COMPARATIVE ANALYSIS OF RHODANESE IN RIPE & UNRIPE PAWPAW SEED AND MESOCARP (*Carica papaya*)

BY

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PSB/14/2349

A FINAL YEAR PROJECT SUBMITTED TO THE DEPARTMENT OF PLANT SCIENCE AND BIOTECHNOLOGY, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF BACHELOR OF SCIENCE (B.Sc.) DEGREE IN PLANT SCIENCE AND BIOTECHNOLOGY

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MARCH 2019

CERTIFICATION

This is to certify that this research project was carried out by Abiodun Barnabas Abidemi with the Matriculation Number PSB/14/2349 under my supervision in the Department of Plant science and Biotechnology, Faculty of science, Federal University Oye-Ekiti, Ekiti State, Nigeria.

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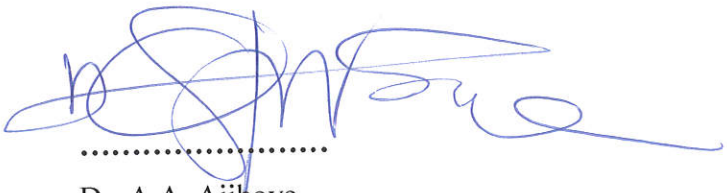

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DEDICATION

This research is dedicated to Almighty God whose word is true and his works are genuine; he only gave me the strength wisdom and enablement to Alpha and Omega this Project. May his Holy name be praised for ever till eternity.

ACKNOWLEDGEMENTS

My appreciation goes to God almighty the beginning and the end of everything for his provision and guidance from the beginning of this project to the very end of completing this project work.

I will like to appreciate my Parents (Dr. and Mrs. Abiodun) for their support, care, love and prayers from the very beginning till this moment and even beyond, I can't forget my siblings too Nathaniel, Priscilla and Peter; you guys have been so wonderful may God continue to bless you all Amen.

I would like to express my profound gratitude to those who facilitated the successful completion of this research work. My special appreciation goes to my wonderful supervisor, my Amiable HOD Dr. Abiodun Ajiboye whose contribution and fatherly love helped immensely to complete this work. Special thanks to Dr. Raphael Okonji, Mr. Seun, Miss Priscilla and Mr. Shola for their relentless effort in contributing to the starting and completion of this project work may God bless you all.

Lastly, I would like to appreciate my colleagues, my friends and my well wishers thank you and God bless you all. How can I forget to acknowledge my hostel brothers and sisters and my landlord (Mr.Olushola Gbenga) who have impacted my life in one way or the other, starting from my beautiful and prudent school daughter Kolawole Mariam, (Ghajini) man like Saucey, Oreoluwa a.k.a Horezy, Blessing, Bisi, Fresh, Young John, Emmanuel-De'Bruyne, AB, sir-T nana, Stephen, Debo, Dunni, Yemisi, John, AY, Toyin, Gybran, Faith funds, Samuel a.k.a Samu Alajo Mr. Shanga, Henry a.k.a Investor H, Sukanmi a.k.a S2dK, Vicky Victoria, Lekan a.k.a Oga lexy, Sir d.k, Jay-Jay, Feyishayor, Jumoke, Gbemisola, Oyinda, Special thanks to Baba and Mama Rasaq, Rosco, Effizy, Luke, Edwin moore, Juwon a.k.a Harrykane, Skrtel, Barry and 1st Ecwa Oye-Ekiti. I love you all guys you are the best…..

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ABSTRACT

Rhodanese was isolated from the mesocarp and seed of ripe and unripe pawpaw *Carica papaya*. Rhodanese is an enzyme which primary function is the protection of the electron transport system from the deleterious effect of cyanide by catalyzing the conversion of the cyanide to thiocyanate, a less toxic compound. This project was aimed at analyzing the activity of rhodanese in ripe and unripe paw-paw seed and mesocarp.

The pawpaw was peeled; the mesocarp and seed were homogenized then sieved. The presence of this enzyme was now tested by putting 0.1ml of the crude sample and 0.5ml of Bradford reagent in a test tube and the readings were taken with the spectrophotometer. The presence of rhodanese was confirmed and then its activities were tested for under different pH, temperature and different sulphur donating compounds like, Mercaptoethanol, $Na_2S_2O_3$, Sodium metabisulphite, Ammonium sulphate, $(NH_4)_2SO_4$. At temp 70°C the ripe paw-paw mesocarp had its optimum activity and had its lowest activity at 100°C while the unripe pawpaw mesocarp had its optimum activity at temp 50°c and its lowest activity at 100°C. The ripe paw-paw seed had its optimum activity at 40°C and it's lowest at 90°C and 100°C while the seed had its optimum activity at temp 60°c and lowest at100°C. Under different pH the optimum activity for the ripe paw-paw mesocarp was at pH6 and no activity at pH9 and pH10 and the unripe pawpaw mesocarp had its own optimum activity at pH6 and showed no activity at pH8, pH9 and pH10. The ripe pawpaw seed it had its optimum activity at pH7 and it's lowest at pH4 while the unripe paw-paw seed had its optimum activity at pH6 and its lowest at pH4.

This study confirmed the presence of rhodanese in the seed and mesocarp of ripe and unripe pawpaw, therefore the consumption of pawpaw can help to reduce the toxicity of cyanide to a less toxic thiocyanate.

Paw-paw was confirmed to be a good source of rhodanese and its consumption can be advised and as it has been known to be a source of rhodanese, it can serve as a supply of rhodanese to the body.

CHAPTER ONE

1.0 INTRODUCTION

Papaya (*Carica papaya* L.), a delicious fruit, belongs to the family Caricaceae. Papaya is sometimes referred to as pawpaw. It is native to tropical America, but it is grown in almost all tropical and subtropical regions of the world.

Papaya is not a tree but an herbaceous succulent plant that possess self-supporting stems (Dick,2003). Papaya is a large perennial herb with a rapid growth rate, the plants are usually short-lived, but can produce fruit for more than 20years. The papaya has a rather complicated means of reproduction. The plants are male, hermaphrodite, or female (Bruce and Peter, 2008).The male trees are uncommon, but sometimes occur when homeowners collect their own seeds. Hermaphrodite trees (flowers with male and female parts) are the commercial standard, producing a pear shaped fruit. These plants are self-pollinated (Jari, 2009).

Carica papaya, commonly known as the paw paw tree is one of the most popular medicinal plants in West and Central Africa (Bouquet, 1969).

Carica papaya plants produce natural compounds (Acetogenins) in leaf bark and twig tissues that possess both highly anti-tumor and pesticidal properties. It was suggested that a potentially lucrative industry based simply on production of plant biomass could develop for production of anti-cancer drugs, pending Food and Drug Agency approval, and natural (botanical) pesticides (Mc Langhlin, 1992). The high level of natural self-defense compounds in the tree makes it highly resistant to insect and disease infestation (Peter, 1991). *Carica papaya* L. leaf tea or extract has a reputation as a tumor-destroying agent. (Walter, 2008) The papaya fruit, as well as all other parts of the plant, contain a

milky juice in which an active principle known as papain is present. Aside from its value as a remedy in dyspepsia and kindred ailments, it has been utilized for the clarification of beer. The juice has been in use on meat to make it tender, (Wilson, 1994). The seed is used for intestinal worms when chewed. The root is chewed and the juice swallowed for cough, bronchitis, and other respiratory diseases. The unripe fruit is used as a remedy for ulcer and impotence, (Elizabeth, 1994). Fresh, green pawpaw leaf is an antiseptic, whilst the brown, dried pawpaw leaf is the best as a tonic and blood purifier. (Atta, 1999). Chewing the seeds of ripe pawpaw fruit also helps to clear nasal congestion, (Elizabeth, 1994). Papaya is considered to be a rich source of provitamin and ascorbic acid (Wenkam and Miller, 1995).

The ripe fruit is consumed fresh for desert and in fruit salad or processed. It is highly accepted worldwide and the demand for fresh papaya fruit is increasing for its high content of vitamin C and provitamin A, which has protective effect against cancer, and its low-calorie status that is recommended for low hypo caloric diets (Lobo and Cano, 1998).

Various part of this plant has been known to have a lot of medicinal properties, in West and Central Africa, the fruits extracts are used by traditional healers for the treatment of hypertension and for the prevention of miscarriages in women (personal communication) (Kamanyi and Eta, 1992),

Carica papaya contains many biologically active compounds which includes Alkaloids, Proteins Fatty oils, glycosides and the presence of Enzymes like Arginase and Rhodanese have been found present in them too (Wilson *et al.*, 2007).

The physiological role of rhodanese (cyanide: thiosulphate sulphur transferase) in animal tissues and perhaps in plants is controversial; particularly its function in the detoxification of acute cyanide exposure (Delvin *et al.*, 1989).

It is generally believed that the major function of rhodanese is cyanide detoxification (Smith and Urbanska, 1986). This function is more prominent in mammals where highly cytotoxic cyanide is converted to a less toxic thiosulphate and excreted through the kidney (Cagianut *et al.*, 1984; Bourdoux *et al.*, 1980).

Cyanide is cytotoxic and kills the cell by inhibiting the mitochondrial electron transport chain enzyme, cytochrome oxidase; it is, thus, an inhibitor of cellular respiration (Ohlen *et al.*, 2016). They are found in certain seeds and fruits like apple, mango, peach, and bitter almonds. In plants, cyanides are usually bound to sugar molecules in the form of cyanogenic glycosides and help to defend the plant against herbivores (Vetter, 2009). Through cytochrome oxidase inhibition, cyanide adversely affects higher plants. Certain host plants also produce cyanogenic compounds in the form of cyanogenic glycosides as Chemical defenses, hence exposing herbivorous insects that feed on such plant to cyanide (Gleadow and Møller, 2014). Cyanide is released to the environment through death and decomposition of plants, but the rate of such production is unknown. With cyanogenic plants, various animals and wildlife are at risk, especially species that feed on such plants and an efficient means of detoxifying cyanide is important to the survival of such animals (Wybouw *et al.*, 2014).

Overtime, it has been reported that exposure to cyanide ions increases the overall mortality of organisms involved (Eisler, 1991), as many industrial products and naturally occurring substances contain cyanide (Egekeza and Oehme, 1980). Social insects, such as

the soldier termites, feed off grasses, decaying woods and plant debris which contain cyanogenic glycosides, leading to the release of cyanide upon ingestion (Gerozisis and Hadlington, 2001). The death of grazing animals can result from the ingestion of such cyanogenic glycosides naturally present in forage crops (Keeler *et al.*, 1978). Similarly, the death of birds can also result from cyanide poisoning which may be through cyanide salts exposure or cyanogenic plants ingestion (Wiemeyer *et al.*, 1986). Aquatic lives are also not left out, as exposure to cyanide ions can cause stress and significantly increase the metabolic load on the aquatic organisms (Eisler, 1991).

Different species of bacteria, fungi, algae and higher plants may serve as natural sources of cyanide if they produce and excrete cyanide (Way, 1984). Plants are exposed to cyanide from soil contaminated with various industrial wastes (Henny *et al.*, 1994). Also, when 1-amino-cyclopropane-1-carboxylic acid is converted to ethylene, cyanide is produced in equimolar quantities as ethylene, thus serving as an endogenous source of cyanide in plants and such cyanide level increases drastically during ripening of fruit and senescence (Yip and Yang, 1988).

Cyanide is toxic to all living things; it reduces ferric cytochrome oxidase (an iron-containing metalloprotein) to ferrous cytochrome oxidase which transfers electron to oxygen (Jeong *et al.*, 2005).

Rhodanese also catalyses the formation of iron-sulphur centers in Escherichia coli, and a physiological role of the enzyme in aerobic metabolism in this organism was suggested (Keith and Volini, 1987).

Many plants and plant products used as food in tropical countries contains cyanogenic glycosides, these plants include cassava, linseed, beans and peas, which are known to

contain linamarin coexisting with lotaustralin. Millet, sorghum, tropical grass and maize are sources of dhurin. Amygdalin is found in plums, cherries, pears, apple and apricots. These compounds are also present in plants such as rice, unripe sugar cane, several species of nuts and certain species of yam (Osuntokun, 1981; Oke, 1979). Most of these plants and their products are staple foods in the tropics. Upon hydrolysis these compounds yield cyanide, a sugar and a ketone or aldehyde. Cyanide is a potent cytotoxic agent that kills the cell by inhibiting cytochrome oxidase of the mitochondrial electron transport chain. When ingested, cyanide activates the body own mechanisms of detoxification, resulting in the transformation of cyanide into a less toxic compound, thiocyanate.

In plants, a close relationship exists between rhodanese activity and cyanogenesis, which suggest that the enzyme provides a mechanism for cyanide detoxification in cyanogenic plants (Smith and Urbanska, 1986).

The capacity of *Bacillus stearothermophilus to* detoxify cyanide could be greatly increased when mutants containing 5 to 6 times rhodanese activity of normal cells were used (Atkinson, 1971).

The distribution of rhodanese in both adult and larvae insects is not restricted to those species that encounter exogenous cyanide through feeding on cyanogenic plants (Beesley *et al.,* 1985). This is an indication that cyanide detoxification may not be the primary role of this enzyme in insects. In insects, it was proposed that the enzyme might be involved in a more important role of sulphur transfer for protein synthesis.

In *Rhodopseudomonas spheroids* rhodanese catalyze the formation of cysteine from cysteine trisulphide (Dexifra *et al.*, 1975).

Rhodanese in its phosphorylated and dephosphorylated forms has been reported to function as a converter enzyme that interact with mitochondrial membrane bound iron–sulphur centers of the mitochondrial electron transport chain where it modulate the rate of respiration (Ogata and Volina,1990). There is an indication of a possible role of rhodanese in providing labile sulphide necessary for the synthesis of ferredoxin in the chloroplast of spinach, parsley, cabbage, and red turnips (Tomati, 1972). It also catalyses the formation of iron-sulphur centers in *Escherichia coli*, and a physiological role of the enzyme in aerobic metabolism in this organism was suggested (Keith and Volina, 1987). Rhodanese was also reported to reconstitute spinach ferredoxin (Pagani *et al.*, 1984); restore durum wheat leaves cyanide inactivated NADH: nitrate reductase activity and if added before cyanide treatment, it protects the enzyme (Tomati et al., 1976). It also restore, partially, the activity of NADH dehydogenase (Pagani and Galante, 1983). It was also fund to increase the activity of malate dehydrogenase (Agro et al., 1976). Restoration of MgATP and chelator inactivated nitrogenase of *Klebsiella pneumoniae* has been reported (Pagani *et al.*, 1987). Possible role of the enzyme in modulating S-amino levulinate synthetase activity has also been reported (Vazquez et al., 1987).

In *Thiobacillus intermedius,* the mechanism of oxidation of thiosulphate to sulphate seems to involve the action of rhodanese (Charles, 1969). At pH 8.8 beef liver rhodanese however catalyses the reduction of thiosulphate to sulphite (Koj, 1968). Its activity was also related to the oxidation of thiosulphate and elemental sulphur to sulphate by the fungus, *Rhizopus oryzae* (Ray *et al.*, 1990).

1.1 STATEMENT OF PROBLEM

Plants are essential for the sustainability of life. They are important to both man, animals and the environment. Plants provide for the essential needs of man such as food, shelter etc.

Fruit crops like paw-paw etc. serve as food, medicine and source of income for man.

Despite how inevitable its importance is, Cyanide tends to make this plants that serve as a source of food poisonous for man and Animal consumption as they produce Cyanogenic glycosides which affects the cells by inhibiting the mitochondrial electron transport chain enzyme. (Gleadow and Møller, 2014)

Hence, Rhodanese is an enzyme that detoxifies the cyanide in plants to form thiocyanate a compound that is less toxic to the cells of man and Animals.

1.2 AIM OF STUDY

The aim of this project work was to study, analyze and make comparison between the rhodanese content and activity in ripe and unripe *carica papaya* (paw-paw).

1.3 OBJECTIVES OF STUDY

- To test for the presence of Rhodanese in ripe and unripe paw-paw (seed and mesocarp).
- To determine the activity of Rhodanese under different temperature and pH.
- To compare the activity of Rhodanese in the seed and mesocarp of ripe and unripe pawpaw

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 ORIGIN, DISTRIBUTION, CLASSIFICATION, PRODUCTION

2.1.1 ORIGIN

Papaya (*Carica papaya* L) is native to Central America and Southern Mexico through the Andes of South America (Samson, 1986). It falls under the family Caricaceae with related species as *Carica stipulata*, *Carica pentagona* and *Carica pubescens* (Samson, 1986).This family is made up of 31 species in the four genera: three genera from America (*Carica, Jacaritia and Jarilla*) and one from equatorial Africa (*Cylicomorpha)*. Paw-paw is the common name in Nigeria, and Nigeria is among the highest producers of paw-paw, producing a respectable 12% of the total world population (FAO). Papaya is an economically important fruit crop in Hawaii, Australia, India, Phiilipines and South-east Asia including Africa. Pawpaw, papayer (French), melonenbaum (German), lechosa (Spanish), mamao, mamaociro (Portuguese), mugua (Chinese) and malakol (Thailand), Paw-paw (Nigeria). In Nigeria different names by different tribes are given to this fruit Yoruba (Ibepe), Igbo (Okworo-gbogbo), Hausa(Gwanda).

2.1.2 HISTORICAL DISTRIBUTION

The papaya spread from the point of origin to the south by the Indians, and throughout the Caribbean with Spanish exploration. The Spanish also carried it to Europe and the pacific Islands. Papaya was introduced into Hawaiian in the 1800s and Hawaii remains the only state in the USA to produce papaya fruit commercially (Pope, 1930). A small industry developed in Florida in the first part of the 20th century but declined rapidly with the infection of viral diseases (Maxwell *et al.*, 1984).

Historically, in the 15th century, the seeds of papaya were transported to West Indies, Philippines, Africa and the Indo-Pak subcontinent before the 17th century (Reid, 1990).

2.1.3 CLASSIFICATION

Kingdom:	Plantae
Subkingdom:	Tracheobionta
Superdivision:	Spermatophyta
Division:	Magnoliophyta
Class:	Magnoliopsida
Subclass:	Dilleniidae
Order:	Violales
Family:	Caricaceae
Genus:	*Carica* L.
Species:	*Carica papaya* L.

2.1.4 PRODUCTION

Carica papaya has exacting climate for vigorous growth and production. It must have warmth throughout the year and will be damaged by frost (Maxwell and Betty, 1984). Brief exposure to 0^{o}c is damaging and prolonged cold without overhead sprinkling will kill the plant. Papaya can tolerate moderate wind if well rooted. Cool temperatures will also alter fruit flavor (Jones *et al.*, 1939). The plant will continue to bear fruits for many years, but yield usually declines as the tree ages, and picking becomes difficult.

In commercial production, fields are usually replanted or abandoned after four to five years.

Papaya makes excellent container and specimen where soil moisture and temperature can be moderate (Samson, 1986). It is cultivated in India, Peru, china, Indonesia, Venezuela, Jamaica,

Brazil and many African countries (FAO, 1984).In the United States, Hawaii is the major producer and supplier of the fruit, with 2500 acres in 1989. It is also estimated that there were 350 acres of papaya grown in Dade County, Florida in 1987-1988 (Statistics of Hawaiian Agriculture, 1991). The United States also imports papaya from foreign countries. Mexico was by far the largest supplier, with 76%of the total. Other major suppliers were the Bahamas with 9.7% and the Dominican Republic, 7.5% (Statistics of Hawaiian Agriculture, 1991).

2.2.0 DESCRIPTION OF PAWPAW (*Carica papaya*)

C. papaya is a perennial, herbaceous plant, with copious milky latex reaching to 20-30ft. (6-10 m) tall, the stem cylindrical to 10 in. (25 cm), and thick, hollow, usually unbranched above the middle and roughened leaf scars. Leaves, clustered around the apex of trunk and branches, have nearly cylindrical stalks, hollow, green, purple-streaked or deep-purple, 10 to 40 in. (25 to 100 cm) long; the leaf blade has 7 to 11 main and some secondary, irregular, pointed lobes and prominent veins; leaf surface is yellow-green to dark-green above, paler beneath.. Flowers emerge singly or in clusters from the main stem among the lower leaves, the female short-stalked, and the male with drooping pedicles 10-40 in. (25-100 cm) long. Corolla is ½ to 1 in. (1.25 - 2.5 cm) long with 5 oblong, recurred white petals. Fruit is extremely variable in shape and size according to variety; it may be nearly round, pear-shaped, oval or oblong; that of wild plants may be as small as a hen's egg. The fruits skin is smooth, relatively thin, deep yellow to orange or salmon-red, sweet and more less musky. The central cavity of the fruit is lined with a dryish, pulpy membrane to which adhere with numerous black, rough, peppery seeds, each with a glistening, transparent, gelatinous coating.

Plate1: matured pawpaw tree with fruits and the leaves

Plate 2a and 2b: Ripe pawpaw fruit with developed seed and mesocarp and unripe pawpaw fruit with undeveloped seed and mesocarp.

2.2.1 NUTRITION

NUTRITIONAL QUALITIES

Carica papaya is considered to be a rich source of provitamin and ascorbic acid (Wenkam and Miller, 1995) and the vitamin is generally associated with carotene. The composition and food value of ripe papaya fruit per 100g of edible flesh is given as 88% moisture, carbohydrates 10%, protein 0.5%, fat 0.1%, acid 0.1%, fibre 0.7%, and ash 0.6%. It also has a calorific value of 40 (Purseglove 1969).

2.2.2 GROWTH HABIT

Papaya is a short-lived perennial fruit crop growing to about 9.14meters high. It is generally unbranched but will branch only when injured (Chia *et al.*, 1989). The deep purple trunk is straight and cylindrical with prominent leaf scars. Its diameter may be from 5.1-7.5 cm to cover a foot at the base (Samson, 1986).

2.2.3 FOLIAGE

The leaves emerge directly from the upper part of the stem in a spiral on nearly horizontal petioles 30-65 cm long. The blade, deeply divided into 5-9 main segments varies from 30-60cm in width and has prominent yellowish ribs and veins. The lifespan of a leaf is 4-6weeks. They also contain papain (Norman, 1976). Chia *et al.* (1989) reported the deeply lobed, palmate leaves are borne on long hollow petioles emerging from the stem apex. Older leaves die and fall as the tree grows.

2.2.4 FLOWERS

Papaya plants are dioecious or hermaphroditic with cultivars producing only female or bisexual flowers preferred in cultivation. Papayas are sometimes said to be trioecious (Norman, 1976). Female and bisexual flowers are waxy, ivory white and borne on short peduncles in leaf axils along the main stem. Each flower contains many ovaries which explain why a single flower can produce multiple fruits (Samson, 1986)

A male papaya is distinguished by the small flowers borne on long stalks while female flowers are pear-shaped when opened, and are also distinguished from bisexual flowers which are cylindrical. Bisexual flowers plants are self-pollinating, but female plants must be cross pollinated by either bisexual or male plant (Nakosone, 1986).

2.2.5 CULTIVATION

Papaya is one of the few rapidly growing and heavily yielding fruit trees. It comes into bearing within a year of planting in the peninsular region and in about a year and a half under North Indian conditions. In the peninsular region it bears fruits nearly throughout the year and in North India it fruits for about 4 months. Area under papaya cultivation all over India is approximately 32,584 thousand hectares with an approximate production of 275,706 thousand tones and a yield of 8,461 kg/hectare (The Wealth of India, 1988). The extensive adaptation of this plant and wide acceptance of the fruit offer considerable promise for papaya as a commercial crop for local and export purpose. Like banana, pineapple and mango, papaya is one of the important cash crops in the tropics and subtropics.

2.2.6 USES

Carica papaya is an important fruit throughout the tropical and sub-tropical countries (Salunkhe and Desai, 1984). At the unripe stage, the fruit is consumed as a cooked vegetable in countries where papaya is widely grown (Mendoza, 2007, Mano *et al.*, 2009).

In west and central Africa, the fruit extracts are used by traditional healers for the treatment of hypertension and for the prevention of miscarriages in women, and it's known to produce uterine relaxation in the rat (Kamanyi and Eta, 1992). In the northern Nigeria, a cold water decoction of the ripe fruit is used to control and calm mentally agitated individuals (Gupta *et al,* 1990).

In Thailand, unripe fruits are used as ingredients in papaya salads and cooked dishes (Sone *et al.*, 1998,). In Puerto Rico, unripe fruits are canned in sugar syrup and sold either in local markets or exported (Morton, 1987). Unripe fruits must be cooked prior to consumption to denature the papain in the latex (Odu *et al.*, 2006; Morton, 1987).

The unripe fruit is used as a remedy for ulcer and impotence, (Elizabeth, 1994). Fresh, green pawpaw leaf is an antiseptic, whilst the brown, dried pawpaw leaf is the best as a tonic and blood purifier. (Atta, 1999). Chewing the seeds of ripe pawpaw fruit also helps to clear nasal congestion, (Elizabeth, 1994). The green unripe pawpaw has a therapeutic value due to its antiseptic quality. It cleans the intestines from bacteria, more so that (only a healthy intestine is able to absorb vitamin and minerals, especially vitamin B12). The tea, prepared with the green papaya leaf, promotes digestion and aids the in treatment of ailments such as chronic indigestion, overweight and obesity, arteriosclerosis, high blood pressure and weakening of the heart (Mantok, 2005).

Papaya is considered to be a rich source of provitamin and ascorbic acid (Wenkam and Miller, 1995).

In addition, papaya fruit is a good source of papain and chymopapain. Both are digestive proteolytic enzymes that digest protein and used as meat tenderizers, as digestive medicine in pharmaceutical, brewing, and tanning industries, and in manufacture of chewing gum (Nakasone and Paul, 1998).

Papaya seeds are sometimes used to adulterate whole black pepper (Morton, 1987).

Papaya puree is also prepared from fully ripe peeled fruits with the seeds removed. Papaya flesh is pulped, passed through a sieve and thermally treated. The puree is an important immediate product in the manufacture of several products such as beverages, ice cream, jam and jelly (Brekke *et al.*; 1972, Ahmed et al., 2002).Powdered or dried papaya can be used as a meat tenderizer or as an ingredient in soup mixes (Singfield, 1998). Papaya pomace, skins, leaves and other by-products of papaya processing may find use in animal feed application (Babu *et al.*; 2003).

2.2.7 Pest and Diseases

The disease of *Carica papaya* includes those that are caused by virus, fungi and nematodes.

2.2.7.1 Damping-off (*Phytium ultimum*)

It is caused by fungi that are present in the soil. This fungus is favoured by high temperature, wet weather and wet soil, wet drainage deep sowing, poor soil aeration, high

nitrogen in the soil and sunshine shortage (Hines *et al.*, 1965). Infected seedlings will wilt, fall and then die (Paull, 1998).

Damping-off can be controlled by using a non-used land (virgin land) or using sterilized soil with steam at 32.3^0C for 30minutes and also protect the plastic films from rain water (Hines *et al.*, 1965) the land can also be treated with 35% Etridiazole to improve the growth of seedlings. (Hines *et al.*, 1965).

2.2.7.2 The Papaya Ring spot Virus

This is one of the most severe papaya diseases and is often the limiting factor in papaya production throughout the world (Nakasone and Paull, 1998). It induces veins- banding mottling and yellowing spots or discoloration of leaf, water soaking streaks on the petioles, and ring spots appear on fruit or even on leaves (Nakasone and Paull, 1998). It stunts the plant and drastically reduces the size of fruits, sugar content, and taste. Some infected plants will bear fruits or production will decline. It spreads very fast and has become the limiting factor in papaya production throughout the world (Nakasone and Paull, 1998). It is mostly transmitted by aphids (Chay-Prove *et al.*, 2000).

It can be controlled by selection and growing tolerant varieties, transplanting at a time when there are relatively few winged aphids around, immediately eradicate and bury the whole infected plant once found, also practice cross protection with specific mild strains, to control aphids on the farm (Nakasone and Paull, 1998).

2.2.7.3 Phytophthora fruit rot (*Phytophthora palmivaro*)

This occurs in hot and humid seasons especially after typhoon attack. It causes root rot on young and adult plants and finally wilts or dies (Ko, 1982). Also it may cause lesions and white mould appears on the fruit and then fruit drops. Phytophthora fruit rot can be controlled by crop rotation, selection of well drain soils, avoid harming root or rogue and deeply bury the diseased fruits (Paull, 1998).

2.2.7.4 Rhizopus Fruit Rot (*Rhizopus stolonifer*)

The fungus invades fruit that are injured only. It causes soft rot and produces masses of visible black sporangia; leakage of cell fluid from the rotting will also occur (Nashijima *et al.*, 1990). It can be treated by heat treatment to kill pathogens and also remove and destroy the rotting fruits in packing sheds (Nishijima, 1990).This means that care must be taken when packaging, transporting and storing our fruits so as to avoid injury.

2.2.7.5 Aphids (*Aphids spp*)

They suck young leaves which become curled and crinkled and even defoliate, especially at seedling stage. Some aphids also transmit the virus diseases (Nakasone and Paull, 1998).

2.2.7.6 Other Insects

Other insects include scales, thrips, beetles, leafhoppers, moths, mealy bugs, white fly, and stink bug (Hunter and Buddenhagen, 1972). These are minor insects, but may occasionally cause certain damage to papaya. These can be controlled by keeping the plantation relatively free of weeds, select the proper insecticides to control the outbreak and harvest all fruits at mature green stage, and then pick and disposed off all soft, ripen and infested fruits promptly to prevent fruit fly infestation and reproduction within the plantation (Hunter and Buddenhagen, 1972).

2.3 RHODANESE

Rhodanese (EC. 2.8.1.1) is a sulfur transferase that catalyses, *in vitro*, the formation of thiocyanate from cyanide and thiosulphate or other suitable sulphur donors (Smith and Urbanska, 1986).

The principal detoxification pathway of cyanide is that catalyzed by a liver mitochondrial enzyme, rhodanese (Cyanide: Thiosulphate Sulphur Transferase; rhodanese is widely distributed in both plants and animal species. Two forms of rhodanese have been demonstrated. These forms are dephospho – and phospho– rhodanese. They were identical with respect to kinetic parameters, amino acid composition amino terminal amino acid, sulphahydryl content, tryptic maps and molecular weights. Both forms utilize β- mercaptopyruvate equally but at 1% efficiency of thiosulphate (Blumenthal and Henrikson, 1971). The phosphorylation is catalyzed by a cAMP – dependent protein kinase utilizing ATP (Ogata et al., 1989).

Similarly, 4 stable forms of the enzyme designated I, II, III, and IV, having the same molecular weights and primary structures with respect to amino acid composition and peptide map were separated from bovine liver. It was speculated that they are conformational isomers originating from the form that predominate in the mitochondrial extract (Cannella et al., 1981).

The physiological role of rhodanese (cyanide: thiosulphate sulphur transferase) in animal tissues and perhaps in plants is controversial; particularly its function in the detoxification of acute cyanide exposure (Delvin et al., 1989; Sylvester and Sander, 1990).

2.4 STRUCTURE OF RHODANESE

Evidences from gel electrophoresis and Electron density map of bovine liver shows that Rhodanese consists of a single polypeptide chain with molecular weight of 32,000 to 33,000 Da (Crawford and Horowitz, 1976; Bergsma *et al.*, 1975). The above reveal clear double domain structure having similar conformation with few structural differences. Each of these domains has four-stranded parallel β-structure, with one helix running anti parallel to the β-sheet (Bergsma *et al.*, 1975). Smith *et al.* (1974) however reported a dimmer with molecular weight of 37,000. These workers found that each monomer has six short helixes and three strands parallel twisted pleated sheet. The carbonyl terminus of one monomer and the amino terminus of the other were found to be in close contact, forming a salt bridge.

2.5 AMINO ACID COMPOSITION OF RHODANASE

Rhodanese purified from chicken liver revealed that the primary structure of the enzyme contain 289 amino acids upon sequential Edman degradation of over lapping peptides obtained by selected chemical and enzymatic cleavages in which 212 of these amino acids matched with those obtained from bovine liver. Observable differences were mainly due to conservative substitutions (Kohanski and Heinrikson, 1990). Between 5 and 11 tryptophan per 26 alanine residues in bovine liver rhodanese was reported (Baillie and Horowitz, 1976). In a more intensive study, involving combination of amino acid analysis, solvent perturbation difference spectroscopy, specific residue modification and direct UV spectra analysis, it was found that the enzyme contain 10 tyrosine, 8 tryptophan and 16 phenylalanine per 26 alanine residues (Baillie and Horowitz, 1976).

2.6 ACTIVE SITE OF RHODANESE

The catalytic site of rhodanese is located in the bottom of the crevice formed by the two domains of the enzyme (Koloczek and Vanderkool, 1987). It has been shown that sulphahydryl (SH) groups are important in the rhodanese catalysis (Wang and Volini, 1968; Keith and Volini, 1987). There is evidence suggesting the presence of a tryptophanyl residue, which is in close proximity with the essential sulphahydryl group in the active site of the enzyme (Wang and Volini, 1968). It was proposed that this SH group is the site of substrate – sulphur binding in the obligatory enzyme – sulphur intermediate (Keith and Volini, 1987). Solution studies indicated that the active site SH group could form an intramolecular disulphide bond with another SH group in the protein.

These groups are however located in such a way that the disulphide bond formation is

very unlikely (Wang and Volini, 1988). Instead the active site SH group can participate in hydrophobic interaction (Horowitz and Westley, 1970). The sulphur – donor substrates, thiosulphate and ethanethiosulphonate utilize the same site in rhodanese but produce different sulphur – substituted enzymes (Jarabak and Westley, 1974).

2.7 MECHANISM OF ACTION OF RHODANESE

Studies of the enzyme have shown that the protein has structural flexibility and that reversible conformational changes accompany catalysis. This is important in the rate limiting binding step (Horowitz and Criscimagna, 1983). Rhodanese form covalent substituted – enzyme intermediate during catalysis. It functions by double displacement mechanism with formation of a covalent enzyme-sulphur intermediate (keith and volini 1987).

This mechanism involves binding of thiosulphate to a metal ion in the enzyme. In this complex, there is electron shift away from the planetary sulphur atom of the thiosulphate with resultant stretching and weakening of the S – S bond, making it more susceptible to attack by a strong enzymic nucleophile which affects the cleavage (Leininger and Westley, 1968). The enzyme substrate [ES] complex, differing in reactivity depending on the nature of the sulphur donor or substrate (Jarabak and Westley, 1974), is formed by discharging sulphite ion from enzyme thiosulphate complex. The acceptor substrate, cyanide ion then combines with the E - S intermediate to form the second product, thiocyanate ion, there by regenerating the free enzyme (Volini and Wang, 1978). The enzyme thus functions through the ping-pong mechanism (Vazquez *et al.*, 1987).

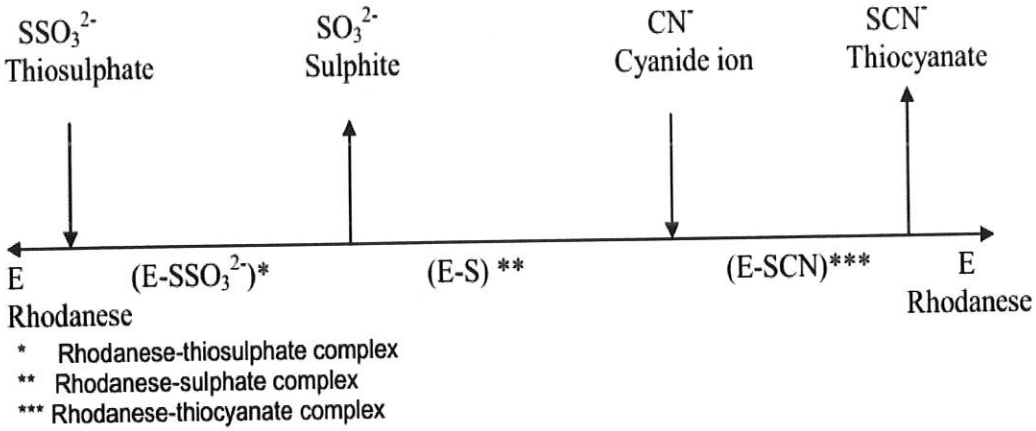

Fig 1: Mechanism of rhodanese action

2.8 BIOLOGICAL FUNCTIONS OF RHODANASE

Rhodanese activity is ubiquitous in nature, suggesting an important physiological role. Besides cyanide detoxification, rhodanese is believed to perform other functions, including formation of iron-sulfur center in proteins (Pagani *et al.*, 1982; Bonomi *et al.*, 1997), participation in energy metabolism (Ogata and Volini, 1990) and functioning as a thioredoxin oxides (Nandi *et al.*,2000). Among all speculative roles, cyanide metabolizing function has been the subject of extensive investigation during last several decades (Lewis *et al.*, 1992; Aminlari and Zohrabi, 2003).

Experimental works have shown that the level of rhodanese in different tissues of animals is correlated with the level of exposure to cyanide (Aminlari *et al.*, 2002), the hepatopancrease which serve the same function as the liver in animals is the richest source of rhodanese, and therefore, a heavy cyanide metabolizing function is performed by this organ. However, the extensive variation in the distribution of rhodanese in different tissues of animals, which in some cases exceed that of liver, indicates that other organs are also involved in cyanide detoxification in a species specific manner. In these

animals, the enzymatic action of microflora of rumen and other parts of the digestive system on cyanogenic glycosides ingested through foodstuff liberates hydrogen cyanide (Wood, 1975). Epithelium of stomach in these animals is the first tissue that is in direct contact with the released cyanide; hence, there is a greater activity of rhodanese in these tissues compared with others.

2.9 CYANIDE

Cyanide (CN-) is a highly toxic compound that is readily absorbed and causes death by preventing the use of oxygen by tissues of animals (Okalie and Osagie, 2000, Okfar *et al.*, 2002; Sausa *et al.*, 2002). This toxicant is widespread in the environment. Many naturally occurring substances as well as industrial products contain cyanide (Chiwona-Karltun *et al.*, 2000). More than 2,000 species of plants are known to contain cyanogenic glycosides which can readily liberate cyanide (Culter and Conn, 1981; Vannesland *et al.*, 1982; Majek, 1992).

Cyanogenic glycosides in forage crops can result in the death of grazing animals (Calabrese, 1983). In addition, CN is formed as a primary product of the biotransformation of several aliphatic nitriles which are used in the manufacturing of synthetic fibers, resins, plastics, pharmaceuticals and vitamins (Willhite and Smith, 1981; Silver *et al.*, 1982; Wiemeyer *et al.*, 1986;Vick, 1991; Majek, 1992; Boron and Baud, 2005). The general population may be exposed to cyanide from ambient air, drinking-water and food.

The primary detoxification reaction is thiocyanate (SCN^-) formation catalyzed by rhodanese. The classic antidotal combination of sodium nitrite and sodium thiosulfate has

been used against cyanide toxicity for years without change. The therapy is aimed in formation of methemoglobin by sodium nitrite, which competes with cytochrome oxidase for the building of CN^- and providing a sulfur donor (thiosulfate) for rhodanese.

2.9.1 RHODANESE ACTIVITY ASSAYING

Rhodanese assay activity can be detected by different methods. The most commonly used assay is based on the colorimetric determination of ferric thiocyanate formed when enzymatically produced thiocyanate reacts with ferric nitrate in the presence of formaldehyde (Sorbo, 1953; Aminlari *et al.*, 2002). Several other methods are available which use different aspects of the enzyme reactivity, including a polarographic procedure, colorimetric determination of sulfite or visualization of precipitation on polyacrylamide gel after electrophoresis (Westley, 1973). A method based on the inclusion of a separate boiled control in the conventional rhodanese assay which corrects for the non-biological contribution to thiocyanate formation. Currently, a new enzyme-coupled assay is being developed in our laboratory which utilizes lactoperoxidase to oxidize thiocyanate with concomitant reduction of hydrogen peroxide. The remaining unreacted hydrogen peroxide can be reduced by peroxidase in the presence of a chromogenic substrate.

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Sample collection

Ripe paw-paw was purchased in a market at Ile-Ife, Osun state, Nigeria while the unripe one was gotten from Oye-Ekiti, Ekiti state, Nigeria.

3.2 METHODS

3.2.1 Preparation of Reagents

3.2.1.1 **50 mM Borate buffer, pH 9.4 (Assay buffer)**

The buffer is made up of 11.403g Sodium borate decahydrate ($Na_2B_4O_7.10H_2O$) and 1.2292g Boric acid (H_3BO_3). To prepare 1000 ml of the buffer, 1.229g of H_3BO_3 and 11.4g of $Na_2B_4O_7.10H_2O$ were dissolved in 200 ml of distilled water and made up to one litre.

3.2.1.2 **50mM Phosphate buffer, pH 7.2 (Homogenization buffer)**

The buffer is made up of 3.02g Sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$) and 10.99g disodium hydrogen phosphate ($Na_2HPO_4.12\ H_2O$). To prepare 1000 ml of the buffer, 3.02g of $NaH_2PO_4.2H_2O$ and 10.99 g of Na_2HPO_4 were dissolved in 200 ml of distilled water and made up to 1000ml.

3.2.1.3 250mM Potassium cyanide (substrate) exactly 1.63g of potassium cyanide (KCN) was weighed and dissolved in 100ml of distilled water

3.2.1.4 250mM Sodium thiosulphate (substrate)

Exactly 6.20g of sodium thiosulphatepentahydrate ($Na_2S_2O_3.5H_2O$) crystals was weighed and dissolved in 100 ml distilled water.

3.2.1.5 15% Formaldehyde

Exactly 15% Formaldehyde was prepared by measuring 39.5 ml of 38% formaldehyde and the volume was made up to 100 ml with distilled water.

3.2.1.6 Sorbo Reagent

Exactly 10.1 g of ferric nitrate salt was weighed into a beaker and 20 ml of nitric acid was added and the preparation was made up to 100 ml with distilled water

3.3.2 Enzyme extraction and Homogenization

About 50g of the ripe paw-paw mesocarp was weighed and it was homogenized with 150ml of phosphate buffer pH 7.2, 65ml of the phosphate buffer was used to homogenize 20.9g of the ripe paw-paw seed. 20g of the unripe paw-paw mesocarp was weighed and homogenized with 150ml phosphate buffer pH 7.2, 20g of the unripe paw-paw seed was homogenized with 60ml of phosphate buffer.

3.3.3 Enzyme Assay

Rhodanese activity was measured according to the method of Lee et al., (1995), as described by Agboola and Okonji (2004), on the principles of the colorimetric determination of thiocyanate formation. The reaction mixture consists of 0.5ml of 50mM Borate buffer (pH 9.4), 0.1ml of 25mM KCN, 0.1ml of 25mM $Na_2S_2O_3$, and 100µl (0.1ml) of the enzyme solution. The mixture was incubated for 1 minute at room temperature and the reaction was stopped by adding 0.25ml of 15% formaldehyde, followed by the addition of 0.75ml of Sorbo reagent (Sorbo, 1995). The absorbance was taken at 460nm.

Rhodanese Assay procedure

Reagents	Test1	Test2	Test3	Blank
50mm borate buffer(pH 9.4, Pka 9.2)	0.25ml	0.25ml	0.25ml	0.25ml
0.25M KCN	0.1ml	0.1ml	0.1ml	0.1ml
0.25M $Na_2S_2O_3$	0.1ml	0.1ml	0.1ml	0.1ml
Buffer (DH_2O)	0.1ml	0.1ml	0.1ml	0.1ml
Sample (crude)	0.1ml	0.1ml	0.1ml	0.1ml

Incubation for 1min at room temperature

15% Formaldehyde	0.25ml	0.25ml	0.25ml	0.25ml
Sorbo reagent	0.25ml	0.25ml	0.25ml	0.25ml

Read at 460nm

15% Formaldehyde: Terminate the reaction

Sorbo reagent: Bring out the colour.

3.3.4 Determination of Rhodanese Activity

$$\text{Rhodanese Activity (RU/min)} = \frac{\text{O. D.}_{460nm} \times \text{D. F (10)}}{1.08 \times \text{S. V.}}$$

O. D._{460nm} = Optical Density at wavelength of 460nm;

D. F. = Dilution Factor (10)

S.V. = Sample Volume;

1.08 = Extinction Coefficient at 460nm

3.3.5 Test for protein and Enzyme

0.1ml of crude sample was put into the test tube and then 0.5m of Bradford regent was added to it.

3.3.6 Effect of Temperature on Enzyme activity

The enzyme was assayed at temperatures between 30^0c and 100^0c to investigate the effect of temperature on the activity of the enzyme and to determine the optimum temperature of the enzyme. The rhodanese activity was assayed routinely as previously discussed.

3.3.7 Effect of pH on Enzyme activity

The effect of pH on the activity of the enzyme activity was determined by using phosphate buffer of different (pH 4-7) to know under which pH the enzyme will have its optimum activity. The rhodanese activity was assayed as described previously.

3.3.8 Substrate specificity

The substrate specificity of the enzyme was determined by using different sulphur compounds such as sodium thiosulphate, ammonium persulphate, sodium bisulphate, Mercaptoethanol.

CHAPTER FOUR

4.0 RESULTS

Table 1: substrate specificity for ripe pawpaw mesocarp

COMPOUNDS	ACTIVITY (1/mol/min)
$NA_2\ S_2\ O_3$	1.02
Mercaptoethanol	0.11
Sodium metabisulphite	0.21
Ammonium per sulphate	0.05
$(NH_4)_2\ SO_4$	0.23

Table 2: substrate specificity for unripe pawpaw mesocarp

COMPOUNDS	ACTIVITY (1/mol/min)
$NA_2\ S_2\ O_3$	0.35
Mercaptoethanol	0.32
Sodium metabisulphite	0.32
Ammonium per sulphate	0.08
$(NH_4)_2\ SO_4$	0.19

Table 3: substrate specificity for ripe pawpaw seed

COMPOUNDS	ACTIVITY(1/mol/min)
$NA_2 S_2 O_3$	0.82
Mercaptoethanol	0.57
Sodium metabisulphite	0.80
Ammonium per sulphate	0.60
$(NH_4)_2 SO_4$	0.13

Table 4: substrate specificity for unripe pawpaw seed

COMPOUNDS	ACTIVITY (1/mol/min)
$NA_2 S_2 O_3$	2.44
Mercaptoethanol	2.00
Sodium metabisulphite	1.6
Ammonium per sulphate	1.25
$(NH_4)_2 SO_4$	1.78

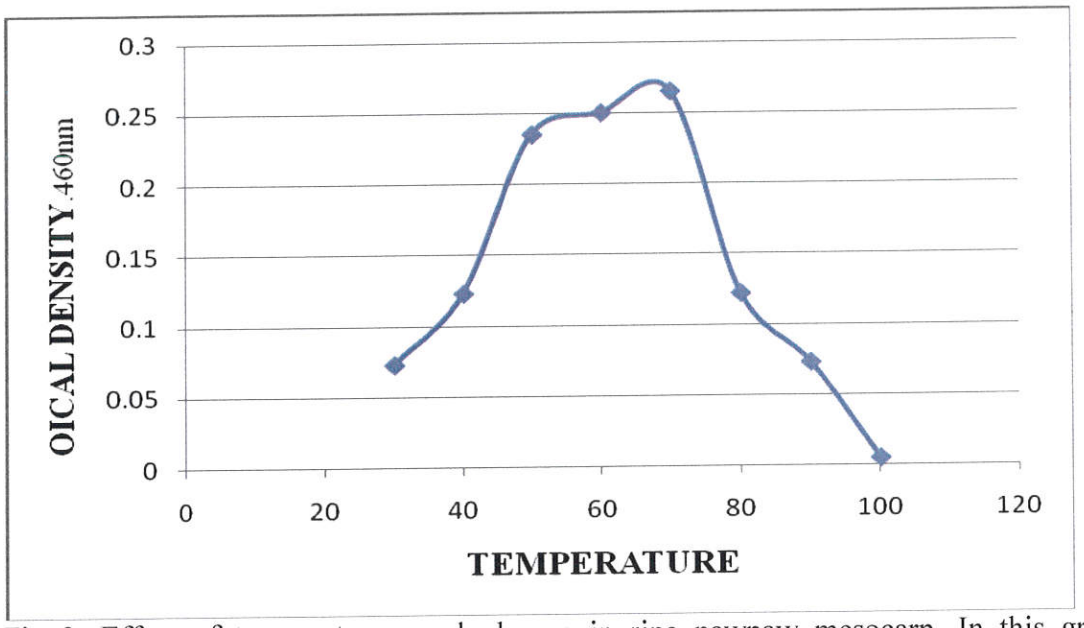

Fig 3: Effect of temperature on rhodanese in ripe pawpaw mesocarp. In this graph Activity of rhodanese under different Temp range (30-100^{0}c) is plotted against temperature, the optimum activity is seen at 70^{0}c a shown by the graph and there was no activity at 100^{0}c.

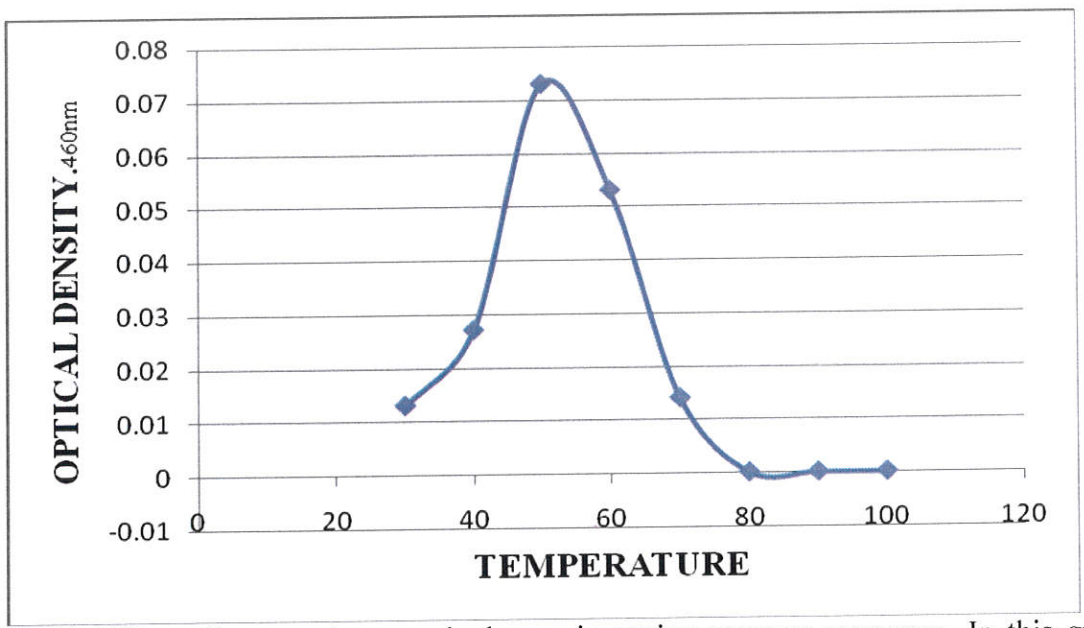

Fig 4: Effect of temperature on rhodanese in unripe pawpaw mesocarp. In this graph Activity of rhodanese under different Temp range (30-100^{0}c) is plotted against temperature, the optimum activity is seen at 50^{0}c a shown by the graph and there was no activity at 90^{0}c and 100^{0}c.

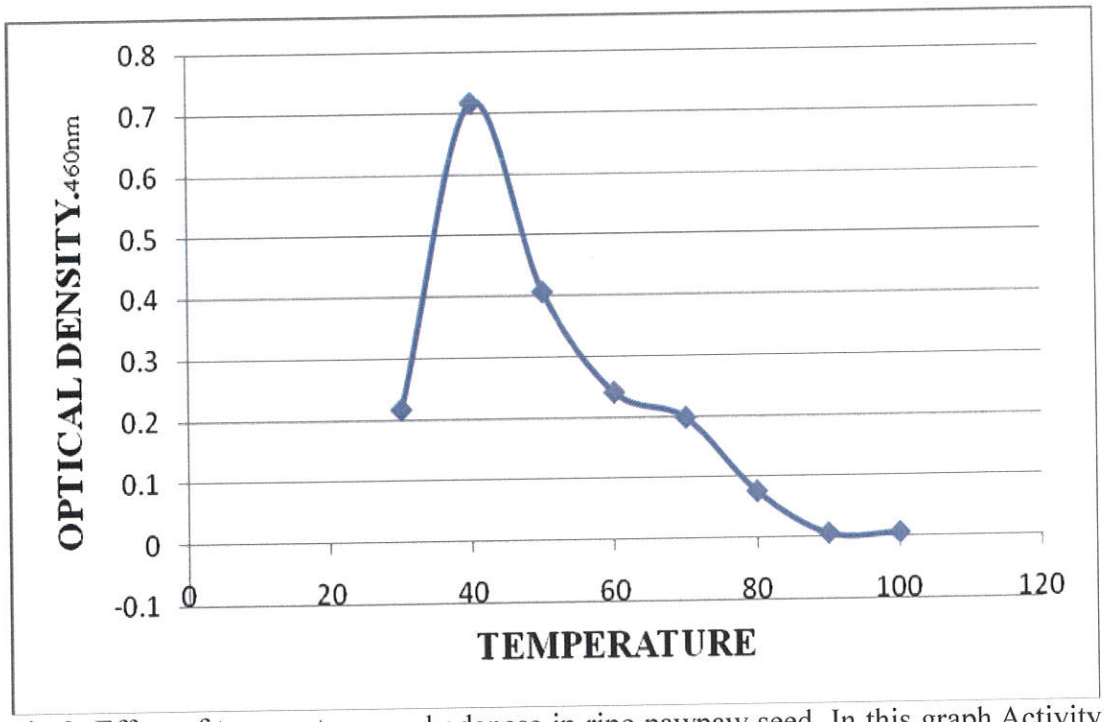

Fig 3: Effect of temperature on rhodanese in ripe pawpaw seed. In this graph Activity of rhodanese under different Temp range (30-100^{0}c) is plotted against temperature, the optimum activity is seen at 40^{0}c a shown by the graph and there was no activity at 90^{0}c and 100^{0}c

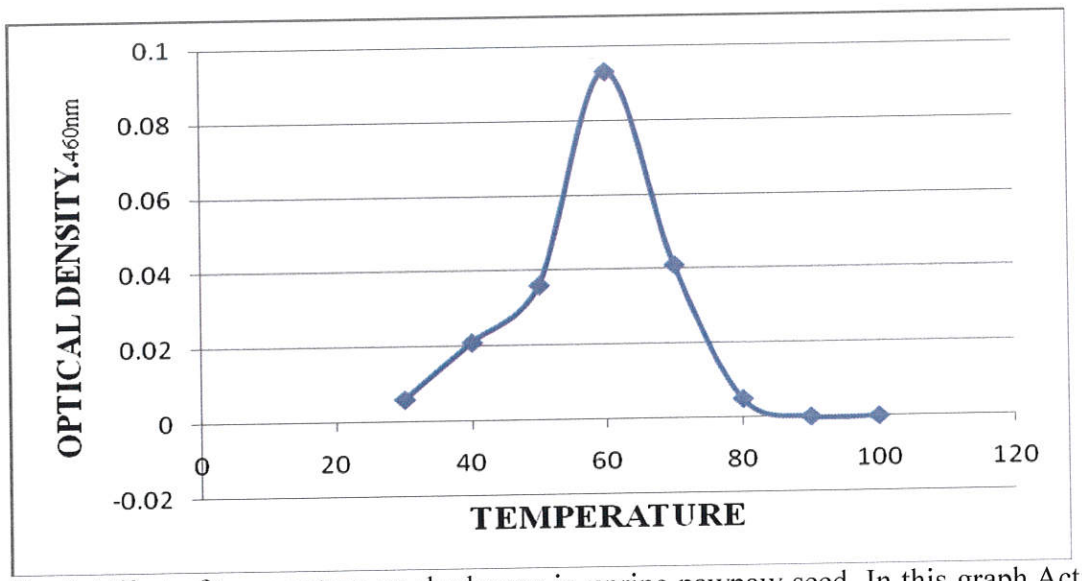

Fig 5: Effect of temperature on rhodanese in unripe pawpaw seed. In this graph Activity of rhodanese under different Temp range (30-100^{0}c) is plotted against temperature, the optimum activity is seen at 50^{0}c a shown by the graph and there was no activity at 90^{0}c and 100^{0}c

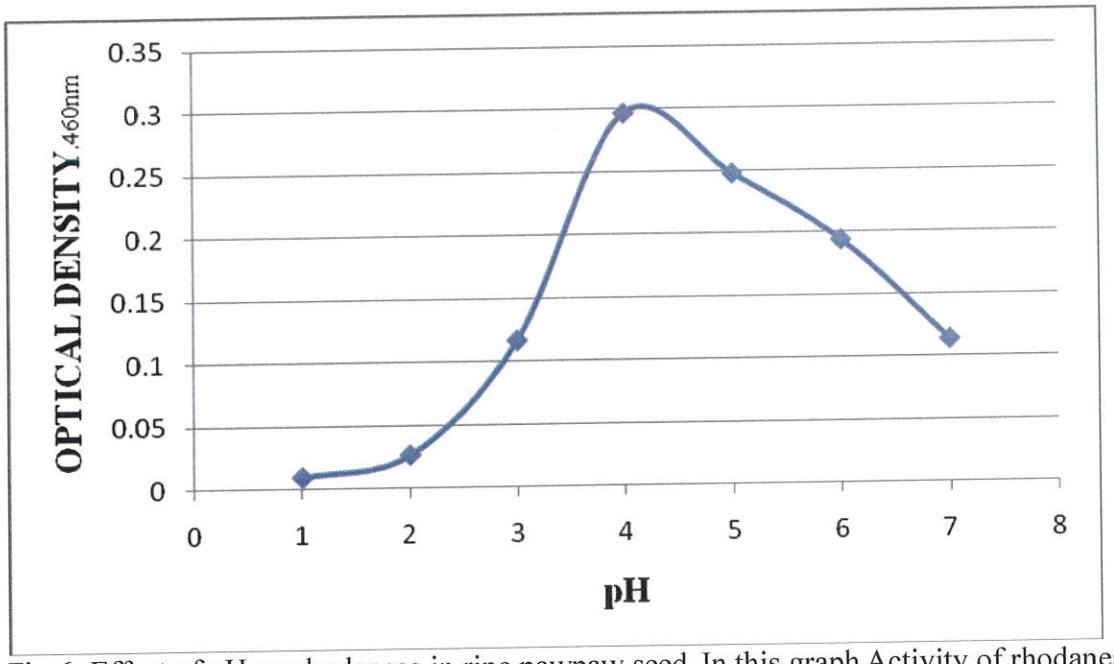

Fig 6: Effect of pH on rhodanese in ripe pawpaw seed. In this graph Activity of rhodanese under different pH range (pH4-pH10) is plotted against pH. Rhodanese shows optimum activity at pH7 and showed its lowest activity at pH10

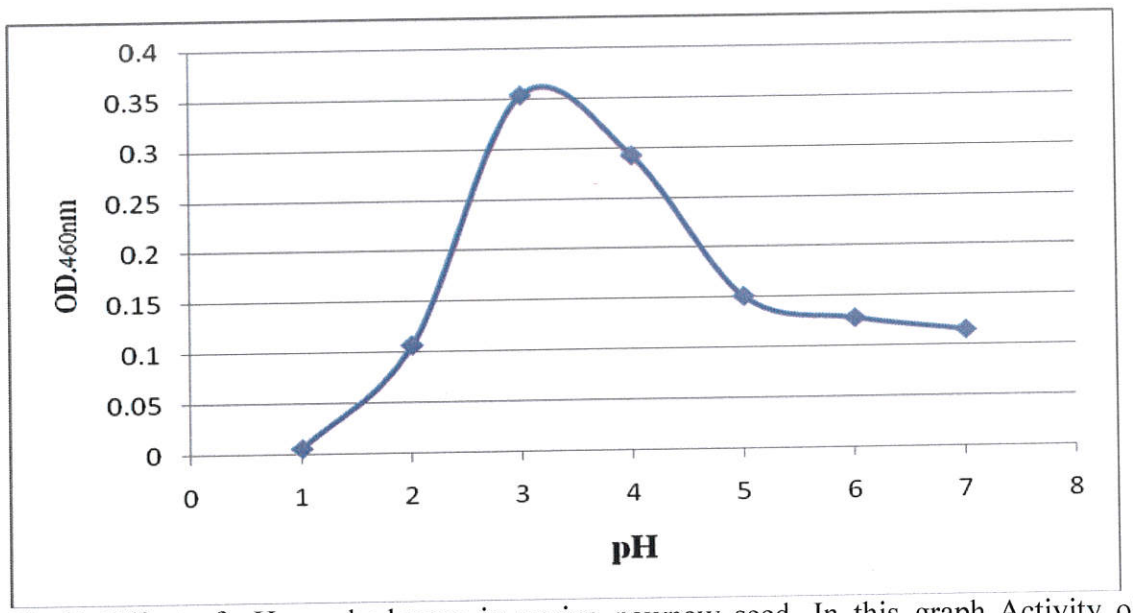

Fig 7: Effect of pH on rhodanese in unripe pawpaw seed. In this graph Activity of rhodanese under different pH range (pH4-pH10) is plotted against pH. Rhodanese shows optimum activity at pH6 and showed its lowest activity at pH10

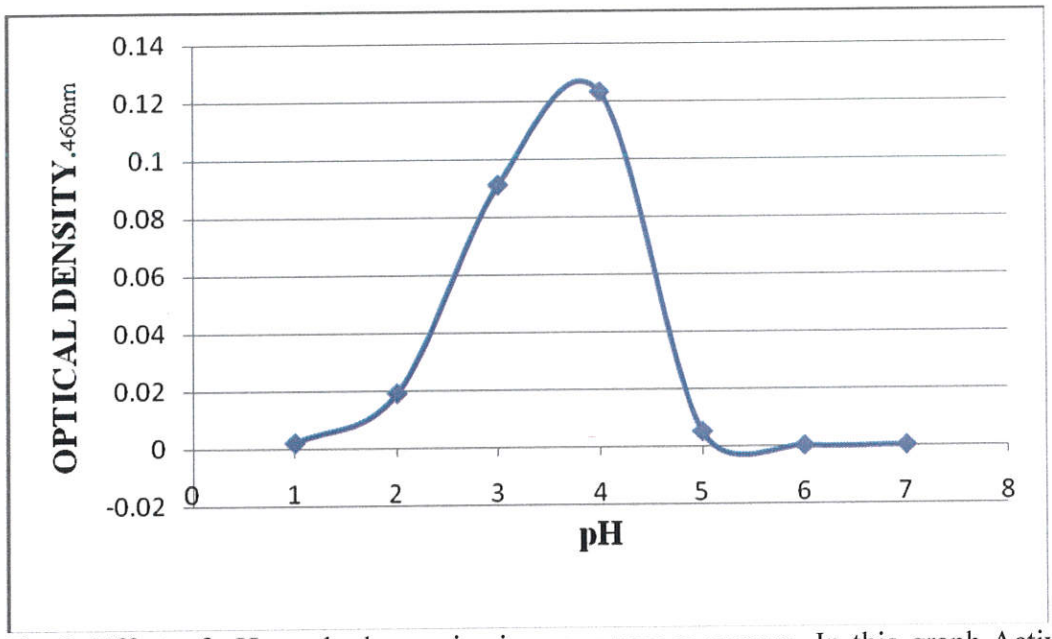

Fig 8: Effect of pH on rhodanese in ripe paw-paw mesocarp. In this graph Activity of rhodanese under different pH range (pH4-pH10) is plotted against pH. Rhodanese shows optimum activity at pH7 and showed no activity at pH9 and pH10

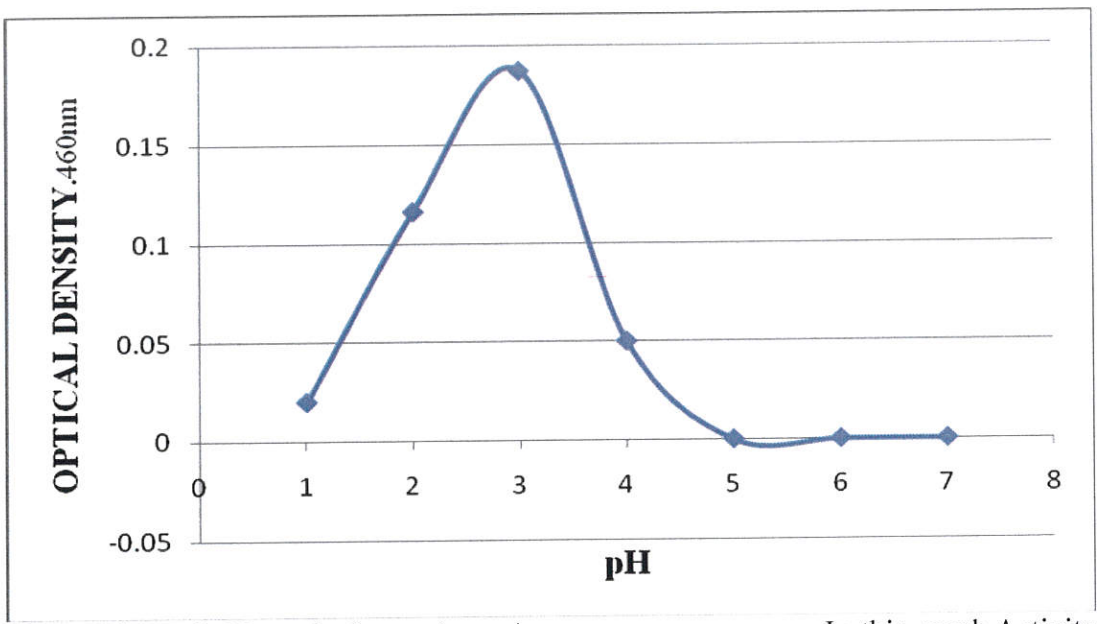

Fig 9: Effect of pH on rhodanese in unripe paw-paw mesocarp. In this graph Activity of rhodanese under different pH range (pH4-pH10) is plotted against pH. Rhodanese shows optimum activity at pH6 and showed no activity at pH9 and pH10

4.1 DISCUSSION

The rhodanese from the seed and mesocarp had its optimum pH of 7.0 (Fig 6 and 8) in agreement with Okonji *et al.,* (2017) who reported an optimum pH of 7.0 for rhodanese gotten from mesocarp and seed of snake tomatoes (*Trichosanthes cucumerina* Linn), while the optimum pH of rhodanese from unripe seed and mesocarp was at 6.0. In recent works by Okonji et al. (2008) and Akinsiku et al. (2009) an optimum pH as low as 6.0 and 6.5 for giant fresh water prawn (*M. rosenbergii*) hepatopancreas and catfish liver, respectively was reported, with both organisms being of aquatic origin. It has also been reported that at low pH (4 to 6) rhodanese is stabilized against inactivation (Kurban and Horowitz, 1991).

The optimum temperature for the rhodanese from the ripe paw-paw seed and the rhodanese from unripe paw-paw mesocarp are both at 50°c (Fig 2 and 3) which is in agreement with that reported from Okonji *et al.,* (2017) from the rhodanese gotten from the seed and mesocarp of snake tomatoes (*Trichosanthes cucumerina* Linn). These results are in agreement with results reported for rhodanese from different sources. Sorbo (1953) reported an optimum temperature of 50°C for bovine liver rhodanese. A wide temperature optimum of 35 to 55°C for rhodanese from different strains of *Trichoderma* was reported by Ezzi et al., (2003), while Okonji et al. (2011) obtained an optimum temperature of 50°C for mudskipper liver rhodanese. While that of the ripe paw-paw mesocarp and unripe paw-paw seed were at different optimum temperature of 70°c and 60°c (Fig 1and 4) is similar to the results obtained by most researchers for rhodanese from different sources and it agrees with optimum temperature of 60°c in rhodanese from

the root of *Pentadiplandra brazzeana* root reported by Okonji *et al,* (2017). Chew and Boey (1972) also obtained optimum temperature of 59°C for cassava leaf rhodanese.

The effect of different substrates (sodium thiosulphate, sodium metabisulphite and ammonium persulphate) showed that rhodanese from both mesocarp and seed was more specific for sodium thiosulphate (Table 1-4). The result showed that only sodium thiosulphate gave the highest activity, while the other sulphur compounds, to some extent, exhibited rhodanese activity.This might be due to the specificity of the enzyme for sodium thiosulphate. Sodium thiosulphate ($Na_2S_2O_3$) has been reported to be highly specific for rhodanese (Sorbo, 1953; Westley, 1980) and the result obtained in this study is in agreement with this finding.

4.2 CONCLUSION

In conclusion, the presence of rhodanese in both ripe & unripe seed and mesocarp of the plant is a suggestion that the enzyme may be functional in many physiological activities in the plant, in which one of them is cyanide detoxification. This study showed the presence of rhodanese activity in the seed and mesocarp of both ripe and unripe pawpaw, therefore the consumption of paw-paw can help to reduce the toxicity of cyanide to a less toxic thiocyanate. The enzyme in both ripe and unripe pawpaw mesocarp and seed showed specificity towards $Na_2S_2O_3$ as sulphur donor.

4.3 RECOMMENDATION

Pawpaw was confirmed to be a good source of rhodanese and its consumption can be advised, as it has been known to be a source of rhodanese which reduces the toxicity of cyanide to a less toxic thiocyanate. It will serve as a supply of rhodanese to the body.

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