BIOCHEMICAL AND MOLECULAR STUDIES ON β-GLYCOSIDASE FROM SORGHUM (*Sorghum bicolor* (L) Moench) AND CASSAVA (*Manihot esculenta* Crantz)

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ZARIA, NIGERIA.

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DEPARTMENT OF BIOCHEMISTRY
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AHMADU BELLO UNIVERSITY
ZARIA, NIGERIA.

DECEMBER, 2012

DECLARATION

I hereby declare that this thesis **"BIOCHEMICAL AND MOLECULAR STUDIES ON β-GLYCOSIDASE FROM SORGHUM (*Sorghum bicolor* (L) Moench) AND CASSAVA (*Manihot esculenta* Crantz)"** has been performed by me in the Department of Biochemistry, Ahmadu Bello University, under the supervision of Professor A.J Nok, Dr. E. Onyike and Dr. B.D. Kashina. The information derived from the literature has been duly acknowledged in the text and the list of references provided. No part of this thesis was previously presented for another degree or diploma at any university.

KWANASHIE ADOBE

…… .....

Name of student Date

CERTIFICATION

This thesis entitled **"BIOCHEMICAL AND MOLECULAR STUDIES ON β-GLYCOSIDASE FROM SORGHUM (*Sorghum bicolor* (L) Moench) AND CASSAVA (*Manihot esculenta* Crantz)"** by Kwanashie Adobe meets the regulations governing the award of the degree of Master of Science Degree in Biochemistry of Ahmadu Bello University, Zaria, Nigeria and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

"No species...possesses a purpose beyond the imperatives created by genetic history...The human mind is a device for survival and reproduction, and reason is just one of its various techniques."

Edward O. Wilson

Dedicated to all voyagers of discovery

ACKNOWLEDGEMENT

This thesis would not have been possible without the guidance and the help of several individuals who in one way or another contributed and extended their valuable assistance in the preparation and completion of this study.

First and foremost, my utmost gratitude to my supervisors, Professor A. J. Nok,
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In the words of Elizabeth Gilbert,

"In the end, though, maybe we must all give up trying to payback the people in this world who sustain our lives. In the end, maybe it's wiser to surrender before the miraculous scope of human generosity and to just keep saying thank you, forever and sincerely, for as long as we have voices."

Very many thanks.

The staff of the Department of Biochemistry for being accommodating and for their untiring effort in teaching and encouraging students to pursue scientific and professional growth, thank you for teaching me that life is the ensemble of functions that resist death.

My colleagues and staff in the Department of Crop Protection for the moral support and encouragement, your sincere and continuous care and concern will forever be cherished.

Class of 2007/2008, it was a great pleasure and privilege to undertake this trip of our voyage of discovery, and now, having been successful I say, congratulations.

Whether by strange coincidence or divine guidance in the course of our life we cross paths with many people, people who make an impact and their memory live on forever. These people we call friends. Mukasa Christopher, many thanks.

"If I have the gift of prophecy and can fathom all mysteries and all knowledge, and if I have a faith that can move mountains, but have not love, I am nothing"

1 Corinthians 13:2.

Gloria, for your unselfish and unfailing support, for your labor of love, thank you very much.

I Love thee, I love but thee. With a love that shall not die. Till the sun grows cold, and the stars grow old. ~William Shakespeare

My family, may God's love continue to encompass you, His grace continue to inundate you and His Spirit continue to shine forth through you. Very many thanks and much love.

To the one above all of us, the omnipresent God, for answering my prayers for giving me the strength to plod on despite my constitution wanting to give up and throw in the towel, thank you so much Dear Lord.

ABSTRACT

Genes encoding linamarase from cassava root cortex and dhurrinase from sorghum seeds were amplified using the polymerase chain reaction. Crude extracts of linamarase and dhurrinase containing most of the intracellular proteins were used to hydrolyze *p*-nitrophenyl-β-D-glucopyranoside (pNP) and linamarin extracted from cassava cortex. A gene fragment corresponding to linamarase (1,504 bp) was amplified. The native enzyme showed an optimum pH of 6.0 for pNP hydrolysis. The optimum temperature observed from the experimental assay showed that cassava linamarase has maximum activity for the hydrolysis of pNP at 65°C. The Q_{10}, calculated between 40°C and 50°C was 1.17. Activation energy (Ea) was found to be 17.32 KJ/mol (4.14 kcal mol^{-1}). The K_M for linamarase was 5.75 x 10^{-3} µmol l^{-1} with a kcat value of 3.41 min^{-1} and Vmax of 1.96 x 10 $^{-2}$ µmol l^{-1} min^{-1}. A 1,695 bp fragment corresponding to dhurrinase gene was also amplified. Dhurrinase enzyme activity was stable at room temperature (25^{o}C) with optimum temperature of 65^{o}C. Temperature coefficient (Q_{10}) calculated between 40^{o}C and 50^{o}C gave a value of 2.53. Activation energy (Ea) of 51.06 KJ/mol was also extrapolated. The pH activity curve put maximal activity for dhurrinase at pH 5.0. The double reciprocal plot of dhurrinase showed a calculated K_M of 1.39 x 10^{-2} µmol l^{-1}, Vmax of 1.01 x 10^{-1} µmol l^{-1} min^{-1} and kcat of 7.27 min^{-1}. Initial velocity data clearly showed that dhurrinase had an index of physiological efficiency (kcat) of 7.27 min^{-1}. Half life of enzyme activity (using linamarin as substrate) extrapolated from steady state data favored dhurrinase (288.79 min), linamarase showing a half life of 117.47 min. Our investigations have shown clearly that dhurrinase holds the capacity to totally detoxify cassava and its products of cyanogenic principles more effectively than linamarase.

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CHAPTER ONE

1.0 INTRODUCTION

Cyanogenic compounds capable of releasing cyanide on chemical breakdown are widespread in the plant kingdom (Bak *et. al*., 2006). More than 2,650 plant species are known to be capable of producing sufficient quantities of cyanogenic compounds that may be important in animal and human health (Bak *et. al*., 2006). These compounds are present in important food and feed crops, including cassava, lima beans, sorghum, almonds, plums, apricots and peaches, to mention but a few. The hydrolysis of these compounds, either by enzyme action or by strong acid is known to release cyanide, which can be very dangerous, if this release occurs in an animal or human body. In order to render them safe and edible, the toxicity associated with these important food crops is reduced by various processing methods, notable among them being various forms of fermentations.

Cassava (*Manihot esculenta* Crantz) is a perennial starchy root crop that is widely grown in the tropical regions of the world. Cassava gives a carbohydrate production per hectare which is about 40% higher than rice and 25% more than maize, thus cassava is the cheapest source of calories for both human nutrition and animal feeding (Tonukari, 2004). Global production of cassava is around 152 million tons per year. Half of the 16 million hectares of cultivated cassava worldwide is devoted to small-scale cassava cultivation in Africa, 30 percent is grown in Asia and 20 percent in Latin America (CIAT, 2001). Small scale farming is characterized by cultivation using traditional methods with little or no inputs and frequent intercropping practices. Cassava's role as a

traditional human food is changing to an efficient industrial crop in some parts of Africa, for instance in Nigeria (Nweke, 2004) and many parts in Asia and Latin America.

Cassava produces potentially toxic levels of cyanogenic glycosides (Linamarin [95%] and lotaustralin [5%]) which are synthesized in the leaves (Koch *et. al.*, 1992) and translocated to all other parts of the plant including the edible tuberous roots (McMahon *et. al.*, 1995). The breakdown of cyanogenic glycosides by the β–glycosidase linamarase results in hydrogen cyanide (HCN) production when cassava tissues are mechanically damaged. HCN in cassava tissues has been medically proven to be a potential health hazard for consumers if the plant is inadequately processed (Tylleskär *et. al.*, 1992; McMahon *et. al.*, 1995).

Sorghum is a genus of numerous species of grasses, one of which, *Sorghum bicolor* (L) Moench (commonly called sorghum), is raised for grain and many of which are used as fodder plants either cultivated or as part of pasture. Sorghum is an important food crop in Africa, Central America, and South Asia and it is the "fifth most important cereal crop grown in the world". (National Academy of Sciences, 1996) Guinea corn (sorghum) is the most widely grown of all the cereal crops produced by farmers in the Savanna zones of Nigeria. Annual production in this part of the country is estimated at over four million tonnes of grains. This crop is used almost entirely for human consumption and provides the staple food for most of the population, particularly in the ecological zones most suited for its cultivation.

The cyanogenic glycoside dhurrin constitutes 30% of the dry weight of sorghum shoot tips but is absent from the seed and roots. The co-distribution of dhurrin and its synthesizing system in the upper portion of the shoot shows that production and storage

sites are located within the same cells (Adewusi, 1990). Sorghum seedlings contain a specific β-glycosidase capable of converting dhurrin into p-hydroxybenzaldehyde, cyanide, and glucose. Upon damage to sorghum tissues, the enzyme and its substrate, which are compartmentalized in intact tissues, come into contact and release a toxic aglycone or a derivative (HCN) (Siritunga and Sayre, 2003). Under these conditions, dhurrin is hydrolyzed by an endogenous β-glycosidase (dhurrinase) to produce *p*-hydroxymandelonitrile, which subsequently disassociates to free HCN and *p*-hydroxybenzaldehyde.

Cassava (*Manihot esculenta* Crantz) is the most important source of dietary carbohydrates for 750 million people around the world, with its starchy root being the main harvested organ (National Academy of Sciences, 1996). Sorghum (*Sorghum bicolor* (L) Moench), king of millets is one of the staple food crops of the world, especially the drier parts of tropical Africa and Asia. It is a principal source of energy, protein, vitamins and minerals for millions of the poorest people in the regions where it is cultivated (National Academy of Sciences, 1996). Both crops contain potentially toxic compounds called cyanogenic glycosides, linamarin and dhurrin respectively, which are broken down by their specific enzymes linamarase and dhurrinase upon disruption of the plant cells to form hydrogen cyanide. In humans, the symptoms of acute cyanide intoxication from inadequately prepared cassava or sorghum can include: rapid respiration, drop in blood pressure, rapid pulse, dizziness, headache, stomach pains, vomiting, diarrhoea, mental confusion, twitching and convulsions (Bokanga *et. al.,* 1994). Therefore, proper processing methods which ensure total liberation of cyanogens must be developed.

1.1 Null Hypothesis

On account of the unique expression of linamarase and dhurrinase in their respective crops and the properties of the enzymes in catalysis of their substrates, a novel approach can be developed for the total hydrolysis of cyanogenic glycosides from cassava in the fermentation process leading to the production of cyanide free products. I therefore assume that the biochemical and molecular parameters measured for linamarase and dhurrinase are dissimilar.

1.2 Justification of Study

Cassava produces potentially toxic levels of cyanogenic glycosides (Linamarin (95%) and lotaustralin (5%) which are synthesized in the leaves and translocated to all other parts of the plant including the edible tuberous roots (McMahon *et. al.*, 1995). The breakdown of cyanogenic glycosides by β–glycosidase enzymes results in hydrogen cyanide (HCN) production when cassava tissues are mechanically damaged. Hydrogen Cyanide in cassava tissues has been medically proven to be a potential health hazard for consumers if the plant is inadequately processed (McMahon *et. al.*, 1995). Sorghum also expresses cyanogenic glycosides but mainly at the seedling stage and only at the shoot tips. Cassava, with the exception of the seeds, expresses in every tissue and at all times. There exist similarities between the actions of β–glycosidase in these two crops on their respective glycosides resulting in the release of hydrogen cyanide. It will be significant to identify any homologous sequences in genes encoding these glycosidases in local varieties of these crops. Considering that intoxication could lead to rapid respiration, drop in blood pressure, rapid pulse, dizziness, headache, stomach pains, vomiting, diarrhoea,

mental confusion and convulsions; even death may occur in some cases, the purpose therefore, is to determine the comparative nature of these toxic principles in their respective crops; the ultimate goal being to be able to produce cyanide free cassava products. The molecular analyses will go a long way to increase the understanding of β-glycosidase gene structure in different plant species.

1.3 Research Questions

In an effort to establish the possible relationship between Cassava linamarase and Sorghum dhurrinase, the following research questions have been considered.

i. What is the size of the gene in both plants and does this correspond to information in the gene bank?
ii. Do the sequences correspond to the gene bank and what similarities exist between the two sequences?
iii. What are the kinetic properties of the native enzyme extracts and how do these properties relate to each other?

1.4 Aim of the Study

Compare the biochemical and molecular parameters for linamarase (α-Hydroxyisobutyronitrile β-D-glucopyranoside) and dhurrinase (4-Hydroxymandelonitrile- β-D-glucoside)

1.5 Objectives of the Study

i. To isolate and quantify total DNA from Cassava (*Manihot esculenta* Crantz) and Sorghum (*Sorghum bicolor* (L) Moench)

ii. To amplify the gene encoding linamarase (α-Hydroxyisobutyronitrile β-D-glucopyranoside) and dhurrinase (4-Hydroxymandelonitrile- β-D-glucoside) from the isolated total DNAs by PCR**.**

iii. To determine kinetic properties of the native enzymes isolated from Sorghum (*Sorghum bicolor* (L) Moench) and Cassava (*Manihot esculenta* Crantz)

1.6 Significance of Study

The results of a study such as this are important in contributing further to the body of knowledge on the gene structure and origin of β-glycosidases in plants. More significantly, it will increase the availability of basic enzymological data obtained by molecular characterization. Also, the use and trial of modified versions of certain assays are expected to contribute to the process of defining more robust, yet simple and easily applicable assays for isolation and amplification of genes. There is also the possibility of contributing to knowledge which could aid in gene expression studies, leading to the selection of local crop varieties for cyanogens.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Sorghum

2.1.1 Origin of Sorghum

Sorghum (*Sorghum bicolor* (L) Moench) is now known almost worldwide. Dhows, which have been crossing the Indian Ocean for some 3,000 years, probably first carried it away from Africa and took it to India more than 2,000 years ago. It was almost certainly put on board as seamen's provisions. The sorghums of India are related to those of the African coast between Somalia and Mozambique. Sorghum probably traveled overland from India and reached China along the silk route about 2,000 years ago. It might also have gone by sea directly from Africa: Chinese seamen reached Africa's east coast more than about 1,000 years ago (probably in the eighth century AD), and they may well have carried some seeds home. This crop comes in such an array of widely different types that various botanists have previously recognized 31 species, 157 varieties, and 571 cultivated forms. However, these all cross readily and without barriers of sterility or differences in genetic balance, so it seems preferable to group them into a single species, *Sorghum bicolor*. Some botanical authorities also include certain wild sorghums, designating them as varieties within the species. (National Academy of Sciences, 1996)

2.1.2 Biology of Sorghum

Sorghum comes in many types. All, however, are cane-like grasses between 50 cm and 6 m tall (Figure 2.0a). Most are annuals; a few are perennials. Their stems are usually erect and may be dry or juicy. The juice may be either insipid or sweet. Most have a single stem, but some varieties tiller profusely, sometimes putting up more than a dozen stems. These extra stems may be produced early or late in the season. Plants that tiller after the

harvest has occurred can be cut back, allowed to re-sprout, and grown without replanting (like sugarcane). Soil permitting, the plant produces a deep tap root. However, a large number of multi-branched lateral roots occupy the upper soil levels, particularly the top meter. They can spread laterally up to 1.5 m. The leaves look much like those of maize. A single plant may have as few as 7 or as many as 24 leaves, according to cultivar. At first they are erect, but later curve downward. During drought they roll their edges together. Rows of "motor cells" in the leaves cause the rolling action and provide this unusual method of reducing desiccation. The flower head is usually a compact panicle. Each carries two types of flowers: one type has no stalk (sessile) and has both male and female parts (perfect); the other is stalked (pedicellate) and is usually male (staminate). Pollination is by wind, but self-pollination is the rule. The degree of cross- pollination depends on the amount of wind and the panicle type, open heads being more liable to cross-pollination than compact ones. Grains are smaller than those of maize but have a similar starchy endosperm. Most are partially covered by husks (glumes). (National Academy of Sciences, 1996)

The seed coat varies in color from pale yellow through purple-brown. Dark-colored types generally taste bitter because of the tannins in the seed coat. The endosperm is usually white and floury as in normal maize, but in some types the outer portion is hard and corneous, as in popcorn. The crop is always grown from seed. Some seeds show dormancy and will not germinate for a month or so after harvesting. It is a little-known fact that the plant can also be propagated by stem cuttings: nodes along the stem have tissues (primordia) that can produce both roots and sprouts and thereby grow a new plant.

a



b



Plate I (a) A mature Sorghum plant; the panicle can be seen bearing maturing seeds.

(b) Harvested Sorghum grains.

Culled from www.wikipeadia.com(accessed 12/5/2012)

The cyanogenic glycoside dhurrin is rapidly synthesized in etiolated seedlings of *Sorghum bicolor* (L.) Moench. The dhurrin content of the seedlings increases sigmoidally with the germination time. Shoots of 10 centimeters height contain 850 nanomoles of dhurrin per shoot corresponding to 6% of the dry weight (Halkier and Moller, 1989). The biosynthetic activity sharply rises upon germination and reaches a maximum level of 10 nanomoles dhurrin/ (hour x shoot) after 48 hours when the shoots are 3 centimeters high (Halkier and Moller, 1989). This maximum level is followed by a sharp decline in activity when germination time exceeds 65 hours. Dhurrin and the dhurrin synthesizing enzyme system are primarily located in the upper part of the etiolated shoot where both are evenly distributed between the coleoptile, the primary leaves and the upper 0.5 centimeter of the first internode including the shoot apex. Dhurrin constitutes 30% of the dry weight of the upper 1.2 centimeter of 10 centimeter high shoots. The seed and root contain neither dhurrin nor the dhurrin-synthesizing enzyme system (Saunders and Conn, 1978). The co-distribution of dhurrin and the enzyme system throughout the seedling indicates that production and storage sites are located within the same cell (Halkier and Moller, 1989).

Cyanogenic β –glycosides have long been known to be involved in the defense against some pathogens and herbivores, releasing the respiratory poison hydrogen cyanide (HCN) upon hydrolysis by β -glycosidase (Poulton, 1993). Upon damage to tissues, the

enzyme and its substrate, which are compartmentalized in intact tissues, come into contact and release a toxic aglycone or a derivative (e.g. HCN) (Selmar, 1993). Under these conditions, dhurrin is hydrolyzed by an endogenous β-glycosidase (dhurrinase) to produce *p*-hydroxymandelonitrile, which subsequently disassociates to free HCN and *p*-hydroxybenzaldehyde (Figure 2.1).

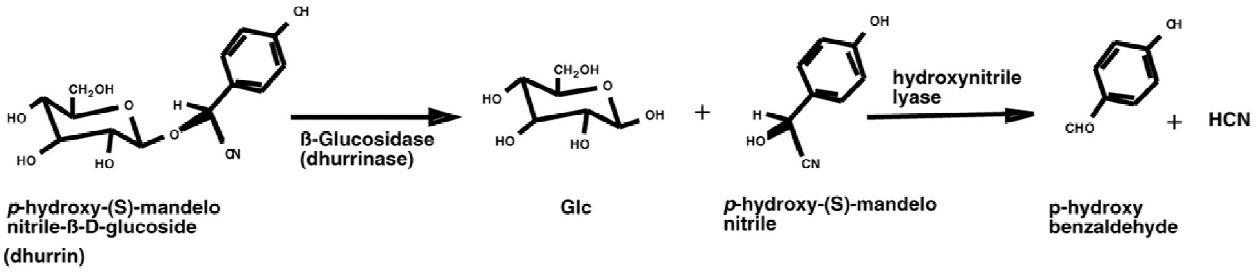


Figure 2.0: Hydrolysis of the natural substrate dhurrin and production of HCN by sorghum β-glycosidase (dhurrinase) Culled from the American Society for Biochemistry and Molecular Biology (2000)

2.1.3 Ecology of Sorghum

Sorghum is adapted to a wider range of ecological conditions than perhaps any other food crop. It is essentially a plant of hot, dry regions but takes cool weather in stride and may also be grown where rainfall is high and even where temporary water logging can occur. Although many cultivars are insensitive to photoperiod, sorghum is basically a short-day species. Most traditional varieties differentiate from vegetative to reproductive growth when day lengths shorten to 12 hours. This switch to flowering often happens just when the rains diminish, and the crop matures in the dry season that follows, a feature that greatly helps the farmer. Some of these traditional forms are extremely susceptible to photoperiod and reach impossible heights if not planted as day lengths shorten. On the

other hand, the dwarf sorghums of the temperate zone are unaffected by day length and can be planted year-round where climate permits. Although part of the crop is grown in rainy regions, sorghum is remarkably drought-resistant and is vitally important where the climate is just too dry for maize. Sorghum is grown from sea level to above 3,000 m. The plant is killed by frost. Optimum growth occurs at about 30°C. It is essentially a plant of the tropics or subtropics, roughly between 40° of the equator (National Academy of Sciences, 1996). Sorghum tolerates an amazing array of soils. It can grow well on heavy clays, especially the deep-cracking and black cotton soils of the tropics. It is equally productive on light and sandy soils. It can withstand a range of soil acidities (from pH 5.0-8.5) and tolerates salinity better than maize.

2.2 Sorghum Products

Sorghum is perhaps the world's most versatile crop. Some types are boiled like rice, some cracked like oats for porridge, some "malted" like barley for beer, some baked like wheat into flat breads, and some popped like popcorn for snacks. A few types have sugary grains and are boiled in the green stage like sweet corn. The whole plant is often used as forage, hay, or silage. The stems of some types are used for building, fencing, weaving, broom-making, and firewood. The stems of other types yield sugar, syrup, and even liquid fuels for powering vehicles or cooking meals. The living plants are used for windbreaks, for cover crops, and for staking yams and other heavy climbers. The seeds are fed to poultry, cattle, and swine. On top of all that, sorghum promises to be a "living factory." Industrial alcohol, vegetable oil, adhesives, waxes, dyes, sizing for paper and cloth, and starches for lubricating oil-well drills are just some of the products that could be obtained.

2.3 Sorghum Cultivation in Nigeria

About 50 % of the total area devoted to cereal crops in Nigeria is occupied by Sorghum. The area estimated at 6.86 million hectares extends northwards from latitude 8^{o}N – 14^{o}N (Aba *et. al*., 2004). Nigeria is the largest producer of sorghum in the West African Sub region, accounting for 90 % of the regional production output in 2008. This also makes Nigeria the single largest producer in Africa, accounting for about 50 % of the continents output.

Table 2.0: 2007/2008 Sorghum Production Figures and projections for the 2009/2010 season.
Culled from World Sorghum Production, 2009 (agrostats.com)

Country/Region	Yield (Metric tons per hectare)				Production (Million metric tons)			
			2009/10 Projected				2009/10 Projected	
	2007/08	Preliminary 2008/09	Jun	Jul	2007/08	Preliminary 2008/09	Jun	Jul
World	1.60	1.57	1.57	1.57	63.26	61.97	61.69	61.69
United States	4.60	4.08	4.01	4.00	12.64	12.00	9.65	9.65
Total Foreign	1.37	1.37	1.41	1.41	50.62	49.97	52.04	52.04
Sub-Saharan Africa								
Nigeria	1.35	1.49	1.53	1.53	10.00	11.00	11.50	11.50
Sudan	0.68	0.71	0.71	0.71	4.50	4.70	4.70	4.70
Ethiopia	1.73	1.69	1.68	1.68	2.66	2.62	2.60	2.60
Burkina	1.04	1.11	1.11	1.11	1.51	1.80	1.80	1.80
Tanzania	1.00	1.00	1.00	1.00	0.90	0.90	0.90	0.90
Niger	0.62	0.67	0.60	0.60	0.93	1.00	0.90	0.90
Uganda	1.58	1.58	1.58	1.58	0.49	0.49	0.49	0.49
Mozambique	0.70	0.75	0.75	0.75	0.35	0.38	0.38	0.38
Ghana	0.75	0.81	0.81	0.81	0.16	0.28	0.28	0.28
South Africa	2.89	3.00	2.56	2.56	0.26	0.27	0.23	0.23

2.4 Cassava

2.4.1 Origin of Cassava

Cassava is originally from South America, probably domesticated in the Amazon region (Olsen, 2004). The process of cassava domestication involved selection for root size, growth habit, number of stems and the ability of clonal propagation through stem cuttings. About 98 species of *Manihot* are known. (Rogers & Appan, 1973). Sexual barriers within the genus appear to be weak, indicating a recent evolution of the genus (Nassar, 1982). Most traditional domestication hypotheses have envisioned the crop to be a "compilospecies" derived from one or more species complexes, either in Mexico and Central America (Rogers & Appan, 1973) or throughout the Neotropics (Sauer, 1993). However, evidence provided from recent findings studying the glyceraldehyd 3-phosphate dehydrogenase (*G3pdh*) gene showed that the crop does not seem to be derived from several progenitor species, and cassava does not share haplotypes with *Manihot pruinosa*, a closely related, potentially hybridizing species (Olsen & Schaal, 1999). The *G3pdh* locus provides high levels of non-coding sequence variation in cassava and its wild relatives. The studies showed that cassava was likely domesticated from wild *M. esculenta* populations along the southern border of the Amazon basin. Furthermore, studies using single nucleotide polymorphisms (SNPs) and simple sequence repeat (SSR) markers showed that cassava was likely domesticated from the wild *Manihot* species, *Manihot esculenta* ssp. *flabellifolia* (Olsen, 2004). These findings have provided the clearest insight to date on cassava's origin.

a



b



Plate II: (a) Young cassava plants (b) Harvested cassava tubers

Culled from www.wikipeadia.com(accessed 12/5/2012)

2.4.2 Ecology of Cassava

Typically the crop is grown between 30° north and 30° south of the equator, in areas where the annual mean temperature is greater than 20°C. It is traditionally grown in a savanna climate, but can be grown in areas with extreme rainfall. In moist areas it does not tolerate flooding. In droughty areas it sheds its leaves to conserve moisture, producing new leaves when rains resume. It takes 18 months or more to produce a crop under adverse conditions such as cool or dry weather. Under more normal conditions the crop reaches maturity in about 9 months. Cassava does not tolerate freezing conditions. In contrast to other staple crops, it grows well under marginal as well as favourable conditions of soil fertility and rainfall. It tolerates a wide range of soil pH, from 4 to 8 (Howeler, 1978), and is most productive in full sun. Cassava can grow indefinitely,

alternating periods of vegetative growth, storage of carbohydrates in the roots and even periods of almost dormancy, brought on by severe climatic conditions such as low temperatures or prolonged water deficit (Alves, 2002). The implication is not only that the crop is found in an array of environments across the continent, but also that it can adapt to variations in relief, soils and cropping systems within the same agro ecological zone.

2.4.3 Biology of Cassava

Cassava (*Manihot esculenta* Crantz) is the only cultigen in the *Euphorbiaceae* family (O'Hair, 1995). It is a shrub that grows erect to heights between 1.5-2.0 m although some varieties are known to reach 4 m (Figure 2.2a). Cassava is monoecious with male and female flowers found on the same plant. The female flowers usually mature one to two weeks earlier than the male flowers (protogyny), a mechanism that enhances out-crossing (Ng and Ng, 2002). Cassava is insect pollinated, mainly by bees (Hahn *et. al.*, 1979). Under natural conditions as well as in plant breeding, propagation by seed is common. Seeds normally undergo a dormancy period lasting months and require relatively high temperatures (30-35 °C) for optimum germination (Ellis *et. al.*, 1982). Cassava is mainly propagated by stem cuttings. It has thickened, knobby stems resulting from shortened internodes, swollen leaf scars and enlarged stipule bases. The thickening, indicative of increased resource allocation to the stem, is thought to reflect artificial selection for ease of vegetative propagation (Ellis *et. al.*, 1982).

Cassava forms fibrous roots which can undergo increased tuberization (Figure 2.2b). Due to cambium activity and deposition of starch grains in secondary xylem tissues, a few fibrous roots swell to produce storage roots. A plant can produce between 5 and 20 tubers

depending on variety and soil conditions, with fewer roots forming in poorer soils. At maturity tuberous roots can be 15-100 cm in length and weigh up to between 0.5-2.0 kg (Knoth, 1993). Cassava storage roots are not reproductive organs and cannot be used as planting material. The roots start undergoing post-harvest physiological deterioration within a day or two after harvest and this consequently results in their short shelf life (Beeching *et al.*, 1998).

Cassava also produces potentially toxic levels of cyanogenic glycosides (Linamarin (95%) and lotaustralin (5%) which are synthesized in the leaves and translocated to all other parts of the plant including the edible tuberous roots (McMahon *et. al.*, 1995). The breakdown of cyanogenic glycosides results in hydrogen cyanide (HCN) production when cassava tissues are mechanically damaged. HCN in cassava tissues has been medically proven to be a potential health hazard for consumers if the plant is inadequately processed (McMahon *et. al.*, 1995). There is a considerable variation in the root content of cyanogenic glycosides among genotypes but the level also depends on the growth environment (Bokanga, 1994). Environmental factors during the growing season contribute significantly to the variation in CNP among genotypes as well as within genotypes and in various parts of the plant (Dixon *et. al.*, 1994). The growth stage of the plant appears to have an effect on the cyanogenic glycoside build up; a high level occurring 120 days after planting (DAP) drops dramatically by 180 DAP coinciding with the beginning of the active root-bulking phase (Bokanga, 1994). Cassava varieties with high cyanogenic glycoside content (>1000 mg Hydrogen cyanide (HCN) equivalent kg-1dry weight) are said to be toxic while cassava with low content of cyanogenic glycosides (<200 mg HCN equivalent kg-1dry weight) are said to be safe for

consumption without processing (Iglesias *et. al.*, 2002). Traditionally, cassava roots are processed by a variety of methods (Ugwu & Ay, 1992) into many different products and used in diverse ways according to local custom and preference. However, some basic steps are followed. After peeling of the roots processing steps consist of grating, crushing, microbial fermentation, enzymatic action or a combination of these. This is usually followed by either heating or drying to reduce moisture content. The final stage in the processing of the roots is to make cassava flour.

Studies have been conducted on the biochemical pathway of cyanogenic glycosides in cassava. Cassava synthesizes and stores two structurally related cyano-glycosides, linamrin and lotaustralin, which accumulate in the leaves and roots in the ratio of 93:7 respectively. The cyano-glycosides are synthesized from the amino acids valine and isoleucine. A small family of cytochrome P450 genes (*CYP79D1* and *CYP79D2*) controls the first step in the conversion of the amino acids in linamarin and lotaustralin synthesis (Andersen *et. al.*, 2000). Multifunctional enzymes convert either valine or isoleucine to their respective oximes which are subsequently converted by another cytochrome P450 (CYP71E) to the respective hydroxynitrile. The hydroxynitrile is then glucosylated by uridine diphosphate glucosyltransferase to make the cyanogenic glycoside. Cyanogenic glycosides are typically stored in cell vacuoles and following tissue damage the glycosides get released allowing them to be de-glycosylated by glycosidase enzymes found in the cell walls (McMahon *et. al.*, 1995). The product of the de-glycosylation, acetone cyanohydrin, is then decomposed to yield cyanide and acetone with the action of hydroxynitrile lyase. The decomposition can also occur spontaneously at pH> 5.0 or at elevated temperatures of > 35^{o}C. Siritunga & Sayre (2003, 2004) developed transgenic

acyanogenic plants, in which the expression of the *CYP79D1* and *CYP79D2* genes were selectively inhibited in the leaves, in a bid to eliminate cyanogens from cassava. (Figure 2.3)

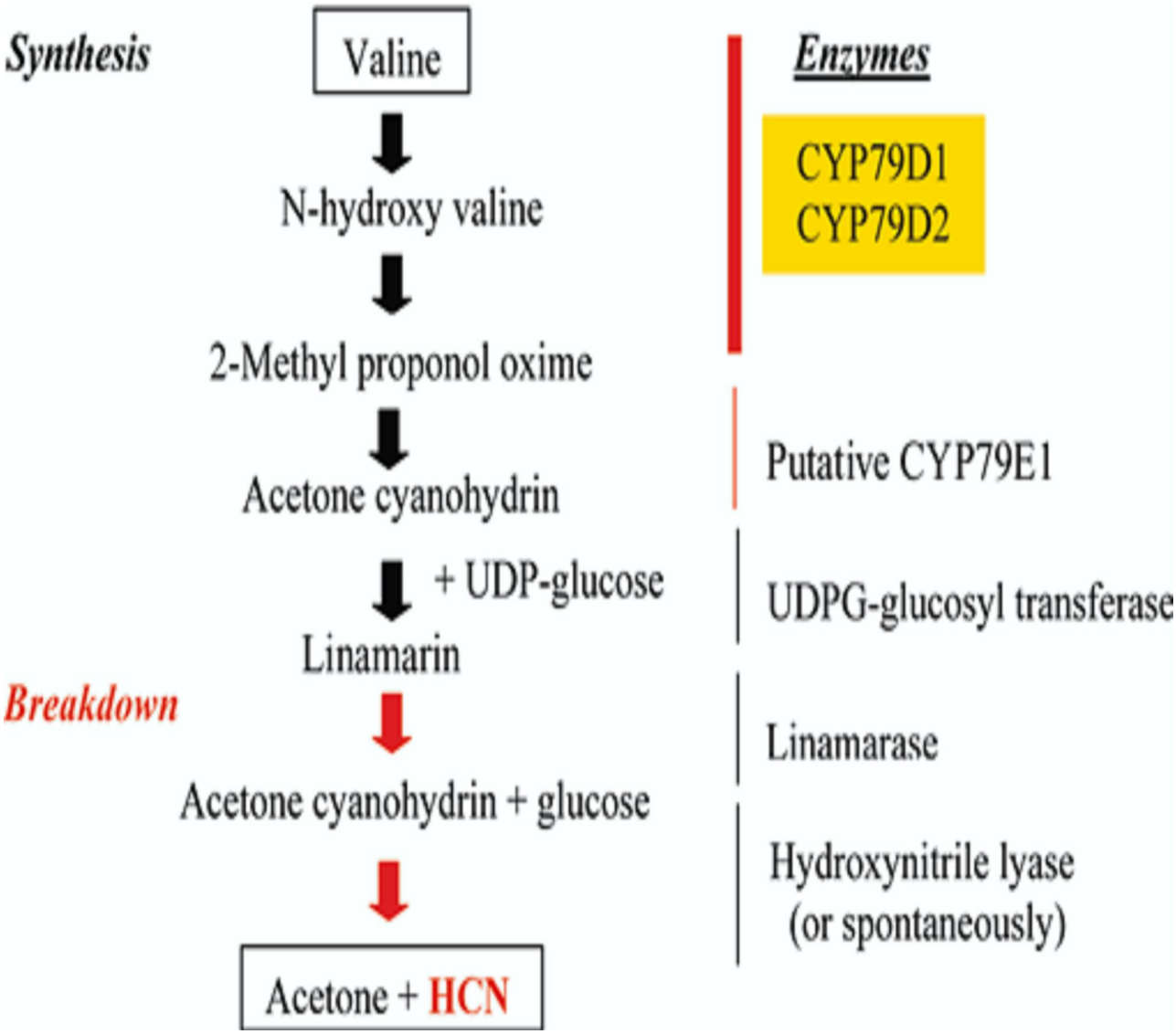


Figure 2.1: Linamarin biosynthesis and breakdown pathway in cassava (*Manihot esculenta*). The CYP79D1 and CYP79D2 enzymes (cytochrome P450) convert both valine and isoleucine to its corresponding oxime for linamarin and lotaustralin production, respectively. Culled from Siritunga and Sayre (2003)

However, the transgenic plants were unable to grow in the absence of reduced nitrogen (NH_3) suggesting that cyanide derived from linamarin is a major source of reduced nitrogen for cassava root protein synthesis (Siritunga and Sayre, 2004). It should be explained that in this experiment linamarin levels were greatly reduced both in the leaves and roots up to 99% in the transgenic plants whereas in non-transgenic sweet cassava varieties glycosides are produced in the leaves but to a much lesser extent transported to the roots as in bitter varieties. Among other cyanogenic species, for instance white clover, cyanogenesis is polymorphic and both cultivars and natural populations contain both cyanogenic and acyanogenic plants. In clover the polymorphisms are under relatively simple genetic control (Hughes *et. al.*, 1984) unlike in cassava where the variation is quantitative and may be polygenically based (Dixon *et. al.*, 1994). So far the only evidence of a differential pest attack on cassava with low versus a high CNP is found with the burrowing bug (*Cyrtomenus bergi*) in South America (Riis *et. al.*, 1994). Formal breeding for low CNP in cassava has been fraught with the confounding effect of the environment on its expression and the long growth cycle of cassava. Since the biosynthesis of the cyanogenic glycosides is complex, with different enzymes involved and probably affected by many genes with influence from the environment, mapping regions in the genome affecting the phenotypic variation (quantitative trait loci, QTL) for the trait has the potential to make formal breeding for variety improvement more efficient.

2.5 Cassava Products

The starchy roots are mainly used as human food, fresh when low in cyanogens, or in many processed forms and products, mostly starch, flour, and for animal feed. Cassava

utilization patterns differ depending on the region of the world. In Africa the majority of cassava produced is used for food consumption with 50% in processed form and 38% in the fresh and boil form. Twelve per cent is used for animal feed. In Latin America between 35 and 40 percent of the cassava production is used for food consumption and a third of the cassava produced is for animal feed, the remaining percentage being used in industry or for export. Starch represents an important use in Latin America; however, this use is more significant in Asia where cassava chips are exported to Europe for use in animal feed (Westby, 2002). Although cassava is primarily grown for its starchy tuberous roots, in some cultures in Africa young leaves are used as vegetables or as a constituent in a form of sauce eaten along with main staple meals (Lancaster and Brooks, 1983). Cassava, however, produces cyanogenic glucosides, which is often rightly or wrongly seen as a health hazard to consumers, particularly the very poor of the population that depend on cassava as a staple crop. In many cassava farming and food systems, cassava varieties are classified based on their perceived toxicity determined by taste (Chiwona-Karltun *et. al.*, 2004). Farmers' varieties with high levels of cyanogenic glucosides have in general bitter taste and are referred to as bitter while those with low levels of cyanogenic glucosides are called sweet or cool varieties. The cyanogens have been observed to protect the plant from herbivory (Riis *et. al.*, 1994) as well as theft (Nweke *et. al.*, 2002) improving food security for the small-scale farmers.

2.6 Cassava in Africa

Total production of cassava in Africa increased from approximately 35 million tonnes in 1965 to over 80 million tonnes in 1995 (FAO, 1998). According to FAO (1999), the rate of increase of cassava production has been higher than any other crop in Africa since the

1980s. It is now one of Africa's major starchy staples second to maize, feeding 200 million people (Nweke, 2004). Most of the increase in cassava production has been due to increase in area under cultivation rather than increases in yield per hectare (Hillocks *et. al.*, 2001). Increase in the cultivation of cassava during the 1990s occurred at least partly in response to declining soil fertility and increased costs of fertilizers for example in countries like Malawi, resulting in replacement of maize cultivation with cassava (Teri *et. al.*, 1999). On the whole, the replacement of maize and other major crops with cassava in Africa had been fostered by the interplay of a wide range of agro-ecological and socio-economic factors which have resulted in a diversity of conditions under which crops are grown, marketed and consumed. The socio-economic factors include declining fallow periods due to population pressure; infrastructure and market access and the ecological factors include different abiotic and biotic stresses (Hillocks *et. al.*, 2001). The interplay of these factors causes farmers to replace more demanding crops with cassava. In the Collaborative Study of Cassava in Africa (COSCA) survey, comprising 80% of the cassava cultivation in Africa, it was found that farmers are also continually abandoning old cassava cultivars and introducing new ones (Nweke and Bokanga, 1994) reflecting a need for better varieties.

2.7 Plant Toxins and Cyanogens

Contrary to the general consumer notion of equating 'natural' with 'safe' when considering plant-based foods and food supplements, scientific literature shows that there is a wide variety of natural plant-derived toxins. These products, generally called phytotoxins, can be present in the fruit, vegetable and tuber components of the diet (Rietjens *et. al*, 2005). Phytotoxins are many and varied in their chemistry, and are

generally regarded as secondary plant metabolites. Groupings of phytotoxins in common food products include glycoalkaloids (like solaline and chaconine) present in potatoes and tomatoes, coumarin in cinnamon, cyanogenic glycosides in cassava, sorghum and linseeds, as well as alkenylbenzenes in herbs like sweet basil, cinnamon and nutmeg (Deshpande, 2002). At the current levels of exposure through foods, most of these compounds do not pose a health threat. However investigations in recent times have shown that some of these products have general toxicity as well as genotoxic and carcinogenic consequences over prolonged use (Tylleskar *et. al.,* 1992). Hence a lot of efforts have been put into researching the mode of action and toxic effects of some of these compounds. In this regard, cyanogenic glycosides have been of great interest (Bokanga *et. al.*, 1994). Cyanogenic glycosides are compounds that on treating with acid or the appropriate enzyme produce hydrocyanic acid (HCN). They are mainly cyanohydrin derivatives of sugars (mainly glucose) or long chain fatty acids (Deshpande, 2002). They have been identified in over 2650 species of higher plants. Plant families such as Leguminosae, Rosaceae, Euphorbiaceae and Graminae, have substantial members which contain important cyanogens (Bak *et. al.*, 2006).

Several cyanogenic glycosides (or cyanogens) have been isolated and well characterized since hydrogen cyanide was first isolated from bitter almonds and from leaves of peach by Scrade in 1802. Some of the well known compounds in this group are amygdalin, dhurrin, linamarin, lotaustralin, prunasin, neolinustatin and taxiphylin (Deshpande, 2002). Cyanogens are important in human and animal nutrition due to their ability to produce/release cyanide in the body, with associated adverse physiological consequences.

2.7.1 Cyanogenic Crops

Cyanogenic glycosides are widely spread natural toxicants, and occur in food crops that are of importance to humans and animals. Some of the common foods containing different levels of cyanogenic glycosides include almonds, peaches, plums, apples, sorghum, cloves, linseed, lima beans, cassava, and cherries among others (Deshpande, 2002). Some of the more common and widely researched cyanogens named above are shown in table 2.1.

Table 2.1: Cyanogenic glycosides in common food crops
Culled from Deshpade, 2002.

Glycoside	Food crops
Amygdalin	Almonds, Cherry, Peach, Plums, Apples
Dhurrin	Sorghum
Linamarin	Linseed, Cloves, Cassava, Lima beans
Lotaustralin	Cassava, Lima beans, White cloves
Linustatin	Linseed
Neolinustatin	Linseed
Prunasin	Almonds, Cherry

2.7.2 Biology of Cyanogens and Enzymes in Plant Tissue

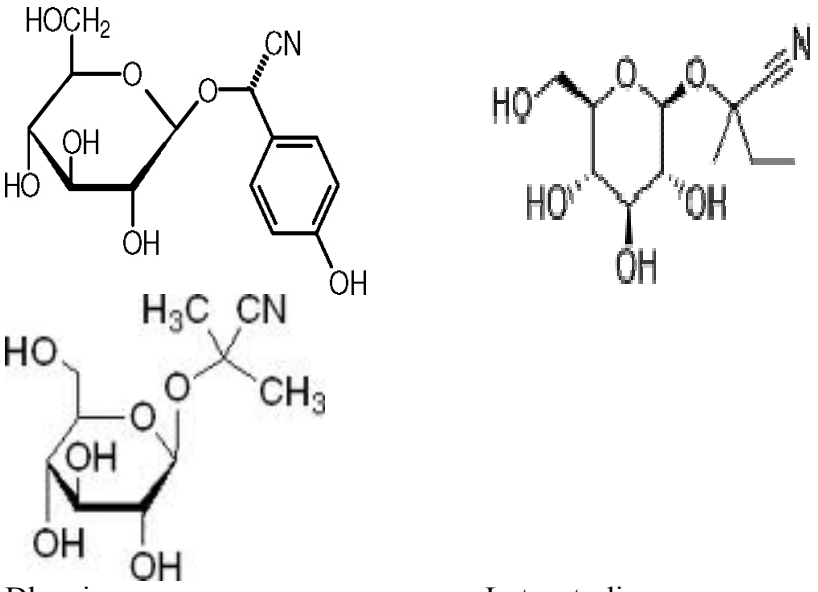
The biological importance of cyanogenic glycosides have been studied and discussed in various plants species. Jones (1988) observed that cyanogenic clovers were less frequently attacked by slugs than their non-cyanogenic counterparts. The inherent

toxicity (due to cyanogenic glycosides) and the bitterness in cyanogenic plants like cassava has been suggested as the cause of their limited use by monkeys and rodents in the wild, though not fully researched and documented (Møller and Seigler, 1998). It has thus been suggested that cyanogens were evolved by these plants to serve as protection from general feeders. However, many organisms have also evolved to withstand the high amounts of cyanogens in these food plants (Nielsen and Møller, 2004).

2.7.3 Biosynthesis (Cyanogenesis) and Structure

Cyanogenesis is a term used to describe the ability of a living organism to produce hydrocyanic acid, though it is also used to describe the process leading to the formation of cyanogenic glycosides in plants. The biosynthesis of these compounds has been investigated extensively in recent decades and is known to involve the conversion of certain amino acids into their corresponding alpha-hydroxynitriles through several intermediates. In the terminal step, these unstable hydroxynitriles are glucosylated to yield cyanogenic glycosides (Vetter, 2000). All of the steps of this channeled synthesis are catalyzed by membrane bound multi-enzyme complex containing multifunctional cytochrome P450 complexes (Bak *et. al.*, 2006). Cyanogenic glycosides are varied in nature and structure, though the pathway for their biosynthesis is similar. The difference mainly lies in the amino acid mostly involved in the synthesis. Thus, use of L-phenylalanine results in prunasin and amygdalin and their structurally related less common glycosides. L-tyrosine usage in the pathway results in the formation of dhurrin, taxiphyllin or such related structures, while L-isoleucine leads to lotaustralin. Linamarin

is the result of L-valine incorporation in the cyanogenesis pathway (Vetter, 2000). The chemical structures of some cyanogens are shown in figure 2.4.



Dhurrin Lotaustralin Linamarin

Figure 2.2: Chemical structure of some cyanogenic glycosides

A summary of the general process as given by Zagrobelny (2004) is shown in figure 2.5.

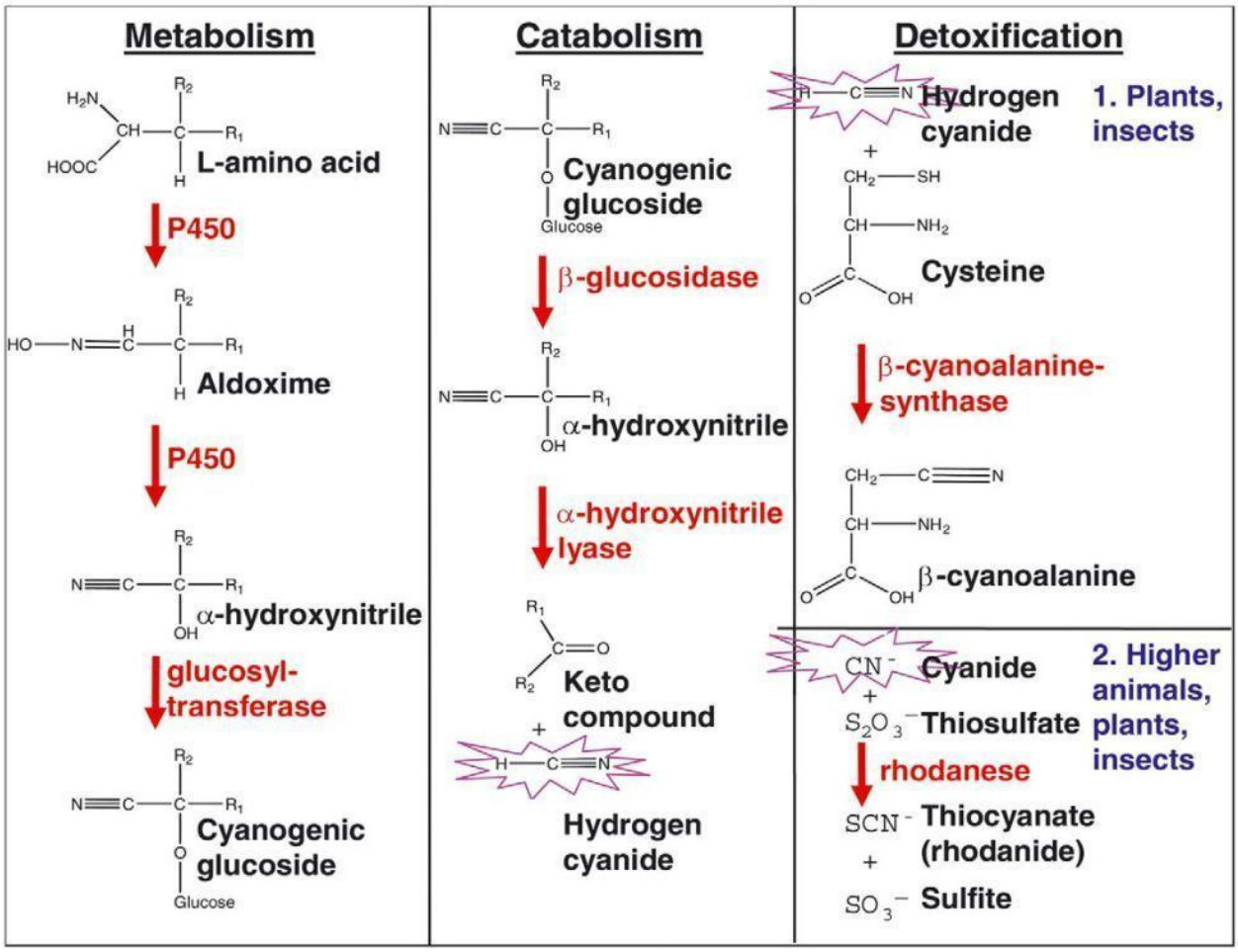


Figure 2.3: Biosynthesis, catabolism and detoxification of cyanogenic glycosides (CNGs) in plants, insects and higher animals. Enzymes involved are shown in red.

2.7.4 Chemistry and Breakdown of Cyanogenic Glycosides

Cyanogenic glycosides are generally stable at neutral pH. They can be hydrolyzed into their component parts- an aldehyde, sugar and HCN, by acid at elevated temperatures. In nature and traditional food processing conditions however, the production of HCN from these glycosides is an enzymatic process. Generally plants that contain cyanogens also have β-glycosidase that can degrade those cyanogens into their corresponding aglycone and sugar. There may also be a second enzyme in some tissues of the plant, such as

hydroxynitrile lyases which catalyse splitting of the aglycone /cyanohydrin to a carbonyl compound and hydrocyanic acid (Vetter, 2000). For instance, the cyanogenesis of linamarin is known to be a two-step reaction. In the first of these steps, linamarinase, a β-glucosidase naturally present in the cell of the cassava hydrolyses the linamarin into D-glucose and acetone cyanohydrin. The cyanohydrin is then further dissociated to acetone and HCN, either spontaneously or catalysed (especially in fermentation of the leaves) by an alpha-hydroxynitrile lyase (McMahon *et. al.,* 1995). This is depicted in Figures 2.1 and 2.3.

In the intact plant tissue, these enzymes and the corresponding cyanogenic substrates are physically separated and thus can only come into contact when the plant tissues are disrupted as during processing (crushing, milling). For instance, in cassava, linamarin is formed and located in the vacuole, while the enzyme linamarase is bound to the cell wall. In the leaf tissue, the enzyme alpha-hydroxynitrile lyase is also present in the cell wall. However their presence and expression in the cassava roots (main food source) is minimal or absent. This difference in enzyme expression and composition in different tissues of the same plant is also present in other cyanogenic crops such as sorghum and flax (White *et. al.*, 1998).

2.7.5 Cyanogens in Food and Health

Cyanogenic food plants are important, mainly because of their ability to release cyanide in the body with its associated health problems. Generally, traditional and modern methods of processing exist that aim at detoxifying the plant product before consumption. The general principle is to use the naturally present enzymes to cause degradation of the

cyanogens and eventually evaporate the HCN, which has a boiling point of about 26^{o}C. However, in many instances, processed food has been found to evolve sub-lethal amounts of cyanide due to improper detoxification (Tyskellar *et. al.*, 1992).

For instance, cassava leaves can contain more than 5.0g linamarin /kg fresh weight, while the roots could contain between 100 and 500mg linamarin/kg fresh weight, depending on the cultivar (White *et. al.*, 1998). Amoa-Awua (1996) reported a cyanogenic potential for 123.2mg/kg fresh edible cassava (grated root), made up of 119.3mg/kg cyanogenic glycosides, 2.8mg/kg cyanohydrin and 1.1mg/kg free cyanide (HCN). Until recently, it was generally accepted that the major residual cyanogens in cassava products was linamarin, as the cyanohydrin was assumed to be unstable. However, Tylleskar *et. al.,* (1992) have shown that, acetone cyanohydrin is rather the dominant cyanogen residual, and White *et. al.,* (1998) have attributed this to the non-availability of the hydroxynitrile lyase in the roots, as opposed to their presence in the leaves.

Once food with HCN or other cyanogens are ingested, several scenarios are possible. Cyanogens can be broken down by intestinal microflora to release cyanide. However, HCN, whether ingested directly or released from cyanogens in the body is readily absorbed into the blood by binding to iron in haemoglobin and quickly distributed to organs such as liver, kidney, brain and the blood tissue (Carlson *et. al.*, 1999). The major pathway for cyanide metabolism in the body is the detoxification process catalysed by the enzyme rhodanese, which is the main defense mechanism. This takes place in the presence of thiosulphate which acts as a sulphur donor, leading to the formation of the less toxic thiocyanate compounds which can be excreted. Other pathways for detoxification involve reactions between cystine and cyanide to form 2-amino-

thiazolidine-4-carboxylic acid which is excreted. Vitamin B12 (hydroxycobalamin) can also combine with cyanide to form cyanocobalamin which is also excreted in the urine and bile (WHO, 1993). About 80% of ingested cyanide (in small quantities) is known to be converted to thiocyanate in the urine after a short period (Rosling, 1987).

The main toxic effect of cyanide has been attributed to its production of histotoxic anoxia by inhibition of cytochrome oxidase, the terminal oxidase of the mitochondrial respiratory chain (Deshpande, 2002). Usually, when this happens, it has been shown that a number of enzymes are equally inhibited. It has thus been inferred that cyanide toxicity may not be a single biochemical lesion, but a complex effect of various enzyme systems involving metaloenzymes and Schiff base intermediates. Most of these cyanide sensitive enzymes have molybdenum, zinc or copper in their structure, as in catalase, nitrate reductase and also myoglobin (Bhattachara, 2000). Cyanide thus generally disrupts energy metabolism at the cellular level, leading to increased blood glucose and lactic acid levels. This is manifested physically as hyperpnoea, dyspnea and eventual convulsive seizure. Acute intoxication may manifest as rapid respiration, drop in blood pressure, rapid pulse, dizziness, headache, stomach pains, vomiting, diarrhoea, mental confusion and convulsions; even death may occur in some cases. The acute lethal dose is reported to be 0.5-3.5mg/kg body weight (about 30-210 mg for a 60kg adult) (WHO, 1965).

Acute toxicity of cyanide from consumption of cyanogenic plant products is however not common. Oke (1980) had estimated that if it was approximated that about 100- 200mg of HCN is the lethal dose for man, as much as 10-20kg of cassava (10- 20mg cyanide/kg) will have to be consumed at a sitting to produce significant toxicity. Thus, chronic intoxication manifestations are more common. There is the widespread pathological

condition (in West Africa) called tropic ataxic neuropathy (TAN) characterized by lesions of skin, mucous membranes, optic and auditory nerves, spinal cord and peripheral nerves (Oluwole *et. al.*, 2003). Though the neuropathology of the disease has been argued to be compatible with effects of chronic cyanide intoxication, recent work has shown that the situation may be more complex than initially assumed; but the role of cyanogens is not ruled out (Oluwole *et. al.*, 2003; Nhassico *et. al*., 2008). Konzo, an upper motor neuron disease, associated with high and sustained cyanogens intake at sub-lethal concentrations from cassava flour and cassava, in combination with low intake of sulphur amino acids (Nhassico *et. al.*, 2008). It is characterized by an irreversible but non-progressive symmetric spastic paralysis that has an abrupt onset. It mostly affects women of child bearing age and children, with observed high urinary thiocyanate concentrations.

There are also nutritional consequences to cyanide exposure from cyanogenic glycosides. Cassava roots are very low in protein content. Thus, when intake of dietary protein from other sources is inadequate, the preferential use of metabolically available sulphur-containing amino acids for cyanide detoxification is believed to hamper protein synthesis and contribute to retarded growth (FSANZ, 2005). There is also the effect of the thiocyanate produced from cyanide detoxification on the thyroid gland and the subsequent expression of cretinism, goitre and myxedema. This is because thiocyanate is similar to the iodine molecule in size and thus, interferes with the uptake of iodine in the thyroid gland (Rosling, 1987). This phenomenon is a major nutritional problem related to growth of children in developing countries where cassava is a staple. Because of the lack of quantitative toxicological and epidemiological information, a safe level of intake of cyanogenic glycosides is difficult to estimate. However, it has been accepted that a level

of up to 10mg/kg HCN as in the Codex Standard for Cassava Flour (CAC, 1991) is not associated with acute toxicity.

2.8 β -Glycosidase

β -glucosidases (EC 3.2.1.21) catalyze the hydrolysis of glycosidic linkages in aryl and alkyl β -glycosides and cellobiose and occur ubiquitously in plants, fungi, animals, and bacteria. β -glycosides and β -glycosidase are ubiquitous in the living world, therefore, one expects to find structural and catalytic properties shared by all β -glycosidases. In fact, a review of the literature indicates that almost all β -glucosidases have subunit molecular weights of 55 to 65 kDa, acidic pH optima (pH 5-6), and an absolute requirement for a β -glycoside (i.e., glucoside, and to a much lesser extent fucoside and galactoside) as substrate (Esen, 1993). β -glycosidase from widely different sources show remarkable similarity in substrate specificity for glycone (glucose) and some nonphysiological aglycones (e.g., nitrophenols and umbelliferone), although they may have widely different physiological glycosidic substrates with different aglycone moieties (Poulton, 1990). In general, β -glycosidase from different orders and kingdoms appear to differ in their specificities for the aglycone (an aryl or alkyl group) linked to the glucosyl group by a β -glycosidic bond. In plants, the β -glycosidase of dicots are localized to the cell wall or protein bodies while the β -glycosidases of monocots are localized to the plastid. In mammals (e.g., humans and mice), acid β -glycosidase is localized to the lysosome while its neutral counterpart is a soluble protein (cytosolic). Plant β -glucosidases have been implicated in a variety of key metabolic events: growth, productivity, and food and feed toxicity-related reactions (Esen, 1993). In humans, one β -glycosidase, commonly known as glycocerebrosidase (β -D-glucosyl-N-acylsphingosine

glycohydrolase) or human acid β -glycosidase, catalyzes the degradation of glucosylceramide in the lysosome. The deficiency of the enzyme leads to an inherited disease, Gaucher's disease, in which the glycosphingolipid glucosylceramide accumulates within the lysosomes of reticuloendothelial cells, producing one of the three forms of this disease (Esen, 2003). The molecular biology of the gene encoding this human β -glucosidase and the physicochemical properties of the enzyme has been studied and are well-understood. Human acid β -glucosidase has potential in the development of therapeutic and diagnostic procedures, particularly for the treatment of Gaucher's disease. β -Glucosidases of cellulolytic organisms (some fungi and bacteria) have been the subject of much past and ongoing research. These enzymes are expected to be targets for genetic engineering to design and select β -glucosidases for specific applications such as biomass conversion.

2.9 Theory of methods

2.9.1 Plant Genomic DNA Extraction

Deoxyribonucleic acid (DNA) must be purified from cellular material in a manner that prevents degradation and because of this; even crude extraction procedures can still be adopted to prepare a sufficient amount of DNA to allow for multiple end uses. Extraction of DNA from plant tissue can vary depending on the material used. Essentially any mechanical means of breaking down the cell wall and membranes to allow access to nuclear material, without its degradation is required. For this, usually an initial grinding stage with liquid nitrogen is employed to break down cell wall material and allow access to DNA while harmful cellular enzymes and chemicals remain inactivated. Once the tissue has been sufficiently ground, it can then be re-suspended in a suitable buffer, such

as Hexadecyl trimethyl-ammonium bromide (CTAB). In order to purify DNA, insoluble particulates are removed through centrifugation while soluble proteins and other material are separated through mixing with chloroform and centrifugation. DNA must then be precipitated from the aqueous phase and washed thoroughly to remove contaminating salts. The purified DNA is then re-suspended and stored in Tris acetate EDTA buffer or sterile distilled water. This method has been shown to give intact genomic DNA from plant tissue. To check the quality of the extracted DNA, a sample is run on an agarose gel, stained with ethidium bromide, and visualised under UV light (Worden, 2009).

2.9.2 Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a molecular technique by which multiple copies of a gene can be generated, literally from a single copy. Copies are made using genetic material such as DNA or RNA, and theoretically, millions of copies can be made from a single gene isolated from a sample. Under controlled conditions, primers (small man-made gene pieces) are used as starting points to generate small sections of DNA by using enzymes (DNA polymerases) to align complementary deoxynucleoside tryphosphates (dNTPs- building blocks of DNA and other genetic material) to a piece of extracted DNA. The template is obtained by heating the extracted DNA to allow separation from a double stranded structure into single strands. These steps are carried out in a repetitive manner to create many copies of the original gene (Phillips, 2009). The major components of a PCR reaction mixture are:

i. A thermostable DNA polymerase which catalyzes the template-dependent synthesis of DNA. The more commonly used enzyme is Taq polymerase

(from *Thermus aquaticus*). These enzymes are able to remain stable when the reaction mixture is heated to 94^{o}C.

ii. A pair of primers, which are synthetic oligonucleotides that start DNA synthesis. Their design is based on knowledge of the sequences at the ends of the gene being amplified.

iii. Deoxynucleoside triphosphates (dNTPs) mix- made up of equimolar amounts of deoxyadenoside-, deoxycytocinoside-, deoxyguanoside- and deoxythyminocide- triphosphates (dATP, dCTP, dGTP and dTTP, respectively). They provide the building blocks which are used by the enzyme to build new gene sequences.

iv. Divalent cations, usually Mg^{2+}, but also Mn^{2+}, which is generally required by DNA polymerases for activity. They are usually used as a salt solution, e.g. $MgCl_2$

v. Template DNA, which is obtained by lysing the cell of the sample by various methods, and consequent separation and purification of the genetic material

vi. Buffer- usually Tris-Cl at pH 8 is added to the mixture to maintain the pH during the reaction. The buffer usually contains KCl which helps with the optimization and thus, yields increase. (Sambrook and Russel, 2001).

vii. Sterile water

The iterative PCR reaction involves 3 main steps:

i. Denaturation of the template DNA by heat to produce single stranded DNA

ii. Annealing of the oligonucleotide primers to the single strand target sequence

iii. Extension of the annealed primers with dNTPs using the thermostable DNA polymerase.

There are now numerous models of automated systems that can run repeated cycles of this reaction protocol within some minutes to a few hours. The temperatures for denaturation and annealing are estimated based on the G+C content of the template and the primer sequences, respectively. Extension temperature is chosen for optimum activity of the DNA polymerase; for Taq polymerase, this is between 72-78^{o}C (Sambrook and Russel, 2001).

2.9.3 Gel electrophoresis and Visualization

To allow visualization of the PCR products, gel electrophoresis is used. By this technique, genetic material is separated using electric current that is applied to a gel matrix usually placed in a chamber containing a buffer such as TBE (Tris-boric acid-EDTA) which contains ions that are used to drive the genetic molecules along with the current. An electromotive force (EMF) is applied to the gel (usually agarose or polyacrylamide- serving as a solid yet porous support) after the genetic material has been loaded to it. The applied current moves the genetic material through the pores of the gel matrix at different rates, largely dependent on the mass, towards either the cathode or anode of the electrophoresis set-up, depending on whether they are positively or negatively charged respectively (Slater *et. al.*, 2000).

For visualization, the gel is stained by immersion in 0.5µg/L Ethidium bromide (EtBr) solution (or using other dyes such as silver or Coomasie blue-R250). The stained gel is

then visualized using UV-illumination. Bands in different lanes that end up at the same distance from the top (starting point) are approximately of the same size as they have passed through the gel at the same speed. Molecular gel markers are usually run alongside the PCR products on the gel to allow estimation of the product size (Sambrook *et. al.*, 2001).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Research Location

Research was carried out at the Centre for Biotechnology Research and Training, Ahmadu Bello University, Zaria.

3.1.2 Reagents

Proteinase K, *p*-nitrophenyl-β-D-glucopyranoside (pNP) and mercaptoethanol were obtained from Sigma-Aldrich (USA). All other extraction and enzyme assay reagents were of analytical grade (98 % - 99.9 % purity). Amplification reagents (primers, dNTPs, Taq polymerase) were obtained from Fermentas, South Africa.

3.1.3 Plant Materials

3.1.3.1 Sorghum

Sorghum (*Sorghum bicolor* L. Moench) grains (NACGRAB 09) were obtained from the Institute for Agricultural Research, Zaria.

3.1.3.2 Cassava

Cassava (*Manihot esculenta* Crantz) cuttings (TMS 96/1632) were obtained from Biological Science Department, Ahmadu Bello University, Zaria. Plants were grown outdoors from stem cuttings for 3 to 15 months before being used as a source of plant tissue.

3.2 Methods

3.2.1 Genomic DNA Extraction

Extraction of plant DNA was conducted using the CTAB (hexadecyltrimethylammonium bromide method of Doyle and Doyle (1990). Two hundred milligrams of plant tissue was ground to a fine paste in 500 µl of pre-heated CTAB-SDS buffer (2% CTAB, 100mM Tris/HCl, pH 7.5, 1.4 M NaCl, 2 % SDS, 2% Polyvinylpyrroliodine (PVP)-40, 20mM Ethylene diamine tetra acetic acid (EDTA) pH 8.0), 10 µl β-mercaptoethanol and 15 µl Proteinase K (10mg/ml) was added immediately prior to use). The CTAB-SDS/plant extract mixture was then transferred to a micro-centrifuge tube and incubated for 1 h at 55^{o} C in a re-circulating water bath. After incubation, the mixture was centrifuged at 13000 rpm (4^{o}C) for 10 min to spin down cell debris. The supernatant was then transferred to clean micro-centrifuge tubes. To each tube, 250 µl of Chloroform: Iso-amyl alcohol (24:1) was added and the solution was mixed by inversion. The tubes were then spun at 13000 rpm for 1 min. The upper aqueous phase only (containing the DNA) was then transferred to a clean micro-centrifuge tube. 50 µl of 7.5 M Ammonium acetate followed by 500 µl of ice-cold absolute ethanol was added. The tubes were inverted slowly several times to precipitate the DNA. The precipitate was isolated by spinning the tube at 13000 rpm for a minute to form a pellet. The supernatant was removed and the DNA pellet washed by adding two changes of ice cold 70 % ethanol. After the wash, the DNA was spun into a pellet by centrifuging at 13000 rpm for 1 min. All the supernatant was removed and the DNA pellet was allowed to dry for approximately 15 min. The DNA was re-dissolved in sterile DNase free water. After re-suspension, the DNA was

incubated at 65^{o} C for 20 min to destroy any DNases that may be present and stored at 4^{o}C.

3.2.2 DNA quality confirmation

Agarose gel electrophoresis and colorimetry were employed to confirm the quality of the isolated DNA. A 1 % solution of agarose was prepared by melting 1 g of agarose in 100 ml of 0.5x TBE buffer in a microwave for approximately 2 min. The mixture was then allowed to cool for a few minutes and then, 3 µl of ethidium bromide was added and stirred to mix. A gel was cast using a gel tray and comb. The gel was then allowed to set for 20 min at room temperature on a flat surface. The wells were loaded with 15 µl of DNA plus loading dye (10 µl sample + 5 µl constituted loading dye). The gel was run for 30 min at 100 V, exposed to UV light and documented.

3.2.3 Polymerase Chain Reaction (PCR)

PCR was performed using a 25 µl reaction volume containing 19 µl distilled water, 2 µl template DNA, 0.5 µl deoxynucleotide triphosphate (dNTPs), 0.5 µl of each primers, 2.5 µl Taq buffer (containing $MgCl_2$) and 0.5 µl Taq DNA polymerase . Amplification was achieved in a thermal cycler programmed for initial denaturation for 5 minutes at 95°C, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 65°C for 1 minute (Dhurrinase Forward and Dhurrinase Reverse), 57^{o}C for 1 minute (Linamarase Forward and Linamarase Reverse) and extension at 72°C for 1 minute. A final extension step at 72°C for 4 minutes was added. Amplification products were separated alongside a molecular weight marker (1 kb ladder) by electrophoresis on 1 % agrose gel run in 1 X TAE (Tris-acetate-EDTA) buffer, stained with Ethidium bromide and visualized under UV light. Gel photograph were documented. Amplification reaction was carried out with

specific primers: Dhurrinase; DF 5′-CCG GAT CCA TGG CTC AAA CGA TAA GCA GTG AG-3′ and DR 5′-AAA GGA TCC TTA GTT AAG CTG GCC GGC CG-3′ and Linamarase; LF 5' GTT GTA AAA CGA CGG CCA GT 3' and LR 5' CAC AGG AAA CAG CTA TGA CC 3'

PCR products were excised from the gel, packaged, labelled and sent for sequencing at Max Planck Institute for Marine Microbiology, University of Bremen, Germany.

3.2.4 Preparation of Crude Enzymes

The method as described by Nok (1982) was used. Forty grams of fresh cassava root cortex was homogenized in 200 ml of 0.1 M acetate buffer, pH 5.5 and steeped for 2 hours. The homogenate was squeezed through two layers of cheese cloth and then centrifuged at 19, 000g for 15 minutes at 4^{o}C. The supernatant was recovered and used as the crude linamarase.

The method of Mao and Anderson (1967) was used with slight modifications to extract crude dhurrinase. Forty grams of sorghum seeds were ground in a porcelain mortar. The resulting powder was extracted with 750 ml of 0.1 M sodium phosphate buffer, pH 7.5 and left to steep at room temperature for 3 hours. The moisture was then squeezed through two layers of cheese cloth and then centrifuged at 19, 000 g for 15 minutes at 4^{o}C. The supernatant was then used as crude dhurrinase. Both extracts were stored at -20^{o}c and used on ice.

3.2.5 Assay for β-glycosidase Activity

The method as described by Nok (1982) was used. *p*-nitrophenyl-β-D-glucopyranoside (pNP) was used as substrate for linamarase activity assay. This was determined by adding 0.2 ml of enzyme solution to 0.5 ml of 0.1 M phosphate buffer, pH 6.5. The reaction was started by adding 0.3 ml pNP (1 mg/ml) and the mixture incubated at room temperature for 1 hour. The reaction was stopped at the end of the incubation period with 2 ml of 0.2 M borate buffer, pH 9.0 and adjusted with distilled water to 4 ml. The absorbance was measured at 490 nm against a blank which contained the same constituents as the reaction mix except the substrate. Dhurrinase activity was determined by adding 0.2 ml of enzyme solution to 0.5 ml of 0.1 M acetate buffer, pH 6.5. The reaction was started by adding 0.3 ml pNP (1 mg/ml) and the mixture incubated at room temperature for 1 hour. The reaction was stopped at the end of the incubation period with 2 ml of 0.2 M borate buffer, pH 9.0 and adjusted with distilled water to 4 ml. The absorbance was measured at 490 nm. In both cases the blank consisted of the respective buffers, 0.2 M borate buffer, pH 9.0 and the enzyme.

One unit of linamarase/dhurrinase activity was defined as the amount of enzyme which produced 1 micromole of p-nitrophenol ion per minute under the described assay conditions.

3.2.6 Effect of pH on β- glycosidase activity

The method as described by Ikediobi and Onyike (1982) with modifications was used. Different pH ranges of 0.1 M Sodium phosphate buffer (pH 3.0, 4.0, 5.0 and 6.0) and 0.1 M acetate buffer (7.0, 8.0, 9.0 and 10.0) were used to determine the activity of the enzymes as a function of pH. The reaction mixture consisted 0.5 ml buffer, 0.2 ml

enzyme and 0.3 ml substrate (pNP). The set up was duplicated for both linamarase and dhurrinase. The reaction was left to incubate for 1 hour and the absorbance at 490 nm was taken after the reaction was stopped with 0.2 M borate buffer, pH 9.0.

3.2.7 Effect of Temperature on β- glycosidase activity

The method as described by Ikediobi and Onyike (1982) with modifications was used. The effect of temperature on reaction rate catalyzed by the enzymes was studied using the pH optimum for each enzyme as determined above (i.e. pH 5.0 for dhurrinase and 6.0 for linamarase). The reaction mixture of 0.5 ml buffer, 0.2 ml enzyme and 0.3 ml substrate (pNP) were incubated at 40^{o}C, 50^{o}C, 60^{o}C, 70^{o}C, 80^{o}C were used in this assay. The reaction was incubated at each temperature setting for 30 minutes and the reaction stopped with 0.2 M borate buffer, pH 9.0. The absorbance was taken at 490 nm. The reaction set up was duplicated for both linamarase and dhurrinase using their respective buffers.

CHAPTER FOUR

4.0 RESULTS

4.1 Molecular Characterization of Genes coding Linamarase and Dhurrinase

4.1.1 Cassava Genomic DNA Extraction

Genomic DNA was extracted from cassava leaves and tuber. The pelletized precipitate from cassava leaves showed the characteristic whitish colour. DNA recovery as measured by the nucleic acid concentration was 67.1 ng/µl. The purity of the DNA extracted was assessed by taking the ratio of the absorbance at 260 and 280 nm (A260/280). The sample was determined to have high purity with an absorbance ($A^{260}/_{280}$) value of 1.4 (Figure 4.1 lane 2).

Total DNA extracted from cassava tuber (Figure 4.1 lane L3) showed the characteristic fluorescence after agarose electrophoresis. The concentration of nucleic acid in the precipitate was determined to be 169.4ng/µl. The purity of the extracted DNA was also measured by the absorbance ($A^{260}/_{280}$) which gave a reading of 1.4.

A gel photograph giving a pictorial representation of the various extracted samples is shown in Figure 4.1.

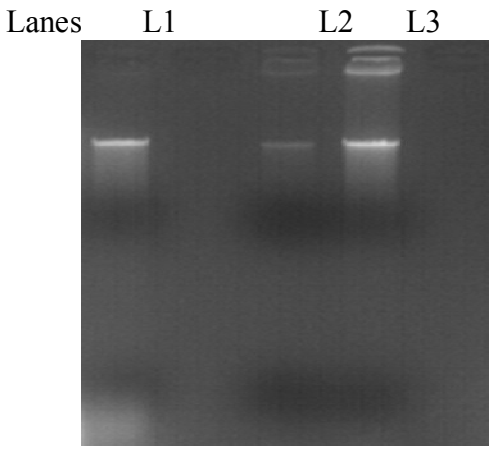


Figure 4.1: Gel photograph showing genomic DNA extracted from cassava and sorghum using the CTAB extraction protocol. L1 – Sorghum genomic DNA, L2 – Cassava genomic DNA (Leaf) and L3 – Cassava genomic DNA (Tuber)

4.1.2 Sorghum Genomic DNA Extraction

DNA recovery as measured by the nucleic acid concentration was 91.7ng/µl. The purity of the DNA extracted was determined by taking the ratio of absorbance at 260 and 280 nm (A260/280). The sample was determined to have good purity with an absorbance ($A^{260}/_{280}$) value of 1.2 (Figure 4.1 lane 1).

4.2 Gene amplification

The gel photograph in Figure 4.2 shows bands separated against a 1 kb DNA ladder. A band corresponding to a linamarase gene (1,504 bp) which was amplified in samples under the experimental conditions used was obtained (Lane 2). The amplicon corresponding to the dhurrinase gene (1695 bp) was also clearly shown (Lane 1)

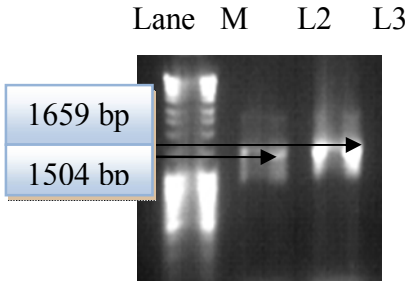


Figure 4.2: Gel photograph showing amplified linamarase and dhurrinase genes using PCR. Amplicons were run on 1 % agarose and viewed/photographed under UV light.

M – DNA 1kb ladder; L2 – linamarase gene; L3 – Dhurrinase gene

4.3 Linamarase and Dhurrinase Assay

4.3.1 Temperature Studies on Linamarase

Linamarase appeared to be relatively stable with an optimum temperature of 65^{o}C (Figure 4.3). Temperature coefficient (Q_{10}) calculated between 40^{o}C and 50^{o}C gave a value of 1.17. This coefficient represents the relative increase in the rate of enzyme catalyzed reactions for every 10^{o}C rise in temperature. Temperature data was subjected to Arrhenius plot. The data obtained from the temperature studies was used to construct the plot of log V against 1/T (Figure 4.4). The intersections on the plot were used to compute activation energy (Ea) and the Arrhenius frequency factor of collision (A). The values computed were 17.32 KJ/mol and 1.5 x 10^{1} $s^{-1}$for activation energy and Arrhenius frequency factor of collision, respectively.

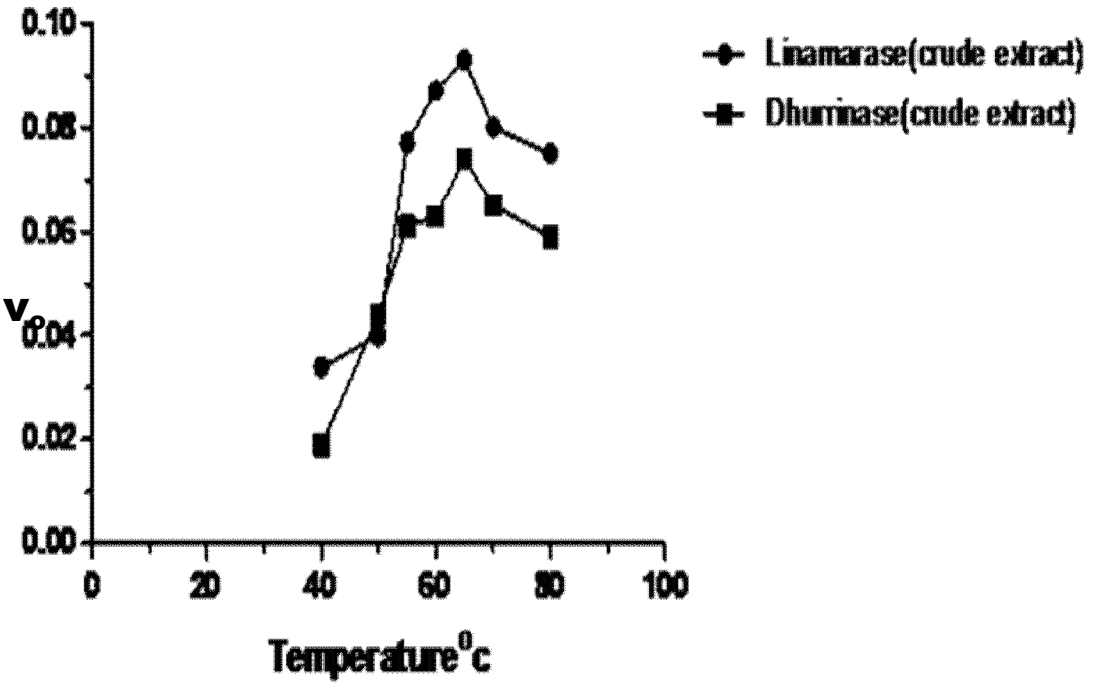


Figure 4.3: Optimum temperature plot for Linamarase and Dhurrinase crude extracts
The effect of temperature on dhurrinase and linamarase activity was followed in 100 mM acetate buffer pH 5.0 for dhurrinase and phosphate buffer pH 6.0 for linamarase over a temperature range of 40 to 80°C using 1.25 mM *p*-nitrophenyl-β-D-glucopyranoside under the standard test conditions.

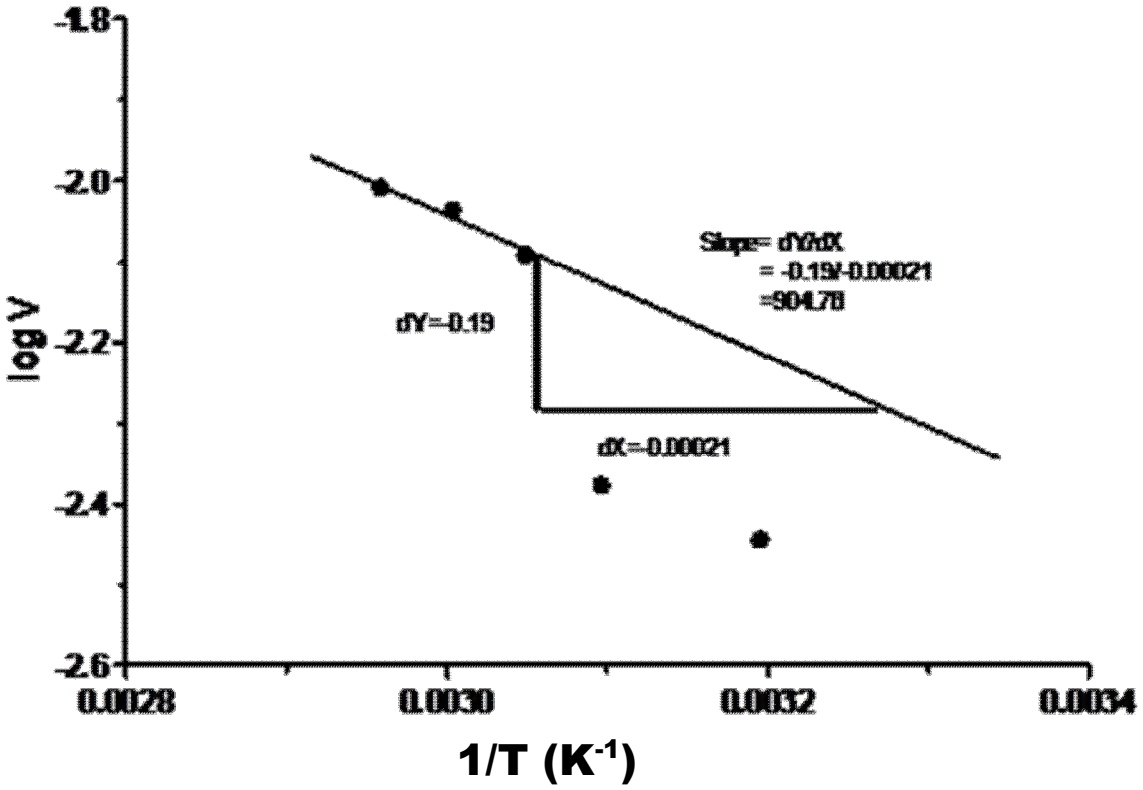


Figure 4.4: Logarithmic plot of initial velocity (V) versus 1/T for linamarase

4.3.2 Temperature Studies on Dhurrinase

The activity of dhurrinase was assayed using *p*-nitrophenyl-β-D-glucopyranoside at temperatures ranging from 40^{o}C to 80^{o}C at 10^{o}C intervals. The plot of the absorbance against temperature showed an optimum temperature of dhurrinase activity to be 65^{o}C (Figure 4.3). Temperature coefficient (Q_{10}) calculated between 40^{o}C and 50^{o}C gave a value of 2.53. This coefficient represents the relative increase in the rate of enzyme catalyzed reactions for every 10^{o}C rise in temperature. The initial velocity data obtained from the temperature studies on dhurrinase was used to construct the plot of log V against 1/T (Figure 4.5). The intersections on the plot were used to compute activation energy (Ea) (this value represents the rate of collision between the reacting molecules) and the Arrhenius frequency factor of collision (A). The values computed were 51.06 KJ/mol and 1.6×10^{1} s^{-1} for activation energy and Arrhenius frequency factor of collision respectively.

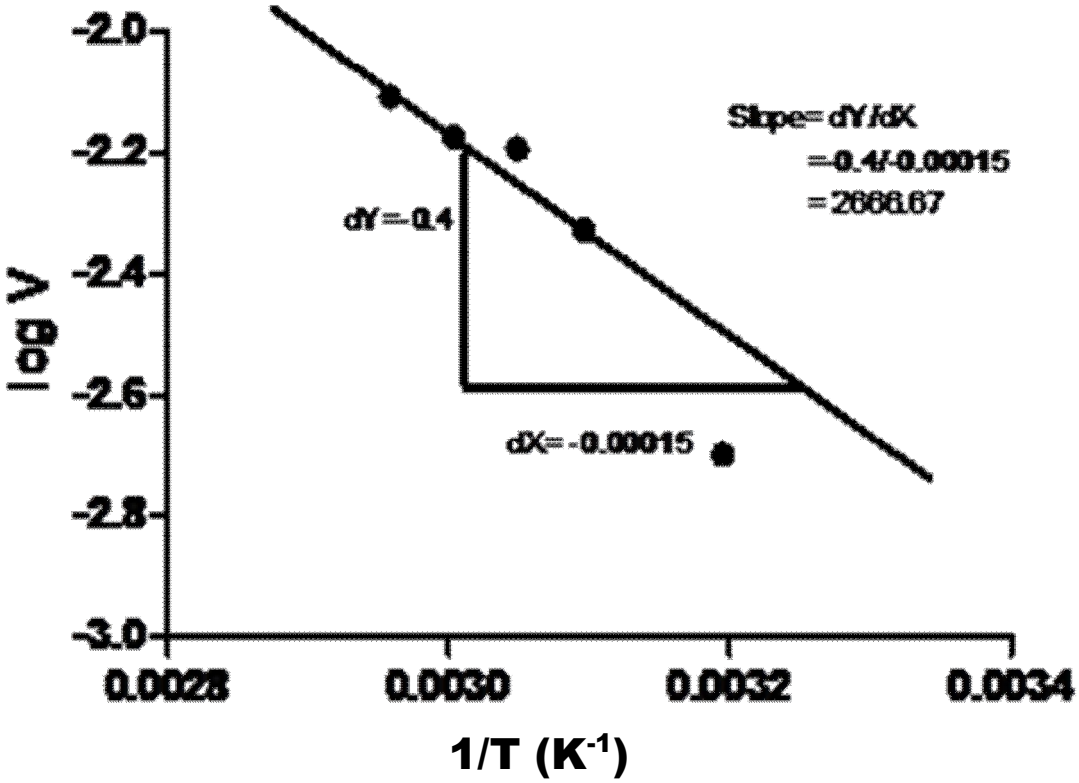


Figure 4.5: Logarithmic plot of initial velocity (V) versus 1/T for dhurrinase.

4.4 pH Optimum of Enzymatic Hydrolysis

4.4.1 pH Studies on Linamarase

The characteristic bell shaped curve (Figure 4.6) of a plot of initial velocity against pH showed that the optimum pH for *p*-nitrophenyl-β-D-glucopyranoside (artificial substrate) hydrolysis with cassava linamarase extracted from root cortex was found to be 6.0. The enzyme retained more than 70% of its activity in the range pH 5.0 to 6.5. Activity was decreased at highly acidic (pH 3) and highly basic (pH 10) conditions following the amphoteric nature of enzymes.

4.4.2 pH Studies on Dhurrinase

The characteristic bell shaped curve (Figure 4.7) of a plot of initial velocity against pH showed that the optimum pH for *p*-nitrophenyl-β-D-glucopyranoside (artificial substrate) hydrolysis with dhurinase extracted from sorghum seeds was found to be 5.0. The enzyme retained more than 70% of its activity in the narrower range pH 4.5 – 5.5. Activity was also decreased at highly acidic (pH 3) and highly basic (pH 10) conditions reflecting the amphoteric nature of enzymes.

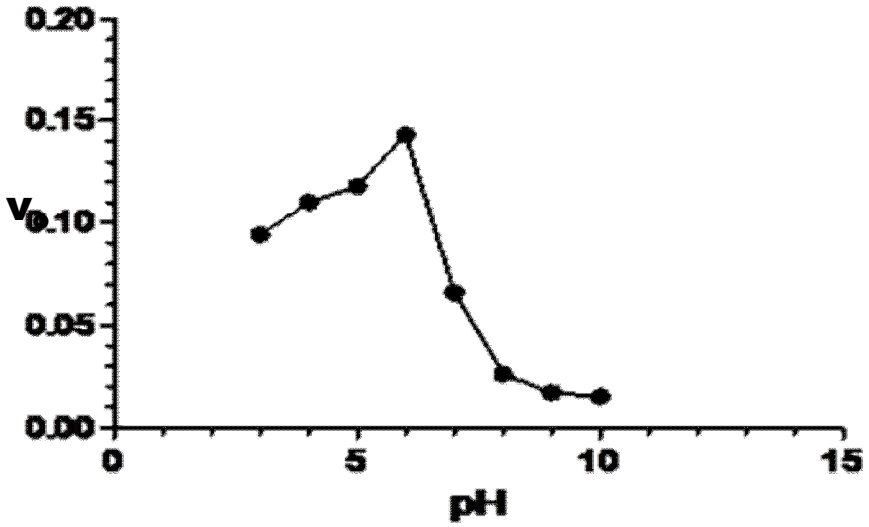


Figure 4.6: Optimum pH plot for Linamarase crude extract

The effect of pH on linamarase activity was obtained by measuring the hydrolysis of *p*-nitrophenyl-β –D-glucopyranoside in a series of buffers at various pH values ranging from pH 4.0 to 10.0. The buffers used were acetate buffer (100 mM) from pH 4.0 to 6.0 and phosphate buffer (100 mM) from pH 6.0 to 10.0. The pH values of each buffer were determined at 37°C. After 1 h incubation at 37°C, the reaction was stopped with borate buffer (pH 9.0). Aliquots were taken and immediately assayed for residual linamarase activity.

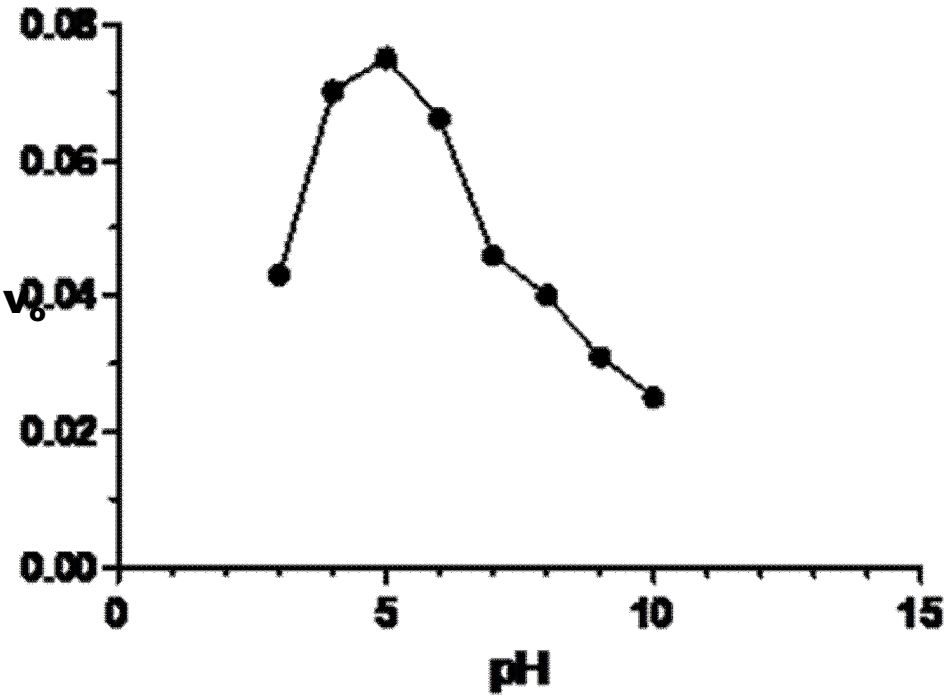


Figure 4.7: Optimum pH plot` for Dhurrinase crude extract

The effect of pH on dhurrinase activity was also obtained by measuring the hydrolysis of *p*-nitrophenyl-β –D-glucopyranoside in a series of buffers at various pH values ranging from pH 4.0 to 10.0. The buffers used were acetate buffer (100 mM) from pH 4.0 to 6.0 and phosphate buffer (100 mM) from pH 6.0 to 10.0. The pH values of each buffer were determined at 37°C. After 1 h incubation at 37°C, the reaction was stopped with borate buffer (pH 9.0). Aliquots were taken and immediately assayed for residual dhurrinase activity.

4.5 Initial Velocity Studies

4.5.1 Determination of K_M and Vmax of Linamarase using pNP-glucoside as substrate

At the optimum pH of 6.0 and temperature of 65^{o}C the linamarase/ *p*-nitrophenyl-β-D-glucopyranoside assay was run. Data obtained from the assay was used to construct a double reciprocal plot. The kinetic parameter K_M, which gives an indication of the binding strength of an enzyme to its substrate was computed from the plot to be 5.75 x 10^{-3} µmol l^{-1}. The V_{max} of the enzyme was determined as 1.96 x 10^{-2} µmol l^{-1} min^{-1}.The kinetic parameter k_{cat}, also known as the turnover number of an enzyme which is a measure of the maximum catalytic production of the product under saturating substrate conditions per unit time per unit enzyme was calculated to be 3.41 min^{-1}.

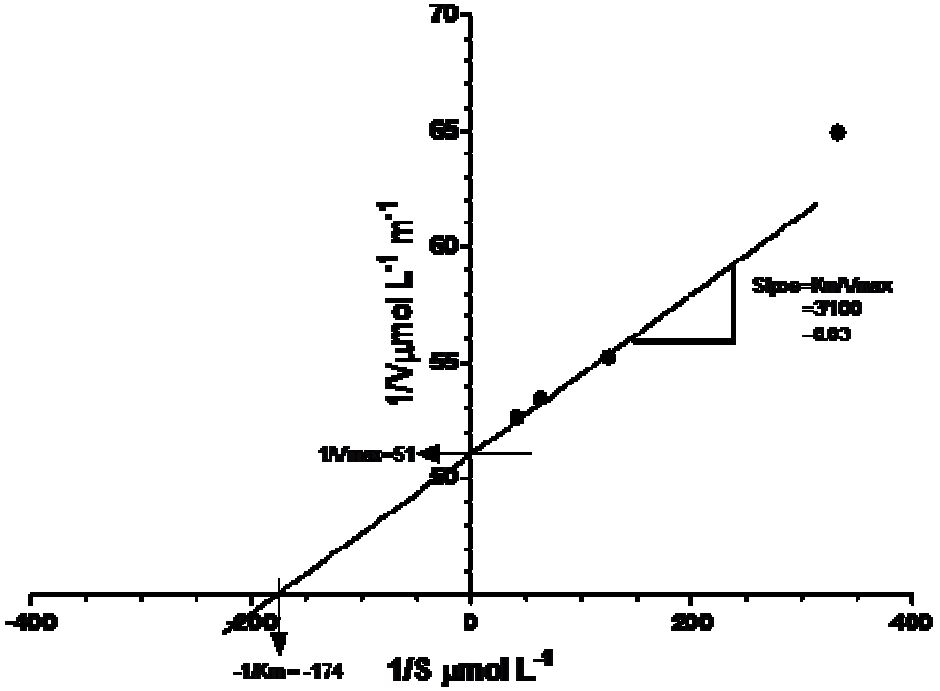


Figure 4.8: Double reciprocal plot for Linamarase crude extract

The kinetic parameters (K_M, Vmax and k_{cat}) were obtained in 100 mM phosphate buffer (pH 6.0) at 65°C. Hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside was quantified on the basis of released *p*-nitrophenol as in the standard enzyme assay. K_M and Vmax were determined from lineweaver-Burk plot using different concentrations of *p*-nitrophenyl- β -D- glucopyranoside.

4.5.2 Determination of K_M and Vmax of Dhurrinase using pNP-glucoside as substrate

At the optimum pH of 5.0 and temperature of 65^{o}C the dhurrinase/ *p*-nitrophenyl-β-D-glucopyranoside assay was run. Initial velocity data obtained from the assay was used to construct a double reciprocal plot. The kinetic parameter K_M which gives an indication of the binding strength of an enzyme to its substrate was calculated from the plot to be 1.39 x 10^{-2} µmol l^{-1}.The kinetic parameter kcat also known as the turnover number of an enzyme which is a measure of the maximum catalytic production of the product under saturating substrate conditions per unit time per unit enzyme was calculated to be 7.27 min^{-1}. The maximum velocity that the enzyme could achieve known as Vmax was calculated to be 1.01 x 10^{-1} µmol l^{-1} min^{-1}.

4.6 Determination of Half Life of Enzymatic Activity

4.6.1 Steady State

Enzyme activity for the determination of half life was conducted at linear range. The graph in figure 4.10 shows the activity over time of linamarase with its natural substrate linamarin and dhurrinase with linamarin as an alternate substrate. Dhurrinase is seen to exhibit better hydrolysis of linamarin (giving absorbance values in the range of 1.86 – 1.90) than its natural enzyme linamarase (with absorbance values ranging from 1.74 – 1.80). (Figure 4.10)

4.6.2 Determination of Linamarase Half Life

The residual activity determined to compute the half life with respect to linamarase was conducted. As observed from figure 4.11 the half life ($t_{1/2}$) of linamarase activity using the natural substrate linamarin calculated from a plot of ln (A) versus time was 117.47 minutes.

4.6.3 Determination of Dhurrinase Half Life using Linamarin as Substrate

Using linamarin as substrate, the half life ($t_{1/2}$) of dhurrinase activity was calculated and a value of 288.79 minutes was obtained (figure 4.12).

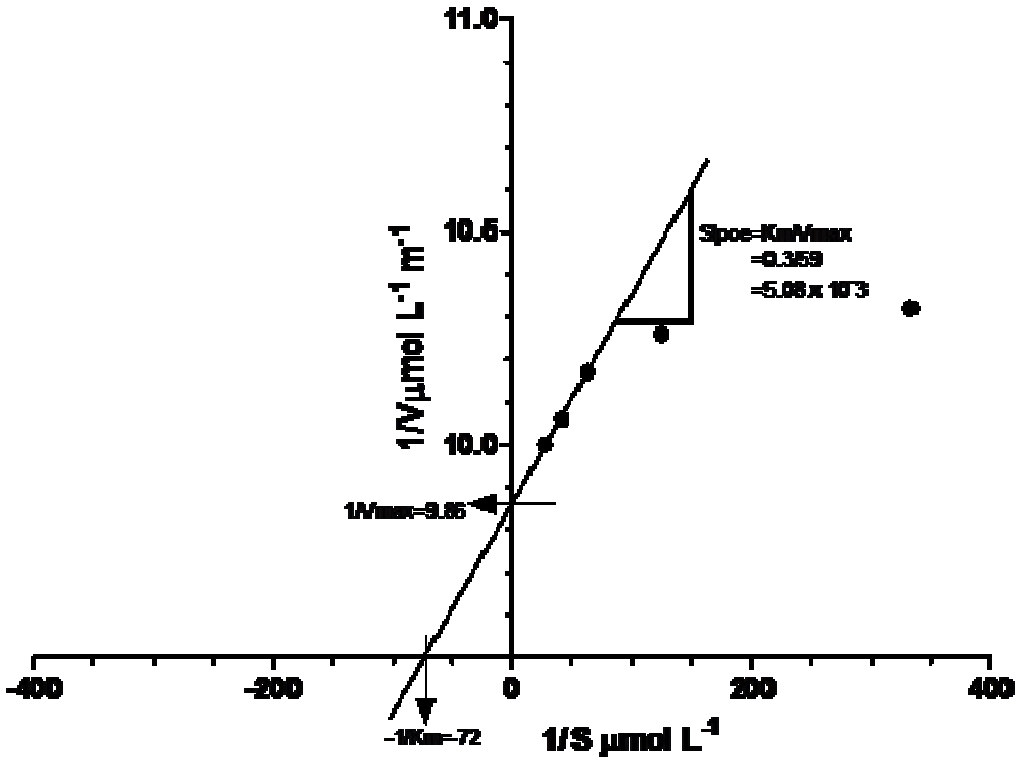


Fig 4.9: Double reciprocal plot for Dhurrinase crude extract

The kinetic parameters for dhurrinase (K_M, Vmax and kcat) were determined in 100 mM acetate buffer (pH 5.0) at 65°C. Hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside was quantified on the basis of released *p*-nitrophenol as in the standard enzyme assay. K_M and Vmax were determined from lineweaver-Burk plot using different concentrations of *p*-nitrophenyl-beta-D- glucopyranoside.

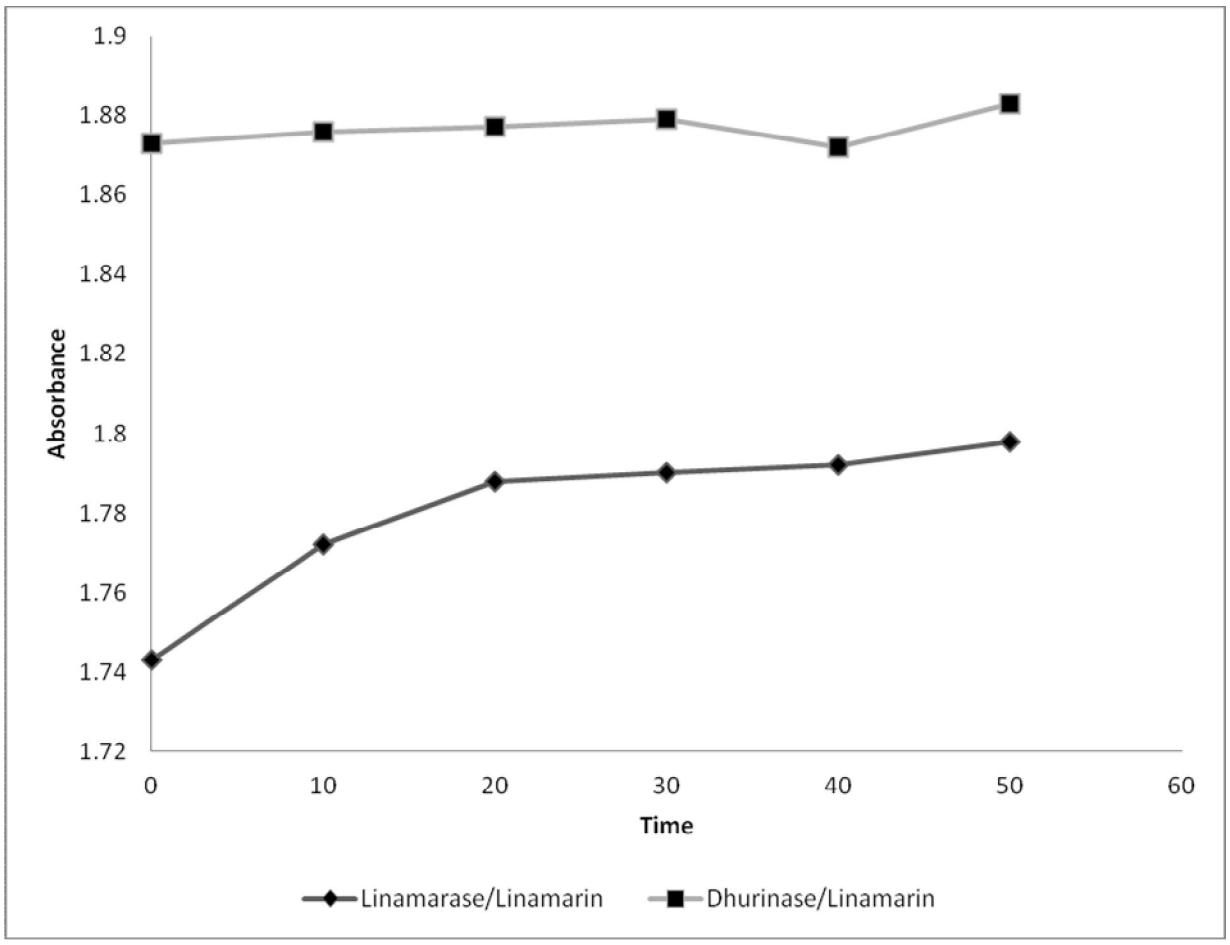


Figure 4.10 Steady state plot of absorbance against time

Dhurrinase activity was determined in 100 mM acetate buffer (pH 5.0) at 65°C. Hydrolysis of linamarin was quantified on the basis of released cyanide. Linamarase activity was determined in 100 mM phosphate buffer (pH 6.0) at 65°C. Hydrolysis of linamarin was quantified on the basis of released cyanide as in the standard enzyme assay.

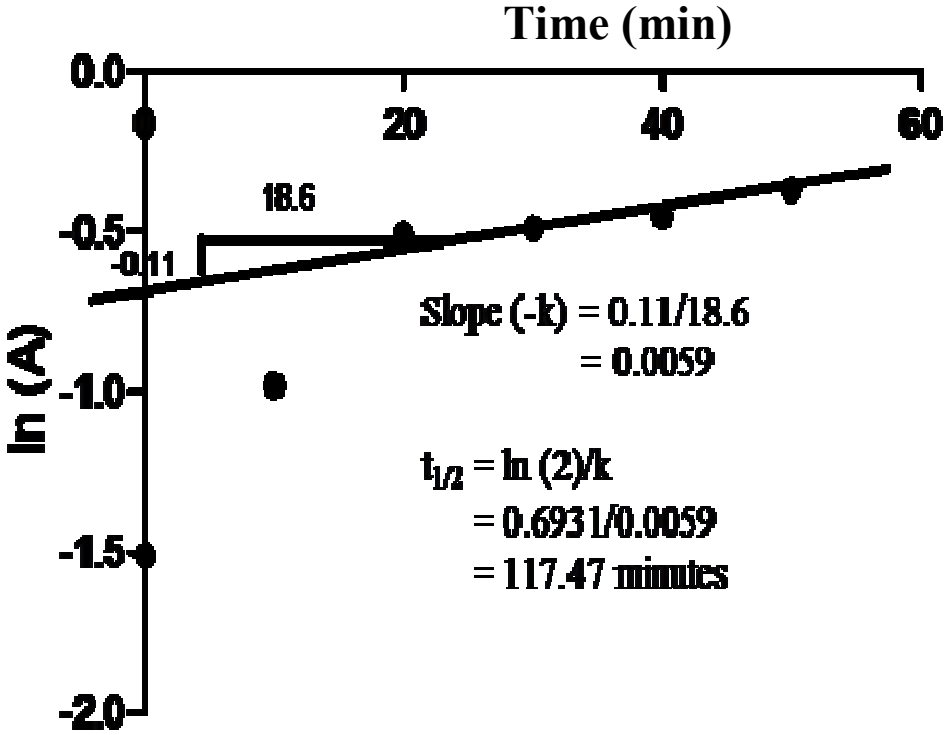


Figure 4.11: Half life plot of linamarase activity using linamarin as substrate.

Time (min)

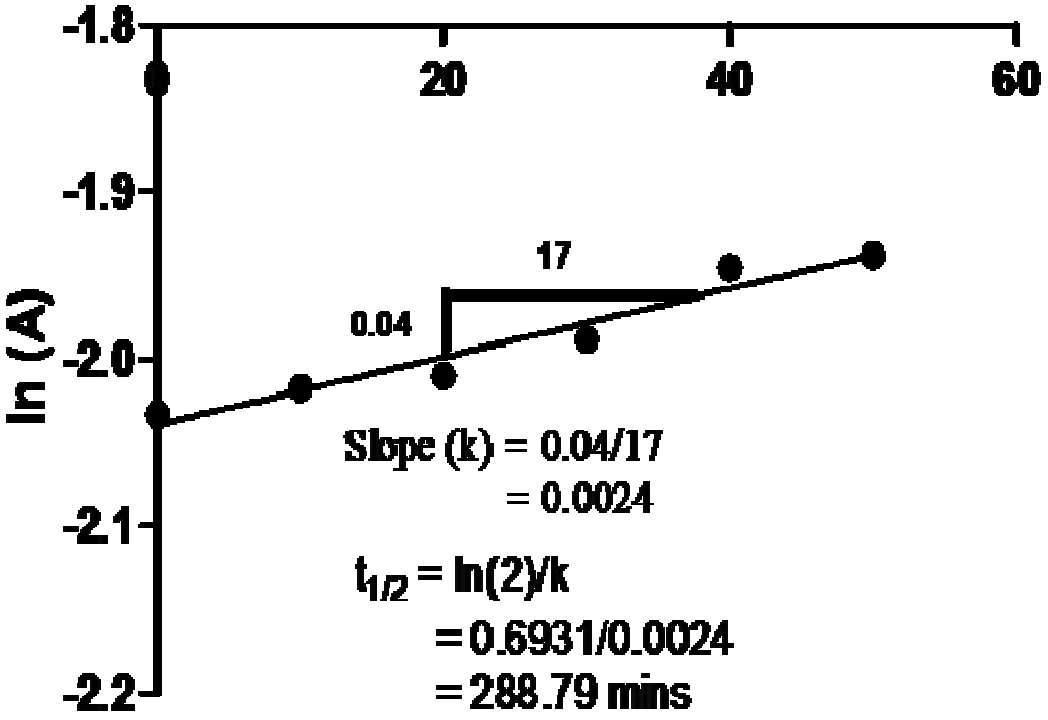


Figure 4.12: Half life plot of dhurrinase activity using linamarin as substrate.

CHAPTER FIVE

5.0 DISCUSSION

Cassava (*Manihot esculenta* Crantz.) roots are quantitatively the third most important food in the tropics, after rice and corn. It is an important source of calories because it covers 60% of the daily calorific needs of the populations in tropical Africa and in Central America (Nweke, 2004). Nigeria produces more cassava than any other country in the world. The crop is abundant in 24 of the 36 states, requires minimum labor and inputs, and remains the most important food security crop for millions of Nigerians. Cassava flour can substitute for at least a third of the 4 million tons in annual imported wheat flour, all of the imported glucose, and starches for food, animal feed, beverages, and pharmaceuticals (another 3 million tons).

One of the major worries for cassava consumption is the potential toxicity of cyanoglycosides (linamarin and lotaustralin) arising from enzymatic degradation to produce hydrogen cyanide, resulting in acute cyanide poisoning. Cyanogen levels can vary widely with cultivar, climatic conditions, plant part and degree of processing. The enzyme responsible (β- glucosidase) may arise from the plant material or from gut micro flora.

Cyanide can be lethal to humans and the acute dose is in the region of 1 mg/kg body weight. Cases of acute poisoning have been associated with misuse, particularly of cassava fermentation.

The fermentation of cassava tubers called retting aims to eliminate the poisonous cyanide components and conserve cassava by lactic acidification. When producing gari or flour of fufu, lactic acidification of cassava is rapid and detoxification is many times incomplete

(Raimboult *et. al*., 1996). Obilie *et al.,* (2004) also reported that fermentation of cassava pulp or dough for 4-5 days results in a decrease in total cyanogens of 52-63%, which leaves quite large amounts of cyanogens in the products. Disruption of cells by grating during the fermentation process also releases the endogenous enzymes allowing them act on the cyanogens (linamarin and lotaustralin) further detoxifying it. But after a few days of fermentation the enzymes are denatured by acidic pH (4.0) rendering the detoxification process incomplete.

The research focused primarily on cyanogenic glycosidases from cassava and sorghum, covering studies on their temperature and pH activity and also on their kinetic parameters. The results showed that both enzymes exhibited similar characteristics relating to the reactions they catalyze. However some contrasts were observed in reaction rates, size and kinetic properties.

Cassava linamarase was extracted from the root cortex of tubers obtained from mature plants. The enzyme showed an optimum pH of 6.0 for *p*-nitrophenyl-β-D-glucopyranoside hydrolysis. This finding concurred with observations made by Pieris (1976). He reported a pH of 6.0 – 6.6 for the A and B forms of linamarase. The enzyme retained more than 70% of its activity in the range pH 5.0 to 6.5. The enzyme exhibited marked reduction in activity at pH 3 and 10.

The optimum temperature observed from the experimental assay showed that cassava linamarase had maximum activity for the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside at 65°C in a 30 minute assay period. Above the optimum temperature the rate decreases as more of the enzyme molecules denature. The thermal energy breaks the hydrogen bonds holding the secondary and tertiary structure of the enzymes together,

so the enzymes lose their shape and become random coils - and the substrate can no longer fit into the active site. This finding is again similar with findings by Peiris (1976) who reported a 60^{o}C optimum. The increase in the ratio of rate with temperature as quantified by the Q_{10}, which is the relative increase in rate for a 10°C rise in temperature, calculated between 40°C and 50°C was 1.17. Up to the optimum temperature the rate increases geometrically with temperature (i.e. the rate of cyanide release by both enzymes increased). Linamarase has a low Q_{10} (1.17) which is much lower than the widely recognized 2.0 or greater for enzyme catalyzed reactions. From the Arrhenius plot, the activation energy was found to be 17.32 KJ/mol (4.14 kcal mol^{-1}). This value concurred with reports from Peiris (1976) who reported activation energies of between 3.3 – 5.7 kcal mol $^{-1}$ for linamarase A and B. This however differed from reports by Nok (1983) who reported activation energies of 11.72 and 8.72 kcal mol $^{-1}$ for linamarase A and B. This disparity could have arisen from source, climatic conditions, soil fertility and period of cultivation of the cassava crop used in the experiment. The Arrhenius frequency factor of collision was determined as 1.5 x 10^{1} s^{-1}. This factor expresses the frequency of molecules that collide in the correct orientation and with enough energy to initiate a reaction.

The K_M for linamarase was 5.75 x 10^{-3} µmol l^{-1} with pNP-glucoside. The implication of this is that linamarase reaches its maximum catalytic activity at low concentrations of *p*-nitrophenyl-beta-D- glucopyranoside making it a more efficient catalyst in the hydrolysis of the substrate. The low K_M indicates that the association rate k_1 (in the induced fit model) is greater than the re-dissociation rate k_{-1} reflecting a higher affinity for the substrate. This high substrate specificity suggests that linamarase could be used as a tool

in the structural analysis of D-glucose containing oligosaccharide chains of glycoproteins, glycolipids, and cellulose. (Yapi *et.al.*, 2009).

The kcat values which reflect the index of physiological efficiency of enzymes present a different inference. Linamarase had a kcat value of 3.41 min^{-1}. This represents the dissociation rate (k_2) of the enzyme-substrate complex. The maximum velocity that the enzyme could achieve known as Vmax was calculated to be 1.96 x 10^{-2} $\mu mol\ l^{-1}\ min^{-1}$.

Sorghum (*Sorghum bicolor* (L) Moench), king of millets is one of the staple food crops of the world, especially the drier parts of tropical Africa and Asia. It is a principal source of energy, protein, vitamins and minerals for millions of the poorest people in the regions where it is cultivated. The protein content (11.3%) is nearly equal and is comparable to that of wheat and maize. Average starch content of the seeds range from 56 to 73 per cent and is relatively rich in iron, phosphorous and vitamin B complex (Mamatha, 2008). Sorghum ranks among the top three important grains in the country with increasing industrial demand particularly in the food, beverage, and livestock feed industries. Nigeria is the second largest producer of sorghum in the world with an annual production in excess of 8 million metric tons (USAID, 2009).

Seedlings of sorghum (*Sorghum bicolor* [L.] Moench) are also known to synthesize the cyanogenic glucoside dhurrin (β-D-glucopyranosyloxy-(S)- p-hydroxymandelonitrile). Sorghum seedlings contain a specific β-glucosidase (dhurrinase) capable of converting dhurrin into p-hydroxybenzaldehyde, cyanide, and glucose. Cyanogenesis in Sorghum, however, is expressed only in the leaves; dhurrin being rapidly synthesized in etiolated seedlings (Halkier and Moller, 1989). The quite similar pathway for cyanogenesis in

cassava prompted the investigation to ascertain the similarities in enzyme activity in the hydrolysis of their natural substrates.

Dhurrinase extracted from sorghum seeds expressed relatively marked differences in reaction rates. pH studies showed an optimum pH of 5.0 with a narrower (70 %) activity retention range of pH 4.5 – 5.5. It showed the same temperature optimum of 65^{o}C as linamarase. Temperature coefficient (Q_{10}) calculated between 40^{o}C and 50^{o}C gave a value of 2.53. Activation energy (Ea) of 51.06 KJ/mol places it higher than linamarase (17.32 KJ/mol). The Arrhenius frequency collision factor was 1.6 x 10 ^{1} s^{-1} while the double reciprocal plot of dhurrinase showed a calculated K_M of 1.39 x 10^{-2} μmol l^{-1}. The apparent Vmax value was 1.01 x 10^{-1} μmol l^{-1} min^{-1} and the kinetic parameter kcat was 7.27 min^{-1}.

Comparing the two enzymes based on the parameters assessed reveals some startling discoveries. Although both enzymes seem to favour the same temperature (65^{o}C) for optimum performance, they differ in their reactivity with respect to pH. While linamarase was shown to prefer a pH of 6.0 dhurrinase showed a preference of 5.0, retaining 70 % activity within 4.5 – 5.5. The significance of this is found in the fermentation process of cassava. Ikediobi *et. al*., (1980) reported a cessation of linamarase activity after 3 days of cassava fermentation when the pH of the fermenting medium was about 4.0. IITA (1990) reported that cassava fermentation for "gari" production takes 5 days. The implication of this being that dhurrinase is likely to retain over 50 % activity over the prescribed fermentation period. This could imply that dhurrinase could be implanted in process technology in the processing of cassava products.

From the Arrhenius plot, the activation energy was found to be 17.32 KJ/mol and 51.06 KJ/mol respectively for cassava linamarase and sorghum dhurrinase. The implication of this is that at 60^{o}c the linamarase/pNP β-D-glucopyranoside reaction (17.32 KJ/mol) proceeded faster than the dhurrinase/pNP β-D-glucopyranoside reaction (51.06 KJ/mol). The increased rate of collisions between enzyme and substrate molecules was greater achieved by dhurrinase. The Arrhenius frequency collision factor was 1.6×10^{-1} s^{-1}, slightly higher than $1.5 \times 10^{-1}s^{-1}$ obtained for linamarase. However, the temperature coefficient (Q10) calculated between 40°C and 50°C was 1.17 and 2.53 respectively, for linamarase and dhurrinase. Linamarase has a low Q_{10} (1.17) which is much lower than the widely recognized 2.0 or greater for enzyme catalyzed reactions. Dhurrinase on the other hand had a Q_{10} of 2.5. Inference from this suggests that even though linamarase showed lower activation energy, in the dhurrinase catalyzed hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside the enzyme and substrate molecules both had more kinetic energy and so collided more often and also because more molecules have sufficient energy to overcome the activation energy yielding more cyanide product.

Again this discovery finds importance in cassava processing. This implies that dhurrinase has the potential to shorten the fermentation period for cassava fermentation because its reaction proceeds faster.

The K_M (which is a parameter reflective of the concentration at which the reaction rate is half its maximum value) for linamarase and dhurrinase was seen to be low. Linamarase, however, had a lower K_M (5.75×10^{-3} µmol l^{-1}) than dhurrinase (1.39×10^{-2} µmol l^{-1}). The implication of this is that linamarase reaches its maximum catalytic activity at low concentrations of *p*-nitrophenyl- β -D- glucopyranoside making it a more efficient

catalyst in the hydrolysis of the substrate. The low Km indicates that the association rate k_1 (in the induced fit model) is greater than the re-dissociation rate k_{-1} reflecting a higher affinity for the substrate. This high substrate specificity suggests that linamarase could be used as a tool in the structural analysis of D-glucose containing oligosaccharide chains of glycoproteins, glycolipids, and cellulose. (Yapi *et.al*, 2009)

The kcat values which reflect the index of physiological efficiency of enzymes present a different inference. Dhurrinase has a higher kcat value (7.27 min^{-1}). This represents the dissociation rate k_2 of the enzyme-substrate complex. This implies that even though linamarase has a higher affinity for the substrate, dhurrinase more readily released *p*-nitrophenol from the substrate *p*-nitrophenyl-β-D-glucopyranoside.

Results from steady state plots clearly showed a higher index of physiological activity for dhurrinase in the hydrolysis of linamarin than linamarase. This gives credence to the deductions that were drawn from reactions with pNP-glucoside. Dhurrinase showed a longer half life of activity (288.79 min) than 117.47 min for linamarase. This shows more than two fold activity retention of dhurrinase under optimum reaction conditions.

Cassava products (Gari, starch, chips, and high quality cassava flour (HQCF)) are produced primarily by the wet milling of fresh cassava roots which includes the following five main stages: preparation (peeling and washing), rasping/pulping/ grating, purification (starch washing), dewatering and drying, and finishing (milling and packaging). Upon tissue disruption, the cyanogenic glucosides are brought in contact with glycosidase and hydroxynitrile lyases and are degraded into cyanohydrins, hydrogen cyanide and ketones. Careful processing therefore, must be done to remove these toxic constituents to avoid chronic cyanide intoxication (Onabolu *et. al*., 2002).

When the roots are completely disrupted, all linamarin will come in contact with its hydrolytic enzyme (linamarase), resulting in hydrolysis and subsequent removal of the breakdown products during washing. Murugan *et. al*., (2012) reported the addition of exogenous linamarase directly into completely disrupted root to sufficiently hydrolyze all the free linamarin. Several investigators have explored linamarase to facilitate the process of cassava cyanide detoxification (Ikediobi and Onyike, 1982; Petruccioli *et. al.,* 1999; Yeoh and Sun, 2001).

Results have shown the potential of dhurrinase as an exogenous source of enzyme in the process of cassava fermentation to totally liberate cyanide. Dhurrinase was shown to have a high affinity for the cassava cyanogen (linamarin), hydrolyzing it to release the toxic hydrogen cyanide. It was able to achieve this at greater efficiency than linamarase and the enzyme retained high activity for a longer period (over the fermentation period).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

In this present study crude enzyme extracts from Cassava (linamarase) and Sorghum (dhurrinase) were isolated and assayed using their artificial substrate (*p*-nitrophenyl-β-D-glucopyranoside) and the cyananogenic glycoside linamarin (from cassava). Genomic DNA was also isolated and genes coding the two enzymes were amplified. The aim was to compare both enzymes and establish similarities or differences between them.

We have partially achieved our target by identifying the variations in enzyme activity. Both enzymes assayed individually with their artificial substrate showed activity similar to previous investigations. Investigations using the natural substrate, cassava linamarin, however showed clearly that dhurrinase holds the capacity to totally detoxify cassava and its products of cyanogenic principles more effectively than linamarase.

The recent introduction of zero tariffs on imports of cassava processing reagents to boost cassava processing and utilization, the drive to promote cassava composite bread with 40 % cassava flour, and the increase of tariffs on wheat imports to 65 % all point to prominence of the crop. Considering the importance of cassava to the Nigerian society, the potent risk of toxicity due to incomplete or improper processing methods should not be overlooked. New methods need to be established for total liberation of cyanide from cassava. Exogenous enzyme application and controlled fermentation presents a viable tool to achieve this. Our investigations have highlighted the potential of dhurrinase to act as an exogenous source of enzyme in the detoxification of cassava and its products provided that reaction conditions are adhered to.

The isolation of the genes coding these enzymes also presents an opportunity to mass synthesize these enzymes using microbial (bacteria) systems where genes are grafted on to plasmids and inserted into bacteria for over expression. Proteins can then be harvested, processed and utilized. Pure enzymes obtained from this process can also be used in the design and development of probes to ensure quick quality assessment of cassava and its products.

6.2 RECOMENDATIONS

1. From the enormous commercial potential of linamarase and dhurrinase as an alternative in cassava detoxification, and bearing in mind the very high threat that cyanide toxicity poses to mankind, further work on possibly scaling up the process for economic fermentation of cassava is thus very pertinent.

2. Because of the sensitive nature of the enzymes to the changes in pH and temperature, extreme handling care must be taken when processing. Fermentation systems could also be designed to accommodate and regulate the desired conditions for maximum enzyme activity.

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3. Future work on linamarase and dhurrinase should be aimed at achieving maximum purity through microbial expression systems.

4. Stability testing of dhurrinase activity on linamarin should be conducted.

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