THERMOSTABILITY OF CASSAVA LINAMARASE

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DEDICATION

I dedicate this work to my family (especially Kosisochukwu Nnebe) and Jyoti Singh.

ABSTRACT

Cassava (*Manihot esculenta*) is a major staple crop for an estimated 700 million people worldwide. Cassava tubers are high in carbohydrates; however, they are an insufficient source of iron, zinc, vitamin A and protein. Additionally, the presence of anti-nutrients, namely cyanogenic glycosides (linamarin 95% and lotasutralin 5%) is an important factor that restricts the consumption of cassava. Cyanogenic glycosides can be hydrolyzed by the endogenous cell wall enzyme linamarase to yield acetone cyanohydrin (which spontaneously breaks down into hydrogen cyanide (HCN) at temperatures above 30°C) and free cyanide. Increasing the breakdown of linamarin into the volatile byproducts by linamarase can reduce the toxicity of cassava. Linamarase is quickly inactivated at high temperatures. Processing methods such as oven drying and boiling, that use high temperatures might reduce the efficiency of the detoxification process and increase the cyanide load in the processed cassava product. The production of High Quality Cassava Flour (HQCF) is an emerging non-traditional use of cassava that employs high temperatures and forgoes fermentation completely. The objective of this study was to investigate thermal stability of linamarase over a temperature range of 35-75°C. Linamrase exhibited highest activity at 45°C and was inactivated at 75°C. Experimental data was fitted by the Arrhenius equation. Two distinct thermal stabilities were present in the Arrhenius plot.

RÉSUMÉ

Le manioc est un aliment de base pour environ 700 million personnes dans le monde. Il s'agit d'un aliment très riche en carbohydrates qui s'avère cependant faible en fer, zinc, vitamine A et en protéines. En outre, la presence d'anti-nutriments, et particulièrement, les glycosides cyanogeniques, en limitent la consommation dans le monde. Cependant l'enzyme linamarase transforme les glycosides cyanogeniques en acétonecyanhydrine et en acide cyanhydrique. Ainsi, plusieurs procédés de transformation alimentaire sont utilisés pour reduire la toxicité du manioc. Ces procédés de transformation exigent toutefois des températures élevées qui peuvent réduire l'efficacité des procédés de detoxification, le linamarase est par exemple rapidement inactivé à des températures élevées. La production du 'High Quality Cassava Flour' (HQCF), un usage non-traditionnel du manioc, utilise des temperatures élevées. Ce projet a pour vocation d'étudier la stabilité thermique de linamarase sur une plage de temperature allant de 35-75°C. Nous trouvons ainsi que le linamarase était rapidement inactivée a des temperatures supérieures à 75°C. D'autre part, les données expérimentales ont été ajusté par l'équation d'Arrhenius et deux stabilités thermiques disntinctes étaient présentes.

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CONTRIBUTION OF AUTHORS

In this work, chapter 2 is a manuscript which has been prepared and will be submitted for publication. The research work reported here was performed and completed by the student Nnedimma Nnebe. She was responsible for the design of experiment, experimental setup, data analysis and preparation of the manuscripts and thesis. Professor Michael O. Ngadi is the thesis supervisor, providing scientific advice and overall technical supervision.

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CHAPTER ONE: INTRODUCTION AND OBJECTIVES

1.1 General introduction

Cassava (*Manihot esculenta*), is a root crop originating from South America and is one of the major staple food crops in West Africa. It is one of the most important sources of dietary energy in tropical and sub-tropical regions of the world; an estimated 700 million people obtain more than 500 cal day^{-1} from cassava (Maziya-Dixon, Dixon et al. 2009). Africa produces more than half of the total world cassava output and Nigeria is now the leading producing country globally (IFAD and FAO 2005). As of 2013, 157,718,952 tonnes of cassava was produced in Africa in 2013 (FAOSTAT 2013). The most dramatic increase was achieved in Ghana and Nigeria with production values of 15,989, 940 and 47,406,770 tonnes, respectively (FAOSTAT 2013).

The crop is grown almost exclusively by low-income smallholder farmers and consumed and processed at the household level (Eriksson 2013). Cassava is drought resistant and can be grown all year round and as such determines the food supply for many smallholder farmers and low-income families. Sweet and bitter varieties of cassava are grown in Nigeria and Ghana. The tubers can be stored in the soil for extended periods, however they must be processed to overcome its perishability and toxicity. All parts of cassava contain varying levels of cyanogenic glycosides which when hydrolyzed by an endogenous enzyme such as linamarase, yields hydrogen cyanide which is harmful to human health.

Processing methods that increase tissue disintegration are very efficient in cyanide removal because they break plant cells of cassava and allow direct contact between the enzyme linamarase found in the cell wall and linamarin (found in the cell vacuole). These methods consist of different combinations of peeling, chopping, grating, soaking, drying, boiling and fermenting (Tewe 1991, Maziya-Dixon, Dixon et al. 2009). The detoxification of cassava is primarily due to the action of linamarase (Ikediobi and Onyike 1982). While all processing methods bring about a reduction in the cyanide load, the extent of the reduction varies considerably due to the combination of methods and the extent to which they inactivate endogenous linamarase (Montagnac, Davis et al. 2009). Cassava tubers can be processed into a variety of dry products with improved safety, durability and stability. Popular foodstuffs include *gari* (granular flour from fermented cassava), *fufu* (fermented cassava paste, fermented flour and high quality cassava flour (HQCF) from unfermented cassava flour(Ferraro, Piccirillo et al. 2015). The use of high quality cassava flour (HQCF) in baking is an emerging non-traditional use of cassava in West Africa, particularly in Nigeria. HQCF is un-fermented, white, smooth and odourless cassava flour processed from freshly harvested roots (Falade and Akingbala 2008, Eriksson 2013). The processing of cassava into HQCF involves peeling, washing, grating, pressing, disintegration, sifting, drying, milling, screening, packaging and storage (Dziedzoave, Graffham et al. 2003). Drying typically occurs at temperatures greater than 65°C. The use of high processing temperatures may reduce the efficiency of the detoxification process; heating at high temperatures may result

in the breakdown of the enzyme (Oke 1984). Inactivation of linamarase at high drying temperature may result in increased retention of toxic cyanogens. The presence of residual cyanogen and their respective cyanohydrins are an important source of dietary exposure to cyanide and are a significant public health concern in areas where cassava products are consumed routinely (Rosling 1986)

1.2 Objectives

In the production of HQCF it is important to maximize linamarase activity in order to produce a final product with low cyanide content. It is therefore important to study the temperature dependency of linamarase at temperatures beyond its optimum. Thus the main objective of this research project was to model the thermal stability of linamarase. The specific objectives were to:

1. Determine the influence of heating temperature and time on the thermal stability of the enzyme linamarase
2. Model kinetics of partially purified linamarase activity variation with respect to temperature

CHAPTER TWO: LITERATURE REVIEW

2.1 Cassava production in West Africa

Cassava (*Manihot esculenta* Crantz) is an extensively cultivated root crop in West Africa. First introduced in the Congo by Portuguese explorers in the 16th century, its consumption rapidly spread across the continent because it provides a reliable source of energy and is drought resilient (IFAD and FAO 2005, Sayre 2011). It can be stored in the soil for up to 3 years (Onyenwoke and Simonyan 2014) and contributes to household food security and food availability in West Africa. It is one of the most important sources of dietary energy in tropical and sub-tropical regions of the world providing an estimated 700 million people with more than 500 cal day^{-1} (Maziya-Dixon, Dixon et al. 2009).

In the early 1960s, Africa accounted for approximately 40% of global cassava production and Brazil was the world's leading producer (IFAD and FAO 2005). Presently, Africa produces more than half of the total world cassava output (IFAD and FAO 2005) and Nigeria is now the leading producing country globally (Table 2.1). The crop is grown almost exclusively by low-income smallholder farmers and consumed and processed at the household level (Eriksson 2013). It is one of the few staple crops that can be produced without need of extensive mechanization and in marginal areas with poor soils and little rainfall (IFAD and FAO 2005).

2.1.1 Area cultivated

Since the early 1960s, total area devoted to cassava cultivation has increased from 5.6 million ha per year to more than 13 million in 2013 according to FAOSTAT (IFAD and FAO 2005). Traditionally, cassava was produced for home consumption but over the past 30-50 years, especially in Nigeria and Ghana, cassava cultivation as a cash crop has made great leaps (IFAD and FAO 2005). In the West African region, more than 6 million ha of land are devoted to cassava cultivation. The countries that account for most of the cassava production in West Africa are Nigeria, Ghana, and Benin (Table 2.1)

Table 2. 1 Production value and area cultivated for cassava in West Africa (2013)

Country	Production (tonnes)[a]	Area harvested (ha)[b]
Nigeria	47,406,770	6,741,300
Ghana	15,989,940	875,185
Benin	3,910,036	265,697
Sierra Leone	3,810,418	352,816
Côte d'Ivoire	2,436,495	360,000
Guinea	1,243,580	126,200
Togo	857,717	256,695
Liberia	520,000	65,000
Niger	156,100	7,800
Senegal	146,031	20,868
Mali	38,000	2,900
Guinea-Bissau	23,037	7,250
Gambia	11,500	3,000
Burkina Faso	4,350	3,200
Cabo Verde	4,123	524

[a,b]Values were obtained from FAOSTAT database

2.1.2 Yield

In 1990 the average cassava yield in Africa was between 9.01 tonnes per ha, in 2013, average yield on the continent was approximately 12.65 tonnes per ha, the average yield in West Africa for the same year is relatively higher at 14.92 tonnes per ha according to data from FAO STAT for 2013. The increasing cassava yields on the continent have been attributed in part to the planting of high-yielding varieties and the adoption of better agronomic practice (IFAD and FAO 2005). Many international bodies are pushing for even greater increases in cassava yields globally, in part because under optimal conditions, cassava yields can reach 80 tonnes per hectare (FAO 2013).

2.1.3 Production

Total cassava production in Africa in the late 1990s was approximately 87 million tonnes (IFAD and FAO 2005) and has since increased to 157 million tonnes in 2013 according to FAO STAT. The most dramatic increase was achieved West Africa where output rose by 60% from 47 million to 76 million (FAO 2013). Growth output was especially noticeable in Ghana and Nigeria; both countries boosted yields by 25% to around 15 tonnes per ha in the space of 11 years (FAO 2013).

2.2 Nutritional profile of cassava

Cassava is the staple food of 250 million marginalized people on the African continent (Gegios, Amthor et al. 2010). In Nigeria, it is one of the most available staple foods and provides the major sources of energy to the most food insecure (Maziya-Dixon, IO. et al. 2004). Following rice, it is also one of the most

affordable source of carbohydrates in Nigeria (Maziya-Dixon, IO. et al. 2004).

Figure 2.1 compares kilocalories per capita of major staple crops in Benin, Ghana, Ivory Coast, and Nigeria.

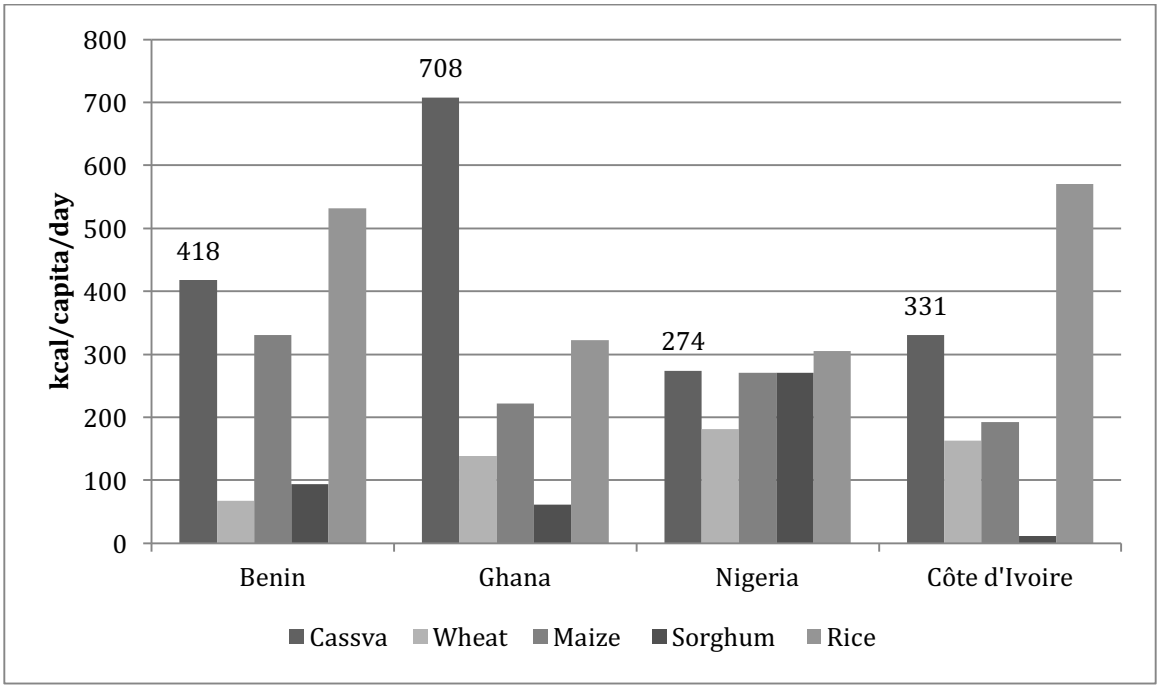

Figure 2.1 Major Food Crop kcals per capita 2011 based on FAOSTAT 2013 data at http://faostat3.fao.org/download/FB/CC/E

Cassava is cultivated primarily for its roots, although the leaves can also be consumed (Fauquet and Taylor 2002). Cassava roots are composed of the non-edible peel and root cortex and the root parenchyma (Figure 2.2)

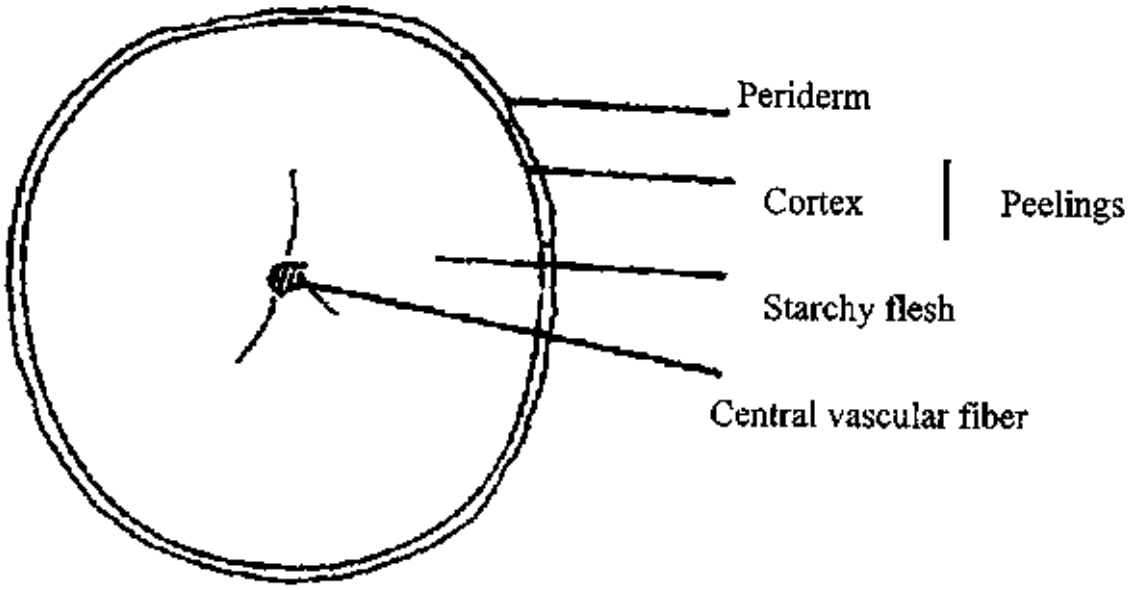

Figure 2. 2 Cross section of a cassava root (adapted from FAO 1998).

The nutritional content of cassava varies according to the cultivars, the age of the plant and environmental conditions (Fauquet and Taylor 2002, Sayre, Beeching et al. 2011). Cassava roots contain 80-90% carbohydrate on dry weight (DW), of which 80% is starch and remaining small quantities of sucrose, glucose, fructose, and maltose (Ferraro, Piccirillo et al. 2015). However, cassava is a poor source of fat (0.1-0.5% crude fat of fresh weight), proteins (1-3% crude proteins), and essential micronutrients such as vitamin A, zinc, and iron that are essential for normal growth, eyesight and cognitive development (Gegios, Amthor et al. 2010, De Moura, Moursi et al. 2015, Ferraro, Piccirillo et al. 2015). Micronutrients are necessary for proper development and growth and deficiencies may lead to severe disease states (Maziya-Dixon, Dixon et al. 2009, Sayre, Beeching et al. 2011). This is an important public health concern for people who consume cassava on a daily basis. In Nigeria, cassava is the most consumed staple crop

(Maziya-Dixon, IO. et al. 2004). Chronic cassava consumption is negatively correlated with vitamin A, zinc, iron and protein intake, and is a risk factor for micronutrient deficiencies and protein energy malnutrition (Gegios, Amthor et al. 2010, Sayre, Beeching et al. 2011). A typical adult sized cassava meal (500g) is an insufficient source of iron, zinc, vitamin A and protein (Gegios, Amthor et al. 2010, Sayre, Beeching et al. 2011).

On the other hand, cassava leaves (which are also edible) have a crude protein content of 5-7%, 1-2% crude fat, 2% mineral (DW) and a well balanced amino acid profile comparable to white, fresh egg, with the exception of methionine, lysine, and isoleucine (Ferraro, Piccirillo et al. 2015). Cassava leaves contain high contents of vitamins, B1, B2, C, carotenoids and minerals like phosphorous, magnesium, potassium and calcium but low contents of iron, zinc, manganese, copper, sodium, and calcium (Adewusi and Bradbury 1993, Wobeto, Corrêa et al. 2006, Letif and Muller 2015). However, cassava roots and leaves have a huge deficit of sulphur-containing amino acids such as methionine and cysteine and nutrients are not well allocated in the plant (Ferraro, Piccirillo et al. 2015). Proximate composition of cassava root and leaves is reported in Table 2.2.

Table 2. 2 Composition of cassava roots and leaves on a fresh weight basis (Montagnac et al., 2009; Bradbury and Holloway, 1988)

Composition	Cassava Root (100 g)	Cassava Leaf (100 g)
Caloric content (Kcal)	110-150	90
Water (g)	46-85	65-89
Protein (g)	0.3-3.5	1-10
Lipid (g)	0.03-0.5	0.2-2.9
Carbohydrates (g)	25-36	7-18
Fiber (g)	0.1-3.7	0.5-10
Ash (g)	0.4-1.7	0.7-4.5
Vitamin		
Ascorbic acid (mg)	15-50	60-370
Niacin (mg)	0.6-1.1	1.3-2.8
Riboflavin (mg)	0.03-0.06	0.21-0.74
Thiamin (mg)	0.03-0.28	0.06-0.31
Vitamin A (µg)	5-35	8300-11800
Minerals		
Calcium (mg)	19-176	34-710
Copper (mg)	0.2-0.6	0.3-1.2
Iron (mg)	0.3-14	0.4-8
Magnesium (mg)	30-80	120-420
Manganese (mg)	0.3-1	7-25
Phosphorous (mg)	6-150	27-210
Potassium (mg)	250-720	350-1230
Sodium (mg)	76-210	50-180
Zinc (mg)	1.4-4	7-25

2.3 Anti-nutrient content

In addition to its nutrient content, cassava roots also contain several anti-nutrients such as phytates, fiber, nitrate, polyphenols, oxalate and saponins (Ferraro, Piccirillo et al. 2015). Some of these compounds have positive effects on health but some may interfere with nutrient absorption and utilization and may have toxic side effects (Wobeto, Corrêa et al. 2007, Ferraro, Piccirillo et al. 2015). Cassava has a phytate content of approximately 199 mg/ 100 g dry weight; phytates interfere with the absorption iron and zinc, which are essential

nutrients (Montagnac, Davis et al. 2009, Lazarte, Carlsson et al. 2015). The polyphenols identified in cassava are catechin and its derivatives. Catechins are associated with cardiovascular health benefits in green tea. Polyphenols are known antioxidants, which may confer positive effects to health, but they may also bind essential minerals making them unavailable for absorption (Montagnac, Davis et al. 2009). Polyphenol concentration in the roots increases as the roots deteriorate (Montagnac, Davis et al. 2009). In the leaves, the polyphenols are condensed tannins that are made of anthocyanidins (Reed, McDowell et al. 1982, Padmaja 1989).

2.3.1 Cyanogenic glycosides

The presence of cyanogenic glycosides is one of the most important factors restricting the consumption of cassava roots and leaves in Sub-Saharan Africa and in the Global North (Montagnac, Davis et al. 2009). Cassava tissues accumulate toxic cyanogenic glycosides (CNG) (linamarin 95% and lotaustralin 5%) which when hydrolyzed or exposed to alkaline conditions release cyanide (HCN) (Fig 2.3) (Nambisan 2011).

Cyanogenic glycosides are present in all cassava tissues at varying concentrations (Balagopalan, Padmaja et al. 1988, Bokanga 1994, Bokanga 1994). Cyanogens are present in the cell vacuoles and are synthesized from amino acid valine (Nambisan 2011, Gleadow and Moller 2014). So called “bitter” cassava may have cyanide levels that exceed the FAO/WHO safe level for cyanide in cassava food products of 10 mg/kg dry weight, this makes raw cassava acutely toxic for human (Ferraro, Piccirillo et al. 2015). The root

parenchyma (the edible portion of the root) has the lowest cyanogen content (Padmaja 1995). The leaf, stem and root cortex contain higher concentration of CNGs than the root parenchyma; leaves have a cyanogenic potential 5 to 20 times higher than that of the peeled root parenchyma (Nambisan 2011, Ferraro, Piccirillo et al. 2015). The cyanogenic potential of cassava cultivars ranges from 53 -1300 mg HCN equivalents/kg DW in leaves (Wobeto, Corrêa et al. 2006) to 10 -500 mg HCN equivalents/kg dry weight (DW) in the roots (Wilson and Dufour 2002, Montagnac, Davis et al. 2009).

The cyanogenic potential of cassava differs among varieties as well and for the same variety increases in drought conditions (McMahon, White et al. 1995, Cardoso, Mirione et al. 2005). “Sweet” cassava varieties have a lower CNG load (< 100 mg HCN equivalents/kg fresh weight) whereas “bitter” cassava varieties, generally have a larger CNG load (100-450 mg HCN equivalents/kg FW) and very bitter roots have a concentration > 450 mg HCN equivalents/kg FW (Bourdoux, Delange et al. 1980, Sundaresan, Nambisan et al. 1987). Cyanide content also varies across tubers of the same variety and within the roots of the same plants (Cooke 1978, Ferraro, Piccirillo et al. 2015). Cyanogen content can vary along the length of the root (Cooke 1978, Bradbury, Egan et al. 1991) and a radial gradient also exists (Cooke 1978); the apical root tip has cyanide content 60% higher than the basal root tip (Cooke 1978, Bokanga 1994, Ferraro, Piccirillo et al. 2015). Cyanogenic gluosides are generally stable in neutral or weak acid conditions (Montagnac, Davis et al. 2009). Through a process commonly referred to as cyanogenesis, they become unstable when the β-

glycosidic linkage is hydrolyzed through the action of a β-glycosidase such as linamarase to yield acetonecyanhidrin (Gleadow and Moller 2014). The labile cyanohydrin can break down through the action of bacterial and/or endogenous β-glucosidase or spontaneously at temperatures above 30°C (McMahon, White et al. 1995, Onabolu, Oluwole et al. 2002, Montagnac, Davis et al. 2009, Gleadow and Moller 2014).

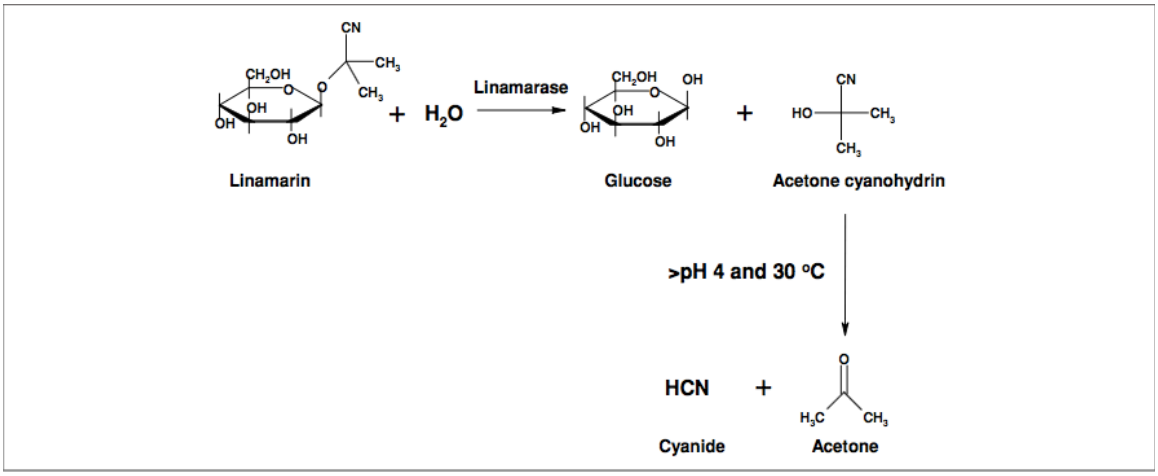

Figure 2.3 Breakdown of linamarain (adapted from Montagnac, Davis et al. 2009)

2.4 Cassava processing

The utilization of cassava roots for human nutrition is therefore limited by the presence of cyanogenic glycosides and the perishability of the root (Maduagwu and Oben 1981, Nambisan 2011). Cassava tubers are extremely perishable; they have a shelf life of less than 3 days after harvesting and rapidly deteriorate and become inedible (USAID/CORAF/SONGHAI 2010, Sayre, Beeching et al. 2011). An estimated 26% (18 million tons) of all the cassava produced in Africa every year is lost to postharvest physiological deterioration (Sayre, Beeching et al.

2011). Therefore, cassava tubers require extensive processing in order to reduce their toxicity, improve their palatability and increase their shelf life (Tewe 1991, Nambisan 2011).

Methods that increase tissue disintegration are very efficient in cyanide removal; they disrupt the structural integrity of the cell and allow direct contact between the enzyme linamarase found in the cell wall and linamarin. These methods consist of different combinations of peeling, chopping, grating, soaking, drying, boiling and fermenting (Tewe 1991, Padmaja 1995, Maziya-Dixon, Dixon et al. 2009). Typical cassava processing steps are shown in Figure 2.4.

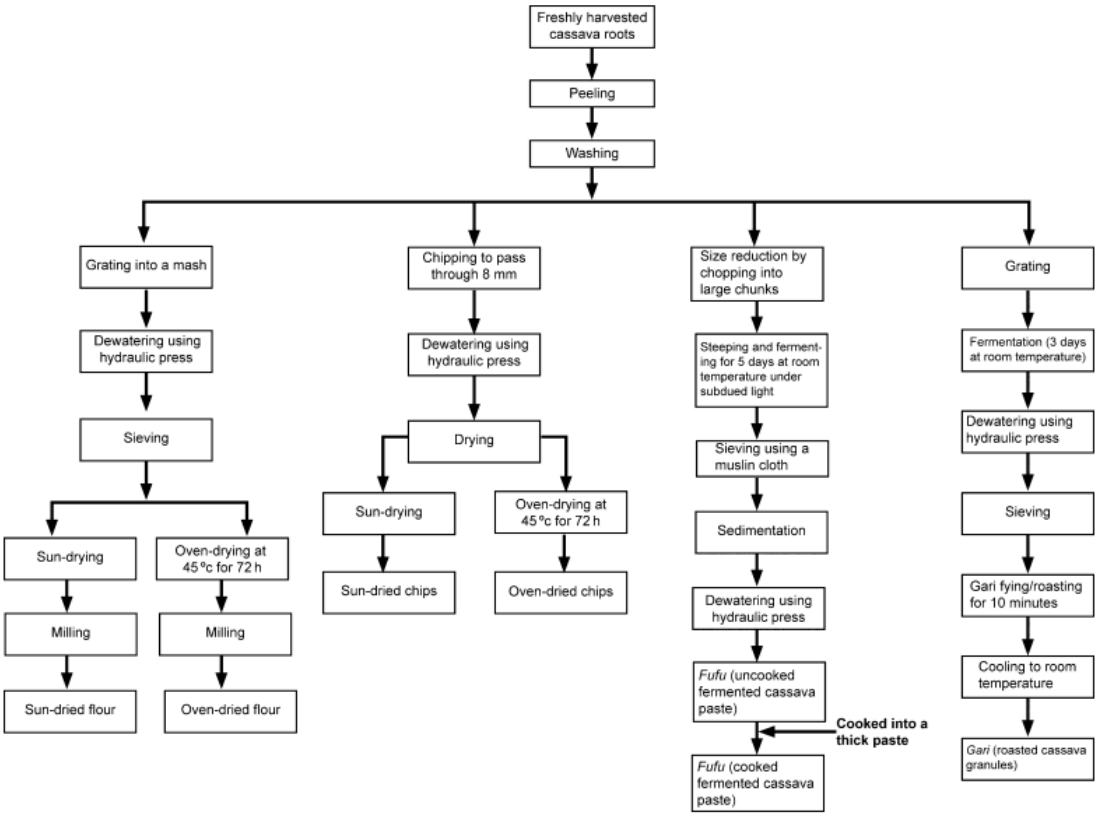

Figure 2.4 Processing of cassava roots (adapted from Maziya-Dixon et al.2009)

The detoxification of cassava is primarily due to the action of linamarase (Ikediobi and Onyike 1982, Vasconcelos, Twiddy et al. 1990). While all processing methods bring about a reduction in the cyanide load, the extent of the reduction varies considerably due to the combination of methods and the extent to which they inactivate endogenous linamarase (Montagnac, Davis et al. 2009). Bitter varieties of cassava in contrast to sweet varieties require more labor intensive processes in order to be palatable and safe for human consumption (Nye 1991). There are large differences in the distribution, activity and properties of linamarase in cassava tissues and amongst different cultivars (Elias, Nambisan et al. 1997, Ravi and Padmaja 1997). Leaf tissues have high linamarase and hydroxynitrile lyase (HNL) activity, tuber cortex has high activity of linamarase and negligible HNL activity, while tuber parenchyma has low levels of linamarase and no HNL activity (Nambisan 2011). Three different isoforms of linamarase, which differ in their specific localization, have previously been identified (McMahon, White et al. 1995, Elias, Nambisan et al. 1997). Linamarase isoforms localized in the leaves and in the root cortex have 30-50 fold and 15 fold higher levels of specific activity, respectively as compared to the linamarase isoform found in the root parenchyma (McMahon, White et al. 1995). All three linamarase isoforms have a lower specific activity than linamarases isolated in other plants (McMahon, White et al. 1995).

Linamarase is stable at a pH range of 5.5-7 and works best at an optimum temperature of 55°C (Yeoh 1989, Keresztessy, Kiss et al. 1994, McMahon, White et al. 1995, Elias, Nambisan et al. 1997, Nambisan 2011). Ikediobi and Onyike

(1982) reported a significant reduction in linamarase activity as fermentation time increased. This is partly due to the pH shift away from the optimum of 5.5 towards lower pH values. Linamarase was completely inactivated once the pH attained a value of 4.2 (Ikediobi and Onyike 1982). The specific effects of various processing technique on linamarase activity and the cyanide content of cassava are discussed below.

2.4.1 Processing methods and their effect on linamarase

2.4.1.1 Peeling

Peeling is usually the first step in the processing of cassava tubers. The peel (including the root cortex) generally contains higher cyanide content than the root parenchyma (Tewe 1991, Padmaja 1995). Removal of the peels therefore reduces the toxicity of the tuber considerably.

2.4.1.2 Boiling and steaming

Linamarin hydrolysis is limited during the boiling of cassava tubers, and detoxification occurs primarily through the leaching of the water soluble linamarin into the boiling water (Nambisan and Sundaresan 1985, Sokari and Karibo 1996). Heating at high temperatures may result in the breakdown of the enzyme (Oke 1984). At 100°C, linamarase is denatured and cannot hydrolyze linamarin into cyanohydrin (Montagnac, Davis et al. 2009). Hydrogen cyanide and cyanohydrin concentrations in boiled cassava roots are very low. Cooke and Maduagwu (1978) observed higher levels of bound cyanide (linamarin) retention in boiled cassva tubers. However, reductions in the chip size or surface area increases the efficiency of the boiling method; cyanogen retention was higher in

50g cassava slices than in 2 gram cassava slices after boiling for 30 minutes (Nambisan and Sundaresan 1985, Montagnac, Davis et al. 2009). Retention of cyanogens in tubers is also high during during steaming; up to 83% of cyanogen content is retained in steamed tubers (Nambisan and Sundaresan 1985). This is due in part to the increased stability of linamarin in neutral or weak acid condition to temperatures greater than 100°C (Bradbury, Egan et al. 1991, Cardoso, Mirione et al. 2005, Montagnac, Davis et al. 2009)

2.4.1.3 Drying, baking, and frying

Drying, baking, and frying are similarly ineffective; Nambisan and Sundaresan (1985) observed higher levels of cyanogen retention in tubers that were dried, baked, and fried. Average cyanogen retention was 86% after baking, 87% after frying, and 73% after drying (Nambisan and Sundaresan 1985). These methods are only suitable for so-called sweet cassava varieties that have low cyanide content (Cardoso, Mirione et al. 2005, Montagnac, Davis et al. 2009). Drying is only marginally better than boiling at reducing linamarin content in cassava roots. High processing temperature during oven drying increases linamarin accumulation in dried cassava foodstuffs (Kemdirim, Chukwu et al. 1995). Cyanide retention during sun drying is lower than in oven drying because temperatures are generally below 55°C. Others have reported free cyanide content of 30% total cyanogens in oven-dried and 60% in sun-dried cassava (Gomez, Valdivieso et al. 1984). Linamarase activity is higher in sun drying because of the lower temperature, this allows for greater hydrolysis of linamarin into cyanohydrin and allows for the accumulation of both the cyanohydrin and

free cyanide, which can subsequently be volatilized. However, drying is only suitable for low cyanide cassava cultivars and cannot be used to detoxify bitter cassava such as occurs in West Africa (Montagnac, Davis et al. 2009).

2.4.1.4 Fermentation

Fermentation by lactic acid bacteria is a common processing method in West Africa. Fermentation is done with grated or soaked cassava roots. Fermentation of grated cassava roots is an effective detoxification method. Westby and Choo (1994) reported that 95% of linamarin was removed within 3 hours of grating. Grating is primarily responsible for linamarin hydrolysis and microorganisms play a minimal role in cyanogen reduction (Vasconcelos, Twiddy et al. 1990). This is in part due to the fact that linamarin is quite stable in acidic conditions(Ikediobi and Onyike 1982). However, though linamarin is rapidly removed by grating, cyanide retention is still relatively high in products of grated and fermented cassava roots. Vasconcelos, Twiddy et al. (1990) reported that high concentrations of cyanohydrin and free cyanide were left in the fermented product. This might be explained by the stability of cyanohydrin at acid pH (Cooke 1978) . Fermentation of soaked roots in water is more effective than that of grated roots in reducing cyanogen content and can reduce cyanogen content in tubers by 90% after 3 days of fermentation (Montagnac, Davis et al. 2009). In the case of fermentation of soaked roots, microbial growth is essential for removing cyanogens (Montagnac, Davis et al. 2009).

Dewatering cassava mash during processing can significantly reduce, cyanogen retention in the final cassava products. Cyanogens are water-soluble; the

addition of water facilitates the leaching of bound cyanide from the cassava tissue and increases the access of linamarase to its substrate (Fomunyam, Adegbola et al. 1985). This reduction is especially pronounced when de-watering is done after the fermentation process. Fermentation followed by drying and/or roasting efficiently reduces the cyanogenic load of the final food product (Kemdirim, Chukwu et al. 1995, Montagnac, Davis et al. 2009). However, Onabolu et al. (2002) reported higher levels of cyanohydrin retention when roasting was done at temperatures above 80°C.

2.4.2 Cassava flour production

Processing of the roots generates a wide range of products with industrial applications (biofuels, paper textiles, plywood, and adhesives) and dry food stuff with improved safety, stability and durability (Ferraro, Piccirillo et al. 2015). Among all the derived products, the most important are high quality cassava flour (HQCF) from unfermented cassava flour, *gari* (granular fermented cassava flour), and fermented cassava flour. The use of HQCF as a replacement of wheat flour in baking is an emerging and potentially profitable non-traditional use of cassava in West Africa. HQCF, is unfermented, colorless and odourless alternative to fermented flour (USAID/CORAF/SONGHAI 2010, Eriksson 2013). This method of flour preparation requires less labour and time; HQCF has to be produced rapidly in order to prevent fermentation (USAID/CORAF/SONGHAI 2010). The process flowchart for the production of HQCF is reported in Figure. 2.5

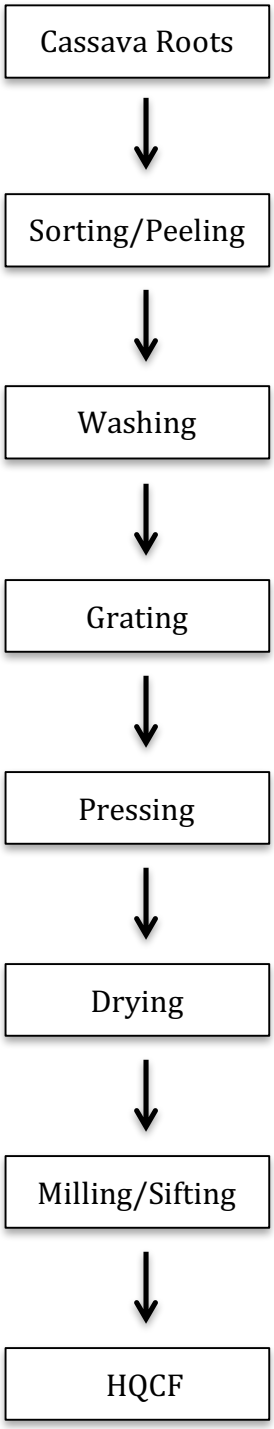

Figure 2. 5 Process flow of High Quality Cassava Flour (HQCF)

The processing of cassava into HQCF involves peeling, washing, grating,

pressing, disintegration, sifting, drying, milling, screening, packaging and storage (Dziedzoave, Graffham et al. 2003). The fresh roots (10-12 months) are peeled, washed and cut into chips. The chips are subsequently grated by a motorized cassava grater that disintegrates the cassava tissue and frees up excess moisture. The cassava mash is then pressed by means of a screw press; pressing is essentially a dewatering process. Pressing time should be short to avoid fermentation of the grated mash. Pressing is critical step in the production of HQCF; inadequate pressing could increase the drying time resulting in fermentation producing a final product with undesirably high acid tastes. Pressing helps to get rid of the yellow coloring in cassava tissues thereby improving the color of the final product. Following pressing, the mash is disintegrated to reduce the pressed cake in to fine grits which is then sifted and dried and transformed into the final product (Dziedzoave, Graffham et al. 2003). Pressing and grating allow for the necessary tissue disintegration that brings linamarase into contact with linamarin (Vasconcelos, Twiddy et al. 1990, Kemdirim, Chukwu et al. 1995). In the absence of fermentation, this step is critical to produce final product with a lowered cyanide content. Drying allows for the remaining cyanide to be volatilized. Oven dryers are often used in the production of HQCF. Processing temperatures normally exceed 65°C. There are concerns that the method of production of HQCF may result in a product with higher cyanide retention as drying may inactivate the endogenous linamarase. Furthermore, this method might not be appropriate when working with bitter varieties of cassava with higher cyanide loads. Indeed the USAID advises against the use of bitter varieties of

cassava for the production of HQCF, because the production process may not afford sufficient contact between linamarase and cyanogens for effective detoxification of the product (USAID/CORAF/SONGHAI 2010). Cardoso, Mirione et al. (2005) developed an equation relating cyanide contents of peeled roots to the percentage of cyanide retention in processed food products. The equation, on the basis on the total cyanogen content in the cassava root parenchyma, estimates total cyanide retention in processed cassava foodstuffs. This equation can be applied to different methods of processing used worldwide. The authors concluded that only sweet cassava varieties can be used to produces cassava flour containing 10 mg HCN equivalents/kg flour, the WHO safe level, by sun drying or heap fermentation. The previously mentioned processing methods do not allow for intimate contact between linamarin and linamarase (Cardoso, Mirione et al. 2005). As consumer demand for HQCF increase it is critical to understand how temperature may affect the activity of linamarase and how this might affect the safety of the final product.

2.4.3 Linamarin metabolism in the human body

Consumption of poorly processed cassava products can lead to chronic dietary cyanogen exposure. The toxicity produced by cassava consumption depends on the cyanogen concentration in the root, the nutritional status of the subject, the quantity of protein consumed, and the amount of the detoxified byproduct formed in the body (Nambisan 2011). The toxic effects of linamarin in the body are poorly understood (Barrett, Hill et al. 1977, Bourdoux, Delange et al. 1980) in part because they are sometimes sub-lethal (Hernández, Lundquist et al. 1995,

Dorea 2004). In humans, intact linamarin is rapidly excreted in urine (Hernández, Lundquist et al. 1995). The residual linamarin can also be broken down by microbial flora in the human intestine to yield acetone cyanohydrin and HCN (Dorea 2004). Under alkaline conditions (pH above 4) such as those observed in the human gut i.e. the small intestine and large intestine acetone cyanohydrin undergoes spontaneous hydrolysis to yield HCN and acetone (Montagnac, Davis et al. 2009, Nambisan 2011).

The human body has two defense mechanisms against cyanide. Cyanide can be temporarily trapped by the methemoglobin fraction in red blood corpuscles, or it can be converted to thiocyanate in a reaction catalyzed by rhodanese (Nambisan 2011). Thiocyanate, the most useful biomarker for cyanide exposure, has known anti-thyroidal effects and has been associated with endemic goiter (Bourdoux, Delange et al. 1980, Chandra, Mukhopadhyay et al. 2004, Dorea 2004). Cyanogenic compounds affect hormone synthesis in the thyroid gland by inhibiting iodine uptake or by modulating the activity of thyroid peroxidase (Chandra, Mukhopadhyay et al. 2004). Dietary cyanide exposure from a monotonous cassava diet has been proposed as a contributing factor in two forms of nutritional neuropathies; tropical ataxic neuropathy (TAN) and epidemic spastic paraparesis also known as konzo (Adamolekun 2011). TAN is characterized by sensory polyneuropathy, sensory ataxia, bilateral optic atrophy and bilateral-sensoria-neural deafness (Adamolekun 2011). Konzo is a symmetrical permanent spastic paraparesis affecting primarily young children and young women (Adamolekun 2011). Clinical symptoms of konzo include;

tachypnea, tachycardia, dizziness, headache, abdominal pain, vomiting, diarrhea, mental confusion, and convulsions; these symptoms occur generally 4-6 hours after ingestion of meals containing cassava (Nzwalo and Cliff 2011). The hallmark of konzo is difficulty in walking (Nzwalo and Cliff 2011). It has been proposed that both neuropathies are associated with 1) high and chronic dietary exposure to cyanogens; 2) low protein intake especially sulfur amino acids (SAAs) such methionine and cysteine needed to detoxify cyanide into thiocyanate (Nzwalo and Cliff 2011). In all recorded epidemics, the appearance of konzo has been associated with a sustained and sub-lethal intake of cassava high in residual cyanogen content in combination with a low SAA intake (Nzwalo and Cliff 2011). There is substantial evidence linking the consumption of poorly processed cassava products to adverse health outcomes. In any attempt to scale up the production of HQCF, it is important to have a better understanding of how high temperatures might affect the activity of linamarase.

2.5 Enzyme inactivation kinetics

There are six different enzyme categories namely EC 1: oxidoreductases; EC 2: transferases; EC 3: hydrolases; EC 4: lyases; EC 5: isomerases and EC 6: ligases (Fraatz, Ruhl et al. 2014). Linamarase is EC 3 as it catalyzes the hydrolysis of a glycosidic linkage to release acetone cyanohydrin and hydrogen cyanide (Gleadow and Moller 2014).

The conversion of the linamarase-linamarin substrate complex into acetone cyanohydrin and free cyanide is a first order reaction (Keresztessy, Kiss et al. 1994). For a first order reaction A->P, the velocity *v* can be expressed as

$$v = \frac{dp}{dt} = -\frac{da}{dt} = ka = k(a_o - p) \quad (1)$$

in which *a* and *p* are the concentration at any time *t* of A and P, and *k* is a first-order rate constant at the start of reaction *t*=0 and *a*= a_0 (Cornish-Bowden 1979). Reaction velocities are strongly influenced by temperature (Cornish-Bowden 1979). Increasing temperature can have two major effects on enzymes; the catalytic rate increases and ultimately denaturation or protein unfolding can occur (Fields 2001). The functional state of globular proteins such as enzymes depends on their unperturbed three dimensional structure (Jaenicke 1991).

The stability of the tertiary structure is largely dependent on environmental conditions such as pH and temperature amongst others (Ludikhuyze, Van Loey et al. 2003). Changing the environmental conditions can lead to changes in the three dimensional conformation of the enzyme or change at or near the active site, resulting in loss of enzyme activity (Ludikhuyze, Van Loey et al. 2003). The manner in which enzymes respond to temperature is fundamental to many areas of food processing technology. Enzyme catalyzed reactions are specific and occur under mild conditions and are of great importance in the food industry.

Previous studies by van't Hoff and Arrhenius demonstrated the temperature dependence of rate constants (Ludikhuyze, Van Loey et al. 2003). Arrhenius postulated that the temperature dependence of any rate constant *k* could be expressed by an equation of the form

$$\frac{d \ln k}{dT} = E_a / RT^2 \quad (2)$$

where E_a is the activation energy, integration with respect to *T* gives

$$\ln K = \ln A - (E_a / RT) \quad (3)$$

where ln *A* is a constant of integration (Cornish-Bowden 1979). This form of the Arrhenius equation is convenient for graphical purposes; it shows that a plot of ln k against 1/T is a straight line of slope $-E_a/R$, or conversely, if log k is plotted against 1/T, the slope is $-E_a/2.303R$. This plot is known as an Arrhenius plot and provides a simple methods of estimating E_a. The activation energy can be used to characterize the temperature dependence of the observed inactivation rate constants. Larger activation energies denote greater temperature dependency.

The thermal inactivation of hydrolases has been studied. Several studies have focused on the effects of thermal processing on the formation and thermal stability of the products of the enzymatic reaction (Munyaka, Verlinde et al. 2010, Wawire, Oey et al. 2012) whereas others have investigated the thermal stability of the enzymes specifically (Xinzhuo, Rui et al. 2014, Spelbrink, Lensing et al. 2015).

Munyaka, Verlinde et al. (2010) investigated the effect of time and temperature on folate poly-γ-glutamate hydrolysis by the endogenous hydrolase Y-glutamyl hydrolase (GGH) in broccoli, a vegetable which is known to contain a high concentration of folate poly-γ-glutamate. Dietary folate predominately occurs in plant based foods as long chain poly-γ-glutamate; hydrolysis by endogenous GGH, generates short chain poly-γ-glutamate which are more bioavailable (Munyaka, Verlinde et al. 2010). Raw crushed broccoli was heated to 25-55°C

for 5- 30 mins and 40 and 60 °C for 30 min. All heat treatments decreased the concentration of long chain folate poly-Ɣ-glutamate, thereby potentially indicating that endogenous GGH was active within this temperature range (Munyaka, Verlinde et al. 2010); the study did not directly investigate the concentration of short chain folate poly-Ɣ-glutamate. Significant folate losses were reported at the highest temperatures tested, 120, and 140°C but non-significant losses were reported at 80, 100°C (Munyaka, Verlinde et al. 2010). It is impossible to draw clear conclusions about the thermostability of GGH from the experimental data. The high folate losses at 120 and 140°C were attributable to the acceleration of oxidation due to heating and may not be directly linked GGH inactivation. Therefore, from the data reported it is impossible determine at what temperature inactivation may occur.

Wawire, Oey et al. (2012) investigated the effect of thermal treatments on folate stability in cowpeas. They reported high levels of short chain folate poly-Ɣ-glutamate at temperatures below 70°C because at these temperatures the enzymatic hydrolysis is enhanced. Lower levels of short chain poly-Ɣ-glutamates were observed at temperatures above 70°C, suggesting that in heat treated samples, the enzyme was unable to break down the long chain poly-Ɣ-glutamates.

Xinzhuo, Rui et al. (2014) investigated the kinetic characteristics of a fungal β-glucosidase from *Neosartorya fischeri* P1, termed NfGBLI. NfBGL1 remained stable at temperatures up to 70°C and over a broad pH range of 3.0-10.0. The recombinant β-glucosidase investigated in this study has characteristics that are

very distinct from the native enzyme, for example the optimum temperature for the recombinant enzyme is 70 whereas other native hydrolyases are inactivated at this temperature (Munyaka, Verlinde et al. 2010, Wawire, Oey et al. 2012). It might be difficult to generalize the findings of this study to other hydrolases.

Spelbrink, Lensing et al. (2015) investigated the inactivation kinetics of patatin, a pototao lipase, in whey. Patatin was substantially inactivated at 74°C and the activation energy in whey was approximately 202 kJ mol^{-1} (Spelbrink, Lensing et al. 2015). Conducting the inactivation studies in whey (a protein) might explain the high activation energy; in the presence of impurities the activation energy of certain enzymes might increase (Ikya, Ariahu et al. 2013).

Few studies have been conducted on linamarase and in contrast to other hydrolases; the inactivation kinetics of linamarase at high temperatures has not been studied extensively.

Yeoh (1989) studied the thermal stability of linamarase at a temperature range of 30-80°C. Linamarase was extracted from the leaf, peel and tuber cortex of cassava. The reported temperature optimum for the three isoforms was 55°C against linamrain and 60*C against the analogue p-nitrophenyl-β-D-glucoside. The tuber cortex linamarase exhibited the highest thermal stability of all three isoforms studies. After heating at 55°C after 30 mins, the tuber cortex enzyme suffered a 10 % loss in activity whereas the leaf and peel enzymes suffered a 34 and 35% loss respectively. After heating at 60°C for 30 minutes, the tuber cortex enzyme lost only 30% of its activity and the leaf and peel lost 80% of their

activity. At 50°C for 1 hour, the leaf and peel enzyme lost 15fi f their initial activity whereas the tuber cortex enzyme retained all its activity. These finding suggest that the tuber cortex enzyme is very thermal stable and can be subjected to temperatures beyond its optimum with only moderate losses in activity. The leaf, peel and tuber cortex linamarase exhibited similar activation energy with respect to linamarin: 12.55, 16.74, and 14.64 kJ mol^{-1} respectively and 27.20 and 24.27 kJ mol^{-1} against the analogue. The activation energy represents the energy that reacting system must surmount in order to convert the initial reactants into the final products (Sizer 2006). The magnitude of the activation energy reported by the author indicates that at the temperature range studied, the progression the reaction is not strongly temperature dependent and increases in temperature do not do much to alter the reaction rate, further indicating that linamarse is largely thermostable.

Petruccioli, Brimer et al. (1999) investigated the thermal stability of two bacterial linamarases (PGI and PGII) from *Pencillium aurantiogriseum* (P35) at a temperature range of 25-70°C. PGI had a temperature optimum of 55°C while 60°C was optimal for PGII. PGI retained its initial activity from 25-50°C and exhibited a loss of 85% at 65°C. PGII retained its initial activity up to 60°C. Both enzymes were completely inactivated after 10 minutes at 75°C. Therefore, once again the findings from this study indicates that bacterial linamarase is equally thermostable at a similar temperature as studied by Yeoh (1989). The authors did not report the activation energies of the bacterial linamarase but one would expect that they would also be low. Nok and Ikediobi (1990), Ikya, Ariahu et al.

(2013) modeled the thermal stability of linamarase at ambient temperatures (10-40°C and 25-35°C respectively.)

Nok and Ikediobi (1990) isolated and purified two isoforms of linamarase and described its temperature dependence at temperature range of 10-40°C according to the Arrhenius equation. They obtained a linear plot for both isoforms determined the activation energies to be 49.04 kJ mol^{-1} and 36.48 kJ mol^{-1}. Similarly to Yeoh (1989),the reported activation energies are low and indicate that linamarase was generally stable at this temperature range. They reported an optimal temperature of 30°C, which is much lower than the generally agreed upon optimum of 55°C (Yeoh 1989, Nambisan 2011). The optimum temperature is the temperature at which the enzyme functions at its maximum velocity (Sizer 2006). However, this temperature optimum is not constant for a given enzyme and varies widely with factors such as enzyme and substrate purity, the presence of activators and inhibitors, and the method used in measuring the rate of the catalyzed reaction (Sizer 2006).

Ikya, Ariahu et al. (2013) verified the temperature dependency of linamarase at ambient temperature (25- 35°C). They reported activation energies in the range of 60.9 to 91.7 kJ mol^{-1} for bacterial linamarase. Immediately, the temperature range used in this study is concerning as it is very narrow and therefore makes it hard to draw clear conclusions from this work. The activation energies reported in this study are higher than those reported by both Yeoh (1989) and (Nok and Ikediobi 1990). This might be due in party to the level of enzyme purity. The authors reported that the degree of enzyme purity had some appreciable effect

on the magnitude of the activation energy. They reported higher activation energy for crude enzyme preparation and lower E_a with increasing enzyme purity (Ikya, Ariahu et al. 2013). Enzyme purity might affect the activation energy by reducing the number of efficient collisions between the enzyme and its substrate thereby increasing the activation barrier (Ikya, Ariahu et al. 2013).

From this initial survey of literature, it possible to define several key parameters that require further investigation. Of the three studies that modeled the thermal stability of linamarase according to the Arrhenius, only one of them looked at thermal stability at temperatures beyond 40°C. Therefore further investigation of the temperature dependency of linamarase at high temperatures is required. Additionally, it would prove helpful to estimate the activation energy at higher temperature in order to determine if it is comparable to the activation energies reported at lower temperatures.

CONNECTING TEXT

The previous chapter established the rationale for cassava processing by detailing the potential toxicity of cassava tubers, namely due to the presence of

cyanogenic glycosides. Furthermore, the efficiency of traditional processing methods was discussed and the temperature dependency of the detoxification process was established. The following chapter will elaborate on the temperature dependency of linamarase as a function of treatment time and temperature. Enzyme solutions were heating in thermal water baths at different temperatures, 35, 45, 55, 65 and 75°C for different time periods 10-40 minutes. Experimental values of the thermal inactivation studies were fitted to the Arrhenius equation with linear regression methods.

CHAPTER 3: INACTIVATION KINECTICS OF LINAMARASE

Abstract

Background: Cassava tubers contain cyanogenic glycosides and the endogenous enzyme linamarase that hydrolyses these compounds to yield hydrogen cyanide, which is toxic to human health. Linamarase is heat labile and is rapidly inactivated at high temperatures. The thermostability of linamarase at a temperature range of 35-75°C was investigated.

Method: Linamarase was extracted from the root cortex of cassava tuber and partially purified with ammonium sulphate. Enzyme extracts were heated at 35, 45, 55, 65 and 75°C for 10, 20, 30, and 40 minutes before activity assays were performed. The thermostability as a function of heating time and temperature was studied.

Results: Temperature had a statistically significant effect on enzyme activity. The enzyme exhibited highest activity at 45°C and activity steadily decreased as the temperature increased up to 75°C after which the enzyme was completely inactivated**.** Treatment time had no statistically significant effect on the activity of the enzyme. Linamarase activity was generally stable across all treatment times. The Arrhenius plot exhibited a break after 55°C with a sharp increase in the activation energy (E_a) at this critical point.

Conclusions: These findings suggest that linamarase is relatively thermostable. Linamarase is inactivated at temperatures above 75°C. Two distinct thermal stabilities were present in the Arrhenius plot. The estimated activation energy (E_a) at 35-55°C and 55-75°C was 1.87 kJ mol^{-1} and 115.9 kJ mol^{-1}, respectively.

3.1 Introduction

Linamarase or β-D-glucosidase (EC 3.2.1.21) from cassava (*Manihot esculenta Crantz*) is a hydrolase that catalyzes the hydrolysis of linamarin to acetone cyanohydrin and subsequently to hydrogen cyanide (Keresztessy, Kiss et al. 1994, Gleadow and Moller 2014). This reaction is of particular interest for food safety and food security in countries where cassava is processed and consumed. Cassava is a major staple crop in West Africa, however, the presence of anti-nutrients, namely cyanogenic glycosides (linamarin 95% and lotasutralin 5%) is an important factor that restricts the consumption of cassava. (McMahon, White et al. 1995, Cardoso, Mirione et al. 2005). Consumption of poorly processed cassava can lead to dietary exposure to cyanohydrin and cyanide (Montagnac, Davis et al. 2009). Dietary cyanide exposure from a monotonous cassava diet has been proposed as a contributing factor in two forms of nutritional neuropathies, tropical ataxic neuropathy and epidemic spastic paraparesis, konzo (Adamolekun 2011). Cassava tubers are processed through a variety of methods in order to reduce this risk(Nye 1991). Some of these methods require high temperatures and may affect the activity of the enzyme. Linamarase is quickly inactivated at high temperatures (Oke 1984, Montagnac, Davis et al. 2009). High Quality Cassava Flour is an emerging non-traditional use of cassava. It is produced without fermentation and relies heavily on grating, pressing and drying at higher temperatures above 65°C to lower the cyanide content of the processed food. Due to high processing temperatures this process may increase

the retention of cyanogens and cyanohydrins in the final product (Montagnac, Davis et al. 2009).

The thermal inactivation of hydrolases has been studied. Several studies have focused on the effects of thermal processing on the formation and thermal stability of the products of the enzymatic reaction (Munyaka, Verlinde et al. 2010, Wawire, Oey et al. 2012) whereas others have investigated the thermal stability of the enzymes specifically (Xinzhuo, Rui et al. 2014, Spelbrink, Lensing et al. 2015). Few studies have been conducted on linamarase and in contrast to other hydrolases; the thermostablity of linamarase at high temperatures has not been studied extensively. Only two studies investigated and modeled the thermal stability of linamarase at ambient temperatures 25-35°C (Ikya, Ariahu et al. 2013) and at 10-40°C (Nok and Ikediobi 1990). The objective of the present study was to investigate the effects of time and temperature on the thermal stability of linamarase at a temperature range of 35-75°C.

3.2 Materials and methods

3.2.1 Chemicals

p-nitropheynl B-D glucoside (PNPG) was purchased from Bioshop Canada, Burlington, Ontario. Ammonium sulphate and potassium acid phthalate, and sodium hydroxide were purchased from Sigma Aldrich, (Oakville, ON, Canada), and Fisher Scientific (New Jersey, USA) respectively. All mentioned chemicals were of analytical grade.

3.2.2 Sample collection and preparation

Cassava tubers were obtained from a local market. The root cortex was separated from the root parenchyma within 24 hours of purchase from the market. Others have shown that linamarase activity is highest in the root cortex (McMahon, White et al. 1995, Nambisan 2011). The root cortex was kept under ice until extraction.

3.2.3 Enzyme extraction

Linamarase was extracted according to Elias, Nambisan et al. (1997) with slight modifications. The root cortex of the cassava tuber was homogenized in pre-chilled 0.1 M phosphate buffer (pH 5.5). It was subsequently filtered through a muslin cloth. The collected homogenate was centrifuged at 8000 × g at 4°C for 45 min. The supernatant was utilized as a crude extract**.** The crude extract was partially purified after addition of ammonium sulphate to 60% saturation. The extract was stored over-night. Subsequently, the extract was centrifuged at 8000 g at 4°C for 45 min. The precipitate was collected and dissolved in phosphate buffer and stored frozen without adverse effects on activity.

3.2.4 Heat treatments

Aliquots of the homogenates were transferred to glass tubes using pipette and held on ice until heated. Samples were heated in a water bath to an indicated temperature (35, 45, 55, 65, and 75°C) for the specified times (10, 20, 30, and 40 min). The temperature of the water bath was verified with a liquid-in bulb thermometer. The come-up time (ie the time samples to set temperature) for the capillary tubes (diameter 1.5 cm) was determined by inserting a thermometer into the solution contained in the tube and recording the time it takes to reach the

water bath temperature. This time was observed to be less than 5 min. Following heating; samples were immediately cooled in iced water and stored on ice until assay. Activity was determined by measuring the difference in absorbance between a treated sample and its corresponding blank using a spectrophotometer at 400 nm (UV-VIS Spectrophotometer, UV1, Thermo Fisher Scientific, Waltham, MA, USA). One unit of enzyme activity is defined as the amount of enzyme that produced 0.001 change in absorbance at 400 nm/min under predetermined assay conditions The reaction mixture contained 0.8 ml of the PNPG in 50 ml distilled water, 1 ml of the homogenization phosphate buffer and 0.5 ml of the enzymatic extract. In the final reaction mixture, the substrate concentration was 20 mM of PNPG. Absorbance over time was recorded and plotted for each temperature and heating treatment combination. The enzyme's activity became stabilized after 20 minutes; the linear part of the graph after two minutes was used to calculate the slope, which was considered as the activity of the enzyme. Assays were performed in triplicates.

Modeling

The kinetics of enzymes can be described by a first order reaction

$$\frac{dA}{dt} = -kA \quad (1)$$

where, *A* is the enzyme activity and *k* is the rate constant. The slope obtained from the linear regression was considered as the rate constant of the reaction. All absorbance values were adjusted by adding the slope of the completely inactivated enzyme according to the following equation

$$A_{T=x} = A_0 + (-k_x t_x) + k_{75} t_x \quad (2)$$

where A is the absorbance at temperature (T) x and A_0 is the intercept at Tx -k_x is the slope at temperature x and t_x is the treatment time at temperature x and k_{75} is the correction factor obtained from the completely inactivated enzyme. Corrected data were fitted to a linear model using Microsoft Excel 2013 (Microsoft Corporation, 2013, Redmond WA, USA).

The temperature dependence of the rate constant k can be expressed by an activation energy, E_a, as given in the Arrhenius equation

$$A = A_0 e^{-\frac{E_a}{RT}} \quad (3)$$

where, R is the gas constant (8.314 J $mol^{-1}K^{-1}$) and T is the temperature in K. According to the Arrhenius equation, the logarithm of the observed rate constant is proportional to the reciprocal absolute temperature (Ludikhuyze, Van Loey et al. 2003). From the slopes of the corresponding logarithmic plots, the activation energy can be derived (Ludikhuyze, Van Loey et al. 2003). In some cases, enzyme fractions with different thermal stabilities can be present.

Data was analyzed and model parameters were estimated performing a linear regression procedure. All figures were drawn using Microsoft Excel 2013 (Microsoft Corporation, 2013, Redmond WA, USA).

Statistical analysis

A full factorial design was used to study the effects of temperature and treatment on the activity of the enzyme. The significance of the effects of temperature and treatment time on reaction rate was determined using the one-way ANOVA using SAS® statistical analysis software version 9.4. Mean comparison of each of the parameters levels and their significant differences were analyzed by Tukey's

honest significant difference (HSD) test is a single-step multiple comparison procedure that compares the means of every treatment to the means of every other treatment. The acceptable level of significance was set at $p< 0.05$.

3.3 Results and discussion

3.3.1. Effect of heating time on linamarase activity

Heating time is known to exert a significant effect on the activity of enzymes such as polyphenoloxidase (PPO) and peroxidase (POD) (Liing and Lund 1978, Fante, Scher et al. 2013, Liu, Niu et al. 2013, Lago and Norena 2014). In the case of tuber cortex linamarase from cassava, treatment time (10, 20, 30, 40 minutes) did not exert a statistically significant ($P>0.0001$) effect on enzyme activity. The results show that at a given temperature, linamarase activity was constant across all heating times. This suggests that linamarase is rather stable at the various temperatures used in this study (Yeoh 1989, Petruccioli, Brimer et al. 1999). Yeoh (1989) observed that root cortex linamarase was generally more thermostable than other isoforms of the enzyme present in the cassava tuber. Yeoh (1989) reported that the leaf and peel linamarase lost about 34 and 45% of their activity after 30 minutes at 55°C while the tuber cortex enzyme only suffered a 10% loss. After 30 minutes at 60°C, the peel and leaf enzyme lost about 80% of their activity and the tuber cortex enzyme lost only 30% and finally at 50°C for an hour, the leaf and peel enzyme lost 50 and 15% of their initial activity, respectively, where as the tuber cortex retain its full activity under these conditions. These variations may be attributable to differences in Km values and differences in the extent of glycosylation present in the enzyme isoforms (Elias,

Nambisan et al. 1997). Longer processing times allows for prolonged contact time between linamarase and the glycosides (Padmaja 1995) and thereby contributes to lower cyanide loads in processed cassava foodstuffs. This finding suggests that increases in processing time may not exert a negative effect on enzyme activity and the rate of cassava detoxification.

3.3.2 Effect of temperature on linamarase activity

Linamarase hydrolyzes toxic cyanogenic glycosides in cassava to yield acetone cyanohydrin. Linamarase is heat labile and this is a major concern for processing methods that require high temperatures. The thermostability of linamarase was investigated at various temperatures and heating conditions. Fig 3.1 shows the average change in absorbance of partially purified linamarase across all heating conditions, which decreases with increasing temperature. Temperature exerted a statistically significant effect on the activity of linamarase.

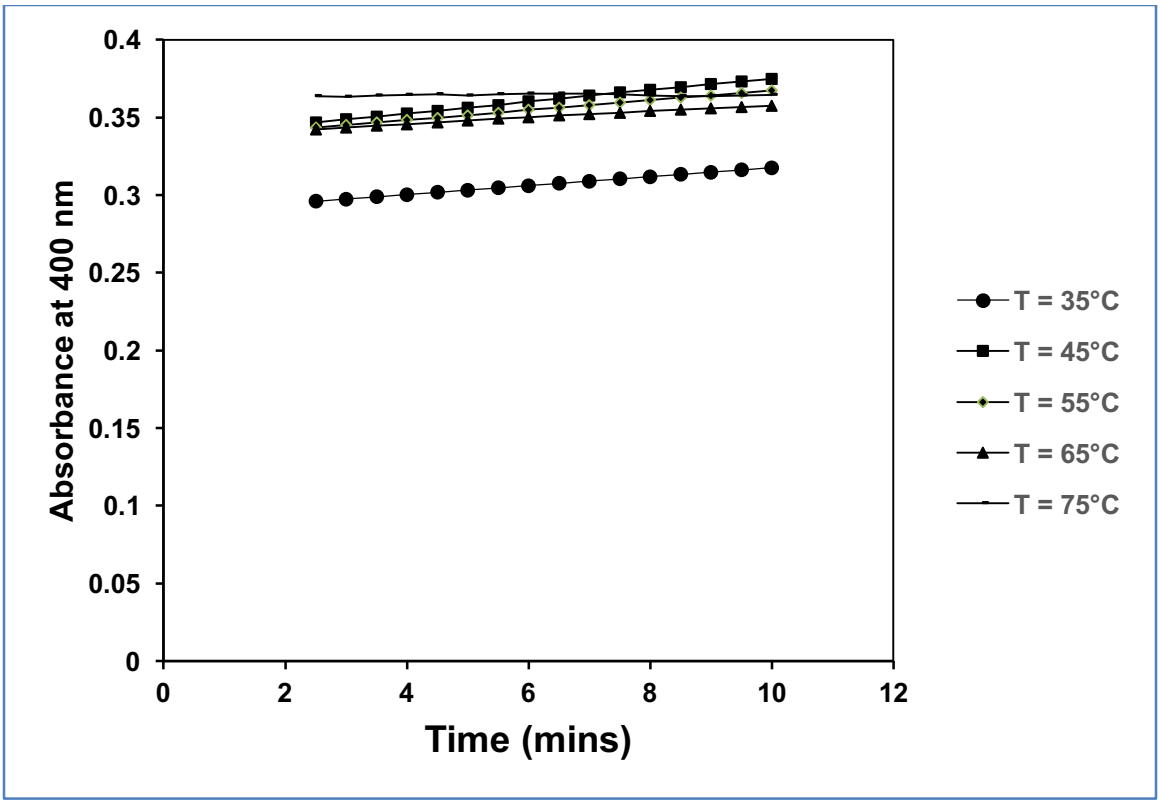

Figure 3. 1 Measured changes in absorbance of partially purified linamarase extracts heated at different temperatures

The enzyme exhibited highest activity at 45°C and the activity steadily decreased until it was completely inactivated at 75°C. There was a sharp drop in enzyme activity between 65°C and 75°C. The temperature optimum for linamarase was 45°C. This is consistent with literature, the reported optimal temperature range of linamarase is within 50-60°C (Yeoh 1989, Petruccioli, Brimer et al. 1999, Nambisan 2011). Additionally, the temperature optimum for a given enzyme is not a constant and can be affected by such factors as enzyme and substrate purity and the method used in measuring the rate of the catalyzed reaction (Sizer 2006). Petruccioli, Brimer et al. (1999) characterized the properties of two bacterial (PGI and PGII) linamarase and also reported a significant drop in

enzyme activity at 65°C and complete inactivation of both enzymes after 10 minutes at 75°C. Other authors have reported similar inactivation temperatures for other hydrolases. Wawire, Oey et al. (2012) investigated the effect of thermal treatments on folate stability in cowpeas. Long-chain folate poly-Ɣ-glutamates are hydrolyzed by the endogenous γ-glutamyl hydrolase to yield bioavailable short chain folate poly-Ɣ-glutamates. The authors reported high levels of short chain folate poly-Ɣ-glutamate at temperatures below 70°C and lower levels of short chain poly-Ɣ-glutamates beyond this critical point. This suggests that in samples subjected to temperatures higher than 70°C, the enzyme was unable to break down the long chain poly-Ɣ-glutamates. Spelbrink, Lensing et al. (2015) investigated the inactivation kinetics of patatin, a pototao lipase, in whey. Patatin was substantially inactivated at 74°C. Interestingly, negative activity were initially obtained at 75°C, which is unusual. However, the data was corrected as summarized in materials and methods and the negative energy observed was considered to be effectively null. These finding suggests that processing methods that require temperatures greater than 65°C might inactivate the enzyme. This would effectively reduce the hydrolysis of linamarin; processed foodstuffs would be low in cyanohydrins and free cyanide but would be high in linamarin. The production of HQCF requires high temperatures beyond the critical point of 75°C reported in this paper. Therefore processing temperatures might rapidly inactivate linamarase thereby increasing the cyanogen load in the final flour sample. Residual linamarin, can be broken down by gut micro-flora to yield acetone cyanohdyrin (Dorea 2004); pH>4, acetone cyanohydrin can

spontaneously breakdown into HCN (Montagnac, Davis et al. 2009). Dietary cyanide exposure from a monotonous cassava diet has been proposed as a contributing factor in two forms of nutritional neuropathies, tropical ataxic neuropathy and epidemic spastic paraparesis, konzo (Adamolekun 2011).

3.3.3 Temperature dependent kinetics

Figure. 3.2 shows the Arrhenius plot of linamarase activity versus heating temperatures. The plot depicts a non-linear relationship with 2 distinct phases.

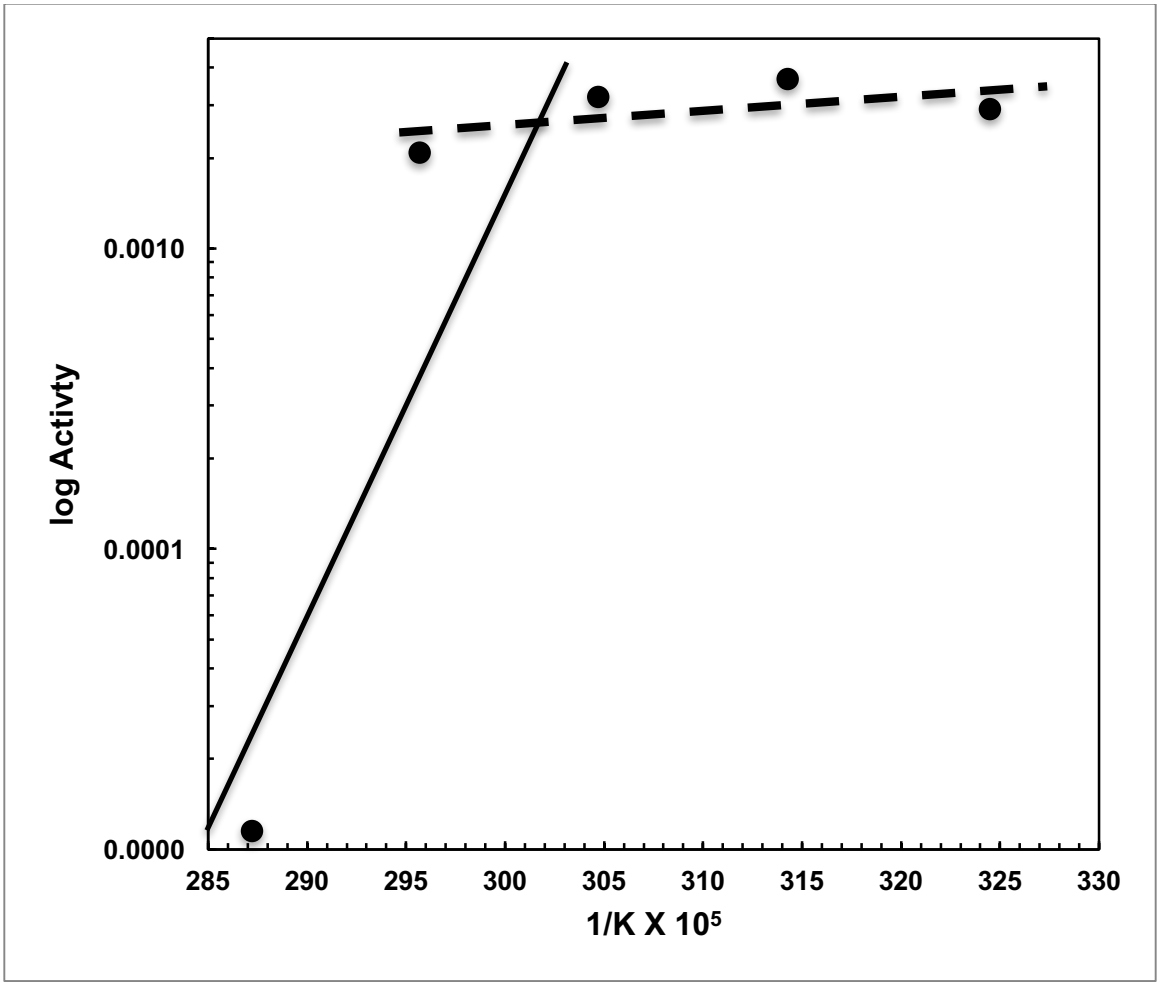

Figure 3.2 Biphasic Arrhenius plot for partially purified linamarase

Non-linear Arrhenius plots have been reported for many enzymes. This non-linearity can appear as a jump (Biosca, Travers et al. 1983), a break (Kumamoto, Raison et al. 1971), or a curvature (Morrison 1975).

The non-linearity indicates that the thermal stability of linamarase might be governed by two distinct temperature dependent mechanisms. The break in the Arrhenius plot for linamarase was at 55°C. There are two possible reasons for a non-linear Arrhenius plot (Massey, Curti et al. 1966, Biosca, Travers et al. 1983). It might be attributable to a change in the rate-limiting step of the enzymatic reaction. As the temperature varies, change in the rate-limiting step can occur without any changes in the conformation of the enzyme's active site (Poonkuzhali, Sathishkumar et al. 2011). The non-linearity of the Arrhenius may also be due to temperature-induced conformation change in a soluble enzyme or phase change in a membrane-associated enzyme(Poonkuzhali, Sathishkumar et al. 2011). The plot is linear from within the temperature range of 35-55°C [324.52 -304.74 1/L 10^5]. Other authors have previously reported similar results. For linamarase, Nok and Ikediobi (1990) investigated the temperature dependence of linamarase against PNPG at a temperature range of 10-40°C and obtained a linear Arrhenius plot. Ikya, Ariahu et al. (2013) also obtained a linear Arrhenius plot at a temperature range of 25-35°C, however the temperature range included in this study is narrow and makes it difficult to draw pertinent conclusion from the data. In Fig 3.2, there's a dip from 55-65°C [304.74-295.73 1/K 10^5] and within the temperature range of 55-75°C [304.74-287.23 1/K 10^5] the plot becomes non-linear. This finding suggests that at the higher temperature

investigated, the temperature dependence of linamarase is governed by a mechanism distinct from that at the 35-55°C. The activation energy at the temperature range of 35- 55°C is 1.87 kJ mol^{-1} and at the temperature range of 55-75°C, the E_a is much higher at 115.19 kJ mol^{-1}. Nok and Ikediobi (1990) isolated and purified two isoforms of linamarase (linamarase A and linamarase B) and described their temperature dependence at a temperature range of 10 – 40°C and determined the activation energies to be 49.04 kJ mol^{-1} and 36.48 kJ mol^{-1}, respectively. Ikya, Ariahu et al. (2013) verified the temperature dependency of bacterial linamarase at ambient temperature (25- 35°C). They reported activation energies in the range of 60.9 to 91.7 kJ mol^{-1}. The variation might be due in part to variations of factors such as enzyme and substrate purity, the presence of activators and inhibitors, and the method used in measuring the rate of the catalyzed reaction (Sizer 2006). The activation energy at the temperature range of 55-75°C is much larger thereby suggesting that the progression of the enzyme-catalyzed reaction is strongly temperature dependent at this range.

The experimental data present here suggests that linamarase is governed by two distinct temperature dependent mechanisms. These findings can be used to understand the effects of temperature on linamarase during the production of HQCF. Application of this model can provide some understanding on the effects of processing on linamarase activity and indicates critical temperature points, which offers the possibility for optimisation.

Dynamic optimization of thermal processing is one of the most important operations in food industry (Banga, Balsa-Canto et al. 2003). This method can be applied to mathematical models describing linamarase temperature dependency and can identify the heating temperature (as a time-dependent profile) that maximizes cyanogen removal from the processed cassava foodstuffs (Banga, Balsa-Canto et al. 2003). These optimization techniques can be implemented at low cost in small scale industries. Optimal operating policies, (also called optimal variable retort temperatures, VRT) are more cost efficient than traditional constant temperature processes. Optimal VRTs can improve final product quality and/or reduce the processing time while ensuring a desired quality level (Banga, Balsa-Canto et al. 2003).

3.5 Conclusion

Linamarase is generally thermostable within the temperature range 35-55°C. However, it exhibited strong temperature dependency within the temperature range of 55-75°C. The enzyme's activity was greatly reduced at 65°C and was completely inactivated at 75°C.The Arrhenius plot was non-linear with curvature. This suggests that linamarase activity may be governed by different temperature dependent mechanisms at the temperature range used in this study. The temperature optimum was at 45°C. The activation energy (Ea) at 35-55°C and 55-75°C is 1.87 kJ mol^{-1} and 115.19 kJ mol^{-1}, respectively.

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CHAPTER 4: SUMMARY AND CONCLUSION

This thesis investigated the thermostability of linamarase from cassava at a temperature range of 35-75°C. In the first part of this work, the underpinnings of cassava production and processing were carefully discussed. Cassava contains varying levels of harmful cyanogenic glycosides which when hydrolyzed by the endogenous enzyme linamarase yields free HCN and acetone cyanohydrin (which can spontaneously break down to free HCN at pH above 4). Careful processing is necessary to make cassava palatable and safe for human consumption. Cassava is primarily detoxified through the action of linamarase, however the enzyme is heat labile and is quickly inactivated at temperatures beyond 55°C (Ikediobi and Onyike 1982, Yeoh 1989, Nok and Ikediobi 1990, Petruccioli, Brimer et al. 1999).

Chapters 1 and 2 explored cassava production and use in West Africa and discussed previous work on the thermostablity of linamarase. This work and other studies have shown that linamarase is thermostable at a temperature range of 10-40°C (Nok and Ikediobi 1990, Ikya, Ariahu et al. 2013) and begins to lose activity beyond 65°C (Yeoh 1989, Petruccioli, Brimer et al. 1999). Nok and Ikediobi (1990) isolated and purified two isoforms linamarase and described its temperature dependence at 10-40°C according to the Arrhenius equation. They obtained a linear plot for both isoforms and obtained activation energies of 49.04 kJ mol^{-1} and 36.48 kJ mol^{-1}. Results from Yeoh (1989) indicate that linamarase exhibited similar activation energy with respect to linamarin: 12.55, 16.74, and 14.64 kJ mol^{-1} respectively and 27.20 and 24.27 kJ mol^{-1} against the analogue p-

nitrophenyl-β-D-glucoside. The magnitude of the reported activation energy indicates that linamarase is thermostable at this temperature range. Petruccioli, Brimer et al. (1999) produced and characterized two β-glucosidase produced by *Pencillium aurantiogriseum* (P35). They investigated the temperature dependency of the enzyme at a temperature range of 25-70°C. The enzymes had a temperature optimum of 55°C and 60°C. Both enzymes maintained their activities from 25-60°C and were completely inactivated after 10 minutes at 75°C. The authors did not model the temperature dependency of the bacterial linamarases according to the Arrhenius equation. Ikya, Ariahu et al. (2013) verified the temperature dependency of linamarase at ambient temperature (25-35°C). They reported activation energies of ranging from 60.9 to 91.7 kJ mol^{-1} for recombinant linamarase. In contrast to work by previous authors this work investigated and modeled the thermal stability of linamarase at higher termperatures and inactivated the enzyme at the highest experimental temperature. All studies reviewed, extracted linamarase form the root cortex, for future studies it would be interesting to study the thermostability of linamarase isoform localized in the root parenchyma which is generally less thermostable (Yeoh 1989) but is the isoform most likely to be present processed cassava foodstuffs.

In this work and previous work by Nok and Ikediobi (1990) and Ikya, Ariahu et al. (2013), the Arrhenius plot was linear at 35-55°C. However in this work, the Arrhenius plot was non-linear after 55°C and exhibited smooth curvature. This finding suggests that the thermostability of linamarase is governed by two distinct

temperature dependent mechanisms. The activation energy (E_a) at 35-55°C and 55-75°C were 1.87 kJ mol^{-1} and 115.19 kJ mol^{-1} respectively. The E_a at 35-55°C is much lower than values previously reported, however these discrepancies may be due in part to the cassava variety and the purity and of the enzyme and substrate and the method used to measure the rates of the catalyzed reactions. The activation energy at the temperature range of 55-75°C suggests that the thermostability and potential inactivation of linamarase at high temperatures is governed by a strongly temperature dependent mechanism.

The results of this work are a stepping-stone to further elucidating the inactivation kinetics of linamarase. Application of this model can provide some understanding on the effects of processing on linamarase activity and indicates critical temperature points, which offers the possibility for optimisation of the HQCF production process.