and Butler, 1969).

The nature of the intermediates between amino acids and the cyanogenic glucosides are still not well established, though feeding experiments have confirmed that radioactive labels on some amino acids can be incorporated efficiently into specific cyanogenic glucosides (see section 2.2) in both sorghum etiolated seedings (Akazawa et al. 1960) and flax seedings (Butler and Conn, 1964).

Feeding experiments using Isobutyronitrile and α-hydroxy-Isobutyronitrile showed them to be more efficiently converted into linamarin, when compared with valine as precursor. Also labelled phenyl acetonitrile proved an efficient precursor of prunasin when compared with labelled phenylalanine. Conversions occurred without randomization of radioactivity (Conn, 1968). Studies with double labelled compounds confirmed the retention of the oximimo nitrogen atom from 2-oximimo isovaleric acid and isobutyraldoxime in the biosynthesis of linamarin. A general pathway from amino acids to cyanogenic glucosides involving N-hydroxyamino acids, aldoximes, nitriles and 2-hydroxy nitriles was proposed (Fig. 2.2).

Tapper et al, (1972) examined linen flax shoots under conditions designed to promote accumulation of Intermediates of linamarin biosynthesis. Their experiments with unlabelled isobutyraldoxime administered together with labelled l-valine, showed that these compounds could be intermediates.

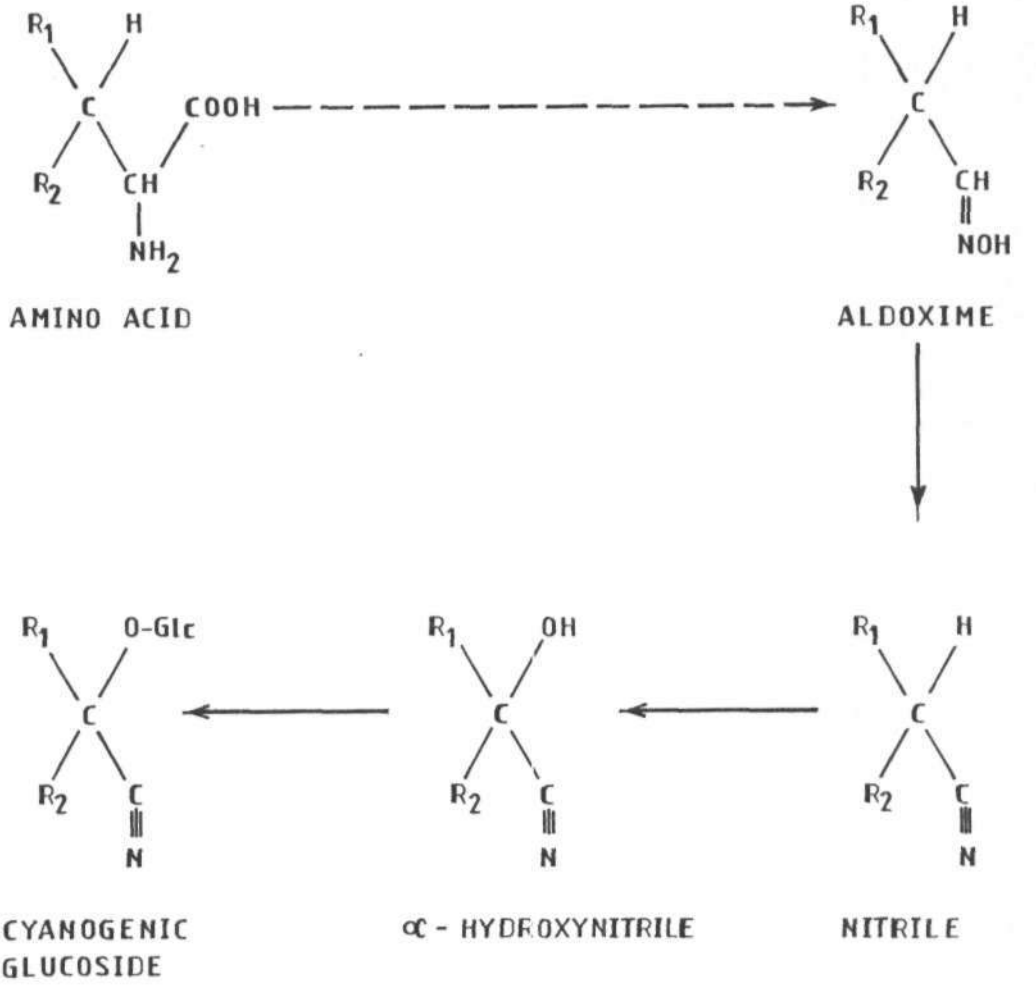


Fig. 2.2. A possible pathway for the biosynthesis of a cyanogenic glucoside from its precursor amino acid.

They also found D-methylthreonine, DL-allo-methylthreonine and DL-2-methoxypropionaldoxime were inhibitors of linamarin biosynthesis, and cause accumulation of an undetected compound which may be isobutyraldoxime-O-glucoside.

Evidence for the proposed pathway for biosynthesis of cyanogenic glucoside (Fig 2.2) was found in the work of Hahlbrock and Conn 1970. An enzyme (UDP-glucose: acetone cyanohydrin transferase) that catalyzes the reaction between acetone cyanohydrin and UDP-glucose to form linamarin was detected and partially purified. The pure enzyme has been shown to be specific for glucosyl donor UDP-glucose, and will not react with ADP-glucose, CDP-glucose, GDP-glucose, IDP-glucose or TDP-glucose. The enzyme is highly specific for the cyanohydrins with which it will react. Thus, it catalyzes maximum reaction rates with ketone cyanohydrins that possess two, three and four methyl or methylene carbon atoms, but as the number of carbon atoms in the cyanohydrin is further increased or decreased there is a rapid decrease in the rate of glucosylation of these compounds. The ketone cyanohydrins possessing two and three methyl or methylene carbon atoms are acetone and butanone, and linamarin and lotaustralin are the glycosides formed from these substrates. Enzymes that catalyzye the formation of other precursors in the pathway (Fig 2.2) have not yet been detected. The pathway (Fig 2.2) will be considered established when these enzymes are detected and observed to exhibit a high degree of specificity.

2.3 Biosynthesis of Cyanogenic Glucosides in Cassava Plant

A common biochemical feature of the cassava plant is that it synthesizes, and accumulates cyanogenic materials in its vegetative tissue especially the edible leaves and tubers (Nartey, 1973). The cyanogenic glucosides in cassava are linamarin and lotaustralin and occur in the ratio linamarin: lotaustralin of 93:7 (Nartey, 1968). Infra-red studies have confirmed they are β-linked (Butler et al., 1965). These glucosides, on hydrolysis give rise to moderate lethal concentrations of hydrocyanic acid or hydrogen cyanide (HCN), a powerful specific and non specific inhibitor of several essential enzymes - catalyzed systems in respiration (Dixon and Webb, 1964).

In different laboratories (Conn, 1969, 1973; Nartey 1968) it has been demonstrated that the aglycone moieties of cyanogenic glycosides may be synthesized from structurally related amino acids. This motivated studies on effectiveness of valine and isoleucine as precursors of the aglycone moieties of linamarin and lotaustralin, respectively (Nartey, 1973). Studies by Nartey (1973) showed that both sweet and bitter cassava seeds contain only trace amounts of these amino acids. However during germination large amounts are accumulated through degradative action of proteolytic enzymes on seed storage proteins. Concurrently, the concentration of linamarin and lotaustralin increased sharply. These showed that linamarin and lotaustralin are synthesized from valine and isoleucine respectively, by the cassava plant. Other

workers (Conn and Butler, 1969; Tapper et al, 1972; Butler, 1965) have shown, through feeding experiments using labelled valine and isoleucine or labelled isobutyronitrile and α-hydroxyisobutyronitrile, the biosynthetic pathway for linamarin and lotaustralin (Fig 2.3). The pathway incorporates data from Conn and Butler (1969), Nartey (1968, 1969) and Tapper et al. (1972).

2.4 Cyanogenesis in cassava

Tissues of all cassava cultivars so far examined contain cyanogenic glucosides, although in varying concentrations. Variations in the HCN concentration in tubers, as well as morphological characteristics of plants, form the basis of a taxonomic differentiation between the bitter (high HCN) and sweet (low HCN) cultivars (Rogers, 1963). This basis for delineation does not offer an adequate means for differentiating cassava cultivars. Since definite metabolite concentrations are not strictly inherited, the variations in the concentrations of cyanogenic glucosides encountered in different cultivars are probably the result of phenotypic differences and factors such as intensity and duration of sunlight, temperature, moisture and nutritional status of the soil (Nartey, 1973).

A comparative study of cyanogenesis in different tissues of sweet and bitter varieties of cassava showed that seeds of the former contained no detectable amounts of cyanogens, whereas seeds of the latter contained low levels of cyanogens

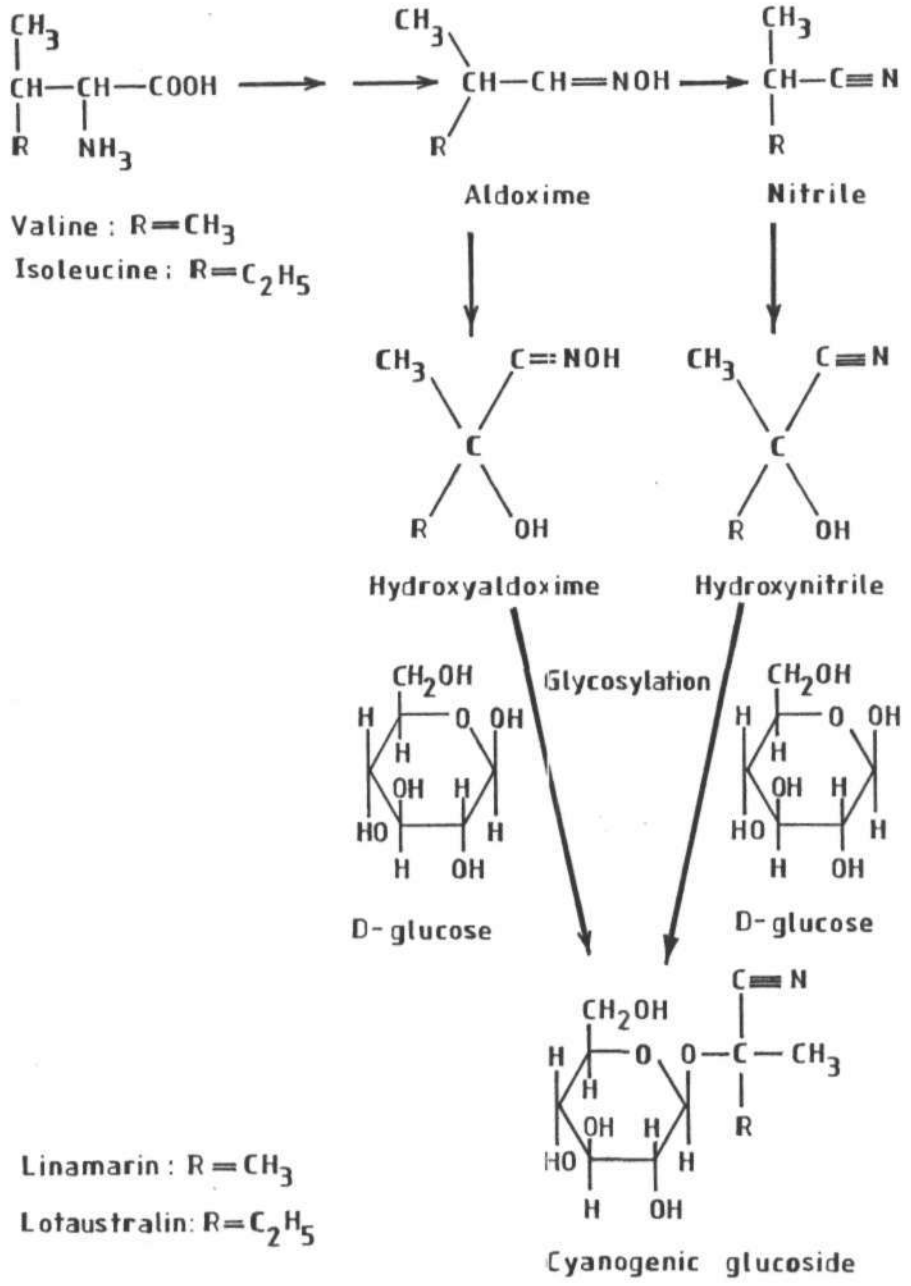


Fig. 2.3. The biosynthesis of linamarin and lotaustralin in cassava. Pathway incorporates data from Conn and Butler, 1969; Nartey, 1968 and 1969; and Tapper et. al., 1972.

(Nartey 1968). It was observed that there were variations in concentrations of HCN evolved from different tissues of six cassava cultivars grown from seed to mature plant. Also it was observed that during germination in the dark, seeds of all six cultivars synthesized high levels of cyanogenic materials. Under continuous illumination, it was observed that the concentration of cyanogenic glucoside increased significantly, but decreased sharply after 2 days. This and subsequent fluctuations did not lead to the release of HCN in the closed system. The fluctuations indicated that cassava cyanogens were dynamic metabolites, and that plants contained enzymes which catalyze biosynthesis of the glucosides as well as those which catalyze their degradation. An enzyme with the later activity was isolated in crude form from various cassava tissues (Nartey, 1968).

2.5 Toxicity of Cyanogenic glucosides in Cassava

Cases of acute cyanide poisoning are well-authenticated in animals grazing on cyanogenic forages, but chronic toxicity from continuous intake of low levels of cyanogenic plant material, including cassava, has not been clearly identified (Hill, 1973).

In a study involving over 360 Nigerians who suffer from Tropical ataxic neuropathy, a disease of the nerves, it was discovered that the disease does not affect children less than ten years old. It affects males and females alike, and the peak incidence of the disease is in the 40-50-years old group.

The highest incidence of the disease is among communities and especially families whose diet consists mostly of cassava products with high cyanide content (Osuntokun, 1973). It was also discovered that the prevalence (2.5%) of goitre in Nigerian patients with Tropical ataxic neuropathy is higher than in the general population and may be related to cassava diet (Osuntokun, 1971). An endemic goitre survey (Ekpechi, 1964) in Nsukka Area of South Eastern Nigeria has shown a correlation between the intake of dry, smoked unfermented cassava and the incidence of goitre. Experimental studies of the possible goitrogenic action of dry, unfermented cassava on rats showed that cassava has an adverse effect on the function of the thyroid (Ekpechi, 1973). 'Gari' and 'Lafun', major cassava products are presently the cheapest source of calories available in Nigeria, and they are consumed in all parts of the country and other West African countries. They are also consumed in large quantities. 'Gari' or 'Lafun' contains substantial quantities of bound cyanide in it. So 'gari' consumers, especially those in the low income group are potential patients of diseases associated with chronic cyanide toxicity since their diets are generally unbalanced and often low in proteins and vitamins.

2.6 Cyanide Detoxification in the Body

There are essentially five ways in which minute amounts of cyanide formed in the human body during metabolism or minute amounts ingested in food are detoxified. When HCN is

converted to thiocyanic acid there is a 200-fold reduction in toxicity (Oke, 1973). Most of the detoxification are due to the conversion of HCN to thiocyanic acid by the action of rhodanese (thiosulphate sulphur transferase) (Lang, 1933a b; Saunders and Himwich, 1950). The enzyme rhodanese, catalyzes the reaction in which cyanide accepts sulphur from inorganic and organic sulphur donor with the resultant formation of thiocyanate (Foos, 1950; Sorbo, 1951a, b, 1953; Himwich and Saunders, 1948). In a review, Nartey (1978) traced the experiments that established that rhodanese catalyzes the formation of thiocyanate. These experiments include injection of cyanide into rabbits which increases their thiocyanate excretion; digestion of liver or muscle tissue from a dog with sodium cyanide which also produced thiocyanate. The liver tissue was more active than the muscle. Therefore rhodanese catalyzes the majority of the reactions (Fig 2.4 and 2.5) which produce thiocyanate.

$$CN^- + (S_2O_3)^{2-} \text{ (rhodanese)} \longrightarrow SCN^- + SO_3^{2-}.$$

The amount of rhodanese in the liver varies with different animals so detoxification in different animals takes place at different rates. This has been established by Himwich and saunders (1948). The activity of the enzyme in parts of the brain and central nervous system seems to be the same for different species of animals.

Cyanocobalamin (Vitamin B_{12}) occurs in the liver to some extent as hydroxocobalamin, (Vitamin $B_{12}a$). This hydroxocobalamin is capable of reacting with cyanide to give cyanocobalamin, providing another independent pathway for cyanide detoxification (Mushet et al, 1952).

3-mercaptopyruvate arising from cysteine by transamination or deamination can provide sulphur as rapidly as thiosulphate for cyanide detoxification (Meister, 1953; Wood and Fielder, 1953). This is made possible by the transfer of the sulfur in 3-mercaptopyruvate to sulphite resulting in the formation of thiosulphate and pyruvic acid (Fig 2.4). The thiosulphate formed then provides sulphur for the conversion of cyanide to thiocyanate by the rhodanese enzyme. The sulphite formed from the rhodanese reaction participates in further sulfur transferase action. A cycle is then formed as shown in Fig 2.4 (Oke, 1973). Cyanide can also react with 3-mercaptopyruvate to yield thiocyanate and pyruvic acid (Fielder and Wood, 1956). The reaction is catalyzed by the enzyme cyanide sulphur transferase (Fig 2.4).

Cyanide can react with cystine to split the disulphide linkage to form cysteine and β-thiocyanoalanine which tautomerizes to 2-aminothiazoline-4-carboxylic acid (Fig 2.5). β-thiocyanoalanine could be a source of thiocyanate in body since it undergoes oxidative deamination to produce thiocyanopyruvic acid which decomposes readily to yield thiocyanate (Schobert et al, 1951).

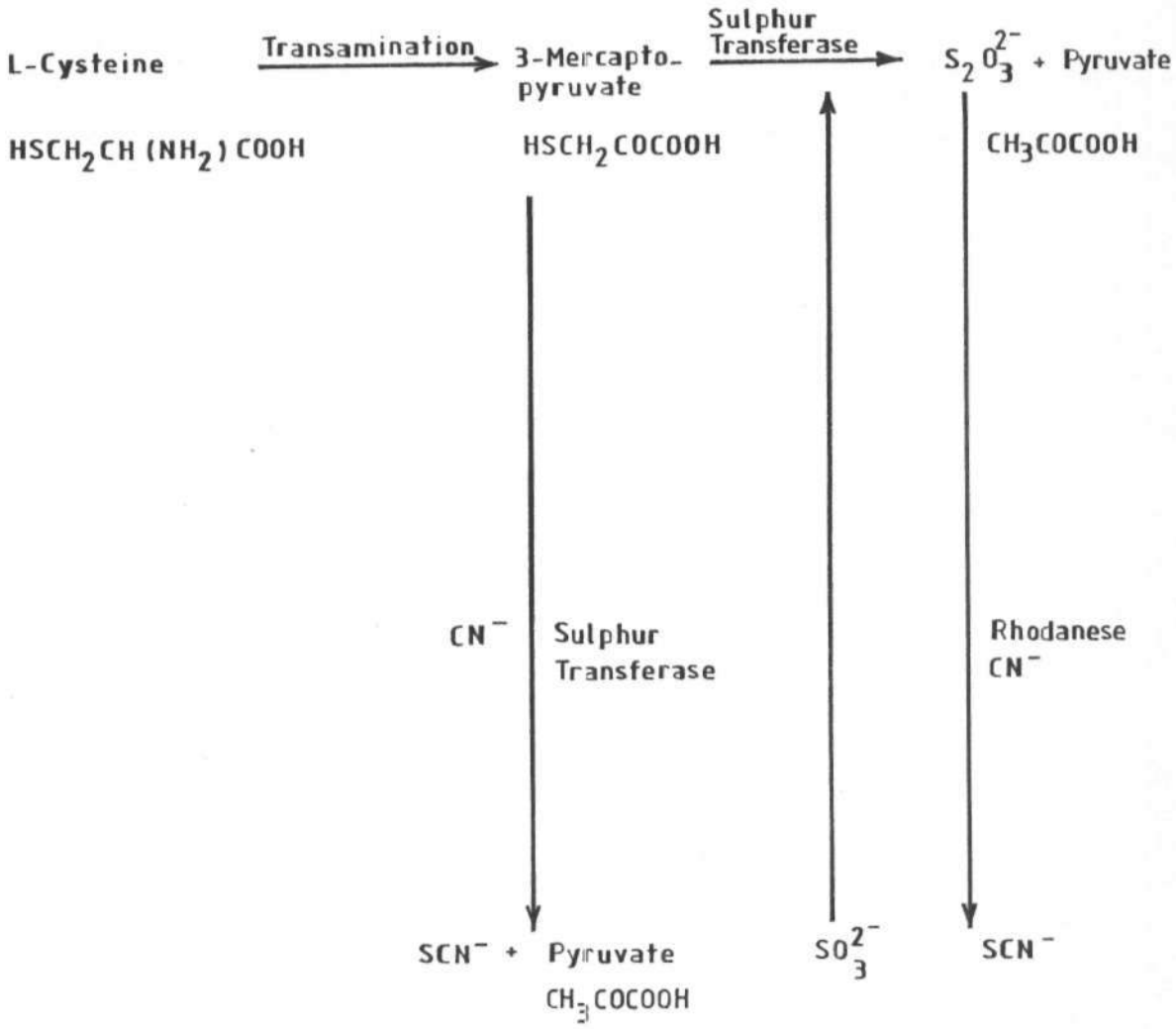


Fig. 2.4. The cycle formed by the reaction of cyanide with 3-Mercaptopyruvate (Oke, 1973).

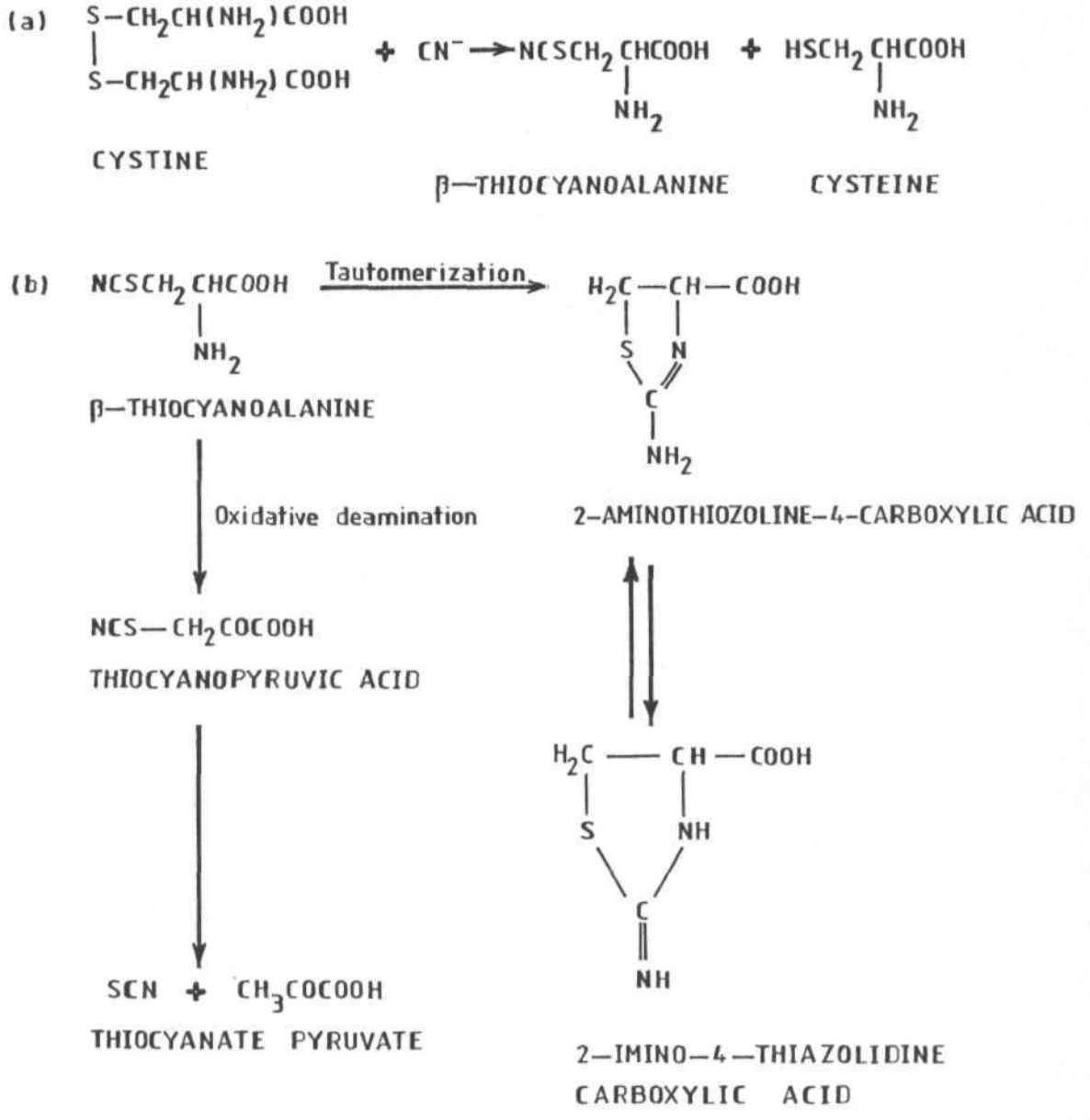


Fig. 2.5. The reaction of cyanide with cystine.

Rabbits injected with acetonitrile excreted 3-5 percent of the acetonitrile and this percentage increased on feeding dessicated thyroid (Bauman et al. (1933). This indicated that the thyroid gland has some detoxificating effect. These same authors observed that thyroidectomy had little effect on the conversion of benzylcyanide to thiocyanate, and were of the opinion that the mechanism of detoxification by thyroid depends on the demethylation of acetonitrile.

Another pathway for cyanide detoxification is provided by methemoglobin which forms complexes with cyanide. The resulting cyanomethemoglobin is itself non toxic and can be slowly metabolized. Since the body contains considerable amounts of hemoglobin, therapy for cyanide-poisoned individuals consists in accelerating methemoglobin formation, e.g. by amyl nitrile administration (White et al. 1978).

2.7 Cyanide and Human Disease

The utilization of cassava roots and products as major staple food presents a variety of health problems (Nartey, 1973).

The high moisture content and the high ratio of carbohydrates to nitrogen make cassava roots an excellent substrate for microbial growth and production of high levels of toxic metabolites. Nartey (1966) has shown that Aspergillus flavus thrives on cassava meal substrate and produces, relatively high levels of aflatoxins.

The chronic uptake of cyanide in small doses, which as single doses do not cause clinical symptoms, is assumed to be decisive for the pathogenesis of a few disease (Baumeister et al., 1975). An inhibition of some routes of cyanide metabolism is probably responsible in some cases (Schievelbein et al., 1969; Baumeister, 1972).

There has been no direct evidence linking human disease with chronic cyanide exposure. In a review by Wilson, (1965). It was shown that the diseases which have been considered to result from abnormal detoxification cyanide are retrobulbar neuritis in pernicious anaemia, tobacco amblyopia, subacute combined degeneration of the cord in vitamin B_{12} deficiency, Lebers hereditary optic atrophy, and dominantly inherited optic atrophy.

Tobacco amblyopia and retrobulbar neuritis in pernicious anaemias are thought to result either from a relative block in cyanide detoxification or from absolute vitamin B_{12} deficiency which leads to a damaging accumulation of cyanide (Wilson, 1966), or from conversions of one of the physiologically active forms of vitamin B_{12} to a physiologically inactive form (Smith et al., 1965). In their experiments, Smith et al. 1965, showed that hydroxycobalamin lowers mortality rate due to cyanide injections and that gross changes in myelin due to injection of cyanide or cyanide plus cyanocobalamin were not seen at any time in rats injected cyanide plus hydroxycobalamin.

Lebers hereditary optic atrophy (Wilson, 1965) is a rare disorder in which visual failure occurs subacutely or inciduously and results in bilateral central scotomato and pallor of the optic discs. The condition occurs typically in young males in their late "teens" or early twenties. These individuals are also unable to detoxify cyanide to thiocyanate normally.

In dominantly inherited optic atrophy the metabolic basis is not known but there is an abnormally high concentration of cyanocobalamin present in the plasma of most patients. Tropical ataxic neuropathy (Osuntokun, 1973), and goitre (Ekpechi, 1967) are two other diseases which have been attributed to chronic consumption of cassava products.

The symptoms of Tropical ataxic neuropathy consist of leisons of skin, mucous membranes, optic and auditory nerves, spinal cord and peripheral nerves, and have been associated with cyanide intoxication due to high cassava diets (Money, 1959; Montgomery et al. 1964; Osuntokun, 1968). A diminution or a complete absence of the sulphur containing amino acids cysteine and methionine in plasma has also been observed in this disease and it seems the detoxification of cyanide via formation of 2-imino-4-thiazolidine carboxylic acid is prevented because of lack of cysteine. The essential neurological components of the disease are myelopathy, bilateral optic atrophy, bilateral perceptive deafness, and polyneuropathy. In about 35% of the patients stomatoglossitis is present. Motor neuron disease, Parkinsons disease,

cerebellar degeneration, psychosis, and dementis have also been found in association with the disease (Asuni, 1967; Osuntokun, 1968). Endemic goitre (Ekpechi, 1964), was found more in high cassava consuming areas of Eastern Nigeria. Cassava has also been shown to have an adverse effect on the functioning of the thyroid gland.

The work of Delange et al (1973) has shown that consumption of cassava by humans induces an inhibition of the penetration of iodine into the thyroid gland as expressed by a considerable drop in radio iodine thyroid uptake in urinary excretion of stable and labelled iodine. Inhibition of iodine uptake by the thyroid is also obtained in rats fed with cassava and this effect is accompanied by a striking rise in the plasma thiocyanate concentration and urinary thiocyanate excretion (Delange et al., 1973).

2.8 Cassava processing technology and detoxification

A wide variety of techniques have been devised in various parts of the world to detoxify the more poisonous varieties of cassava. Most traditional food preparation techniques appear to be designed to bring together enzyme and substrate (linamarin) by cell rupture, followed by elimination of liberated HCN by either volatilization or solution in water. In some processes, the initial hydrolysis of the linamarin is assisted by the use of fermentation processes (Coursey, 1973). The traditional methods include drying, soaking, boiling and fermenting whole or fragmented roots; all of these reduce the

total cyanide content of the material (Coursey, 1973).

In Nigeria, sundrying of cassava (root, slices, chips, flour) is one of the popular processing methods. The materials are spread out, usually on mats, concrete floors, or unused portions of main roads, and left in the sun to dry gradually with occasional turning. The amount of cyanide that is lost from the materials depends on such factors as shape and size for fragments (Thani and Lohani, 1978) and relative humidity, air temperature and wind velocity (Rao and Cock, 1973).

The HCN level (milligram HCN per kilogram) of samples of a number of African cassava based food products have been given by Oke (1968) as follows: Fresh cassava 380, Gari 19, Fufu 25, Lafun 10, and Kpokpogari 11. Wood (1965) has given a value of 25mg HCN/kg in fresh gari, this falling to 2mg HCN/kg after storage for 1 month. During the extensive investigation conducted in Nigeria in connection with the mechanized production of gari (Akinrele et al. 1962), a value of 30mg HCN/kg was regarded as acceptable in the product. In Brazil, where cassava flours are extensively used in the baking Industry, Paula and Rangel (1939) stated that crude flours made from bitter cassava contain 10-200mg HCN/kg, but the bitter grade of table flour are free from HCN. Nemoto (1940) gives 27-37mg HCN/kg as the normal range, but grated manioc flour may contain as much as 125mg HCN/kg. Both results state that these residual quantities of HCN are destroyed in the baking process when the flours are

incorporated in the bread. However, in studies on the manufacture of rotis, (a roasted product) with cassava flour containing 57-118mg HCN/kg, Joachim and Panditteskere (1944) found losses ranging from 47 to 80% during cooking, which would indicate high degree of retention.

The changes in concentration of free cyanide (non-glycosidic) and bound cyanide (cyanogenic glucoside) in fresh cassava chips during dehydration, boiling or soaking in water have been studied (Cooke and Maduagwu 1978). Their findings show that all of these processes rapidly removed free cyanide from the chips, but that only 8-12% of the total cyanide present is free cyanide. Air drying in the oven showed that 29% of the bound cyanide was removed by drying at 46.5^{0}C. Smaller loses were recorded at higher temperatures. Greater losses occurred on sundrying, presumably because of the longer drying time at moisture contents and temperatures at which endogenous linamarase is active. The corresponding loss of free cyanide was 82.5% (Cooke and Maduagwu, 1978).

On soaking (leaching) and boiling of fresh cassava chips, the same authors found that the free cyanide was rapidly removed in boiling water (over 90% removed within 15 minuets); the bound cyanide was removed at a slower rate but a thorough cooking for 25 minutes removed over 50% of bound cyanide. During sundrying of cassava slices, Maduagwu and Adewale (1980) reported a progressive loss of cyanide with time, most of it within the first 8 hours, during which time 46-58% free and 69-74% bound cyanide disappeared from the "bitter" variety

and about 74% of bound cyanide disappeared from the "sweet" cultivar. These losses, they suggested could be due to optimal hydrolytic activity of the β-glycosidic, linamarase.

Nambisan and Sundaresan (1985) on evaluating the efficiency of different processes in reducing the cyanogenic glucoside content of cassava found that the maximum retention of cyanogenic glucoside (80%) was observed in baked, fried and steamed tubers. Cyanogenic glucoside retention in sundried chips varied from 30-60%, the retention being governed by the chip thickness. In the case of cassava boiled in water, smaller chip size and sufficient water were found to be the ideal conditions for maximum cyanogenic glucoside removal (25-75% retention) depending on the chip size. It was found that the most effective method for cyanogenic glucoside removal was by crushing fresh tubers and subsequent sun-drying, whereby 95% of the cyanogenic glucoside was eliminated.

The extent of loss of hydrocyanic acid during fermentation of cassava roots for 3-4 days (in the traditional) and 8 days have been studied (El-Tinay et al. 1984). Loss of the cyanide from whole tubers was 80-87% after 8 days and was 51-53% after 4 days. When water was added to the crushed pulp the reduction in cyanide was 83-91% with marked decrease in bound cyanide in the 1st day of fermentation. This result suggests that autohydrolysis is enhanced by addition of water to the crushed pulp (El-Tinay et al. 1984). The high cyanide of the water necessitates dewatering of the pulp before consumption (El-Tinay et al.

1984; Cooke and Maduagwu 1978). Maduagwu and Oben (1981) used a screw press for dewatering fermented cassava and reported a 63.8% reduction in cyanide content as a result of the processing step.

The variable and inconsistent literature data for cyanide losses during processing are probably because of problems associated with assay methodologies (Zitnak, 1973; Cooke, 1978). Important process parameters such as cassava piece size, dehydration rates, temperatures, were not specified. It thus appears that cassava food products, as commonly prepared by many of the various detcxification methods, can still contain appreciable traces of HCN (Coursey, 1973; Cooke and Maduagwu, 1980; and Oben, 1981).

2.9 Cassava fermentation

Fermentation is a very important stage in the processing of cassava for production of "gari" a popular food among Nigerians of all classes, both low and high income group. The significance of fermentation as already reported (Collard and Levi, 1959) is that it brings about the detoxification of cassava by the liberation of hydrocyanic acid at low pH, through the combined activities of Corynebacterium manihot and Geotricum candida, and the development of characteristic flavour of gari. This fermentation which normally takes place 3-4 days during village processing can be shortened to 24 hours by seeding a fresh pulp with 4-day old cassava juice (Akinrele, 1964). Akinrele (1964) and Ikediobi and Onyike

(1982b) have confirmed this by actually seeding a fresh pulp with 4-day old cassava juice, or adding exogenous linamarase from cassava cortex to fresh pulp. It is reported (Akinrele, 1964) that the fermentation of cassava proceeds in two stages during which the mash is generally sterilized against adventitious microbial growth. During the first phase, cassava bacteria Corynebacterium manihot attack the starch with the production of lactic and formic acids, a reaction accompanied by the evolution of heat. When the pH of the medium has fallen to about 4.25, a mould, Geotricum candida begins to proliferate rapidly bringing about further acidification and the production of the characteristic aroma of gari.

Earlier studies (Collard and Levi, 1959; Collard, 1963; Akinrele, 1964) of cassava fermentation have attributed detoxification to the spontaneous hydrolysis of the constituent cyanogenic glucosides principally linamarin, by the organic acids produced during starch degradation by the fermentative action of Corynebacterium manihot at low pH. More recent work (Wood, 1966; Okafor, 1977) has demonstrated the unusual stability of linamarin in dilute acid even at $100^{0}C$, thus making it unlikely that detoxification is due solely to chemical hydrolysis by the weak acids of microbial fermentation. Ikediobi and Onyike (1982b) have shown that the detoxification is due mainly to the action of linamarase on the cyanogenic glucoside of fermenting cassava. It has also been observed that some of the formic acid is broken down by

a hydrogenase system to form carbondioxide and probably hydrogen. All these gases tend to render the medium anaerobic (Akinrele, 1964). Fermentation seems to proceed best at a temperature of about 35^{0}C. Frequent mixing of the mash and exposure to light appear to accelerate fermentation particularly during the second stage. A continuous system is possible and in fact, gives a better fermentation than does the batch process. Exposure to air or oxygen and contact with iron should be reduced to a minimum to avoid discoloration (Akinrele, 1964).

2.10 Use of linamarase in cassava detoxification during fermentation

The use of exogenous linamarase to assist cassava detoxification during fermentation has been reported (Wood, 1966; de Bruijn, 1973; Ikediobi and Onyike 1982a). Fermenting grated cassava was detoxified 87. 5% when exogenous linamarase was added to the fermenting mash, as opposed to 50% without any enzyme addition. This is an indication of the enzymatic basis of the detoxification process (Ikediobi and Onyike, 1982a). This work showed that higher fold detoxification is achieved after 24 hours of fermentation in the presence of added linamarase than after 5 days of fermentation without enzyme addition. This work also indicated that very little additional detoxification is achieved by adding more enzyme to the fermenting mash beyond the first 24 hours. These observations are explained by the development of a pH favourable for linamarase activity within the first 24 hours

of fermentation and accounts for the rather high cyanide detectable within this period (Ikediobi and Onyike, 1982a; Tewe, 1975).

2.11 Assay Methods for Hydrocyanic Acids in Plant Tissues and their Application in Studies of Cyanogenic Glucosides in Cassava

Most research data are reported in terms of potential cyanide yields rather than the glucoside content itself. The assays are based on the determination of HCN liberated by enzyme or acid hydrolysis (Zitnak, 1973).

The assays of cyanogenic glucosides bound with aromatic aglycone (Table 2.1) are less difficult to perform as these glucosides are easily hydrolyzed by acid or exogenous β-glycosidase which are readily available from commercial sources (Gullingham at al., 1969). The assays of linamarin in flax and cassava are rendered more difficult owing to the apparent requirement of a specific enzyme, linamarase, which is not readily available and is particularly needed for hydrolysis of prepared or processed food material (Wood, 1965, 1966).

Early work on cyanogenic glucosides stimulated by acute and sometimes lethal toxicity cases (Montgomery, 1969) placed fewer demands on cyanide detection limit, since it was sufficient to determine potentially toxic levels. Recent concern about chronic toxicity through continuous intake of low quantities of cyanide in food and forages and the necessity to study the physiological effects of such intake,

stimulated the development of procedures which would allow detection and quantitation of trace amount of cyanide (Zitnak, 1973).

The most recent method for analytical determination of cyanogenic glucoside incorporates linamarase in its assay protocol and has detection limits down to 0.16 mg/ml (Ikediobi et al, 1980) and less than 0.01mg cyanide per 100g fresh weight and peeled root (Cooke, 1978)>

There are 3 distinct steps in the HCN assay:

1. Liberation of cyanide through acid or enzymatic hydrolysis.
2. Extraction and isolation of the cyanide from the substrate and
3. Determination of the Isolated cyanide.

Cooke (1978) has established an enzymatic assay of total cyanide content of cassava parenchymal tissue (peeled root), or cassava leaves.

Ikediobi et al, (1980) improved the alkaline picrate test for cyanide by incorporating an enzymatic step to make the assay more specific and quantitative. In both methods (Cooke, 1978; and Ikediobi et al, 1980) the assay protocol involves incubating the cassava extract with specific amount of the enzyme, linamarase, at 30^0C or room temperature for 10-15 minutes.

The cassava extracts were prepared using 0.IM orthophosphric acid, which inactivates the endogenous linamarase. These extracts were then either filtered or

centrifuged, and subsequently neutralized before incubation with enzyme. The cyanogenic glucosides present are hydrolyzed to free cyanide which is estimated spectrophotometrically by a modified chloramine/pyridine/pyrazolone method (Cooke et al, 1978). In the Ikediobi et al (1980) method, 30-40 units of activity are needed for an assay, whereas in the Cooke (1978) method 0.23-0.3 unit of activity is needed per assay.

Recently (Dartnal et al, 1987) a method for measuring cyanide and cyanogenic glucosides in sand culture and soil has been devised. This microdifussion technique depends on the enzymic conversion of linamarin and lotaustralin to HCN, the release of HCN following acidification and incubation, and its detection in sodium hydroxide. Using this technique silt loam soil was shown to contain 5.47 nmol cyanide per gram air dried soil.

2.12 β-Glucosiases

When plant tissues containing cyanogenic glucosides are crushed or autolyzed, an enzymic hydrolysis takes place, releasing the sugar moiety and the aglycone (Butler et al, 1973). The crushing of the plants probably allows the glycosidases and glucosides to diffuse together and react. The enzymes hydrolyzing these glycosides are β-glucosidases with differing degrees of specificity for the aglycone portion of the compound (Butler et al, 1973).

β-glucosidases have broad specificity. Their ability to hydrolyze the cyanogenic glucoside depends on the structure of

the cyanogenic glucoside. In general, the β-glucosidases are known to be specific for the aglycone moiety but they also hydrolyze cyanogenic glucosides with different aglycone moieties (Butler et al, 1965). The degree of hydrolysis depends on the structure of the aglycone.

A survey of β-glucosidase activities of enzyme extracts from a variety of higher plants revealed that the ability to hydrolyze linamarin and lotaustralin was very limited (Butler et al., 1965). Extracts from linseed were fractionated for β-glucosidase activity using DEAE- Chromatography, gel diffusion, and column electrophoresis. Column electrophoresis proved particularly successful by allowing 105 fold purification of linamarase in one step. Substrate specificity of purified linseed linamarase was examined for a number of glucosides and the result is shown on Table 2.2.

Table 2.2 Hydrolysis of Glucosides by Purified Linseed Linamarase (Butler et al, 1965)

Glucoside	% Hydrolysis
Linamarin	76.2
Prunasin	51.6
Amygdalin	0.0
Phloridzin	30.0
Arbutin	34.2
Salicin	32.7
β-Methyl glucoside	1.1

The activity of linseed linamarase was also tested against two isomeric cyanoglucosides, dhurrin and taxiphyllin. Dhurrin and taxiphyllin were hydrolyzed at the same rate with crude linamarase. It was also observed that emulsin hydrolyzed linamarin and lotaustralin, but very low amount of hydrogen cyanide compared to glucose was found (Butler et al, 1965) The disparity between the glucose and hydrogen cyanide yields was not observed when crude linamarase was used

Several β-glucosidases have been purified and characterized. β-glucosidases from sorghum vegetative tissues and seed have been partially purified and characterized (Mao and Anderson, 1967). Two distinct β-D-glucopyranosidase activities (glucosidase I and II) with distinct aglycone specificities were characterized. D-glucose, D- glucose-1, 4-lactone and D-glucono-1, 5 lactone were inhibitors for both glucosidase I and II. Dhurrin was found to be inhibitor for glucosidases I and p-nitrophenyl glucoside as an inhibitor for glucosidase II. All Inhibitions were competitive. The enzymatic degradation of dhurrin can be summarized as follows (Mao and Anderson, 1967):

Dhurrin.....> Glucose + P- hydroxy- mandelonitrile.....>
(glucosidase II) (glucosidase I)

P-hydroxybenzaldehyde + Cyanide.

Recently, Ikediobi and Terry (1989) have separated β-glucosidase II (Dhurinase) from β-glucosidase I by treatment

of sorghum seed extract with acetone to precipitate impure dhurinase which was recovered and solubilized in a minimal amount of sodium phosphate buffer, pH. 6.8. This was purified first on hydroxyapatite and later on DEAE cellulose. The dhurrinase- catalyzed hydrolysis of dhurrin, when compared to alkaline hydrolysis was shown to lead to release of additional 8-17% of CN- from dhurrin and thus gives a more accurate assessment of the dhurrin content of sorghum and sorghum products.

Studies on almond emulsin β-glucosidase by Grover et al. (1977) have shown that β-D-glucoside glucohydrolase, isolated from almond emulsin catalyzed the hydrolysis of β-D-glucoside and β-D galactopyranoside. The Km, turnover rate and activation energies are different for the β-D-glucopyranoside and β-D-galactopyranoside.

β-glucosidases from barley grain extract have been separated from one another by Sephadex G-150 gel filtration and CM cellulose chromatography (Del, 1977). The glucosidase types were varied. The multifunctional nature of the enzymes were demonstrated by inhibition studies, gel electrophoresis, pH and thermal stability studies (Dey, 1977). Using chromatography on DEAE-cellulose, 2 distinct β-glucosidase enzymes have been identified in white clover. One form is primarily associated with the seed, the other with young leaves (Hughes, 1968). The β-galactosidase and β-glucosidase components of Medicago sativa L (Alfalfa seed) were separated using heat denaturation studies and reaction with specific

inhibitors. The separate identity of the two enzymes were thus determined (Robinson, 1966).

2.13 Purification and Characterization of Glucosidase From Fusarium equiseti

Fusarium equiseti was first observed to be capable of producing β-glucosidase by its ability to hydrolyze both p-nitrophenyl β-D-glucoside and linamarin (Ikediobi and Ogundu, 1985).

Linamarase from (F. equiseti) has also been purified 56-fold by ammonium sulphate fractionation, gel filtration on sephadex G-150, and ion exchange chromatography on DEAE-Sephadex A50-120 (Ikediobi et al, 1987). The enzyme has a Km of 0.36mM and 0.51mM with respect to the artificial substrate p-nitrophenyl β-D-glucoside and linamarin respectively. γ-gluconolactone is a strong competitive inhibitor of the enzyme and yielded Ki of 0.2mM, when p-nitrophenyl glucoside was used as substrate. The activity of the enzyme is affected by ionic strength. Optimum ionic strength was found to be 0.50, above which the enzyme activity begins to decline due to primary and secondary salt effects (Foster, 1980). The pH optimum of the enzyme is 6.0 and further analysis indicates that the carboxyl of aspartate or glutamate, imidazole of histidine and the sulphydryl group of cysteine may be involved in enzyme catalysis. The Fusarium equiseti linamarase was shown to be significantly inhibited by such thiol-group reagents as iodoacetate, p-hydroxymercuribenzoate and

iodobenzoate, indicating that a sulphydryl group may be present at the active site of the enzyme. The enzyme was found to be stable up to 45^{0}C, this corresponding approximately with the optimum temperature of the linamarase-catalyzed hydrolysis. The activation energy, Ea, was found to be 8.21 Kcal/mole.

2.14 Purification of Cassava Linamarase

Linamarase from parenchymal tissue of cassava was purified and characterized. The extraction was achieved with acetate buffer. Fractionation of the crude extract from cassava parenchymal tissue with 60% $(NH_4)_2SO_4$ gave a 14-fold increase in specific activity. Elution of the $(NH_4)_2SO_4$ fraction using DEAE-cellulose gave 32-fold increase in specific activity and 67% yield, and elution of the $(NH_4)_2SO_4$ fraction using Sepharose 6B gave 350-fold increase in specific activity and 35% yield (Cooke et al, 1978).

Preliminary studies on the extraction of linamarase from cassava peel, confirmed that a greater concentration is present in the peel than in the parenchyma (de Bruijn, 1973). The peel enzyme resembles the parenchymal enzyme in the pH and ionic strength dependence, and Km for linamarin. The specific activity of the crude extract and initial $(NH_4)_2SO_4$ concentrate are much higher than the corresponding figures for the parenchymal enzyme. The final purity of the peel enzyme is uncertain (Cooke et al, 1978).

β-Glucosidases can be stabilized using polyhydric alcohols (Bernier et al., 1988). The authors have been able to stabilize β-glucosidases from Thermonospora curvata lysed cells extracts. Culture solids and lyophilized cells were increased 9-16 fold by polyhydric alcohols. Sorbitol doubled the recovery of glucosidases.

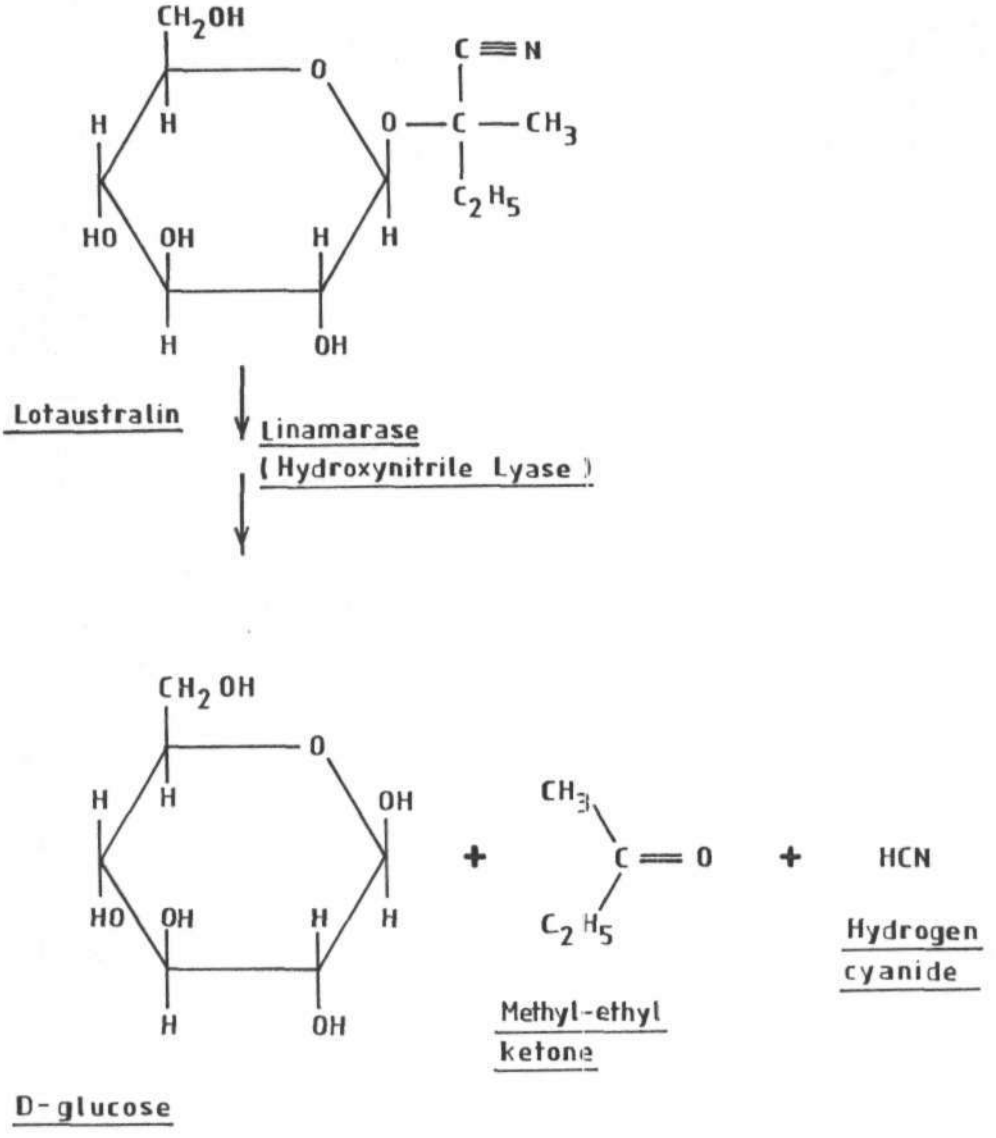


Fig. 2.6. Enzymatic hydrolysis of lotaustralin.

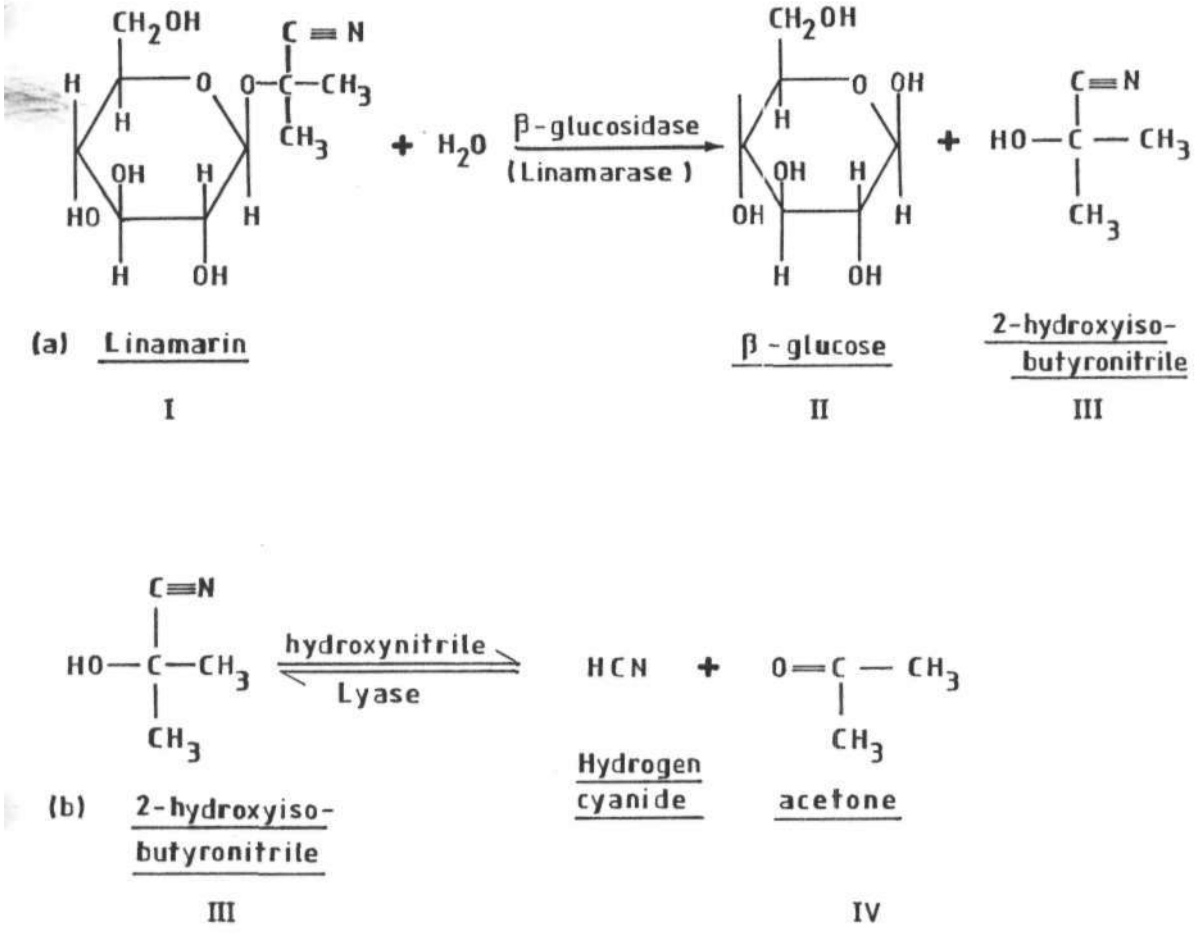


Fig. 2.7. Enzymatic hydrolysis of linamarin.

CHAPTER 3

MATERIALS AND METHODS

3.1 Yeast Isolates

Yeast Isolates used were obtained from Dr Ameh of the Department of Microbiology, Ahmadu Bello University, Zaria. The yeast isolates and their code numbers are as follows:-

Candida	magnoliae	K-F-1
Candida	rhaggi	B-82
Saccharomyces	Kluvveri	B-1
Saccharomyces	cerevisiae	J-82
Saccharomyces	cerevisiae	B-2
Kluvveromyces	maxianus	R-1

3.2 Media Materials

Glucose, bacteriological agar, peptone, and yeast extract are from Hopkin an William Co., Chadwell, Heath Essex, England.

3.3 Chemicals

P-nitrophenyl-β-D-glucoside (PNP glucoside) and linamarin used for enzyme assay were obtained from Sigma Chemicals Company, st Louis, MO. U.S.A. Dextrose for preparing culture media, bovine serum albumin for preparing protein standard curve, and ammonium sulphate for precipitating proteins were products of Hopkin and Williams company, chadwell, Essex, England.

Potassium cyanide, pyridine, boric acid, chloramine T, sodium dihydrogen orthophosphate, acetic acid, and sodium acetate were purchased from British Drug House (BDH), Poole, England. Sephadex G-150, and DEAE-Sephadex A50 were from Pharmacia Fine Chemicals, Uppsala, Sweden. Bispyrozolone and 3-methyl-1-phenyl-5-pyrazolone were from Calbiochem, Behring Corporation, Lajolla, California, U.S.A.

3.4 Nutrient Broth and Agar

3.4.1 Nutrient Broth

Three hundred milligramme yeast extract, 2.00 grammes glucose, 0.50 gramme peptone and 0.10 gramme inducer (cassava powder videinfra) were weighed into a conical flask and dissolved in 100ml of distilled water. The flask and contents were placed in a hot water bath and swirled gently until the contents dissolved well. The broth was dispensed into smaller conical flasks which were carefully plugged with wads of cotton wool and the cotton wool covered with aluminum foil. These flasks with broth aliquots were then autoclaved at 121^0C

under a pressure of 15 pounds per square inch for 15 minute and there after, allowed to cool.

3.4.2 Nutrient Agar

Four hundred and fifty milligrammes yeast extract, 3.00 grammes of glucose, 0.75 gramme peptone, and 2.25 grammes of bacteriological agar were weighed into a conical flask and mixed in 150 mls of distilled water. The flask and contents were placed in a hot water bath and swirled until well dissolved. The nutrient agar was dispensed into Bijou bottles which were then capped loosely and autoclaved at 121^0C under pressure of 15 pounds per square inch for 15 minute. While still hot, the Bijou bottles were slanted, using a wooden support and then allowed to cool.

3.4.3 Cassava Powder (Inducer)

Fresh cassava parenchyma was cut into 0.5cm cubes, soaked in 0.1M orthophosphoric acid, kept over-night at 4^0C and dried in the over at 80^0c for about 18 hours, then it was ground into powder.

3.5 Yeast growth

The yeast isolates were inoculated onto the various agar slants in a sterilized inoculating room. The yeast cultures were allowed to grow at room temperature (26 ± 2^0C) for 3 days. Each culture was then transferred from nutrient agar into the nutrient broth in a 250ml conical flask. The conical flasks

were placed in a thermostated rotary orbital shaker and maintained at a speed of 100 revs/min at room temperature for 10 days.

3.6 Assay of Crude Yeast Extracellular linamarase

At the end of every 24 hours an aliquot of 2-5ml of the content of each growth flask was taken out and centrifuged at 5,00 X g for 15 minutes. The supernatant was assayed for linamarase while the pellet was discarded.

3.7 Preparation of crude Linamarase from dried cassava cortex

The thin brown and dry outer skin of freshly harvested cassava tubers was carefully removed, revealing the underlying cortex. The latter was removed and dried partially in the sun and at room temperature. The dried cassava cortex was ground into a powdery flour. One hundred grammes of the cassava cortex flour was steeped in 200ml of 0.IM acetate buffer of pH 5.50 for 24-48 hours, and then was homogenized. The homogenate was centrifuged at 19,000g for twenty minutes at 4^0C in a Beckman model J-21B refrigerated centrifuge. The supernatant was saved and the pellet (consisting mainly of fibrous tissues of cassava cortex) was discarded.

3.8 Ammonium sulphate Fractionation

Enough solid ammonium sulphate was added to the crude extract (supernatant from 3.7) with stirring to give 60% saturation as described by Dixon and Webb (1964). The crude

extract with dissolved ammonium sulphate was kept for 15-20 minutes at room temperature, and then centrifuged at 20,000g for 20 minutes. The resulting pellet was dissolved in 20ml 5mM sodium phosphate buffer of pH 6.8. The supernatant was assayed for linamarase activity. More solid ammonium sulphate was added with stirring to the supernatant to bring it up to 62% saturation. It was centrifuged again at (20,000g) for 20 minutes. The supernatant was further assayed for linamarase activity and more solid ammonium sulphate was added with stirring to the supernatant to bring it up to 65% saturation. It was centrifuged again at 20,000g for 20 minutes but no pellet was obtained. The supernatant was discarded.

There was no other available refrigerated centrifuge which could spin at higher speeds. All the linamarase in the crude extract was thus precipitated out as much as practicable with 62% ammonium sulphate.

3.9 Gel filtration Chromatography

The enzyme preparation from the $(NH_4)_2SO_4$ step was applied to a Sephadex G-150 column (2.2 x 25cm) pre-equilibrated with 0.01M sodium phosphate buffer of pH 6.8. Elution was with the same buffer and 6ml fractions were collected. The enzymatically active fractions were pooled and referred to as the purified enzyme. This was for subsequent physicochemical studies.

3.10 Ion - Exchange Chromatography

The pooled eluate from the gel-filtration step was concentrated down to about 10ml and applied to a DEAE-sephadex A50-120 column (2.2 x 25cm) pre-equilibrated with 5mM potassium phosphate buffer pH 7.50. Elution was with a linear gradient from 5mM potassium phosphate buffer to 1M NaCl in the same buffer; 5ml fractions were routinely collected and enzymatically active fractions (Ikediobi et al., 1987), were pooled. The enzyme collected from this stage had little or no activity.

3.11 Protein determination

Protein content of the crude extract and eluted fractions was determined according to the procedure of Lowry et al (1951). Bovine serum albumin was used as standard. The reagents used were:

A. 50% Folin-Ciocalteau reagent (1:1% H_2O)

B. 2% Sodium potassium tartarate

C. 1% $C_uSO_4.5H_2O$

D. 2% Na_2CO_3 in O.IN NaOH

E. Bovine serum albumin (BSA) stock solution contained 200mg BSA/ml.

Standard solutions of BSA (Concentration range O-200mg/ml) were place in different test tubes; 5ml of a mixture (50ml reagent D plus 0.5ml each of B and C) was added to each test tube which was let to stand for 10 minutes at room temperature, after thorough mixing. Then

0.5ml of the diluted Folin - Cicocalteau reagent (freshly prepared) was added to each tube, which was then shaken vigorously and allowed to stand for 30 minutes for colour development. Absorbance of the contents of each tube was read at 750nm against a blank which had no BSA. A calibration curve was prepared by plotting absorbance readings against (BSA) concentration. (Appendix 2A).

The protein content of enzyme extracts was determined in the same way and the corresponding protein concentrations were read from the calibration curve.

3.12 Assay of Linamarase Activity and Kinetic constants

3.12.1 Assay protocol

p-Nitrophenyl-β-D-glucoside (PNP-glucoside) and linamarin were used as substrates for the enzymes. Assay of linamarase activity with PNP-glucoside as substrate was conducted according to the method described in Ikediobi *et al*. (1980). One millilitre of a solution containing 10. 50 mg PNP-glucoside in 0. 01M sodium phosphate buffer pH 6.8 was incubated for one hour at room temperature (26 ± 2^{0}C) with 0.1ml of the enzyme. At the end of incubation period, the reaction was stopped by addition of 0.2M borate buffer pH 9.8. The reaction solution was then made up to 4ml with distilled water and the absorbance of the resulting solution read at 400nm on a spectrophotometer (model W.P.A-S101). One unit of linamarase produced 1 mole of p-nitrophenolate ion per minute

under the described assay condition. ϵ_{400} for para-nitrophenolate is 1.81×10^4 cm^{-1} M^{-1}). Assays were carried out in duplicate.

3.12.2 Determination of Kinetic Constants (Km and Vmax)

Twenty-one milligrammes p-Nitrophenyl-β-D glucoside (PNP-G) was dissolved in 7ml of 0.01M sodium phosphate of pH 6.8. This served as the stock solution (10mM). The stock solution was diluted to lower concentrations (2.50-0.50mM):

Three millilitres of the appropriately diluted substrate was placed in a cuvette and 0.05ml of the enzyme preparation was added and mixed. The cuvette was then placed in the spectrophotometer and the absorbance was read at 400nm at twenty-second intervals. The absorbance readings were used to calculate activity of linamarase (see Appendix 1A). Double reciprocal plots showing effect of PNP-G concentrations on linamarase activity were drawn from the data obtained. The assays were carried out in duplicate at each substrate concentration.

3.12.3 Effect of pH on Kinetic constants

For pH 4.5 - 5.5, 0. 1M acetate buffer was used for preparing the different substrate concentrations using the acetate buffer at appropriate pH. For pH 6.0-8.5, 0.01M phosphate buffer was used for preparing all the different concentrations using the buffer at appropriate pH. For pH 9.0-

10.0, 0.1M borate buffer was used for preparing substrate concentrations. Preparation of substrate at different concentrations was as described in (3.13.2)

For each pH and for different substrate concentrations of substrate, 3ml of substrate solution was placed in a cuvette and 0.05ml of purified enzyme added and mixed, and the cuvette placed in the spectrophotometer. Absorbance readings were taken at 400nm at 30-second intervals. Double reciprocal plots showing the effect of PNP-G concentration on linamarase activity at different pH values were drawn from the data. Graphs of pKm versus pH, log Vmax versus pH, and log Vmax/Km versus pH were plotted.

3.12.4 Effect of Temperature on Kinetic Constants

The different substrate concentrations were as described in (3.13.2). For each temperature tested (26 ± 2 - 47^{0}C), 1Ml of the substrate was placed in the assay tubes and the assay placed in a hot water bath at the appropriate temperature to equilibrate. 0. 05ml of purified enzyme was added, and the assay tube placed back in the water bath at the appropriate temperature. The reaction was stopped after 30-second using 0.20M borate buffer at pH 9.8. Absorbance was read at 400nm on the spectrophotometer. Double reciprocal plots showing the effect of PNP-G concentrations on linamarase activity at different temperatures were constructed from the data. Graph of pKm, log Vmax and log Vmax/Km versus reciprocal of absolute temperature were also plotted from the data obtained. All

assays were carried out in duplicate.

3.12.5 Effect of D-gluconolactone on kinetic constants

Ten millimolar stock solution of PNP-G was prepared as described in 3.13.2. Eighteen milligrammes of D-gluconolactone was dissolved in 10ml phosphate buffer of pH 6.8. This gave a solution of 10mM concentration. To fixed substrate, PNP-G concentrations, different concentrations of D-gluconolactone (0.20 -1.0mM) were added. 0.05ml of purified enzyme was added to the mixture of PNP-G + D-gluconolactone in a cuvette and mixed. The absorbance at 400nm was read at intervals of 30 seconds. Double reciprocal plots showing the effect of D-gluconolactone on linamarase activity at different concentrations of PNP-G were drawn from the data. Assays were carried out in duplicate.

3.13 Effect of temperature on linamarase activity

One millilitre of a solution containing 10.50mg PNP-glucoside in 0.01M sodium phosphate buffer pH 6.8 was incubated for 20 minutes at different temperatures (26 $\pm$ 2 - 52.0^{0}C), with 0.1ml of the enzyme. At the end of the incubation period, the reaction was stopped by addition of 2ml of 0.2M sodium borate buffer pH 9.8. The final volume was brought up to 4ml with distilled water and the absorbance read at 400nm in the spectrophotometer. Assays were in duplicate. Arrhenius plot was constructed from which the energy of activation was determined.

3.14 Effect of Various Chemicals on Linamarase Activity

0.9ml of 5mM PNP-G was placed in several test tubes containing 0.1ml of the following reagents in duplicate.

1. 15. 3mM potassium cyanide
2. 10. 30mM L-cysteine
3. 9. 52mM citric acid
4. 10.0mM E D T A
5. 21.4mM sodium chloride
6. 10.0mM β-D-galactoside
7. 10.0mM α-D-glucoside
8. 10.0mM sodium metabisulphite

0.1ml of the pure enzyme was added to each test tube, and after 5 minutes, 2ml of 0.2M borate buffer pH 9.8 was added, to stop the reaction. Absorbance was taken at 400nm after the final volume was adjusted to 4ml. The relative activity was determined from the absorbance reading obtained.

3.15 Calibration of Cyanide Standard Curve (Cooke et al. 1978)

A standard stock solution of 3.2 mM KCN containing 80mg CN-per ml corresponding to 0.1ml to 0.6ml were measured into different stoppered test-tubes. Each was made up to 0.5ml with distilled water. 0.6ml of 2N sodium hydroxide was added and the contents mixed. 6.8ml of 50mM sodium phosphate buffer was added followed by O. 5% (W/V) Chloramine T. The tubes were mixed and stood in iced water for 5 min. 1.6ml of a solution containing 0.2g bispyrazolone 3,3'dimethyl-1, 1'-diphenyl- (4,4' -bis-pyrazoline) - 5,5'-dione) (4,4' -bi-2-pyrazoline) -5,5' -

dione) and 1 gram of 3-methy 1-1-phenyl 1-5 -pyrazolone in 200ml pyridine was then added and mixed. Absorbance of the blue colour developed after storage for 90 min at room temperature and was measured at 620nm. A calibration curve was prepared by plotting absorbances against concentrations of CN- (Appendix 2B).

3.16 Preparation of Cassava and Cassava Product Extract for cyanide Analysis

Twenty grammes of fresh cassava parenchymal tissue was homogenized in 50ml of 0.1M orthophosphoric acid for 3 min. The homogenate was centrifuged. The supernatant was saved and stored in the freezer until analyzed. To cassava chips, cassava powder, or garri was added 0.1M 0-phosphoric acid and these were allowed to stand for 30min to achieve rehydration of the cassava or cassava product in a sealed beaker. After rehydration, the cassava or cassava product was homogenized and extracted.

3.17 Quantitative determination of free and total Cyanide in cassava and cassava products

About 0.1 - 0.2ml of cassava extract or its product extract was neutralized with 0.2-0.4ml, 0.2M sodium phosphate buffer pH 7, and incubated with 0.1ml of linamarase preparation containing 0.3-0.8 unit of activity for 10-15 minute at room temperature, in a tall stoppered test tube. The reaction was stopped after 30 minutes by addition of 0.6ml of 2N NaOH. The HCN released was estimated by a modification

of Epstein's method as described in Cooke et al. (1978) (see 3.15 above) The determination of free cyanide was performed as described above, except that the exogenous linamarase preparation was not added to the aliquot of the extract to be analyzed for free cyanide. Instead an equivalent amount of 0.2M sodium phosphate buffer was added. The difference between the total and free cyanide gave the bound cyanide or unhydrolyzed cyanogenic glucosides.

CHAPTER 4

Results

4.1 β-D glucosidase production from some yeast stains

The yeast stains screened; Condida magnoliae, Candida rhhaggi, Saccharomyces kluyveri Saccaharomyces cerevisiae sp, and Kluyveromyces marxianus were grown in yeast extract broth. Samples were taken out and assayed for β-D glucosidases daily for 10 days.

Maximum β-glucosidase activity was obtained on the 5th day for all the strains. But on the 8th day, there was further increase in β-glucosidase activity for Candida magnoliae Kluyveromyces marximus and Candida rhaggi. The results in Appendix 2.2 represent β-glucosidase activity as well as the enzyme specific activity, for each organism.

4.2 β- D- glucosidase production from some yeast isolates from burkutu, pito, palm wine and grape fruit.

Twenty five yeast isolates from burkutu, pito, (locally brewed wine), palm wine and grape fruit were grown in yeast extract broth. β-D-glucosidase activity was assayed for in the medium daily. Maximum activity for each isolate was obtained after 4 days.

Results in Appendix 2.3, 2.4, and 2.5 show that the yeast isolates; G II, $GIII_2$ BIII, BIV, B II_4, BII_3, BI_3, $BIII_3$, BIV_4, PI_3, PII_3, PII_4, PVI, PV_3, PI_1, PII_2, PI_2, $PWII_2$, PW_1II_2, $PWII_2$,

PWI_3, PWI_2, $PWII_3$, $PWII_5$ produced little or no β-D-glucosidase even after including malt in the (yeast extract) growth medium (Appendix 2.4), and reducing the pH (to make the growth media more acidic).

4.3 Enzyme purification

Table 4.7 shows the purification data of linamarase from dried cassava cortex. The total activity in crude extract was high, 11, 100 μmole/min. Fractionation of the crude extract with 62% $(NH_4)_2SO_4$ gave an 11 fold increase in specific activity and 47% yield. Gel filtration Chromatography gave an early elution of the enzyme between factions 6 and 10 (Fig. 4.1)). This step gave a 97 fold increase in specific activity and an 8-fold increase in the previous step, $(NH_4)_2SO_4$ fractionation step. Most of the enzyme activity was lost on the ion exchange chromatography column during the DEAE Sephadex step (Table 4.7).

4.4 Effect of substrate concentration on linamarase activity

Fig. 4.2 shows the effect of substrate concentration on linamarase activity with paranitrophenyl-β-D- glucoside and linamarin as substrate respectively. Their Km values are 0.75mM and 2.22mM respectively.

4.5 Effect of inhibitor (D-gluconolactone) on linamarase activity

The inhibition of linamarase activity by D-gluconolactone is linear competitive (Fig 4.3). The inhibition constant Ki

is 2.22mM

4.6 Effect of pH on linamarase activity

The pH activity profile is shown in Fig 4.4. Lineweaver-Burk plots to determine Vmax and Km of linamarase at different pH values are shown in Fig 4.5A, B, C, and D (Appendix 4.5A_1 and A_2, 4.5B, 4.5C_1 and C_2, 4.5D_1 and D_2). The kinetic constants; Km and Vmax were deduced from each graph. Graph of log Vmax/Km versus pH, pKm versus pH and log Vmax versus pH were constructed (Fig. 4.6. A,B, & C). From the graph, the following pK values were deduced: 8.4, 6.2 and 7.2, 6.3. These values indicate that groups whose pKa corresponds to these ones may be present at the active site.

4.7 Effect of temperature on linamarase activity

Lineweaver-Burk plots of reciprocal initial velocity data to determine Vmax and Km of linamarase at different temperatures are shown in fig 4.7 A,B,C,D and E (see Appendix 3.5, 3.6, 3.7, 3.8, 3.9).

Arrhenius plots for the determination of the energy of activation of linamarase-catalyzed hydrolysis of PNP-glucoside are shown in Fig. 4.8 A (pKm against reciprocal of absolute temperature), 4.8B (log Vmax against reciprocal of absolute temperature), 4.8C (log Vmax/Km against reciprocal of absolute temperature).

4.8 Effect of various chemicals on linamarase activity

Table 4.8 shows the effect of various chemicals on linamarase activity; potassium cyanide and citric acid have strong inhibitory effects on linamarase. Sodium metabisulphite, NaCl, EDTA, L-cysteine, phenyl-β-D-galactoside and phenyl-α-D-glucoside had no effect on the activity of linamarase.

4.9 The use of linamarase in the determination of the cyanide content of some cassava products.

Table 4.9 shows the cyanide content of some cassava products and fresh cassava in parts per million (ppm). The fresh cassava was kept for 2 days at room temperature, (in transit from the south-eastern part of Nigeria) and 4 days at 4^0C (Refrigeration temperature). The cyanide content was calculated in parts per million (ppm).

Table 4.7 PURIFICATION TABLE OF LINAMARASE FROM DRIED CASSAVA CORTEX

Stage	Vol. (ml)	Protein conc. (mg/ml)	Total prot.	Activity (μmole/min.)	Total activity μmole/min.)	Specific Activity μmole/mg	Purification fold	Yield (%)
1. Crude Extract	150	2.60	390	74	11,100	28.46	1	100
2. Ammonium Sulphate fraction	15	1.10	16.50	347.8	5,217	316.18	1211.09	47
3. Gel filtration on sephadex G-150	10	0.16	1.60	444	4,440	277.5	97.36	40
4. DEAE-SEPHADEX		0.03		1.85		62		

Table 4.8 Effect of various chemicals on linamarase activity

Chemical	*Relative Activity %
Control	100.00
KCN	41.47
Sodium metabisulphete	100.00
Sodium chloride	103.10
Citric	45.00
EDTA	101.55
L-cysteine	103.10
Phenyl-β-D-galactoside	100.00
Phenyl-α-glucoside	100.00

* The concentration of each chemical was between 9.50 - 10mM except KCN and NaCL which are 15.3mM and 21.4mM respective. The different concentrations did not affect the relative activity of linamarase.

Table 4.9 Cyanide content of fresh cassava and some cassava product

Product	Use	Cyanide content (ppm)
White sour gari	Human food	17.20
White gari	Human food	29.51
Red gari	Human food	26.72
Lafun (fermented and dried cassava flour)	Human food	48.72
Lafun (After 5 months)	Human food	35.85
Cassava treated with O-phosphoric acid and dried at 90^0C	Research	271.74
Fresh cassava (kept for 2 days at room temperature) and 4 days at 4^0C	Comparison	246.5

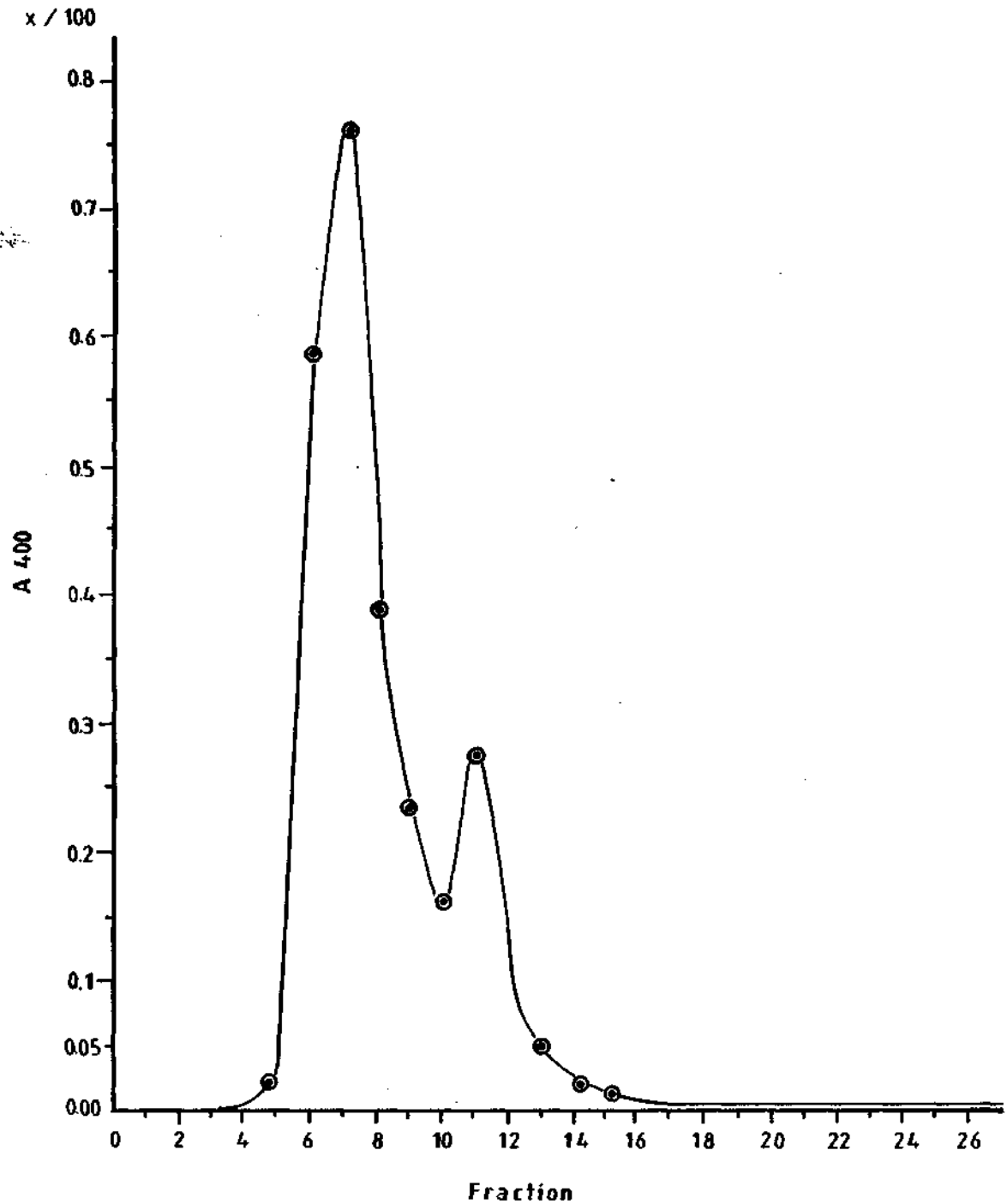


Fig. 4.1. Gel filtration profile of cassava cortex linamarase on sephadex G-150 column. The enzyme preparation from the $(NH_4)_2 SO_4$ step (10ml) was applied to sephadex G-150 column (2.6 x 40cm) pre-equilibrated with 0.01M sodium phosphate buffer of pH 6.80. Elution was with the same buffer and 6ml fractions were collected ; -⊙—⊙- enzyme activity (A_{400}).

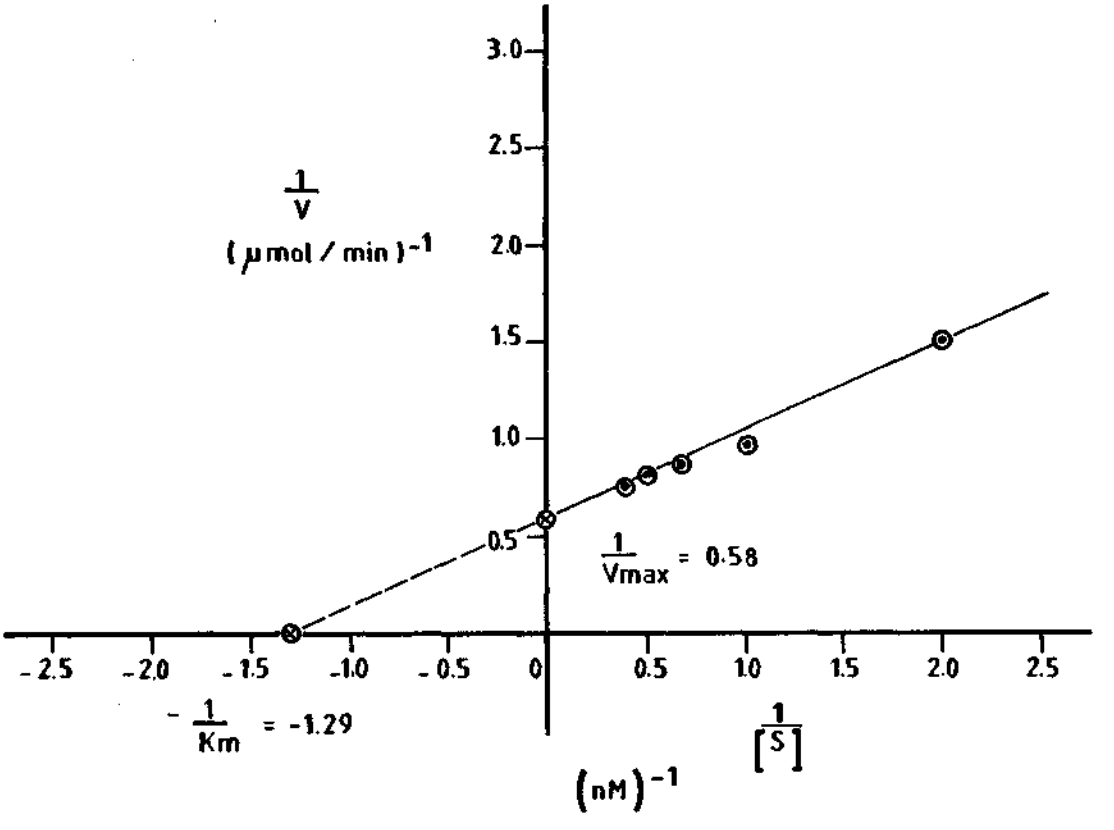


Fig. 4.2A Double reciprocal plot showing the effect of PNP glucoside ; ⊗——⊗——⊗ concentration on linamarase activity, ⊗--------⊗ calculated value . (Line of best fit was by least square method).

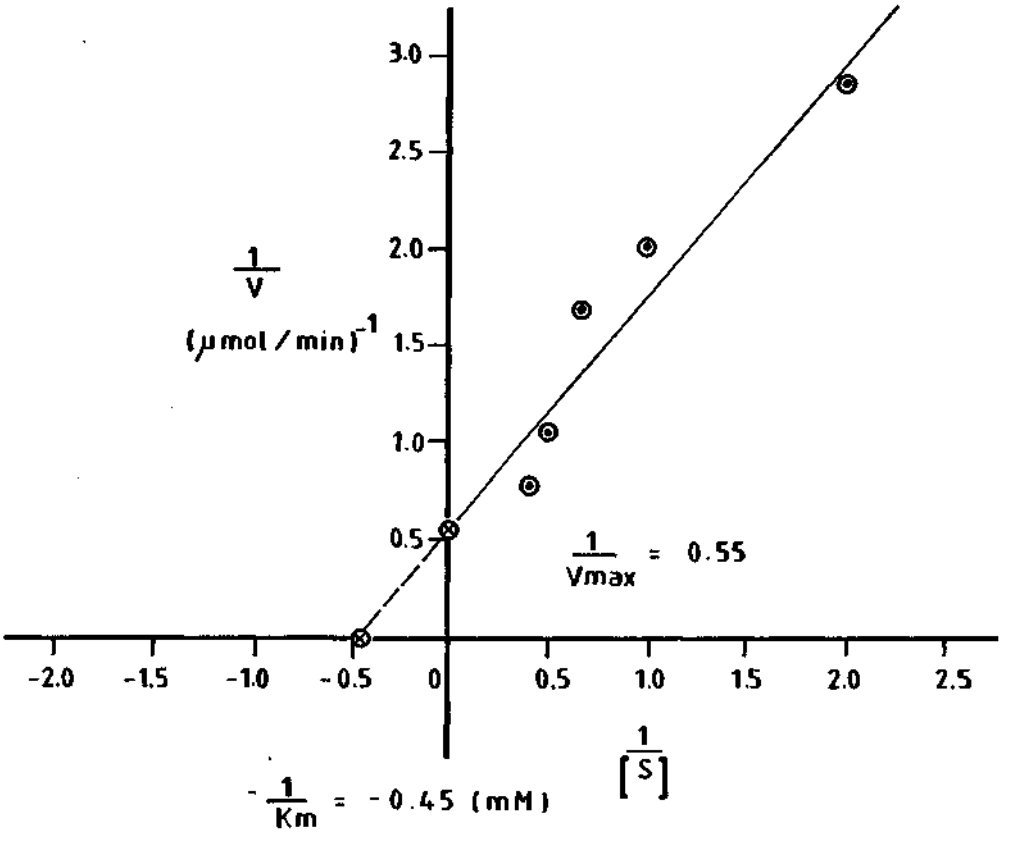


Fig. 4.2B. Double reciprocal plot showing the effect of linamarin; ⊙——⊙——⊙ concentration on linamarase activity, ⊗- - - -⊗ calculated value. (Line of best fit was by least square method).

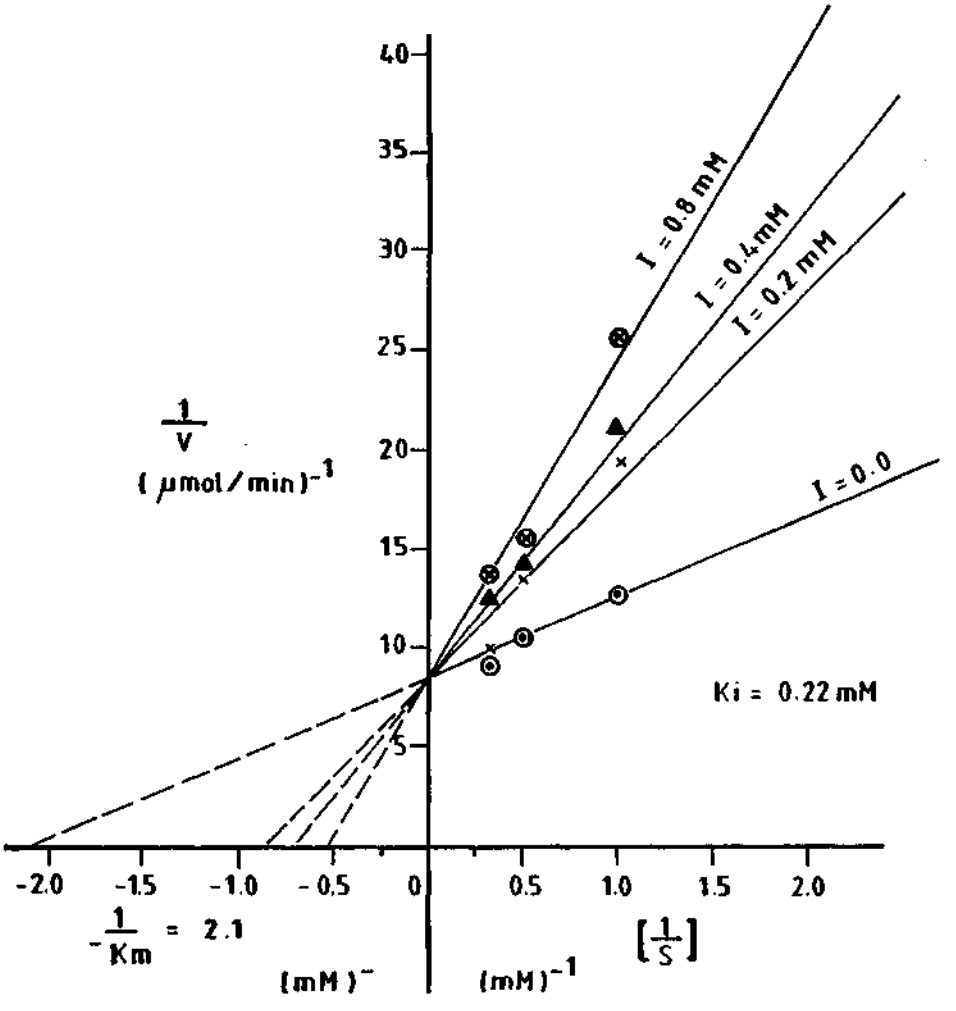


Fig. 4.3. Inhibition of linamarase by D-Gluconolactone at the following concentrations : ⊗ = 0.8 mM; ▲ = 0.4 mM; x = 0.2 mM; ⊙ = no inhibitor.

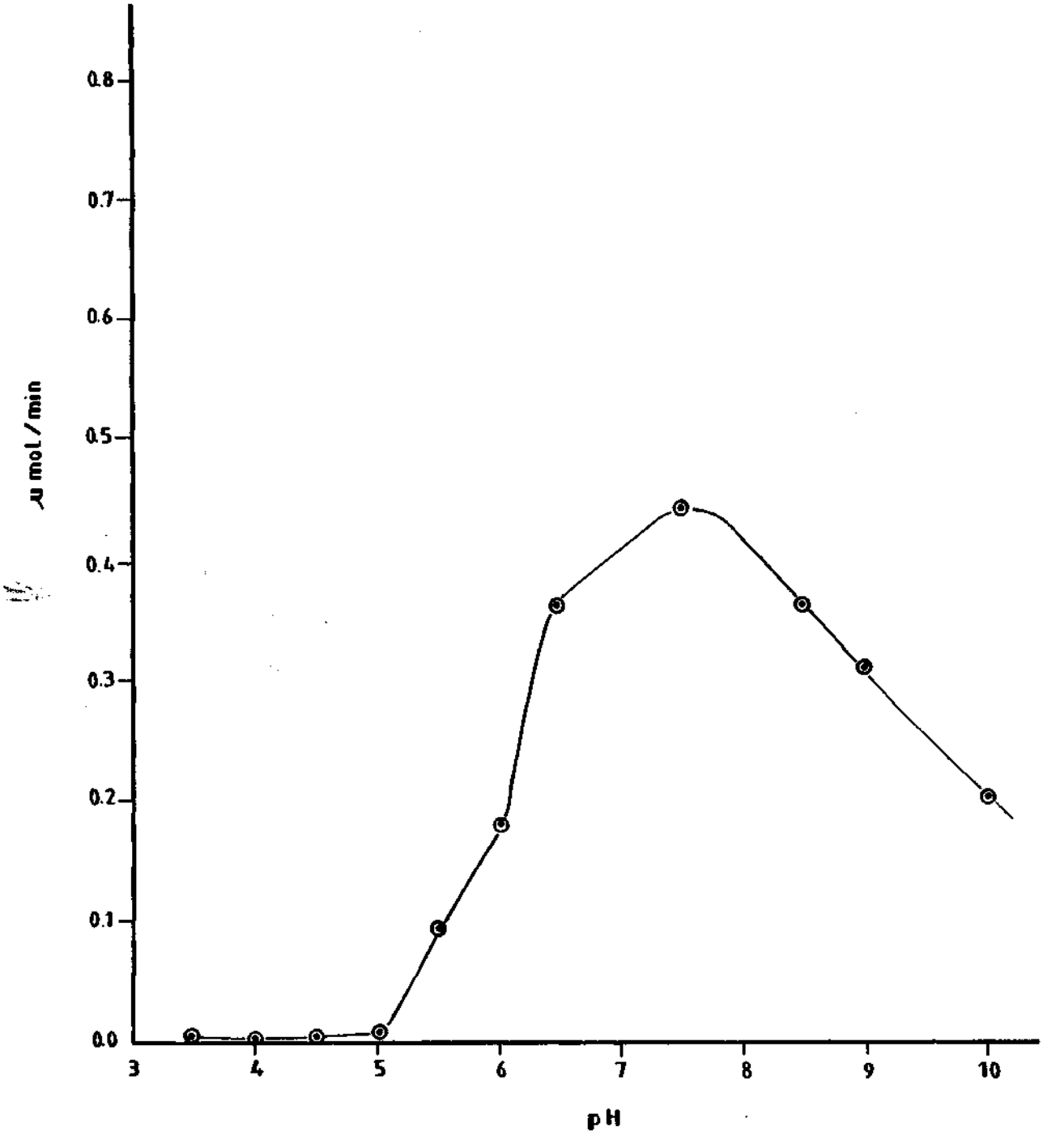


Fig. 4.4. pH - Activity profile of linamarse .

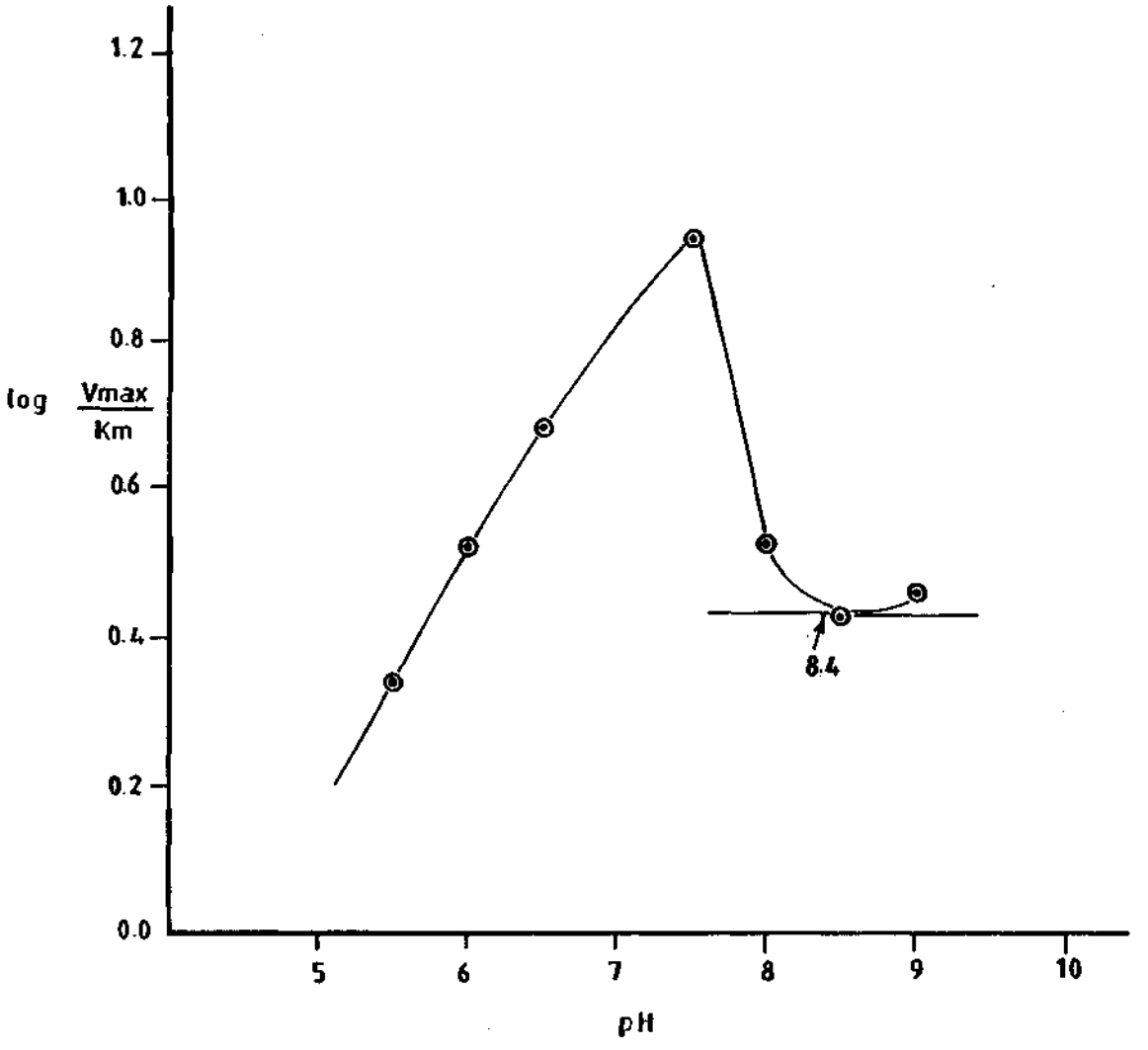


Fig. 4.6A. Effect of pH on log $\frac{Vmax}{Km}$

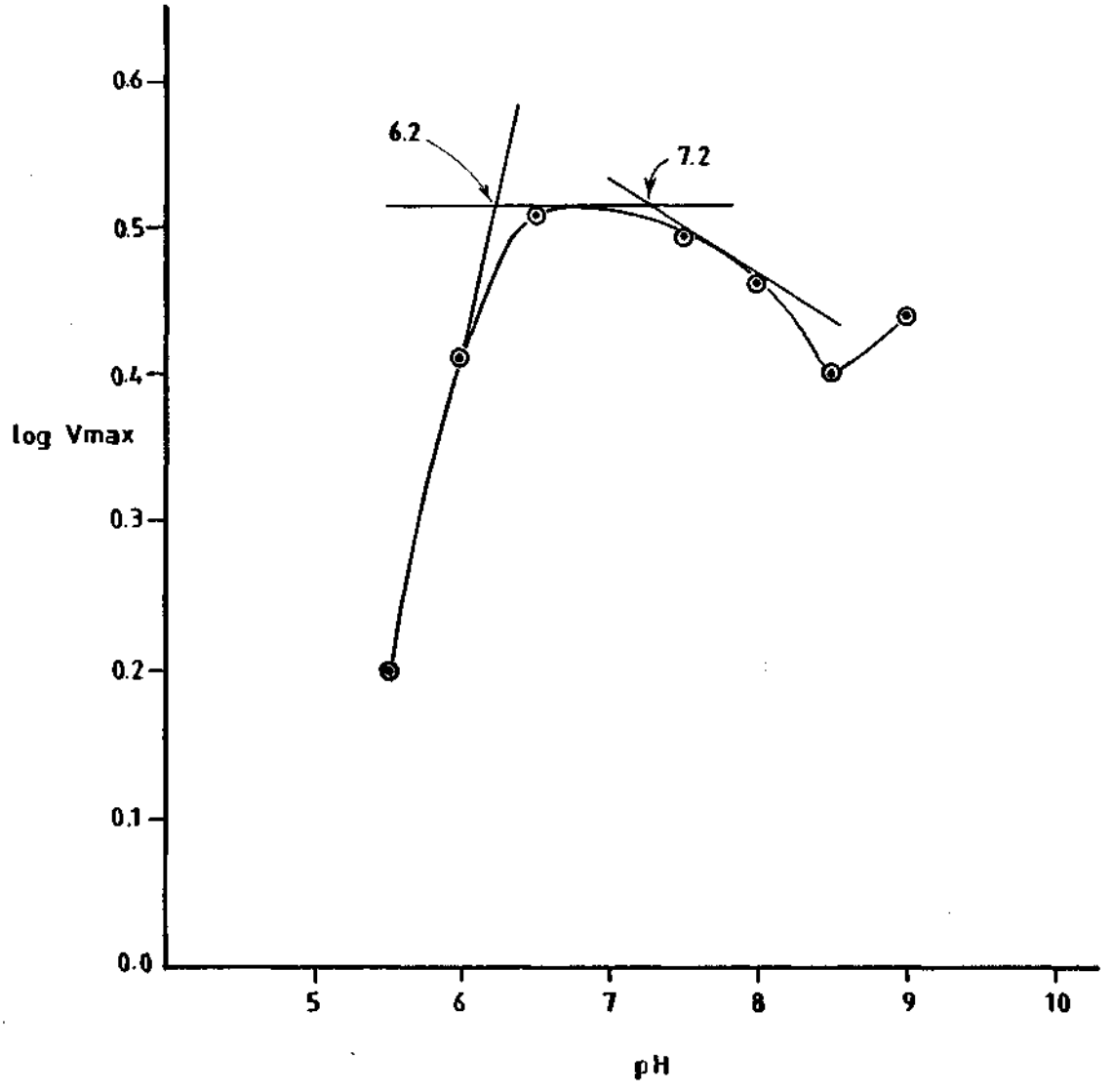


Fig. 4.6B. Effect of pH on log Vmax.

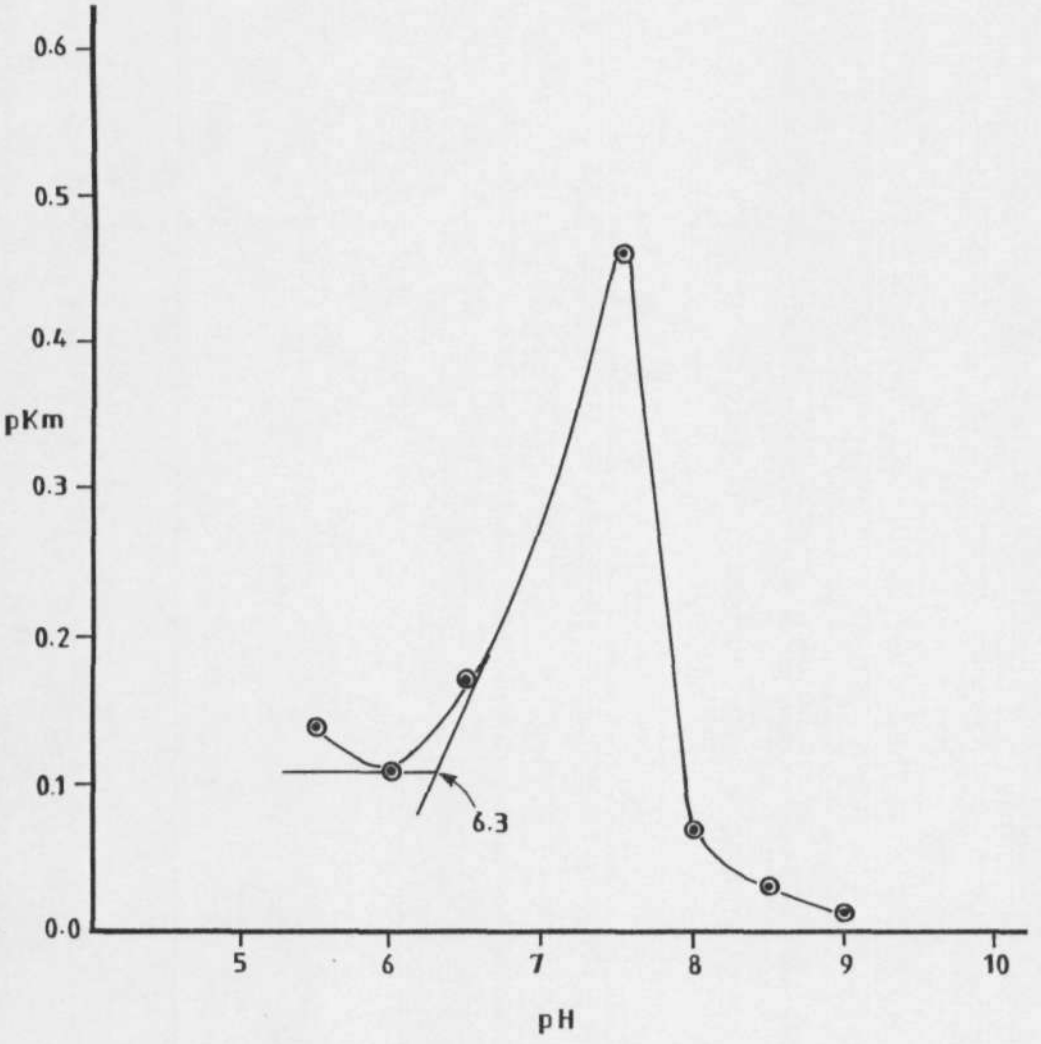


Fig. 4.6C. Effect of pH on pKm.

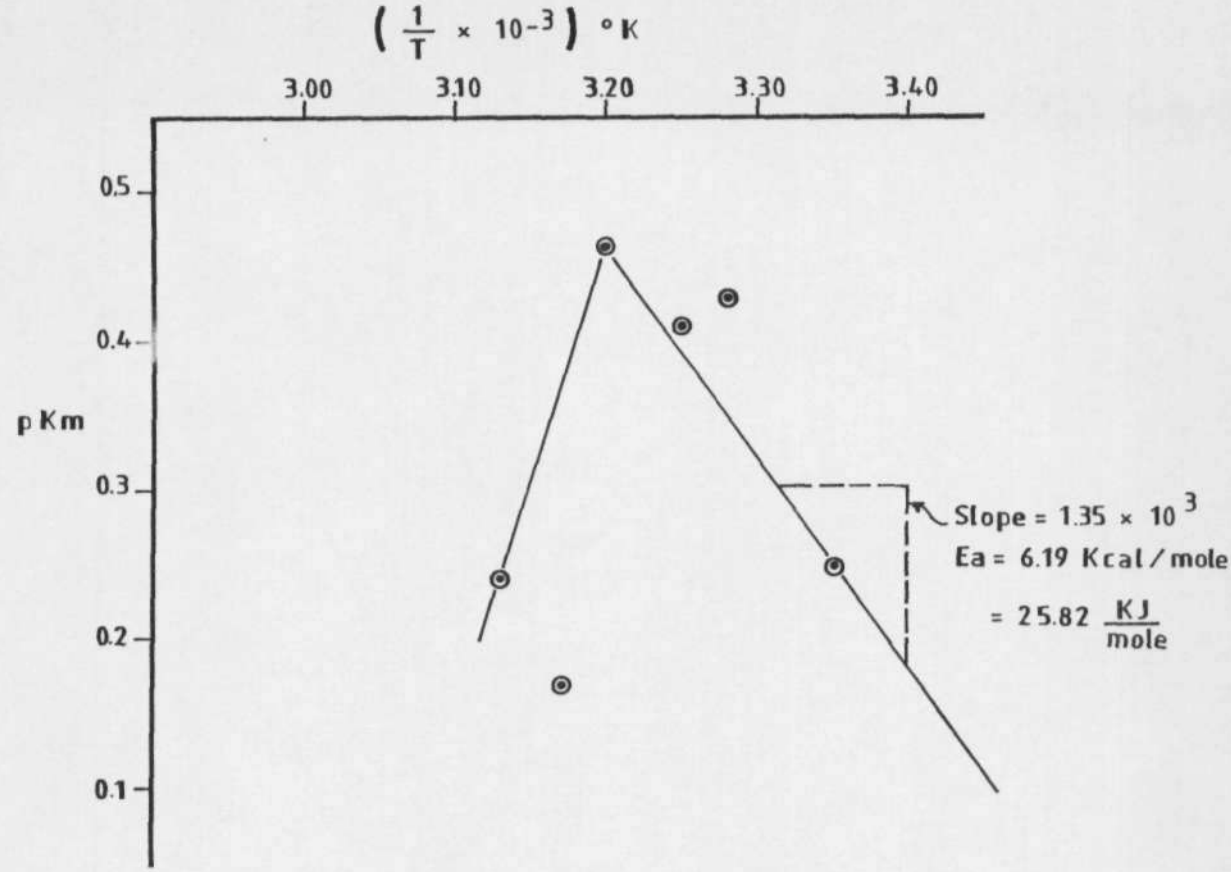


Fig. 4.8A. Effect of reciprocal of absolute temperature on Km.

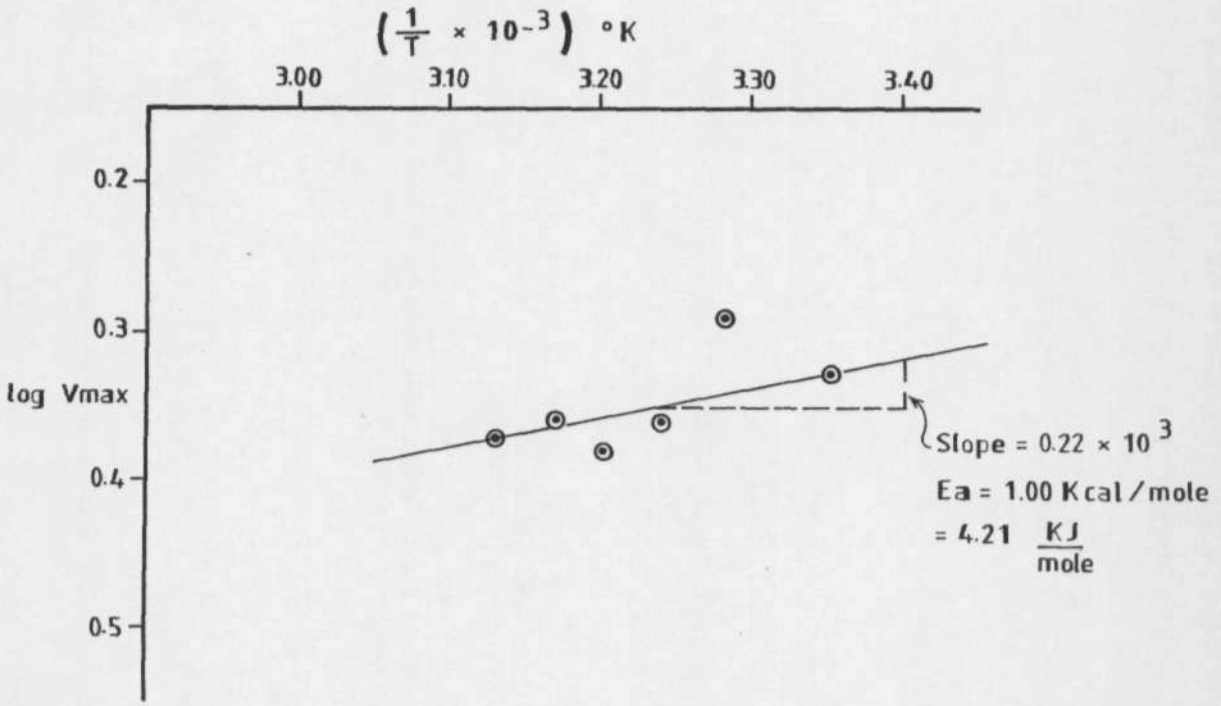


Fig. 4.8B. Effect of reciprocal of absolute temperature on Vmax.

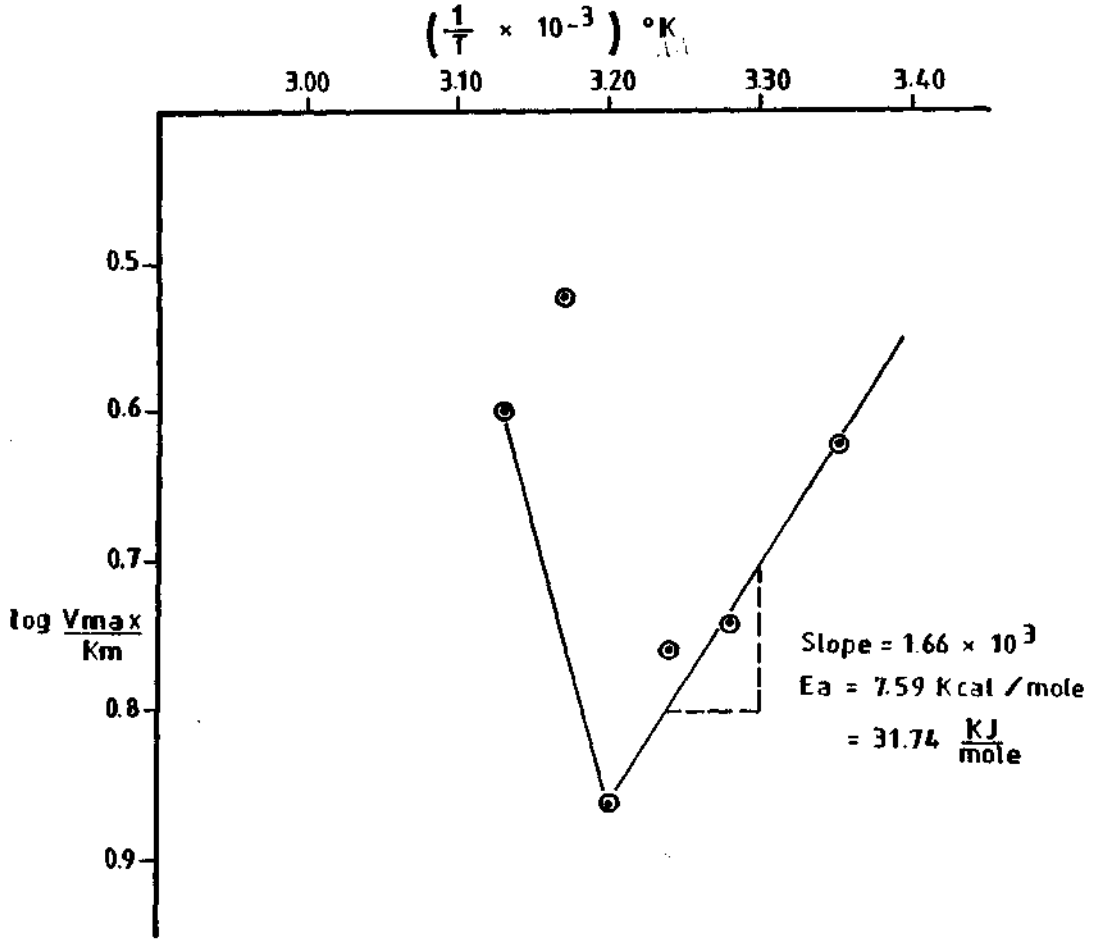


Fig. 4.8C. Effect of reciprocal temperature on $\frac{Vmax}{Km}$

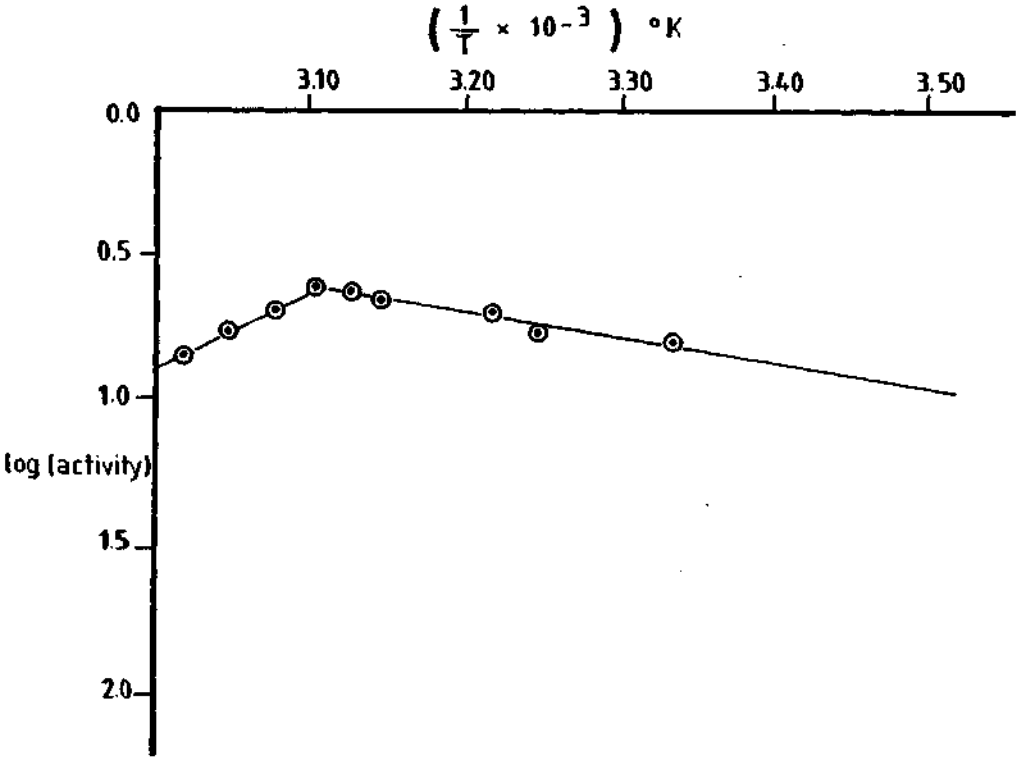


Fig. 4.8D. Effect of temperature on activity.

CHAPTER 5

DISCUSSION

5.1 Linamarase production by yeast strains and isolates.

The 6 yeast strains and 24 yeast isolates from burkutu, pito, palmwine, and grape fruit could not produce linamarase activity, neither could they produce substantial β-glucosidase activity (see Appendix 2.2 and 2.3 & Tables 4.2, 4.4). One of the yeast strains, Candida magnoliae produced β-glucosidase activity slightly higher than the other strains screened, but it did not hydrolyze linamarin.

The need to search for an inexpensive convenient, economical and safe source of linamarase arose following the work of Cooke and colleagues (Cooke, 1978; Cook et al, 1978) who introduced the used of linamarase in estimation total cyanide in cassava tubers and cassava leaves. The work of Ikediobi, et al (1980) also increased impetus for this search by incorporating an enzymatic step to make the assay more specific and quantitative. In addition, Ikediobi and Onyike (1982b) have successfully used exogenously added linamarase preparations to accelerate detoxification of grated cassava during fermentation to produce virtually cyanide-free gari. Several unsuccessful attempts have been made to produced linamarase through a microbial source. Some of these attempts are embodied in the work of Ikediobi and Onyike (1982b), Ikediobi and Ogundu (1985), Ikediobi, et al (1987). These

researchers screened and characterized some fungal isolates. Five of the 17 fungal isolates screened showed linamarase activity, and one of them, the enzyme for Fusarium equiseti, was characterized (Ikediobi et al, 1987). Recently, however, it has been shown that Fusarium equiseti enzyme can not hydrolyze linamarin, but could only hydrolyze para-nitrophenly-β-D- glucoside (Ogundu, 1988). So, its enzyme is not a linamarase but, rather a β-glucosidase.

Okafor (1977) isolated bacteria and yeast from cassava pulp. The bacteria were identified as Leuconostoc spp, lactobacillus spp, Alcaligenes spp, Corynebacterium spp, while the yeast isolates were all Condida species. Okafor and Ejiofor (1985) have also produced and partially characterized linamarase from Leuconostoc messenteroides.

There is very little information on the production of linamarase by yeast. Thus more work is needed in this area, including screening for more yeast isolates from different sources, and also inducing some others to produce the enzyme. Other areas of investigation to address include monitoring the conditions that can best lead to induction of the enzyme. Recently, molecular biology research on linamarase production is in progress, (personal communication, Ikediobi, 1991). This involves isolation of the gene that codes for linamarase from cassava, with a view to cloning it in a suitable organism.

5.2 Linamarase from cassava cortex

Presently, cassava itself is best source of linamarase. Cassava peel (cortex) which is normally discarded has now been shown to be a good source of linamarase. About 200grammes of dried powdered cassava cortex is capable of producing 1.6mg linamarase with specific activity of about 2,700 µmole/mg. This can be used for the assay of 2,000 samples of different cassava products. The activity of linamarase in 0.01M sodium phosphate buffer pH 6.8 decreases with time, even when kept in the refrigerator at 4-5^{0}C. When kept in the refrigerator for one week, 70-80% of linamarase activity is retained; when kept for two weeks, 50% activity is retained while 50% is lost. When kept for about 4 weeks (one Month) only 10-15% activity is retained.

The Cassava products assayed have substantial quantities of cyanide in them thus "Gari" has 26-30 ppm while "lafun" has 40-45 ppm, whereas "sour gari" which is generally considered to have little or no bound cyanide has 17.20 ppm (Table 4.9). With the use of purified linamarase, it has been possible to determine the exact quantities of bound cyanide in these cassava products. This will help the consumer to know on average how much bound cyanide he has taken in from a specific product at a specific time, and the manufacturers to seek to improve their processing techniques to achieve a zero cyanide level.

Simple processing techniques like boiling and sundrying and addition of water to crushed cassava, have been shown to

substantially decrease the bound cyanide of cassava (Cooke and Maduagwu, 1978; Maduagwu and Adewale, 1980; Nambisan and Sundaresan, 1985; El Tinay et al, 1984; Maduagwu and Oben, 1981). Infact, Nambisan and Sundaresan (1985) specifically found that the most effective method for cyanogenic glucoside removal was crushing fresh tubers and subsequent sundrying, where 95% of the cyanogenic glucoside was eliminated. The 5% cyanogenic glucoside that remains in the cassava product after crushing and sundrying is substantial when the cassava is the bitter variety and also when chronic cyanide toxicity is considered.

None of the processing techniques have been able to remove all the cyanogenic glycosides in cassava. The reported values of cyanogenic glucosides in cassava products vary because of differences in varieties of cassava used, and also because of the methods of cyanide determination (Zitnak, 1973, Cooke and maduagwu, 1978). Therefore linamarase is a crucial requirement for quality control of any cassava food product in the manufacturing industry. Since its use will make determination of the quantity of the bound cyanide in any cassava food product uniform.

More information is needed on the implication of consuming cassava products especially "gari" and "lafun" without knowing the quantity of cyanogenic glucoside in them. Many consume 'gari' because it is the cheapest source of calories. There is an urgent need for quality control for all cassava products sold in the local markets. Consumers have a

right to know what they are eating.

Consuming large quantities of cassava products leads to gradual accumulation of small quantities of cyanide in the system. This could lead to chronic cyanide intoxication, which is not readily obvious at the onset (see 2.7).

5.3 Purification of linamarase

Most of the linamarase was salted out between 60% and 65% ammonium sulphate saturation. The 11 fold purification obtained in this step is close to the 14 fold reported by Cooke et al (1978) for the fresh cassava cortex enzyme. Gel filtration chromatography (Fig 4.1) revealed an early elution of the enzyme between fractions 6 and 10. This is due to its high apparent molecular weight, 600,000 daltons (Cooke et al, 1978) which is outside the exclusion limit of sephadex G-150. There was a 9-fold increase in the previous purification step, $(NH_4)_2SO_4$ fractionation. This is as a result of the elimination of almost all the unwanted proteins. Ion-exchange chromatography of the enzyme, unlike gel filtration yielded an enzyme that had lost most of its activity. The reason for the loss in activity was associated to the working condition of the experiment. 3 other trials were made and they produced much reduced activity. Ion-exchange chromatography on the DEAE sephadex column after dialysis yielded a poorer result in the form of further loss in activity of the enzyme. Reports in the literature have shown that this step does not always give high purification. Thus Cooke et al (1978) showed that

DEAE cellulose step gave a 2 fold purification from the previous step, the ammonium sulphate fractionation step, whereas the sepharose 6B gel filtration step gave a 25 fold purification from the ammonium sulphate step, and 350 fold purification with 35% yield. Ikediobi et al 1987 obtained 56 fold purification with 3.9% yield after DEAE sephadex step. Nok et al, (1989) obtained 148 fold purification with DEAE sephadex and the yield was 29%. Butler et al, (1965) found linamarase from both DEAE-cellulose and sephadex columns to be still contaminated with polysacharides, but found column electrophoresis on sephadex G-25 to be a much better technique for linamarase purification. Ninety-seven fold purification was obtained in the present work. The working conditions for this work contributed to the kind of results obtained. The high temperature of the room ($26\pm2^{0}C$) possibly led to the loss in activity before the enzyme was assayed. Enzymes are more stable when they contain some impurities and very unstable at higher temperature in their pure state (Ukoha and Ikediobi, 1985).

5.4 Effect of substrate concentrations and inhibitors on linamarase activity

Linamarase from cassava cortex follows the Michaelis-Menten kinetics with Km 0.75mM with respect to paranitrophenyl glucoside and Km 2.22mM for linamarin.

D-gluconolactone known to be a potent inhibitor of plant glucosidases (Mao and Anderson, 1967) was first observed to interfere with the detoxification of grated fermenting cassava

during "gari" manufacture (Ikediobi and Onyike, 1982b) presumably by inhibiting the endogenous detoxifying enzyme, linamarase.

Fig 4.3 presents further evidence that the inhibition is linear competitive.

5.5 Effect of pH on kinetic constants

The optimum pH was found to be 6.5 (Fig 4.2 A & B and 4.6A, B & C). This is in agreement with other reports in the literature (Wood, 1966; Cooke, 1987; Ikediobi and Onyike, 1982a; Ogundu 1988). In Fig 4.6 A, B and C are possible curves for a diprotic system (Dixon and Webb, 1964; Segel, 1975). Fig 4.6A indicates that a group with pKa value of 8.4 may be present in the enzyme forms that exist within the pH range examined (Dixon 1953). Fig 4.6B on the other hand shows that groups with pKa values 6.2 and 7.2 are involved in the ionization of the enzyme substrate complex, while fig 4.6C appears to implicate group with pKa 6.30 in the ionization of free enzyme. Therefore, if linamarase exists in two ionizable forms and the substrate PNP-glucoside is not ionized (medium fairly acidic) it would seem that the side chain groups with pKa values 8.4, 6.2-7.2 and 6.30 are involved in the catalysis by the enzyme. These groups could be aspartate or glutamate (pKa 4.0±0.3) or Histidine (pKa 6.2-7.2).

5.6 Effect of various chemical substances

Potassium cyanide and citric acid reduced the activity of linamarase drastically (Table 4.8). These are therefore potent inhibitors of linamarase. Other chemicals like sodium metabisulphite, sodium chloride, Phenyl-β-D galactoside and phenyl α-D-glucoside showed no inhibition on linamarase activity. Reduction by citric acid indicates the presence of a metal cofactor in the enzyme linamarase. Potassium cyanide inhibitory action on linamarase suggests that a possible product inhibition regulatory mechanism may be in operation. In its natural environment, the enzyme reacts, with cyanide from linamarin or lotaustralin. The cyanide then decreases the activity of the enzyme.

5.7 Effect of temperature on linamarase activity

The effect of temperature on enzyme activity is a composite of two effects:

(a) The effect on the chemical reaction catalyzed by the enzyme and (b) The effect on the enzyme as a protein molecule. Linamarase was found to be stable up to 48^0C when incubated with substrate for 20 minutes at appropriate temperature. When initial velocity experiments were carried out, it was found to be stable up to 36^0C. The Arrhenius plots of the temperature - activity data showed a biphasic curve, (Fig 4.8D) which is explicable in terms of enzyme protein denaturation above the critical temperature 48^0C. The Increase in temperature imparts more kinetic energy to the

reactant molecules which results in production of more useful collision per unit time. As the temperature further increases the molecules absorb too much energy, the tertiary structure will disrupt and the enzyme will be denatured, that is loose activity. Thus as the temperature increases, the expected increase in rate of production resulting form increased enzyme and substrate collisions is offset by increasing rate of denaturation (Laidler and Branko 1979; Segel, 1975). In Fig 4.8A the reactant molecules, linamarase and p-nitrophenol had their highest collisions at 36^0C and the rate of production of the product, p-nitrophenolate ion is offset by the increasing rate of denaturation of the enzyme molecules. In Fig. 4.8B Vmax eliminates the effect of temperature-dependence of Km. The points above 48^0C were not recorded because linamarase activity decreased drastically at temperatures above 48^0C when initial velocity experiments were carried out (Fig 4.8A, B and C).

The energy of activation of the enzyme reaction was computed to be 3.92 Kcal per mole.

pKa values obtained from the study of pH effects on kinetic constants and values for energy of activation obtained from studies on temperature effects on kinetic constants clearly show that a histidine residue is likely to be present at the active site of linamarase. The carboxyl group of aspartate or glutamate also seem to be present. The presence of these residues suggests acid-base catalysis as the most likely mechanism of action of linamarase, with the imidazole

group (histidine) acting as an election sink and the carboxyl group as the nucleophile (Metzler, 1977). A charge- relay system may be present in the active site; carboxyl group transfers electron to the imidazole nitrogen and p-nitrophenolate ion is formed (Metzler, 1977).

5.8 Conclusion

In most technologically advanced countries new products, especially food and drugs have to meet some national established standards in addition to having to be accepted by consumers. These established standards are a series of quality control criteria for assessing the food/drug item. They differ for different food stuffs and drugs. At present, most locally produced foodstuffs in Nigeria do not go through any rigorous tests to enable their classification as having satisfied any established quality criteria. The consumer acceptance, or the cost of the food item, determines whether it will, or will not be sold. There is therefore an urgent need to establish quality criteria for food stuff produced locally. The cyanide content is one of the good indices for assessing cassava products. The present critical situation in the food supply in the country, and the world at large demands that all our agricultural resources be utilized to the fullest.

Improving the quality of cassava will also improve the health of the nation. Eating a properly processed cassava product reduces the risk of chronic cyanide intoxication.

As Nig and other African countries grow in technology there is a need to have basic scientific knowledge on our locally available materials: food, drugs and others. Scientists in the advanced countries would not be concerned about studying what we have in our environment except they are interested in it or we invite them to study it for us. Basic knowledge on the biochemistry of linamarase is necessary at this time. From this work an others (Ikediobi et al 1987; Nok and Ikediobi, 1989) a histidine residue is likely to be one of the groups at the active site. Acid-base catalysis may be the mechanism of action.

5.9 Suggestions for further work:

(1) Further work is needed on basic research on the synthesis of linamarase and environmental factors influencing it's synthesis and secretion by yeast and other organisms.

(2) There is need for further purification and characterization of linamarase from yeast, bacteria and cassava.

(3) Also, determination of amino acid composition of linamarase from different sources for homology studies.

(4) Studies on the appropriate storage condition for linamarase.

APPENDIXES

APPENDIX 1: CALCULATION OF LINAMARASE ACTIVITY

APPENDIX 1.1 Using p-nitrophenyl β-D-glucoside as substrate

A = ECL

Where A = Absorbance at 400nm

C = Concentration

L = Path Length of cuvette

ϵ = Molar extinction

$$\text{Enzyme unit} = \frac{A \times V \times 10^6}{\epsilon \times t \times 1000} \text{ micromoles/min}$$

Where A = Absorbance at 400nm

V = Reaction volume = 4ml

T = Incubation time = 60mins

ϵ = Molar extinction coefficient = 1.8×10^4 Mol^{-1} cm^{-1}

1 unit of linamarase activity is defined as that which catalyzes the formation of 1 micromole of p-nitrophenolate ion from P-nitrophenyl β-D-glucoside per minute under the described assay conditions (Appendix Fig. 3)

Total Activity = Total volume x Activity/ml

$$\text{Specific Activity} = \frac{\text{Activity (units/ml)}}{\text{Protein content (mg/ml)}}$$

Appendix 1.2: Using Linamarin as substrate

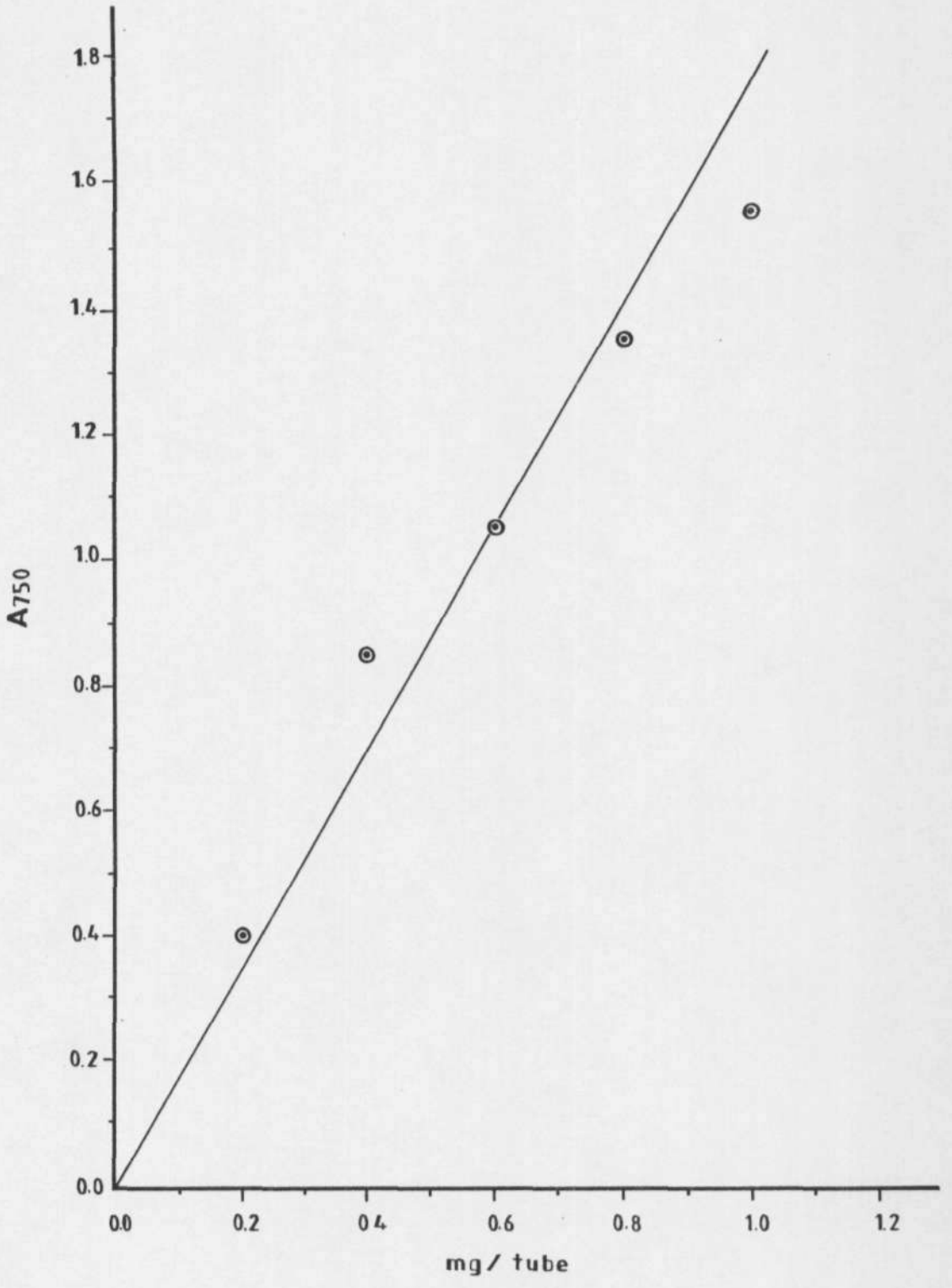
Micromoles of CN- produced/min

$$= \frac{x}{t \quad x \quad 26}$$

Where x = Microgramme CN- Per reaction mixture for the standard curve

1 unit linamarase activity is define as that which will catalyze the breakdown of 1 micromole of linamarin per minute under the described assay conditions.

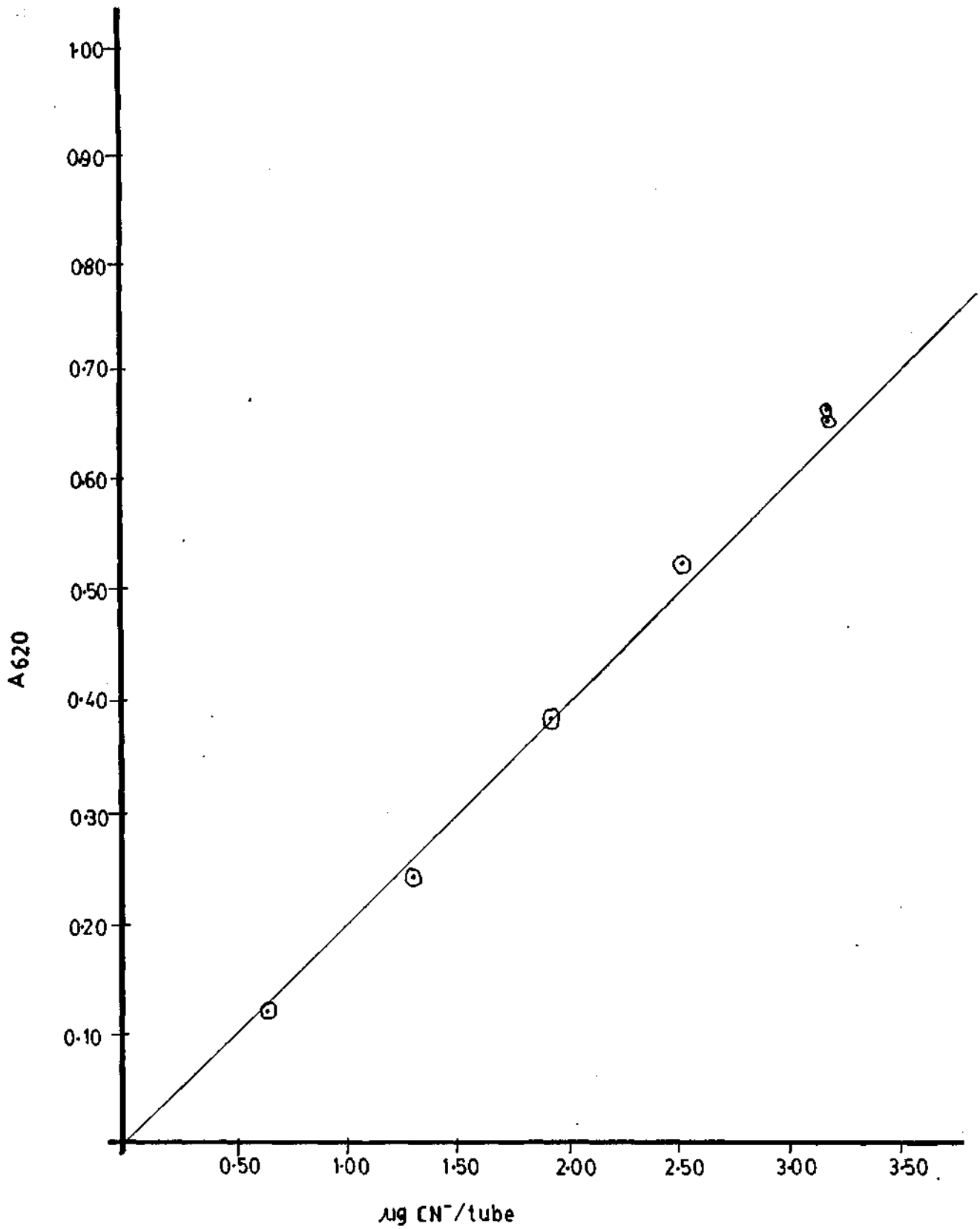
This unit of enzyme activity can be expressed in terms of either the rate of product formation, or that of substrate degradation.

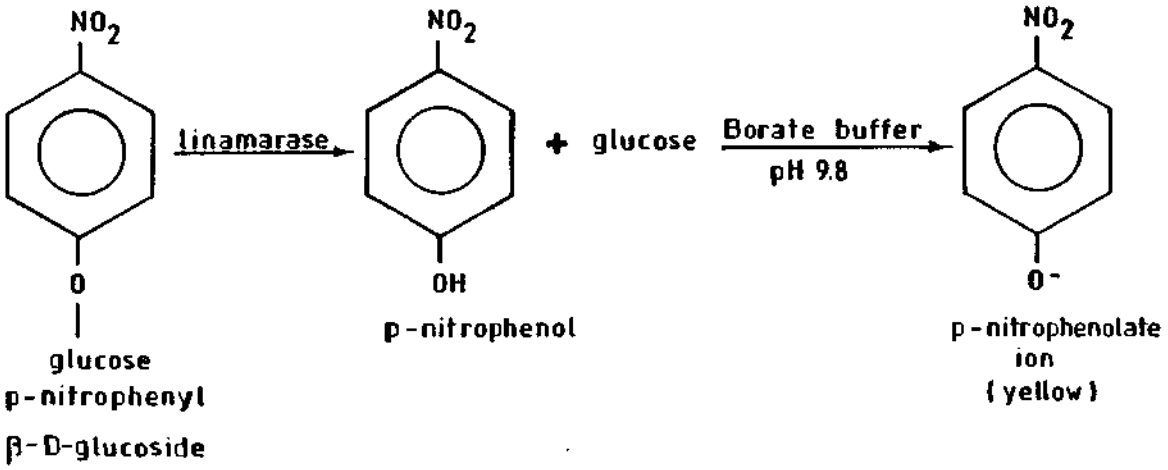


Appendix 1A: Lowry standard curve for protein.

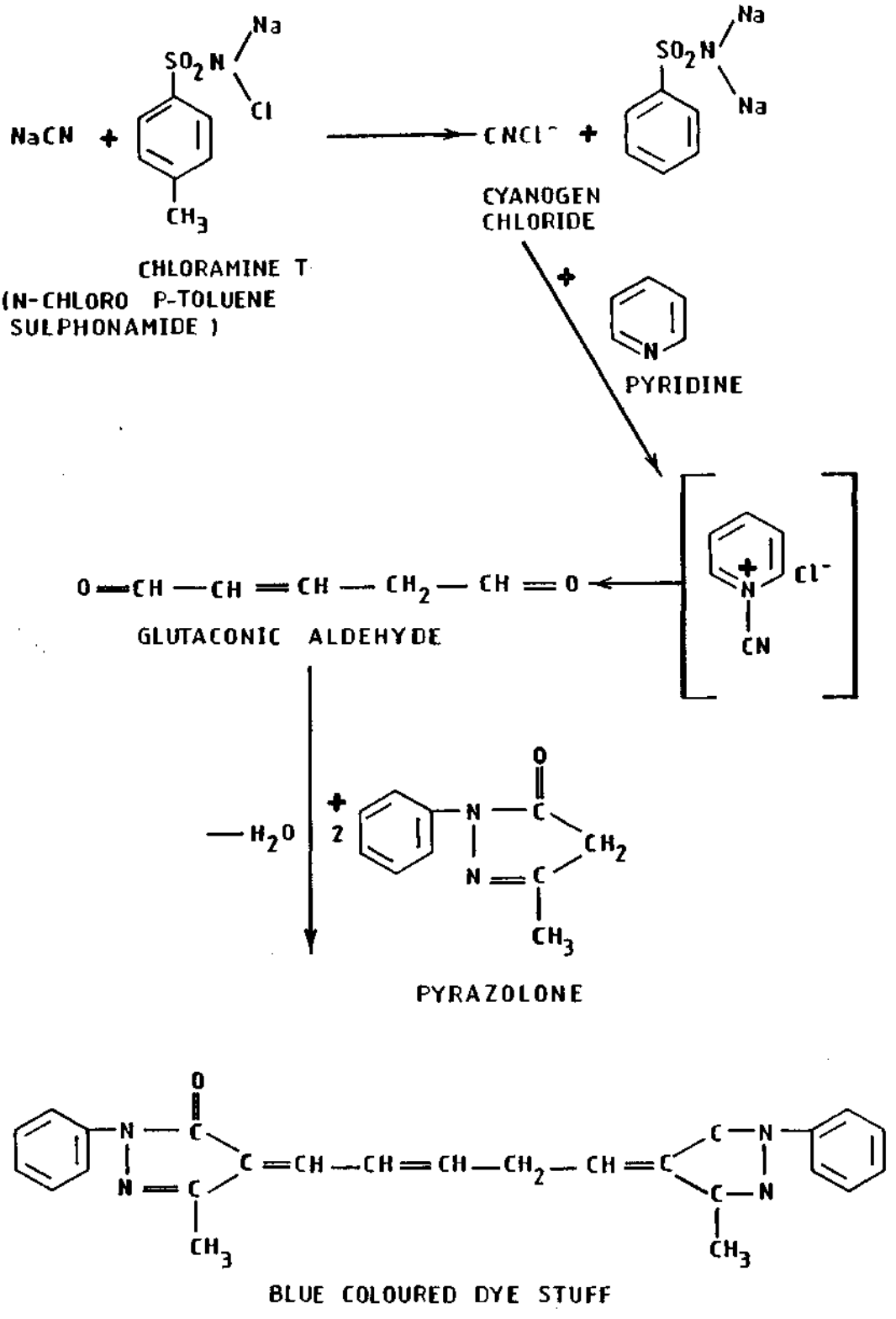
APPENDIX 1B

Cyanide standard curve.





Appendix Fig. 3. Enzymatic hydrolysis of p-nitrophenyl β-D-glucoside.



Appendix Fig. 4. Reactions for determination of cyanide using cooke's method.

Appendix: 2:0 Yeast strains and isolates

Appendix 2.1. code numbers

B	III_1
B	IV_3
B	II_4
B	IV_4
B	III_3
B	I_3
B	II_3
B	VI
P	III_2
P	II
P	I_3
P	VI
P	II_4
P	II_3
P	I_1
P	V_3
P	II_2
PW	II_2
PW	III_2
PW	I_2
PW	I_3
PW	II_3
PW	II_5

KEY

G = Grape fruit

B = Burkutu

P = Pito

PW = Palm wine

Appendix 2.2

Table 4.2: Specific activity of crude β-D-glucosidase from yeast strains grown in yeast extract broth.

Source of enzyme	Specific activity (μm/mg protein)
Candida magnoliae	3.16
Candida rhaggi	2.52
Saccharomyces kluyveri	2.35
Saccharomyces cerevisiae(1)	0.84
Saccharomyces cerevisiae(2)	2.32
Kluyveromyces maxianus	3.20
Control	1.576

Appendix 2.3

Table 4.4 Specific activity of crude extracellular β-D-glucosidase produced by yeast isolates grown in yeast extract broth, pH 5.8-6.00

Source of enzyme	Specific activity (μmol/mg)
G II	2.46
G III_2	2.62
B III	2.96
B IV	3.10
B VI	3.10
B II_4	3.86
B II_3	3.44
B I_3	3.05
B III_3	2.58
B IV_4	3.3.4
P I_3	3.22
P II_3	4.29
P II_4	2.86
P IV	3.90
P V_3	3.59
P I_1	2.41
P II_2	2.82
P I_2	4.16
P W II_2	1.97
P W1 II_2	2.51
P W III_2	2.61

P W I_3	2.34
P W I_2	4.48
P W II_3	3.60
P W II_5	2.38
Control	2.22

Appendix 2.4

Table 4.5 The Specific activity of extracellular β-D-glucosidase produced by yeast isolates grown at pH 5.2

Source of enzyme	Specific activity (µmol/mg
G II	3.90
G III_2	3.31
B III	3.92
B IV	4.09
B VI	4.19
B II_4	3.89
B II_3	4.01
B I_3	4.29
B III_3	3.51
B IV_4	3.60
P I_3	3.39
P II_3	3.70
P II_4	3.80
P IV	3.43
P V_3	3.70
P I_1	3.97
P II_2	3.41
P I_2	4.54
P W II_2	3.95
P W1 II_2	4.55
P W III_2	3.31
P W I_3	4.09

P W I_2	3.65
P W II_3	3.51
P W II_5	3.61
Control	3.20

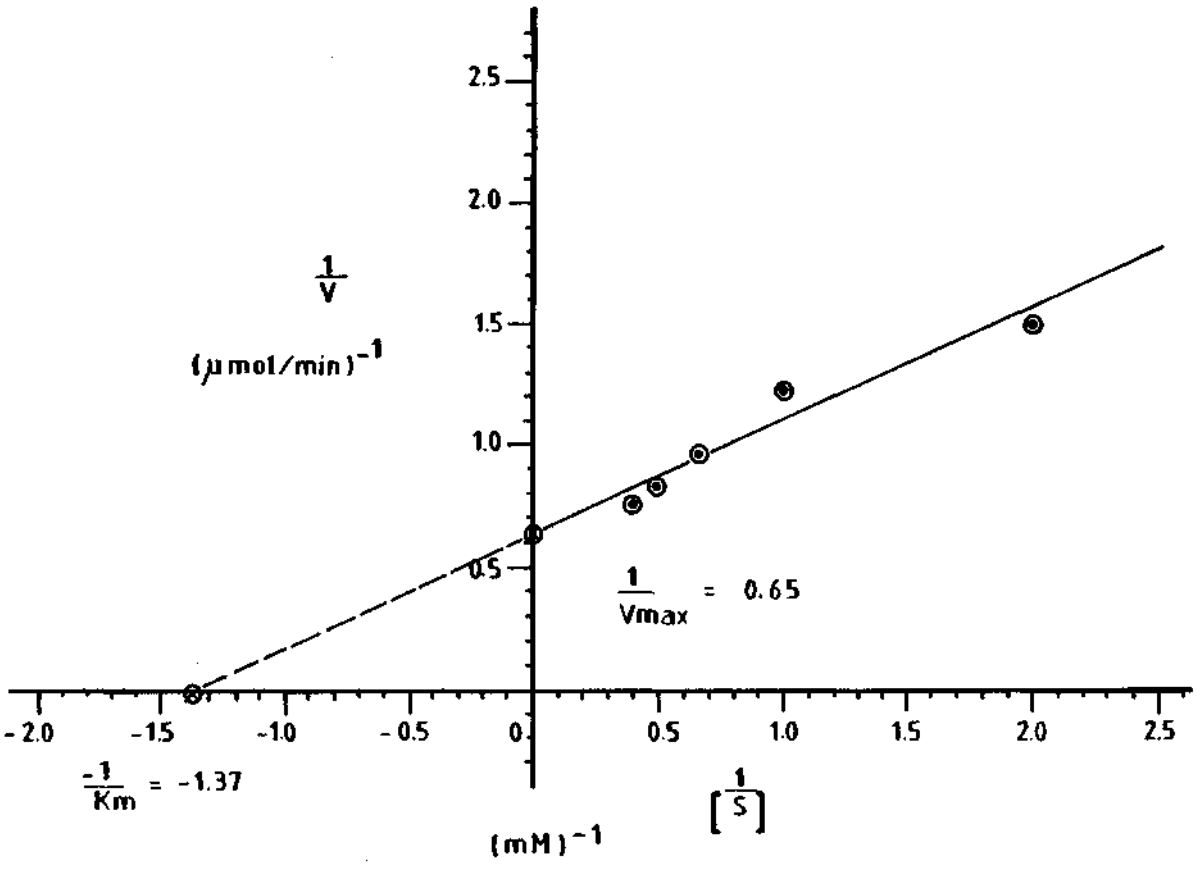
Appendix 2.5

Table 4.6 The Specific activity of extracellular β-D-glucosidase produced by yeast isolates grown at pH 5.2 with malt extract.

Source of enzyme	Specific activity (µmol/mg
G II	4.59
G III_2	4.48
B III	4.00
B IV	4.19
B VI	4.35
B II_4	4.21
B II_3	4.09
B I_3	3.55
B III_3	3.21
B IV_4	3.49
P I_3	4.26
P II_3	3.87
P II_4	4.19
P IV	4.02
P V_3	3.64
P I_1	3.55
P II_2	3.32
P I_2	3.64
P W II_2	3.70
P W1 II_2	3.90
P W III_2	3.16
P W I_3	3.28

P W I_2	2.68
P W II_3	4.28
P W II_5	3.97
Control	3.01

Assays were in duplicates.



APPENDIX 3.1.

Fig. 4.5A1. Lineweaver-Burk plots of initial velocity data to determine Vmax and Km of linamarase for PNP-glucoside at room temperature (26 ± 2)°C and pH 5.5. (Line of best fit was by least square method).

⊗---⊗ Calculated value ; ●—⊙ Observed value .

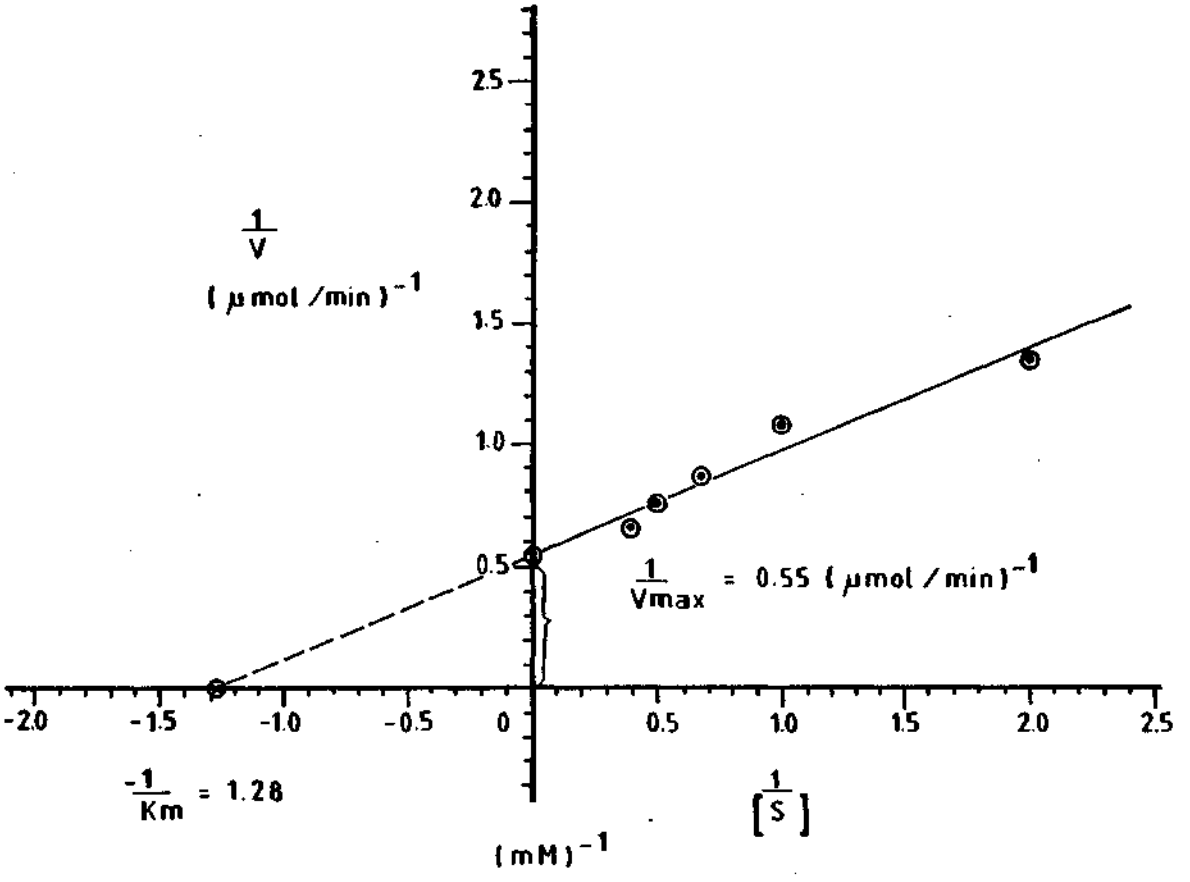
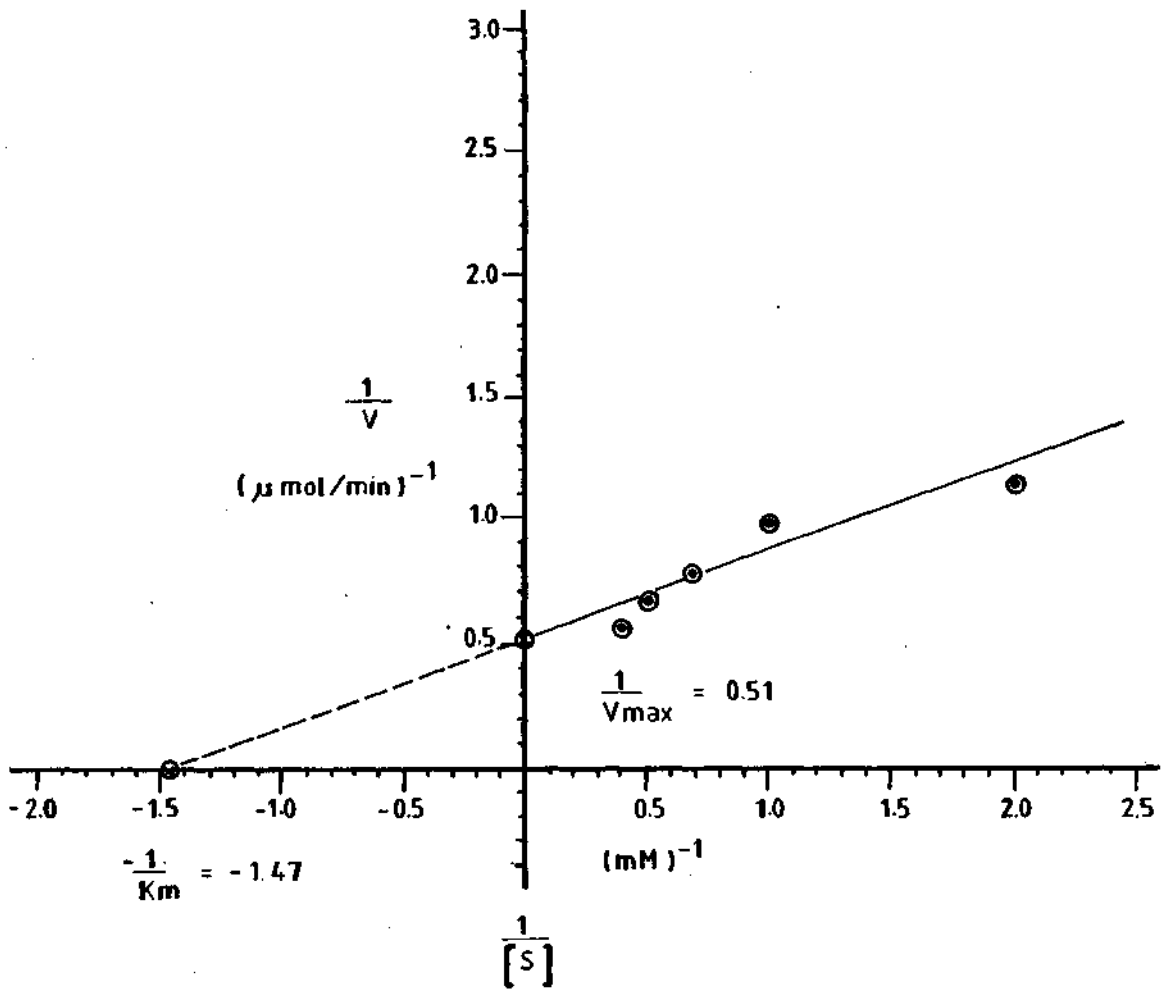
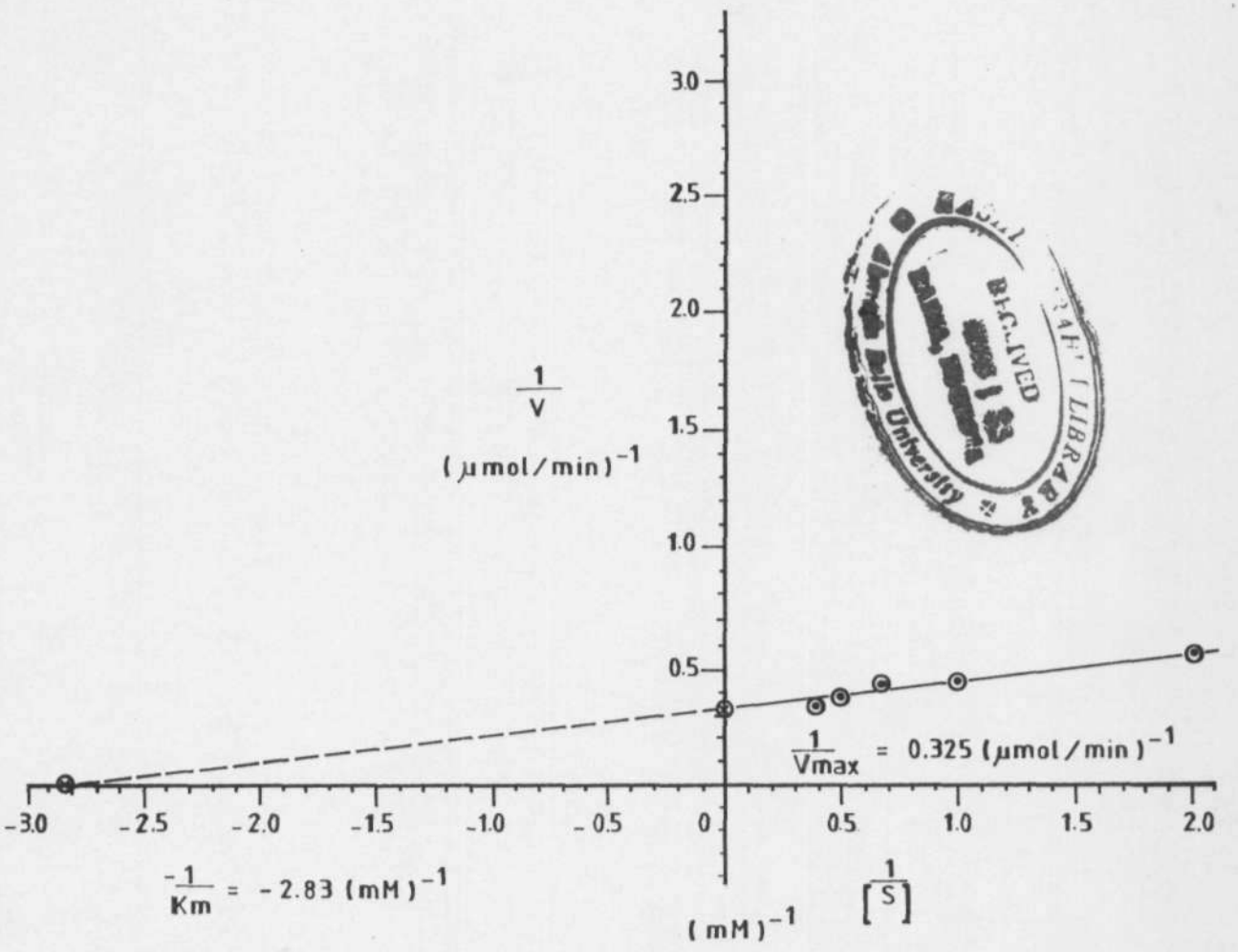


Fig. 4.5A2. Lineweaver-Burk plots of initial velocity data to determine Vmax and Km of linamarase for PNP-glucoside at room temperature (26 ± 2)°C and pH 6.0. (Line of best fit was by least square method).
⊗---⊗ Calculated value; ⊙——⊙ Observed value.



APPENDIX 3.2:

Fig. 4.5B. Lineweaver-Burk plots of initial velocity data to determine Vmax and Km of linamarase for PNP-glucoside at room temperature (26 ± 2) °C and pH 6.5. (Line of best fit was by least square method). ⊗---⊗ Calculated value; ⊗——⊗ Observed value.



APPENDIX 3.3:

Fig. 4.5C1. Lineweaver-Burk plots of initial velocity data to determine Vmax and Km of linamarase for PNP-glucoside at room temperature (26 ± 2)°C and pH 7.5. (Line of best fit was by least square method). ⊗---⊗ Calculated value; ⊙——⊙ Observed value.

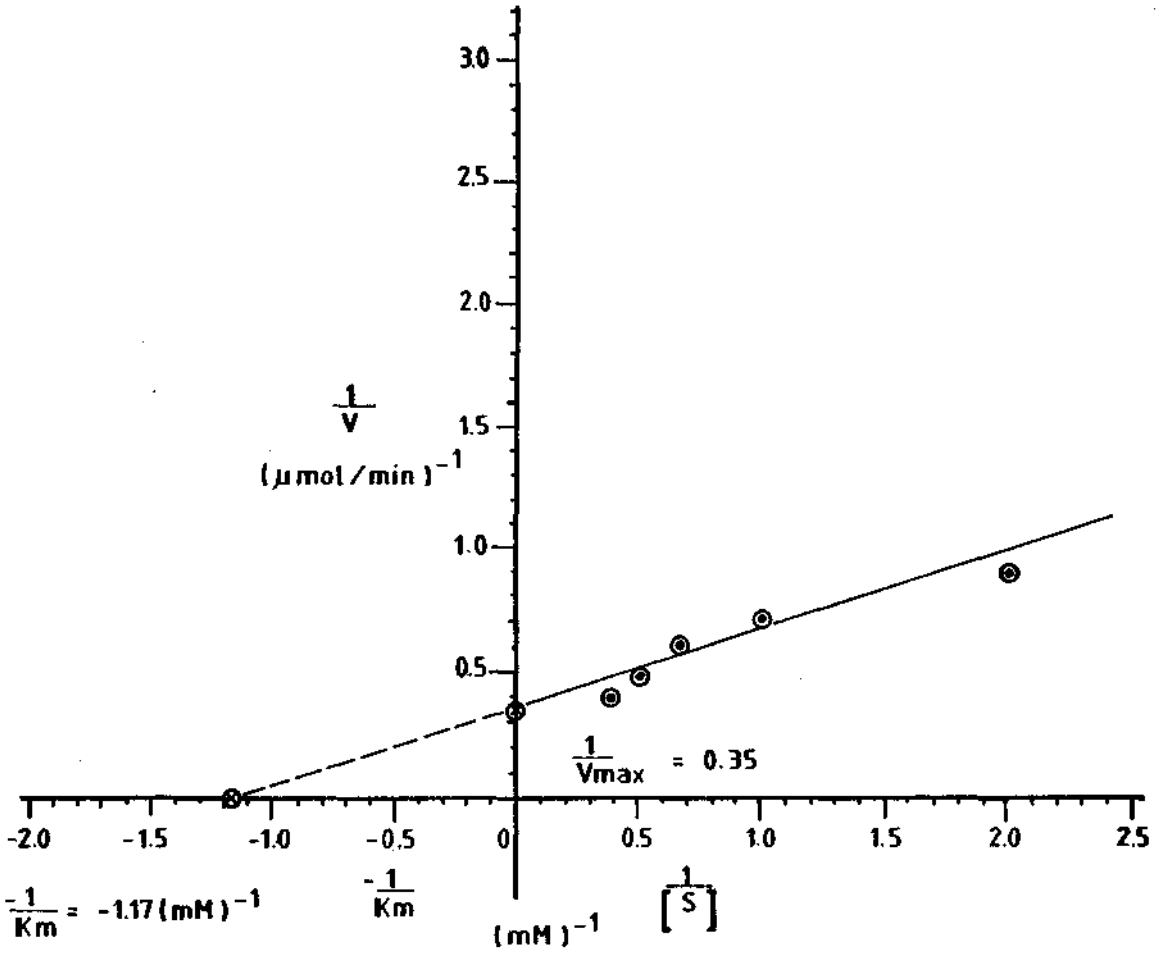
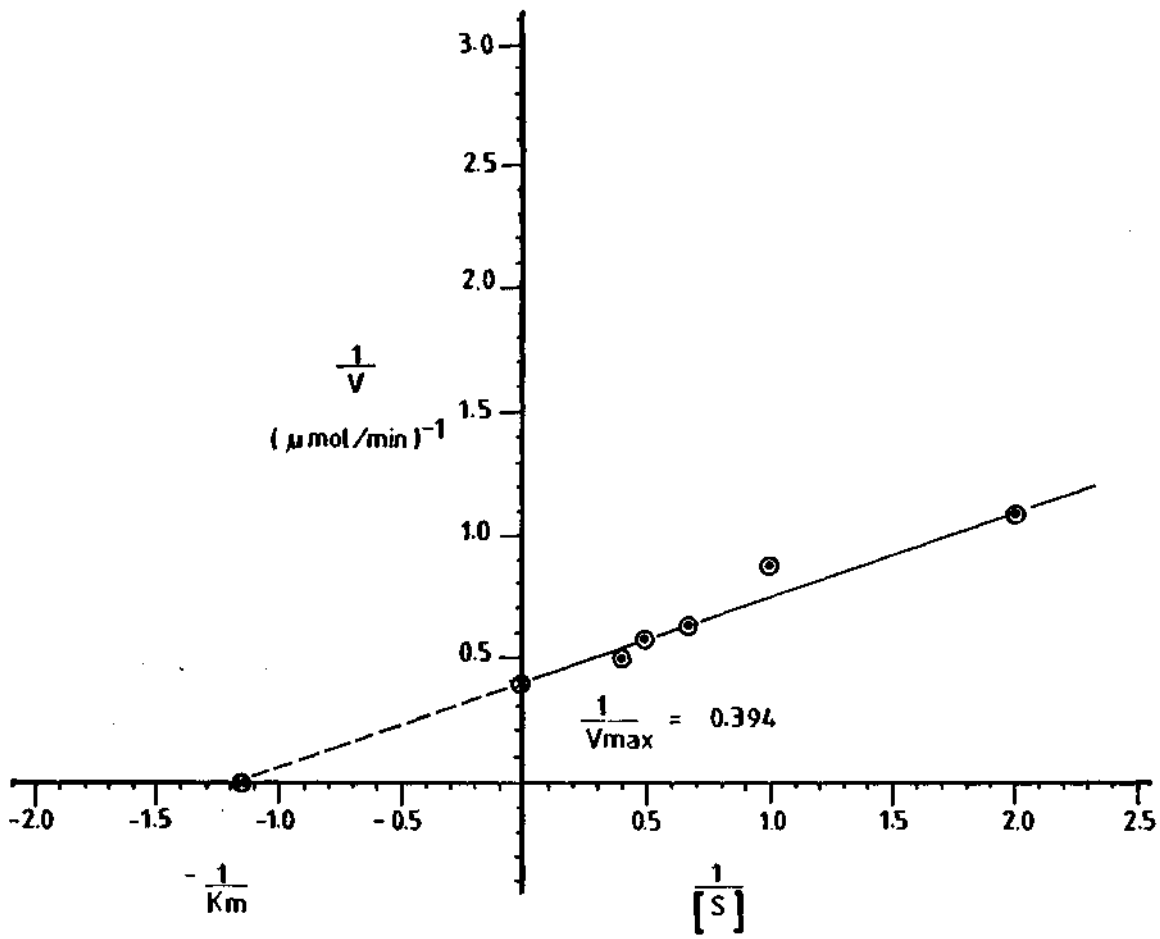


Fig. 4.5C2. Lineweaver-Burk plots of initial velocity data to determine Vmax and Km of linamarase for PNP-glucoside at room temperature (26 ± 2)°C and pH 8.0. (Line of best fit was by least square method). ⊛---⊛ Calculated value; ⊛——⊛ Observed value.



APPENDIX 3.4:

Fig. 4.5D1. Lineweaver-Burk plots of initial velocity data to determine Vmax and Km of linamarase for PNP-glucoside at room temperature (26 ± 2)°C and pH 8.5. (Line of best fit was by least square method). ⊗----⊗ Calculated value; ⊙—⊙ Observed value.

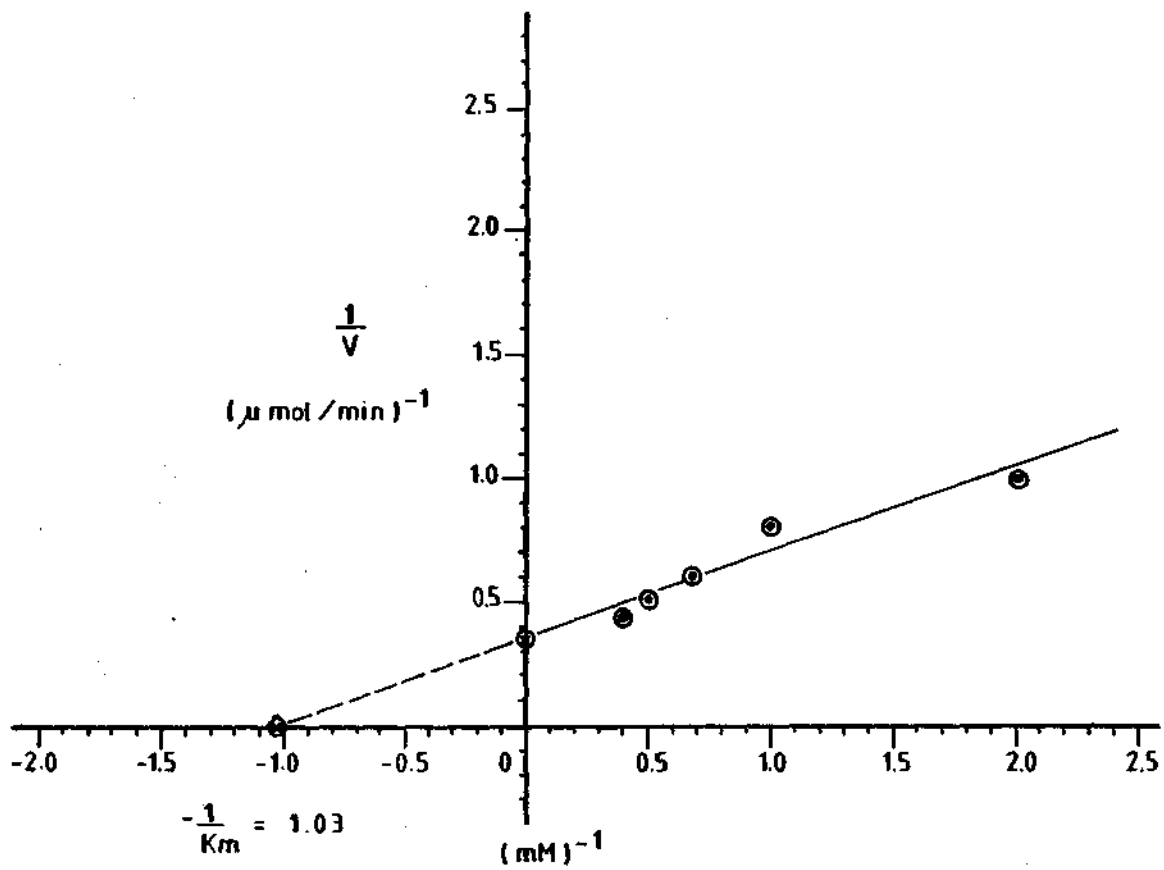
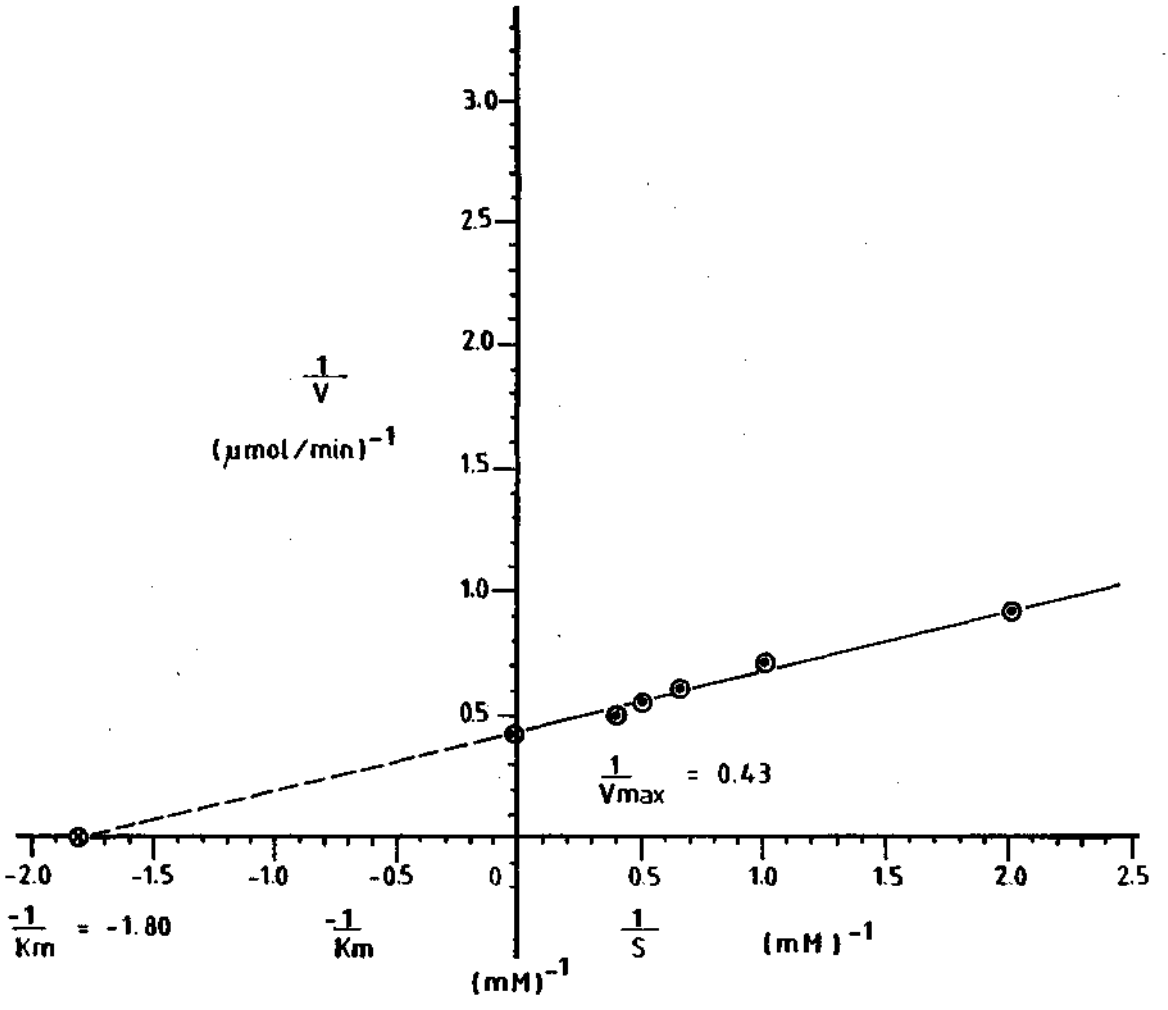
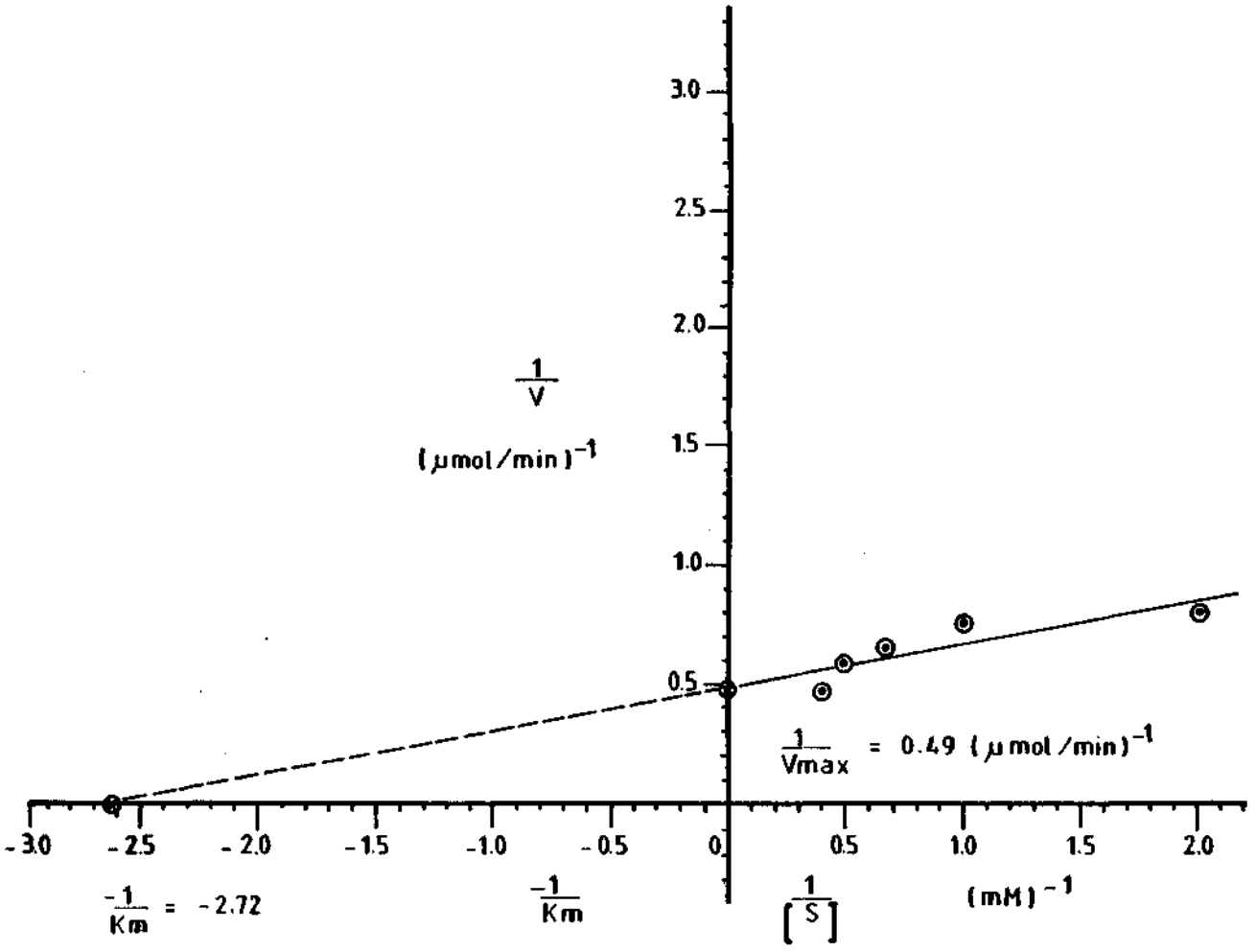


Fig. 4.5D2 Lineweaver-Burk plots of initial velocity data to determine Vmax and Km linamarase for PNP-glucoside at room temperature (26 ± 2) °C and pH 9.0. (Line of best was by least square method).
⊗---⊗ Calculated value; ⊗—⊗ Observed value.



APPENDIX 3.5:

Fig. 4.7A. Lineweaver-Burk (double reciprocal) plot of initial velocity data to determine Vmax and Km of linamarase for PNP-glucoside at 26 °C and pH 6.8. (Line of best fit was by least square method). ⊛---⊛ Calculated value; ⊛——⊛ Observed value.



APPENDIX 3.6:

Fig. 4.7B1. Lineweaver-Burk (double reciprocal) plot of initial velocity data to determine Vmax and Km of linamarase for PNP-glucoside at 32°C and pH 6.8. (Line of best fit was by least square method). ⊗——⊗ Calculated value; ⊙——⊙ Observed value.

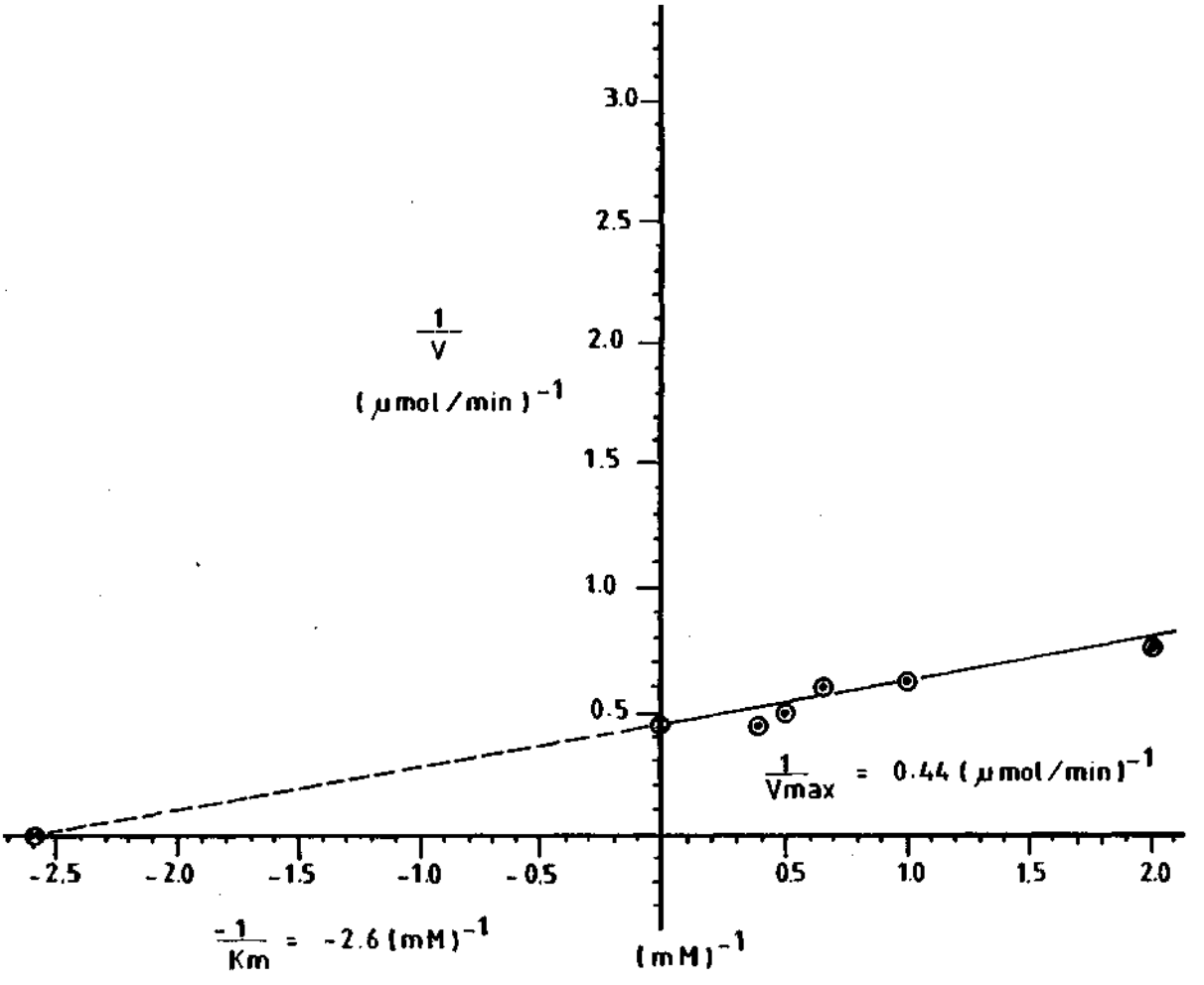
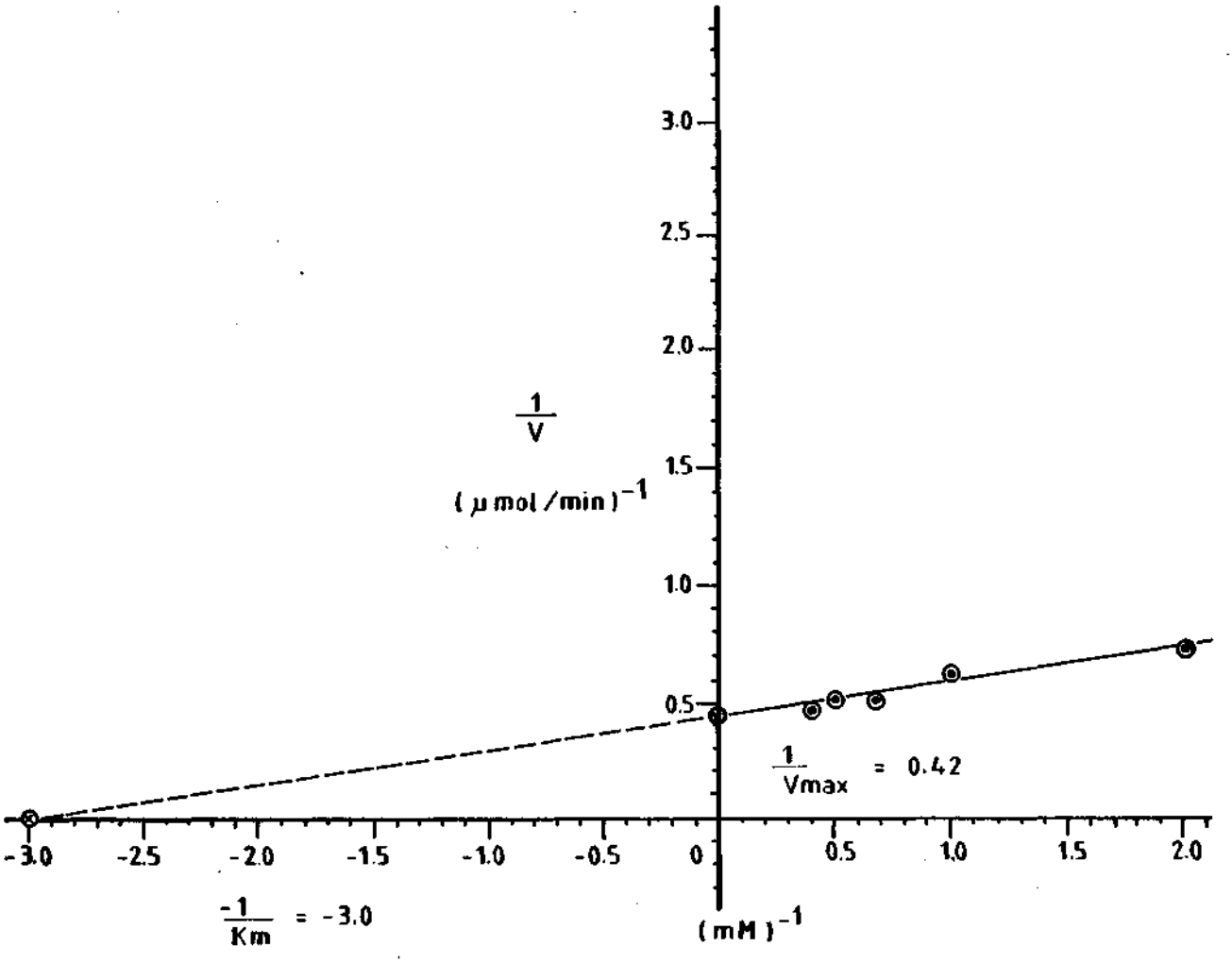
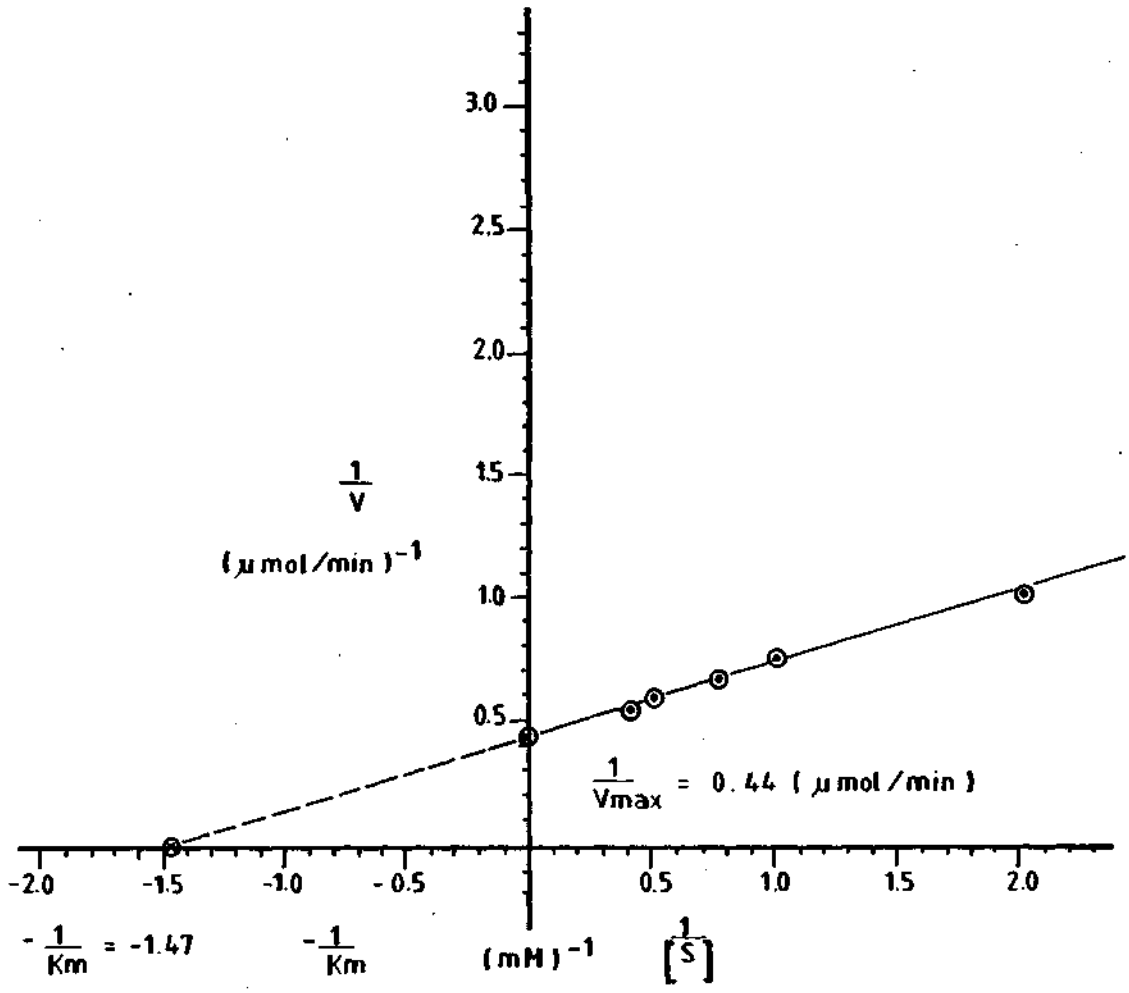


Fig. 4.7B2. Lineweaver-Burk (double reciprocal) plot of initial velocity data to determine Vmax and Km of linamarase for PNP-glucoside at 36 °C and pH 6.8. (Line of best fit was by least square method). ⊗---⊗ Calculated value; ⊙——⊙ Observed value.



APPENDIX 3.7.

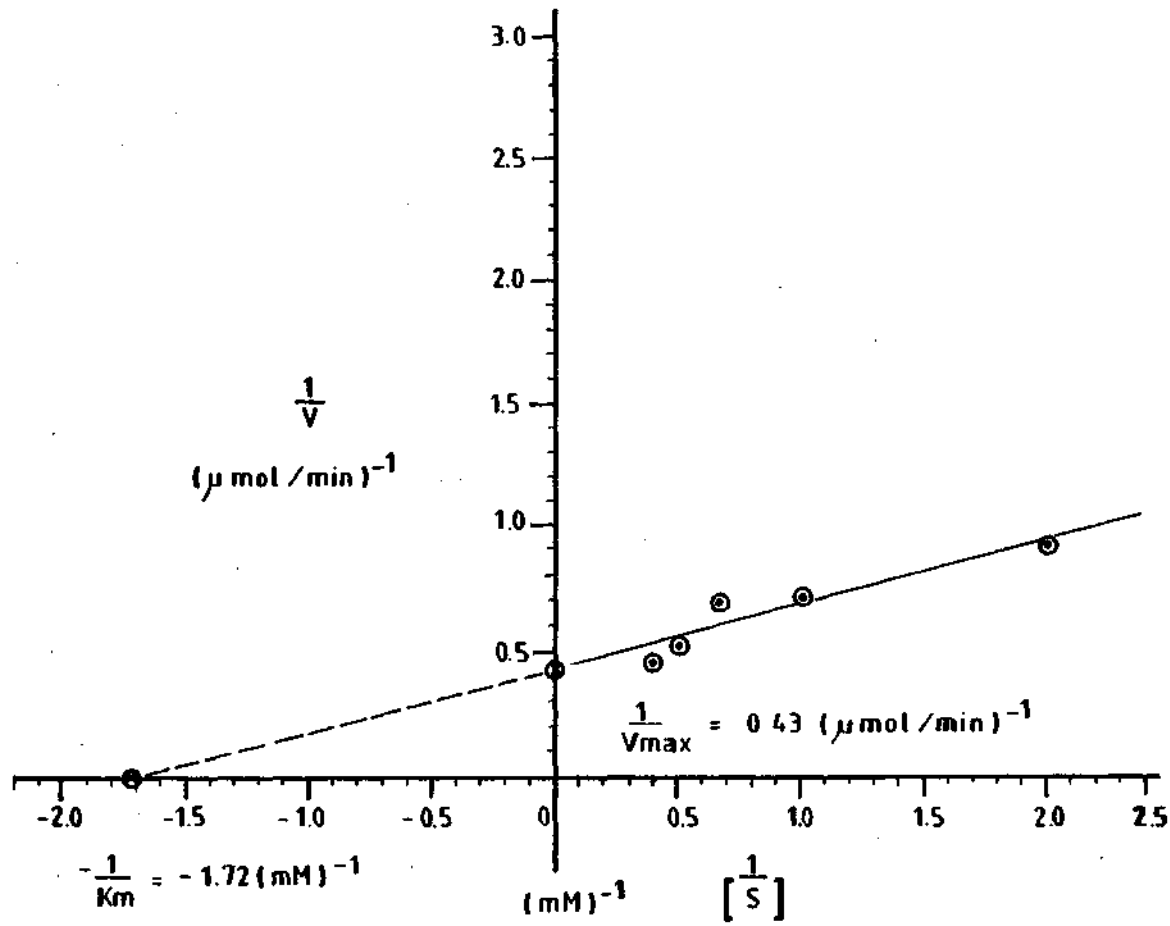
Fig. 4.7C. Lineweaver-Burk (double reciprocal) plot of initial velocity data to determine Vmax and Km of linamarase for PNP-glucoside at 40 °C and pH 6.8. (Line of best fit was by least square method). ⊛---⊛ Calculated value ; ●——● Observed value.



APPENDIX 3.8.

Fig. 4.7D. Lineweaver-Burk (double reciprocal) plot of initial velocity data to determine Vmax and Km of linamarase for PNP-glucoside at 43 °C and pH 6.8. (Line of best fit was by least square method).

⊗---⊗ Calculated value; ⊗—⊗ Observed value.



APPENDIX 3.9:

Fig. 4.7E. Lineweaver-Burk (double reciprocal) plot of initial velocity data to determine Vmax and Km of linamarase for PNP-glucoside at 47°C and pH 6.8. (Line of best fit was by least square method). ⊙--⊙ Calculated value ; ⊙——⊙ Observed value.

REFERENCES

Akazawa, T., Miljanich, P., Conn, E.E. (1960). Studies on cyanogenic glycoside of sorghum vulgare. Plant Physiol. 35: 535-58.

Akinrele, I.A. (1964). Fermentation for Cassava. Sci. Fd. Agric. 15:589-594.

Akinrele et al (1962). see Akinrele, I.A. Cooke, A.S. and Holgate, R.A. (1962).

Akinrele, I.A., Cooke, A.S. and Holgate, R.A. (1962). The manufacture of gari from cassava in Nigeria. Proc. 1st Int. Congress fed. Technol. London pp. 632-644.

Asuni, T. (1967). Tropical neuropathy and psychosis. Brit. Psychiat. 113: 1031-1033.

Bauman, C.A., Sternbock, W.M., and Rupel, I.W.(1933). Fat soluble vitamins. Biol. Chem. 105:167-176.

Baumeister, R.H, Schievelbein, H. and Zickgraf-Rudel, A. (1975). Toxicological and clinical aspects of cyanide metabolism. Arnei-forsch. 25:1056-1064.

Ben-Yohoshua, S. and Conn, E.E (1964). cited by Nartey, (1978) Bernier, R.F. and stutzenberger (1988). Stabilization of β-glucosidase by Polyhydric alcohol - J.Biotechnol 7 (4): 293-298.

Bleichert, E.F., Neish, A. and Towers, G.H.N. (1966). Biosynthesis of taxiphyllum in Taxus, P. 119-127.

In G. Billek (ed.) Biosynthesis of aromatic compounds, Proc. 2nd Meeting of Fed. Eur. Biochem. Soc. Vol.3 Pergamon Press, Oxford.

Butler, G.W. (1965). The distribution of the cyanogenic glucosides, Linamarin and lotaustralin in higher plants. Phytochemistry 4: 127-131.

Butler, W.H. and Barnes, J.M. (1963). Toxic effects of groundnut meal containing aflatoxin to rats and guinea pigs. Brit. Cancer. 17: 699-710.

Butler, G.W. and Butler, B.G. (1960). Biosynthesis of linamarin and lotaustralin in white clover. Nature 187: 780-781.

Butler, G.W. and Conn, E.E. (1964). Biosynthesis of the cyanogenic glucosides, linamarin and lotaustralin. Labelling studies in vivo with Limum usitalissium. J.Biol. Chem. Vol 239: 1674-1679.

Butler, G.W. Bailey, R.W. and Kennedy, L.D. (1965). Studies on the glucosidase "Linamarase". Phytochemistry 4:369-381.

Butler, G.W. et al (1965) see Butler, G.W., Bailey, R.W., and Kennedy, L.D (1965)

Butler, G.W. Reay, P.F. and Tapper, B.A. (1973) Physiological and Genetic aspects of cyanogenesis in cassava and other plants. In Chronic cassava toxicity. B. Nestle and R. Macintyre (eds.). ID.R.C -010e, Ottawa, Canada, Pp. 65-71.

Cock, J.H (1985). Cassava: World production in cassava; New potential for a neglected crop. West view press/Boulder and London pp. 3-5.

Collard, P. (1963) Species of corynebacterium Isolated from fermenting cassava roots. J. Applied Bacteriology 26: 115-116.

Collard, P. and levi, S. (1959). A 2 stage fermentation of cassava Nature 163:620-621

Conn, E.E. (1969). Cyanogenic glucosides. J. Agric Fd Chem. 17 (3): 519-526.

Conn, E.E. (1973). Cyanogenic glucosides: Their Occurrence, biosynthesis and function. In Chronic Cassava Toxicity. B. Nestle and R. MacIntyre (eds). I.D.R.C. 010e, Ottawa Canada. pp.55-63.

Conn, E.E. and Akazawa, T. (1958). Federation proc. 17, 205. cited by Conn, E.E. (1969). J.Agr. Food Chem. 17 (3): 519 - 526.

Conn, E.E. and Butler, G.W. (1969). The biosynthesis of cyanogenic glucoside and other nitrogen Compounds. Perspective in phytochemistry. Academic press London. PP 47 - 74.

Cooke, R.D. (1978) An enzymatic assay for the total cyanide content of cassava Manihot esculenta crantz). J. Sc. Fd Agric. 29:345-352

Cooke, R.D., Blake, G.G. and Battershill, J.M. (1978). Purification of cassava Linamarase. Phytochemistry 17: 381-383.

Cooke, R.D. and Maduagwu, E.N. (1978), The effects of simple processing on the cyanide content of cassava chips. J. Fd Technol. 13:299-306.

Cooke, et al., (1978) see Cooke, R.D., Blake, G.G. and Battershill, J.M. (1978).

Coursey, D.G. (1973). Cassava as food: Toxicity and Technology. In Chronic Cassava Toxicity. B. Nestle and R. MacIntyre (eds). I.D.R.C 010e, Ottawa, Canada. PP 27-36.

Dartnal, A.M and Burns, R.G. (1987). Sensitive method for measuring cyanide and cyanogenic glucoside in sand culture and soul. Biol. fertil soils 5 (2): 141-147.

De Bruijn, G.H. (1973). The cyanogenic character of cassava (Manihot esulenta), In Chronic Cassava Toxicity. B. Nestle and R. MacIntyre (eds). I.D.R.C. - 010e, Ottawa, Canada. PP. 147-157.

Delange, F. Thilly, C. and Ermans, A.M. (1968). Iodine deficiency, a permissive condition in the development of endemic goitre. J. Clin Endocrn. 28:114.

Delange, F. Vander Valden, M. and Ermans, A.M. (1973). In Chronic Cassava toxicity B. Nestle and R. MacIntyre (eds). IDRC 010e, ottawa, Canada. PP. 147-151.

Dey P.M. (1977). Polymorphism of some glycosidases from Barley. Phyt 16: 323.

Dixon, M. (1953). The Effect of pH on the Affirnities of Enzymes for Substrates and inhibitors. Biochem J. 55: 161-170.

Dixon, M. and Webb, E.C. (1964). Enzymes 2nd Edition. Longmans, New york, PP. 116-166.

Duerkson, J.D. and Haverson, H. (1958) Purification and properties of an Inducible β-Glucosidase of yeast. J. Biol. chem. 233(5): 1113-1120

Ejiofor, M.A.N. and Okafor, N. (1983) Microbial breakdown of Linamarin in fermenting cassava pulp. In Tropical root crops: Production and uses in Africa Ed. by Terry, E.R., Doku, E.V., Arenx O.B. and Mahungu, N.M. Proceedings of the 2nd triennial symposium of the internation society for tropical root crops Africa branch. Ottawa Canada, IDRC 22e PP 105-107.

Ekpechi, O.L. (1964) Ph.D thesis London University. Cited by Ekpechi 1973.

Ekpechi, O.L. (1967) Pathogenesis of endemic goitre in Eastern Nigeria. Brit. J. Nutr., 21: 537-545.

Ekpechi, O.L. (1973) Endemic goitre and high cassava diets in Eastern Nigeria. In Chronic cassava toxicity. B. Nestle and R. MacIntyre (eds). I.D.R.C. -010e PP. 139-145

El-Tinay, A.H., Bureng, P.L. and Yas A.E. (1984). Hydrocyanic acid levels in fermented cassava. J. Fd. Technol. 197-202

EL-Tinay, A.H. et al (1984). see El-Tinay, A.H., Beureng, P.L. and Yas, A.E. (1984).

Fielder, H. and Wood, J.L. (1956). Specificity studies on the β-Mercaptopyruvate-cyanide transulphuration system. J. Biol. Chem. 222: 387-397.

Foos, O. (1950) The prevalence of unbranched sulphut chains in Polysulphide and polythionic compounds. Acts Chem Scand. 4: 404-415.

Foster R.C (1980) The Nature of Enzymology. Croom and Helin Ltd. London.

Gander, J.E. (1958). Rederation proc. 17, 226. Cited by Conn, E.E (1969). Cyanogenic glucosides. J.Fd Agric 17(3): 519-23.

Grover, A.K., Macmurchie, D. and Cushley, R.J. (1977). Studies on Almond Emulsin β-D-glucosidase. Biochim. Biophys Acta. 482: 98-108.

Gullingham, J.T., Shirer, M.M., Starnes, J.J. Page, N.R. and Clain, E.F. (1969). Relative occurence of toxic concentrations of cyanide and nitrate in varieties of sudangrass and sorghum sudan-grass hybrids. Agron. J. 61: 727-730.

Hahlbrock, K. and Conn, E.E. (1970). Purification and properties of a uridine diphosphate glucose-ketone cyanohybrin β-glucosyltransferases from Linum usitatissimum J. Biol Chem. 245: 917-922.

Hahlbrock, K., Tapper, B.A., Butler, G.W., and Conn, E.E (1968) Conversion of nitriles and α-Hydroxynitriles to cyanogenic glucosides in flax seedlings and cherry laurel leaves. Arch. Biochem. Biophys. 125: 1013 - 1016.

Hill, D.C (1973). Physiological and Biochemical responses of rats given pottassium cyanide or linamarin. In cassava as animal feed. B. Nestle and M. Graham ed. Proceedings of a workshop held at the University of Guelph. International Development Research Centre. IDRC- 095e PP.

33-42.

Himwich, W.A and Saunders J.P. (1948). Enzymatic conversion of cyanide to thiocyanate, Amer. J. Physiol. 153: 348-354.

Hughes, M.A. (1968) "studies on β-glucosidase production of Trifolium repens L. J. Exp. Botany 19: 427-434

Hunt, (1905-1906). Hefflers hard brunch der experimentallen Phamakologia, Berlin Julius sprinker 133: 702-725. (Cited by Oke (1973).

Ikediobi, C.O. and Terry D.E. (1989). Enzyme-based assay of total cyanide in sorghum (sorghum bicolor L. Moench) and sorghum products. (in press).

Ikediobi, C.O., Ibrahim S. and Ogbonna, A.I. (1987) Linamarase from Fusarium equiseti. Appl. Microbiol. Biotechnol 25: 327-333.

Ikediobi, C.O. and Onyike, E. (1982a). Linamarase activity and Detoxification of cassava (Manihot esculenta. during fermentation for gari production. Agric. Biol. Chem. 46: 1667-1669.

Ikediobi, C.O. and Onyike, E, (1982b). The use of linamarase in gari production. Process Biochem. 17(4): 2-5.

Ikediobi, C.O. and Ogundu, E.C. (1985a). Screening of some fungal isolates for linamarase production. Nigerian food J. 3: 165-168.

Ikediobi, C.O. and Ogundu, E.C. (1985b). Production of linamarase by Aspergillus sydowi and Fusarium equiseti. Process Biochem. 20 (4): 99-102.

Ikediobi, C.O. and Fashagba, E. (1985a). A method for cyanide assay in cassava (Manihot esculenta Crantz and cassava products using barbiturate-pyridine reagent. Nigerian J. Biochem. 2: 124-132.

Ikediobi, C.O., Onyia, G.O.C, and Eluwah C.E. (1980). A rapid and inexpensive enzymatic assay for total cyanide in cassava (Manihot eculenta Crantz) and cassava products. Agric. Biol. Chem. 44: 2803-2809.

Ikediobi, et at. (1980). See Ikediobi, C.O., Onyia, G.O.C., and Eluwah, C.E. (1980)

Ikediobi, C.O. and Ukoha, A.I. (1985). Enzymology, Principles and applications. Department of Biochemistry A.B.U Zaria, copyright 1985, by the authors.

Joachim, A.W.R. and Panditteschker,.D.G.(1944). Investigations on the hydrocyanic acid content of manioc. Trop. Agriculturist 100: 150-163.

Laidler, K.J. and Branko, F.P. (1979). Temperature Effects in Enzyme Kinetics. In Methods in Enzymology. Daniel L. Purich (eds). Academic Press New York. Vol. 63, Part A, pp. 234-257.

Lowry, O.H, Rosebrough, N.J. and Randall, R.J. (1951). protein measurement with the folin phenol, reagent. J. Biol chem. 193: 265-275.

Maduagwu, E.N. and Adewale, A.F. (1980). Loss of Hydrocyanic acid and its dervatives during sundrying of cassava. In tropical Root Crops: Research strategies for the 1980's In E.R. Terry, K.A. Oduru, and F. Caveness (eds)proceedings of the First Triennial Root crops symposium of the International society for Tropical Root Crops (Africa Branch), Ibadan. International Development Research Centre (IDRC) Ottawa, Ont. Canada. 163e pp. 149-151.

Maduagwu, E.N. and Oben, D.H.E. (1981). Effects of processign of grated cassava roots by the screw press and by traditional fermentation methods on the cyanide content of gari. J. Fd. Technol. 16: 299-302.

Mao, C.H. and Anderson, L. (1967). Cyanogenesis in Sorghum vulgare III. partial purification and characterization of two β-glucosidases from sorghum tissues. Phytochemistry, 6: 473-483.

Meister, A. (1953). Preparation and enzymic reactions of the keto analozines of asparagine and glutamine. Federation proceedings 12: 245.

Mentzer and Favre-Bonvin (1961) cited by Conn, E.E. (1969) Cyanogenic glucosides. J. Agr. Food Chem. 17 (3):519-526.

Metzler, D.E. (1977). Biochemistry: The chemical reactions of the living cells. Academy press New York. p. 370-376.

Money, G.L. (1959). Clinical aspect of tropical ataxic neuropathies related to malnutrition. West Afri. Med. J. 8:3-17.

Montgomery, R.D. (1964), Observation on the cyanide content and toxicity of tropical pulses. W. Indian Med. J. 13: 1-11.

Mushet, C. W. Kelley, K.L., Boxer, G.E. and Richards, J. C. (1952). Antidotal efficiency of vitamin B_{12} in experimental poisoning. Proc. Soc. Exp. Biol. N.Y 81:233-236.

Nambisan, B. and Sundaresan, S. (1985). Effect of processing on the cyanoglucoside content of cassava. J. Sc. Food Agric. 36: 1197-1203.

Nartey F. (1966). Aflatoxins of Aspergillus flavus grown on cassava. Physiol plant 19: 818-822.

Nartey, F. (1968). Studies on cassava Manihot utilissima pohl. I. cyanogenesis and the biosynthesis of linamarin and lotaustralin in etiolated seedlings. Phytochemistry 7: 1307-1312.

Nartey, F. (1969). Studies on cassava Manihot utilissima II. Biosynthesis of asparagine 14 C-lebelled bydrogencyanide and its relations with cyanogenesis. Physiol. Plant 22: 1085-1096.

Nartey, F. (1973). Biosynthesis of cyanogenic glucosides in cassava (Manihot spp). In Chronic Cassava Toxicity. B. Nestle and R. MacIntrye (Eds) I.D.R.C-010e, Ottawa, Canada, pp. 73-87.

Nartey, F. (1978). Manihot esculenta (Cassava) "cyanogenesis, ultrastructure and seed germination" Muns gaard press Copen hagan Denmark.

Nemoto, Y. (1940). Toxicity of bread prepared with grated manioc flour constaining hydrocyanic acid. Rev. Aliment. Chem. Indust. 4: 5-7

Nok, J.N. and Ikediobi, C.O. (1989). Some properties of linamarase from cassava (Manihot esculenta) cortex J. Food. Biochem. 14: 477-489.

Ogundu, E.C. (1988). The performance of animals on linamarase processed cassava and aspects of cyanogenic glucoside metabolism. Ph.D Thesis. Ahmadu Bello University, Zaria, Nigeria.

Okafor, N. (1977) Micro-organisms associated with cassava fermentation for gari production. J. Appl bacteriology. 42: 279-284.

Okafor, N. and Ejifor, M.A.N. (1985). The linamarase of Leuconostoc mesenterodes: production, isolation and some properties. J.Sci. Fd. Agric 36: 669-678.

Oke, O.L. (1973). The mode of cyanide detoxification. In Chronic cassava toxicity. B. Nestle and R. Macintyre (Eds) I.D.R.C -010e, Ottawa, Canada, pp. 99-104.

Oke, O.L. (1968). Cassava as food in Nigeria. World Review of Nutrition and Diet 9: 227-250.

Osuntokun, B.O. (1968). An ataxic neuropathy in Nigeria. A clinical biochemical and electrophysiological study. Brain. 91: 215-248.

Osuntokun, B.O. (1970). Cassava diet and cyanide metabolism in wistar rats. Brit. J. Nutrition 24:377-380

Osuntokun, B.O. (1973). Ataxic Neuropathy associated with high cassava diets in West Africa. In Chronic cassava toxicity. B. Nestle and R. MacIntyre (eds). International Development and Research Centre, Ottawa, Canada, I.D.R.C 010e pp. 127-138

Osuntokun, B.O and Aladetoyinbo, M.A. (1970) Plasmic cyanide and hepatic thiosulphate-cyanide sulphur transferase (rhodanese) activity in Nigeria ataxic neuropathy. Nig. J. Sci. 4: 209-230.

Paula, R.D. De G. and Rangel, J. 1939. HCN or the poison of bitter or sweet manioc. Rev. Alimentar. (Rio do Janiora). 3: 215-217. Cited by Oke, O.L. (1973). The mode of cyanide detoxification. In Chronic cassava toxicity B. Nestle and R. MacIntrye eds. IDRC. 010e pp 99-104.

Rao, G. and Cock, J.H. (1973). Natural drying of cassava. Paper resented at the 3rd symposium for the international society for Tropical Root Crops, IITA, Ibadan, Nigeria 2-9 December 1973.

Robinson, D. (1966). The β-glucosidase and β-galactosidase components of Medicago sativa L. Phytochem. 5:699.

Rogers, D.J. (1963). studies on Manioc esculenta and related species. "Bull" Torry Bot, Club 90: 43-54.

Schievelbein, H., Baumeister R., Vogel, B. (1969). Naturwiss enehaffen 56: 416. Cited by Baumeister et al 1975.

Schobert, A. Kawoli, M and Hann, R. (1951), Grun getarble menshliche and tievesche II. Chem. Ber 84: 571-573.

Segel, I. H. (1975) Enzyme kinetics: Behaviour and analysis of Rapid Equilibrium and steady state enzyme systems. John Wiley and Sons Inc, New york, PP 884-926.

Smith, A.D.M. and Duckett, S. (1965), Cyanide, vitamin B_{12}, experimental demelination and tobbacco amblyopia. Brit J. Exp. Path 46: 615-622.

Sorbo, B.H. (1951a). Crystalline Rhodanese I Acta. Chem. scand 5: 724-726.

Sorbo, B.H. (1951b). Crystalline rhodanese II. The enzyme catalyzed reaction. Acta Chem. Scand. 5:1218-1219.

Sorbo, B.H. (1953). Studies on Rhodanese I. Acta. Chem. scand 7:238-241.

Saunders, J.P. and Himwich, W.A. (1950). Am. J. Physiol. 163, 404. Cited by Osuntokun, B.O. (1970). Cassava diet and cyanide metabolism in wistar rats. Br. J. Nutr. 24,797-800.

Tapper, B.A. Zilg H. and Conn E.E (1972). 2-hydroxaldoximes as possible precursors in the biosynthesis of cyanogenic glucosides. Phytochemistry 11: 1047-1053.

Tewe, O.O (1975). ph.D. Thesis, University of Ibadan, Nigeria, Cited by Cooke, R.D. 1978.

Thani, N.C. and Lohani, B.N. (1978). Cassava chipping and drying in Thailand. Cassava Harvesting and processing: Weber, E. J., Cock, J.H and Chouinard, A (eds). Proceedings of a workshop held at CIAT, Cali, columbia I.D.R.C. Ottawa, Canada IDRC-114e pp. 21-25.

Uribe L. and Conn, E.E. (1966). The metabolism of aromatic compounds in higher plants. J.Biol. Chem. 241: 92-94

White, A., Handler, P., Smith, E.L., Hil, R.L., Lehman, I.R. (1978). Hemoglobin and the chemistry of Respiration. Principles of Biochemistry 6th edition McGraw-Hill Book company, New York, pp. 974.

Wilson, J. (1965). Leber's heriditary optic atrophy: A possible defect of cyanide metabolism. Clin. Sci 29:505-515.

Wilson, J. (1965). Cyanide and human diseases. In Chronic Cassava Toxicity, B. Nestle and R. MacIntyre (eds) I.D.R.C-010e Ottawa, Canada. pp. 121-125.

Wilson, J. and D.M. Mattews (1966). Metabolic Inter-relationships between cyanide, thiocyanate and vitamin B_{12} in smokers and non smokers. Clin. Sci 31: 1-7.

Wood, T. (1965b). The cyanogenic glucoside content of cassava and cassava products. J. Sci. Fd. Agric 16:300-305.

Wood, T. (1966). The isolation, properties and enzymatic breakdown of linamarin from cassava. J. Sci. Fd Agric. 17: 85-90.

Wood, J.L. and Fielder, H. (1953). β-mercaptopyruvate, a substrate for rhodanese. J. Biol. Chem. 205: 231-234.

Wood, W. Wilson F., Benbow R., Hood L. E (1974). Biochemistry: A problems approach, W. A Benjamin Inc, publishers, menlo park California.

Zitnak, A. (1973.) Assay methods for hydrocyanic acid in plant tissue and their application in studies of cyanogenic glucosides in Manihot esculenta. In Chronic Cassava Toxicity. B. Nestle and R, MacIntye (eds) I.D.R.C., Ottawa, Canada IDRC 010e pp. 89-96.