

**EFFICACY OF LOCALLY PRODUCED PAPAIN ENZYME FOR THE  
PRODUCTION OF PROTEIN BAIT FOR *BACTROCERA INVADENS*  
(DIPTERA: TEPHRITIDAE) CONTROL IN GHANA**

A thesis presented to the:

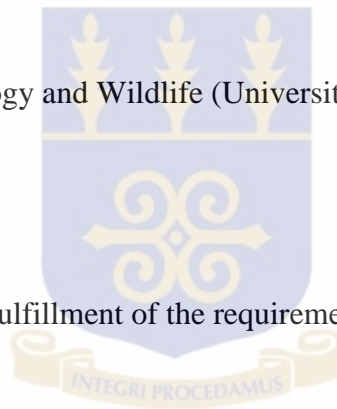
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## DECLARATION

The entire thesis is an outcome of research work undertaken by Rosemary Aggrey-Korsah in the Department of Nuclear Agriculture and Radiation Processing, School of Nuclear and Allied Sciences, and the Department of Biochemistry, Cell and Molecular Biology, University of Ghana, under the supervision of Dr. Delphina A. M. Adabie-Gomez and Dr. Charles E. Annoh.

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## **DEDICATION**

I dedicate this work to Mr. Michael Effah Asamoah (Makosa).



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**ABBREVIATIONS/CHEMICAL SYMBOLS**

APHIS	Animal and Plant Health Inspection Service
BNARI	Biotechnology and Nuclear Agriculture Research Institute
BSA	Bovine Serum Albumin
CTA	Centre for Technical Agriculture
EPPO	European Plant Protection Organisation
EDTA	Ethylene Diamine Tetraacetic Acid
GAEC	Ghana Atomic Energy Commission
HCl	Hydrochloric acid
IPM	Integrated Pest Management
KDa	Kilo Dalton
MAT	Male Annihilation Technique
NaOH	Sodium hydroxide
PAGE	Polyacrylamide Gel Electrophoresis
REPMC	Radiation Entomology and Pest Management Centre
RTC	Radiation Technology Centre
SDS	Sodium Dodecyl Sulphate
TCA	Trichloroacetic Acid
TEMED	N,N,N,N'-Tetramethylenediamine
WHO	World Health Organisation
U	Tyrosine Units
μ	Micro
μl	Micro litre
K	Kilo
ECB	Ethanol chlorobenzene
pH	Podus hydronus
OH	Hydroxide
CuSO <sub>4</sub> .5H <sub>2</sub> O	Copper (II) sulphate pentahydrate

$\text{Na}_2\text{CO}_3$	Sodium carbonate
m	Metre
PEG	Polyethylene Glycol
$(\text{NH}_4)_2\text{SO}_4$	Ammonium sulphate
$\text{H}_2\text{O}_2$	Hydrogen peroxide
KGy	Kilo Gray
mg	Milligram
ml	Millilitre
$\text{NH}_3$	Ammonium
g	Gram
mm	Millimetre
N	North
W	West
°	Degree
°C	Degrees Celsius
UV	Ultra violet
VIS	Visible spectrum
nm	Nanometre
cm	Centimetre

## ABSTRACT

Autolysed brewery yeast waste is currently being used as cost effective protein bait for *Bactrocera invadens* control the world over to replace commercial protein hydrolysate bait formulations. However, significant reduction in production cost can be achieved when all the production materials are from local sources. This experiment was aimed at assessing the efficacy of locally produced papain extracted from “Red lady” pawpaw fruit latex and skin peel to be used for protein bait production. Aqueous two-phase extraction of papain from pawpaw fruit latex with 15 %  $(\text{NH}_4)_2\text{SO}_4$  - 8 % PEG recovered  $64.72 \pm 2.08$  % papain into the supernatant with 7.33 % proteolytic activity yield and a fold purification of  $58.11 \pm 1.67$ . Proteolytic activity and protein concentration measured for the aqueous two-phase extracts of pawpaw skin peel were significantly higher ( $p= 0.00$ ) than crude extracts of skin peel. However, the aqueous two phase extraction of papain from skin peel needs to be optimised further since SDS-PAGE showed no visible bands in the different phase extracts. Gamma irradiation at 10 KGy increased the proteolytic activity of crude papain by 21.69 % of the non-irradiated papain and subsequently increased the specific activity by 18.51% but the protein concentration was not affected. Protein baits prepared with crude papain extracted from the pawpaw fruit latex and skin peels were evaluated in laboratory bioassays with wild flies reared from field collected infested mangoes. The source of papain did not affect the protein bait recovery, the pH and protein concentration though colour of bait differed for crude fruit latex papain bait (dark brown) and skin peel papain bait (light brown). The bait preparations had equal attractance to male and female *B. invadens*. Mean attractance to protein baits produced

with fruit latex and skin peel papain baits were between  $25.00 \pm 7.56 \%$  and  $47.50 \pm 11.09 \%$  respectively for males,  $25.00 \pm 13.13 \%$  and  $32.86 \pm 8.23 \%$  for female flies. Similarly, female flies showed equal affinity to feed on the different protein baits in a choice assay. However, flies reared on the no papain protein bait (control) were more fecund than flies reared on the local laboratory diet formulation. There was a relation between survival duration and fecundity; high fecundity resulted in reduced survival duration for flies reared on the different protein baits and laboratory diet formulation.

Papain extracted from the “Red lady” pawpaw fruit latex and skin peels are as effective as the commercial papain for proteolysis of proteins, therefore, local protein baits can now be prepared using the extracted local papain for *Bactrocera invadens* control in Ghana. In conclusion, an aqueous two-phase extraction of 15 %  $(\text{NH}_4)_2\text{SO}_4$  – 8 % PEG is effective for papain extraction from the “Red lady” pawpaw fruit latex which can be used to produce local protein baits. Crude papain from local pawpaw variety is as effective as commercial papain for the proteolysis of protein bait. The phase concentration of the aqueous two-phase extraction system should be evaluated to improve especially the papain extraction from the pawpaw fruit skin peel. Cobalt-60 gamma radiation did not improve the purity of the crude papain, however, it can be a suitable treatment to enhance or maintain the proteolytic activity.

## CHAPTER ONE

### 1.0 INTRODUCTION

#### 1.1 Background of study

The African continent serves as a home and a point of introduction for several fruit fly species, especially since the major route for fruit flies to spread from one region to another is through the international movement of infested fruits (Ekesi and Mohamed, 2010). Thus, the liberalisation of fruit and vegetable trade has made the African continent vulnerable to alien fruit fly species invasion, such as, *Bactrocera zonata* in Egypt (1997); *B. invadens* in Kenya (2003) and *B. latifrons* in Tanzania (2003) (Ekesi, 2010a). Considering all the different fruit fly species of the continent, *B. invadens* is recognised for its highly destructive nature as it displaced all native fruit fly species within the first year of its introduction into Africa. Evidence of competitive displacement of *Ceratitis* species (natives) has been observed in many countries since the first detection of *Bactrocera invadens* in Africa in 2003 (Abdullahi *et al.*, 2011; Ekesi and Mohamed, 2010). Bioecological studies of *B. invadens* in Kenya revealed that *B. invadens* frequently co-infested the fruits with native *Ceratitis cosyra* but the population densities of the natives were always low (Ekesi *et al.*, 2006; Rwomushana, 2011). Consequently, this would result in the complete displacement of the native *C. cosyra* especially because *B. invadens* have a high K-selection oriented life history (Meyer *et al.*, 2014).

Fruit flies are recognised worldwide as pests of economic importance in horticultural industries, causing damages worth millions of dollars each year to the agriculture industry (Muchemi *et al.*, 2011). Throughout the tropics, fruit flies are the major pests of several cultivated fruits and vegetables with the growing international trade further increasing the significance of fruit flies (Allwood, 1997; Yeboah, 2012). Therefore, a successful control of these fruit flies can improve the productivity and quality of fruits and vegetables which can ultimately enhance food security, employment and trade opportunities (Fruit fly info, 2010).

Early detection is the best means to achieve speedy and cost-effective eradication of fruit flies, and traps are among the most used tools for fruit fly monitoring. Traps are usually used in association with attractants such as methyl eugenol, protein baits, and cue lures among others. Control measures based on the use of methyl eugenol, insecticide sprays, poisoned protein baits and orchard sanitation are most effective when used in an integrated pest management programme (Bugs for bugs, 2013). Though insecticide sprays and protein baits are among the most used control strategies, the bait system is more acceptable for an integral control due to the minimal pesticides use and its target specificity (Dekker and Messing, 1999). Unlike insecticide sprays, adult fruit flies require protein for development and therefore protein baits hold a greater potential for fruit fly control. In effect, recent control measures have been directed towards an area-wide management which seeks to incorporate all these control measures into a single programme (Sookar *et al.*, 2006).

Protein baits are food attractants and their effectiveness relies on the fact that immature males and females require protein for reproductive development (Secretariat of the Pacific Community, 2002). These proteins are detected by fruit flies over short distances (10 - 15 m) though some commercial baits are combined with other volatile odours to make them attractive over greater distances (African Fruit Fly Programme, 2014).

Protein bait incorporated with insecticides is approved globally as an environmentally friendly control strategy due to the minimal insecticide usage and thus poses little threat to the environment and non-target organisms. Furthermore, these protein baits with insecticides can be applied at resting and feeding spots of the fruit flies (spot spraying) instead of spraying the whole crop (cover spraying) to minimize waste (Dekker and Messing, 1999). Protein baits are effective for *B. invadens* control but their use in the Ghanaian agro-industry is low due to several reasons. Currently, few types of bait are available on the Ghanaian market and these few available types of bait are sold at high prices because they are not produced locally (Yeboah, 2012). Apart from the cost of importation of these baits, clearance duties and delays at the port are enough to deter farmers from patronising protein baits. Producing protein baits from local materials can possibly minimise the cost of baits and the inconvenience of importation could be dealt with adequately.

In Ghana, scientists have prepared protein baits from brewery yeast waste as it has been done in several parts of the world including Australia and Mauritius where yeast autolysate from brewery yeast waste are being used for protein bait preparation with great success (Chinajariwong *et al.*, 2003; Sookar *et al.*, 2003). The yeast cells of the brewery

waste are autolysed with heat followed by proteolysis with an enzyme to breakdown the proteins which enables the release of volatiles attractive to the insects (Secretariat of the Pacific Community, 2002). Thus, the proteolytic enzyme is as important as the protein. Therefore, though replacing the protein source of protein baits with brewery yeast waste can minimise cost of baits production, total reduction of cost can be achieved if the proteolytic enzyme used is less expensive (Gopaul and Price, 1999).

The proteolytic enzyme used in protein bait production is papain (Monti *et al.*, 2000), which breaks down peptide chains into free amino acids (Saha and Swati 2013). Papain exhibits a broad specificity, hydrolyses esters and amides, cleaves to peptide bonds of amino acids and can digest most protein substrates more extensively than the pancreatic proteases (Sigma-Aldrich, 2014). In terms of the effect of papain in protein bait production, the higher the concentration of the enzyme, the higher the proteolysis of the yeast cells and thus the higher the amount of free amino acids made available in the bait (Sookar *et al.*, 2003). Papain is however not produced locally and is therefore associated with similar challenges of costs, clearance duties and delays at the port like the commercial protein baits. For instance, 25 gram laboratory grade papain is sold on the Ghanaian market at GH¢ 103.50 (January, 2014). More so, the 25 gram papain based on the Gopaul and Price, 1999 protocol is only enough to autolyse 6.5 litres of brewery yeast waste to produce approximately 3.25 litres of bait which is not enough for a commercial farmer who is interested in controlling or eradicating *B. invadens*.

Therefore, a successful production of the papain enzyme in Ghana can contribute significantly to *B. invadens* control in the country.

## 1.2 Statement of the Problem

Fruit flies rank high on global quarantine lists and *Bactrocera invadens* is no exception (Ekesi and Mohamed, 2010). With the expanding international fruit trade, fruit flies have become significant quarantine pests of fruits and vegetables. Infestation by adult and larvae of *Bactrocera invadens* directly reduces the quantity and quality of the fruits and vegetables produced (Muchemi *et al.*, 2011). Beside the direct damage to fruits, indirect losses due to quarantine restrictions experienced as loss of export trade opportunity is on the rise. A major setback in the global horticultural industry has been the restriction of free trade and export of fresh horticultural fruits from a country because of infestation by *B. invadens* or even mere presence of *B. invadens* in the country (Ekesi and Mohamed, 2010).

Quarantine restrictions imposed on fruit fly-infested fruits has limited the export of fruits to lucrative markets in Europe, Japan, the Middle East, South Africa and USA, where fruit flies are considered as quarantine pests (Ekesi, 2010b). The European Plant Protection Organization (EPPO) for instance has categorized *B. invadens* as an A1 pest because it is a serious pest, while the Animal and Plant Health Inspection Service (APHIS) of the United States of America has issued alerts on *B. invadens* to the countries trading with the USA (Muchemi *et al.*, 2011). As a result, whole consignments will be destroyed at the port of entry if even a single fly or larva is detected in the batch. The destruction of a batch of mango meant for the international market in 2008 cost € 3000 per container and this cost had to be supported by the exporter (Ndiaye *et al.*, 2008).

Fruit and vegetable production is significant to the economy of many countries as they ensure food security, source of income and also create employments. Effects of *Bactrocera invadens* on human lives and livelihoods may be categorised into food loss, quarantine restrictions and economic loss. In many countries, post-harvest losses due to the action of fruit flies are high enough to threaten household food security, result in poor human nutrition and increase poverty (Drew, 2001).

Yield loss on mango across Africa was estimated to be 30 - 80 % since the arrival of *B. invadens* (Ekesi *et al.*, 2006; Vayssières *et al.*, 2009; Rwomushana *et al.*, 2008), while direct damage on citrus is estimated between 8 - 34 % (Vayssières *et al.*, 2008). Yield losses on several cultivars of mango for instance, have increased with the presence of *Bactrocera invadens* in Africa. In Kenya, Tanzania and Uganda, yield loss on mangoes due to the indigenous fruit fly *Ceratitis capitata* can range between 30 - 70 % depending on the location, season, and variety. However, the presence of *B. invadens* has increased damage to 40 - 80 % notably in lowland areas where *B. invadens* is currently key fruit fly pest (Ekesi, 2010b). In 2005, about 40 % of Africa's two million tonnes of mango harvested was ruined by fruit flies and Senegalese farmers had to cut short the mango growing season by a month due to the activities of fruit flies (IRIN Africa, 2014). The damage of fruit and vegetables due to the activities of fruit flies is so devastating that some local farmers attribute it to the wrath of God (IRIN Africa, 2014). In Kenya, annual ban imposed on fruits exported to South Africa cost the Kenyan horticultural industry up to KSh 477.6 million (\$5,638,758.13) (Muchemi *et al.*, 2011).

Within the first 4 years (2003 - 2007) *B. invadens* displaced the indigenous fruit fly, *Ceratitis cosyra* and is currently an important quarantine pest on the African continent. In 2003, infestation by *C. cosyra* was estimated at 18 % as against 80 % infestation by *B. invadens* after it had been successfully established in 2007 (Ekesi *et al.*, 2009). *B. invadens* is capable of wiping out harvestable crops and economies worldwide if left uncontrolled and West Africa's horticultural industry is most vulnerable due to the patchy nature of fruit production, poor phytosanitary standards, trans-border agricultural trade and lack of management skills (Africa Geographical Media, 2008; IRIN Africa, 2014).

In Ghana, *B. invadens* is still widespread despite all eradication efforts (Yeboah, 2013a). The country loses approximately 80 % of its exportable crops to *Bactrocera invadens*, a situation which deprives farmer's access to international markets despite their individual huge cost incurred in controlling the fly (Danso, 2013; Yeboah, 2013a). Furthermore, Ghana is currently branded as a fruit fly endemic zone along with some other countries in the West African sub-region (Yeboah, 2013a). As a result, the International Mango Market Community, which includes Europe, South Africa, and the United States of America have refused to patronise fruits from Ghana (Yeboah, 2013a). The export restrictions from these countries and many others have been enormous since Ghana is unable to meet their quality standards (Abdullahi *et al.*, 2011). The USA market for instance requires an almost impossible strategy of cultivating crops in glass houses to ensure protection against all fruits flies or irradiating the produce before export (Yeboah, 2013b).

The development of an effective method of controlling *B. invadens* in Ghana has been very paramount to mango farmers in particular and this has led to calls for mass spraying against fruit flies as done for cocoa farmers (Yeboah, 2013a). However, the use of insecticide sprays usually affects non-target organisms, pollutes the environment and leaves chemical residue on the fruits especially when spraying is done few months to harvest.

### **1.3 Justification of study**

Enhancing the productivity and quality of fruits and vegetables can be achieved through the effective control of fruit flies (Asian Institute of Technology, 2013). Several control measures are available for fruit fly management and control worldwide. These include Male Annihilation Techniques (MAT), insecticide sprays, use of entomopathogenic fungi, and use of food baits, especially protein baits.

The MAT works by selectively attracting male flies to a para-pheromone (methyl eugenol) poisoned with insecticide (Asian Institute of Technology, 2013). Population reduction results when flies ingest the para-pheromone along with an insecticide and die. Though methyl eugenol is acceptable for control and monitoring of fruit flies, it is expensive for many farmers (Kumar *et al.*, 2011).

Insecticide sprays, on the other hand, is a widely used control method due to the ease of accessibility and cost, but, these insecticide sprays are characterised by broad spectrum activity which poses issues of environmental concern such as, environmental pollution

and adverse effects on non-targets (Kumar *et al.*, 2011). In addition to the above, the cover spraying mode of application (spraying whole plants) requires frequent applications of chemicals which leave chemical residue in the produce and also wastes chemicals (Secretariat of the Pacific Community, 2002). The negative effects of chemical insecticides in recent times have influenced increased emphasis on the use of biological control agents as well as control programmes that require minimal insecticide usage (Infonet-biovision, 2012).

The use of entomopathogenic fungi is a biological control method which involves the inoculation of specific fungi into the soil to kill the pupae (Kumar *et al.*, 2011; Ouna, 2011). Though this control method is environmentally safe, it has a high level of specificity, and several modes of action (useful to minimise insect resistance), it is too costly for large scale applications. Furthermore, these entomopathogenic fungi have short shelf life, require specific environmental conditions for application and cannot be used as a preventive tool since the pest must be identified in a location before pathogen can be applied (Kumar *et al.*, 2011).

Protein bait sprays, like chemical insecticides are suitable for controlling both the female and male adult fruit flies as these baits operate on the fact that both male and female *B. invadens* require protein for development (Lyon, 2014). The minimal amount of insecticide required for poisoning makes protein baits environmentally safe. Furthermore, spot spraying parts of the tree such as stems or leaves with the poisoned protein baits prevents waste of insecticide and leave no chemical residue on fruits since the baits are not applied directly to fruits (Secretariat of the Pacific Community, 2002). Protein baits

are comparatively less expensive thus suitable for use in large scale control programmes or in an integrated pest management programme. Efficient fruit fly management and control is achieved through an Integrated Pest Management (IPM) based programme which incorporates the use of food baits, traps and spot treatments.

Currently, global efforts at fruit fly suppression is mainly based on the use of food baits (hydrolyzed proteins or their ammonium mimics) mixed with a killing agent due to the specificity and safe mode of action of these food baits (Galun *et al.*, 1983; Ekesi and Mohamed, 2010). However, increasing cost of these commercial baits has made it inaccessible to farmers in many parts of the world, most especially in the developing countries, including Ghana. A recent advance at producing protein hydrolysate substitutes from brewery yeast waste is significant to reducing the cost of bait production (Allwood 1997; Sookar *et al.*, 2003; Yeboah, 2012). In Australia, protein bait produced from yeast autolysate has been approved for the suppression and control of fruit flies at low population densities (Australian Centre for International Agricultural Research, 2014). In Malaysia, a yeast autolysate, Promar, has been used to successfully control large populations of fruit flies in Carambola Plantations (Australian Centre for International Agricultural Research, 2014). The Royal Tongalure has also been produced in Tonga using yeast autolysate. Protein bait sprays conducted in Fiji under field experimental conditions revealed that these locally produced protein baits can be used to control fruit flies effectively in both commercial and wild stands of guava (Australian Centre for International Agricultural Research, 2014).

The brewery yeast waste which is the major component of most local protein baits is a rich source of B-complex vitamins, proteins (providing all essential amino acids) and minerals making them suitable attractants (Sookar *et al.*, 2002). Brewery yeast waste that was originally disposed into streams and other water bodies can now be put to good use. The successful production of the protein bait from brewery yeast waste is an important step to making fruit fly control cheap and effective.

Despite this effort, cost of production could still be high due to the proteolytic enzyme, papain, required for the proteolysis of the autolysed yeast cells (Gopaul and Price, 1999). The use of imported food-grade or industrial-grade papain can reduce production cost of baits than using the laboratory-grade papain (Gopaul and Price, 1999); however, producing the papain locally with local raw materials would result in significant cost reduction.

In Ghana, none of the papain grades on the market is produced locally. The additional cost of importation, clearance duties, coupled with delays in clearance at ports and other requirements would not make local protein bait production using imported papain any less costly or accessible to the resource-poor farmers. The selection of control methods for an Integrated Pest Management is based on the cost of the individual control methods, hence, the need to develop cost effective control measures. Therefore, the ability to produce a control measure based on local raw materials can help sustain fruit fly control activities in any country (Sookar *et al.*, 2002). In Ghana, a successful production of papain locally would be very significant to the production of yeast autolysed protein baits for these fruit farmers who are losing tonnes of fruits daily.

## 1.4 Objectives

### 1.4.1 Overall objective

To assess the efficacy of locally produced papain enzyme for the production of protein bait suitable for *Bactrocera invadens* control.

### 1.4.2 Specific objectives

- ❖ To evaluate a standard protocol for extracting pure papain from pawpaw fruit latex and skin peels.
- ❖ To assess the effects of irradiation on the locally-produced crude papain.
- ❖ To assess the efficacy of protein baits prepared with locally-produced crude papain.

## 1.5 Hypotheses

- ❖ Papain produced from local source is as effective as commercial papain for proteolysis of protein baits.
- ❖ Irradiation has no effect on the proteolytic activity of papain.
- ❖ The source of papain does not affect the efficacy of protein baits.

## CHAPTER TWO

### 2.0 LITERATURE REVIEW

#### 2.1 Morphological and behavioural attributes of *Bactrocera invadens*

*Bactrocera invadens* possess distinct morphological features from other related species. Plate 2.1a shows the morphological features of the male *B. invadens*. They are characterised by a pair of blue-black compound eyes (Plate 2.1b) and a thorax with a blue-black base and distinct yellow patches on the sides (Plate 2.1c). The thorax is separated from the post pronotal lobe or shoulder (Plate 2.1c) and its dorsal surface varies from red-brown to black, often red-brown with black streaks (Plate 2.1d) (Meyer *et al.*, 2014). Adult *B. invadens* possess a pair of wings with dark prominent leading edges (Plate 2.1e) (Meyer *et al.*, 2014). On the abdomen are a couple of pale strips; terga iii-v are marked with a distinct black mid-longitudinal line (Plate 2.1f) (Meyer *et al.*, 2014). The female aculeus (piercing apical segment of the ovipositor) is also pointed when extended.



a. Male *B. invadens*



b. The head showing large compound eye, aristate antenna and retracted haustellate mouthparts.



c. Lateral view of thorax showing distinct yellow patches on the sides.



d. Dorsal view of thorax showing the red-brown background with black streaks.



e. Wing showing dark prominent leading edge



f. Abdomen of *B. invadens* showing the distinct black mid-longitudinal line.

**Plate 2.1: Morphological features of adult *B. invadens***

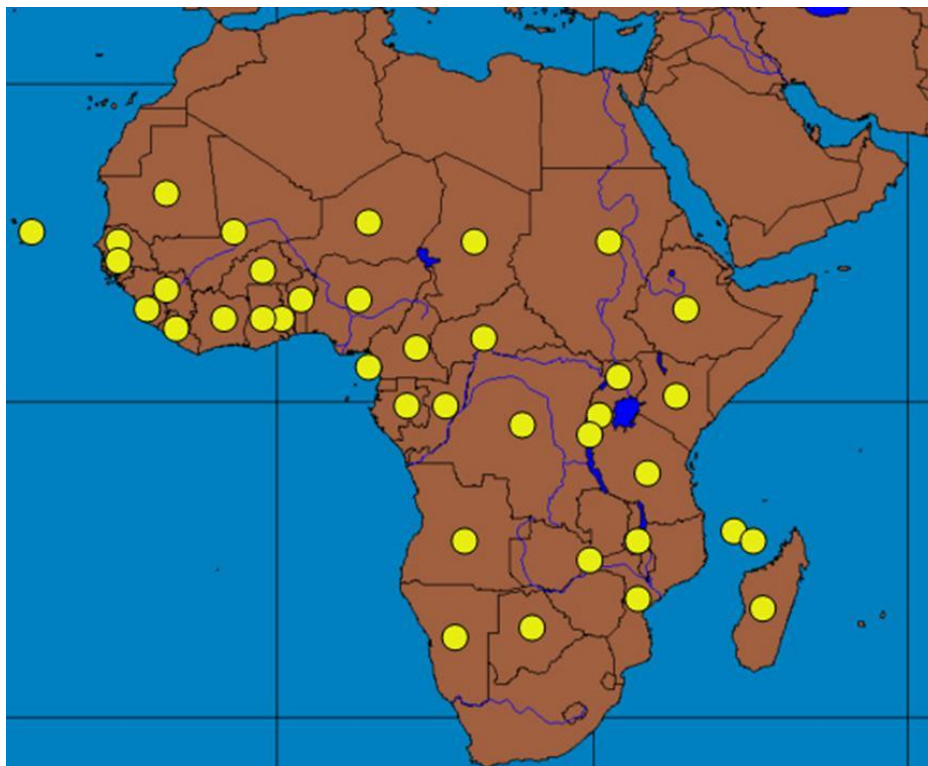
In terms of behavioural biology, the species has adult traits that include high mobility, high reproductive rates (multivoltine), extreme polyphagy and high dispersive powers (Ekesi *et al.*, 2007) which have contributed to the successful establishment of the species.

## 2.2 Origin of *Bactrocera invadens*

The African Invader fly, *Bactrocera invadens* (Drew, Tsurata and White) (Diptera: Tephritidae) is an invasive species (Vayssières *et al.*, 2008) which was initially thought to belong to the *Bactrocera dorsalis* Hendel complex of tropical fruit flies (Drew *et al.*, 2005). *B. invadens* was later recognized as a separate species whose native range was from Sri Lanka, Asia (Meyer *et al.*, 2014). *B. invadens* was introduced into Africa through trade which is the main pathway for the spread of *B. invadens* from one country to another (Ekesi and Mohamed, 2010). Though the African continent is the home of several fruit fly species, it has also become the entry point of many serious alien fruit fly species (Ekesi and Mohamed, 2010). In Africa, *B. invadens* was first reported in Kenya in 2003 (Ekesi and Mohamed, 2010; Meyer *et al.*, 2014).

## 2.3 Geographical Distribution

The African Invader fly, *Bactrocera invadens* is mainly a lowland pest though it is increasingly being observed at higher elevations (Wax, 2010). *B. invadens* occurs at low and mid-altitudes of 380-520 metres above sea level, but at high altitudes of 1650 metres above sea level the incidence of *B. invadens* is temporal (Mwatawala *et al.*, 2009; Ekesi and Mohamed, 2010). Apart from Kenya, *B. invadens* has been reported in Benin, Burkina Faso, Chad, Congo, Gabon, Ghana, Nigeria, Tanzania and Uganda among others (Vayssières *et al.*, 2008; Wax, 2010). Currently *Bactrocera invadens* is reported in over 35 countries in Sub-Saharan Africa (Plate 2.2) where it causes extensive economic losses to horticultural crops throughout the continent.



**Plate 2.2: Countries in Africa from which *B. invadens* has been reported (Meyer *et al.*, 2014).**

## 2.4 Host Plants

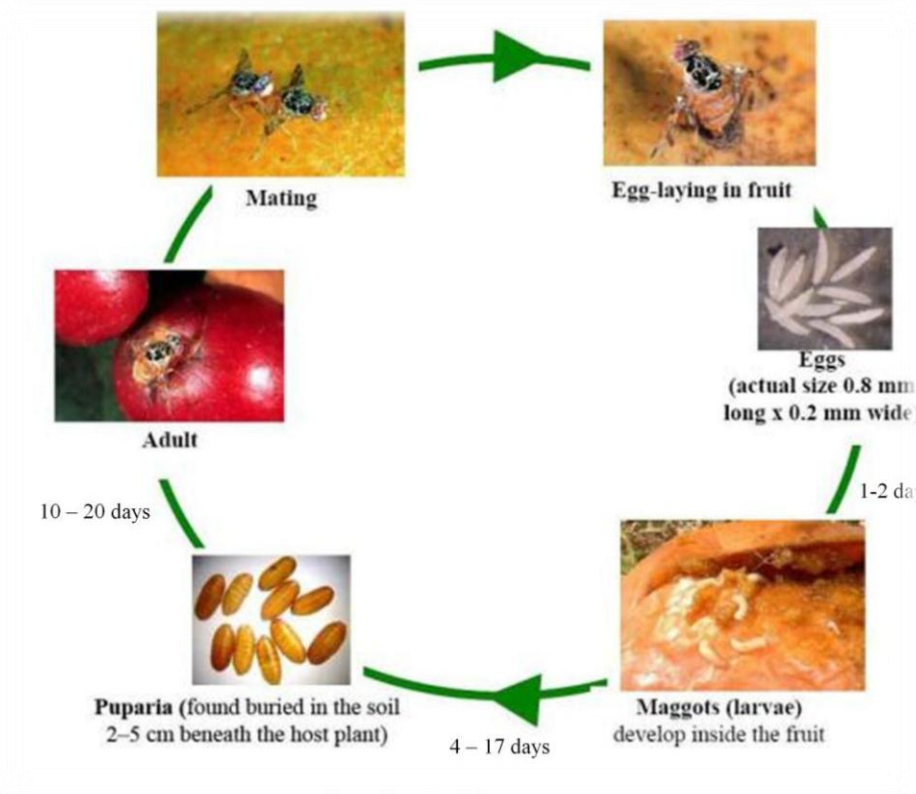
*Bactrocera invadens* is a highly polyphagous insect with several cultivated and wild plants as hosts. It attacks a wide range of unrelated families of crops (Meyer *et al.*, 2014). Though its entire host range is not known, mango (*Mangifera indica*) is its most preferred host (Wax, 2010). Other cultivated hosts include: guava (*Psidium guajava*), citrus fruits (*Citrus* spp.) pawpaw (*Carica papaya*), bush mango (*Irvingia gabonensis*), avocado (*Persea americana*), star apple (*Chrysophyllum albidum*), badamier (*Terminalia catappa*), tomato (*Lycopersicon esculentum*), banana (*Musa acuminata*). Wild species

such as Cider tree (*Sclerocarya birrea*), and Shea tree (*Vitellaria paradoxa*) act as reservoirs (Ekesi and Mohamed, 2010; Muchemi *et al.*, 2011). The wild reservoirs of *B. invadens* usually act as hosts when the target cultivated crop is not in season (Wax, 2010).

## **2.5 Biology and Ecology of fruit flies**

The life cycle of *B. invadens* like most fruit flies follow four stages; egg, larvae, pupae and adult (Plate 2.3). The female after mating lays eggs under the skin of the fruit which hatch into the larval stage (three larval instars), pupate and develop to the adult stage (CTA Practical Guide, 2007).

Adult females use their ovipositor to pierce both ripe and green fruits to deposit their eggs. Bacteria from the intestines of females are introduced into the fruits during egg deposition which causes necrosis of the tissues surrounding the egg (Infonet-biovision, 2012), whiles, the breached skin serves as a pathway for further bacteria invasion. Those localised rotten tissues become food for the young larvae when the eggs hatch.



**Plate 2.3: Typical life cycle of fruit flies (Wax, 2010)**

Eggs are white, slender; about 0.8 mm long x 0.2 mm wide and are laid under the fruit skin in groups of 3 - 8 (Infonet-biovision, 2012). They hatch within a day or two into the first instar larvae (Kumar *et al.*, 2011).

The larvae (also referred to as maggots) are whitish and feed within the fruit flesh creating more damaging galleries (Infonet-biovision, 2012). The larvae moult twice before leaving the fruit. The first instar larva is delicate, the second instar is more robust while the third instar is stout and tough (Kumar *et al.*, 2011). The third instar is about ten (10) mm in length and distinguished from the first and second instars by its creamy white appearance (Weems *et al.*, 2012). After about 4 - 17 days of feeding within the fruit, the

matured third instar larvae crawl out of the fruit to sheltered spots usually the soil to pupate (Infonet-biovision, 2012). Infected fruits, distinguished by their premature ripening and localised rot at the sites of ovipositor sting may drop to the ground just before the larva pupates.

Pupae are often found buried 2 - 5 cm within the soil just beneath the host plant. They are white but with age they become brown or black due to the level of sclerotisation and about 4 – 12cm long. Depending on the climate, the adult flies may emerge between 10-20 days to repeat the cycle (Infonet-biovision, 2012).

## **2.6 Papain production**

Generally, papain is obtained from the latex of unripe pawpaw fruits (Cholish, 2010) though alternative sources from fruit peels, leaves, petioles, stems and bark are still being investigated for papain activity (Baeza *et al.*, 1990; Espin and Islam, 1998). Large scale production of papain has made the study into other parts of the pawpaw plant necessary as the latex extraction from fruit is laborious and time consuming (Chaiwut *et al.*, 2007). Studies on the extracts obtained from pawpaw peel have shown the presence of proteolytic activity and the possibility of crude papain production from pawpaw peels (Arimura, 1989; Espin and Islam 1998; Chaiwut *et al.*, 2007). Other proteolytic enzymes that are not originally in the pawpaw fruit latex have also been observed in the latex from the non-fruit parts (Chaiwut *et al.*, 2007). Furthermore, differences in proteolytic activity

have been observed in the proteases extracted from newly wounded fruits and existing wounds (Brocklehurst and Salih, 1985).

Crude papain is usually extracted from the fully matured but unripe fruits while they are still on the tree (Shaimi, 2010). Ripe pawpaw contains less latex which is attributable to the cessation of the latex production or breakdown of the latex producing cells (Shaimi, 2010). Collected latex may be processed directly by drying (sun, oven or spray drying) to obtain crude latex, which is further processed into other papain grades or even purified to obtain enzymes with higher proteolytic activities.

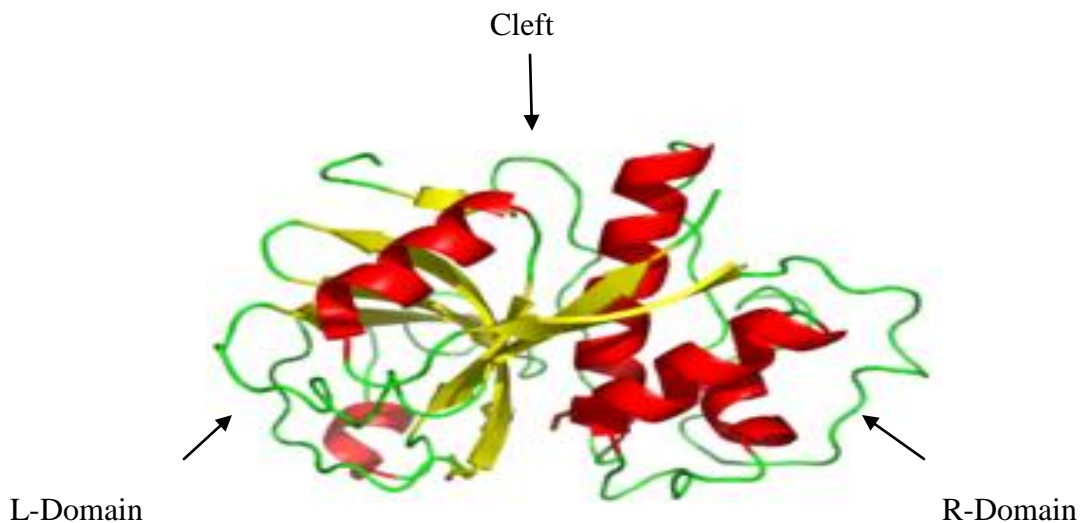
### **2.6.1 Molecular structure of papain**

Papain or papaya peptidase I is a cysteine protease of the peptidase C1 family sourced from pawpaw (Sigma-Aldrich, 2014). Pawpaw latex is composed of four cysteine proteases which contribute 69-89 % of total protein; 26 - 30 % chymopapain, 23 - 28 % glycyl endopeptidase, 14 - 26 % caricain and less than 10 % papain (Shaimi, 2010). However the percentage composition of these proteolytic enzymes vary in the fruit, leaves and roots (Shaimi, 2010). Though papain is the least enzyme in terms of amount present in the pawpaw latex, it is the most studied because it is easy to purify (Worthington Biochemical Corporation, 2014).

The related proteins of papain are endopeptidases, aminopeptidases, dipeptidyl peptidases and enzymes with both exo- and endo-peptidase activity (Rawlings and Barrett, 1994). The enzyme commission number of papain is EC 3.4.22.2. Papain is EC 3 because it can break peptide bonds in the presence of water (hydrolytically), EC 3.4 because it acts on

peptide bonds and EC 3.4.22 because it is a cysteine endopeptidases. Papain has a molecular weight of 23, 406 daltons (amino acid sequence), an optimum temperature range of 65 - 80 °C and an optimum pH range of 6 - 7 (Sigma - Aldrich, 2014). Papain is activated by chemicals such as cysteine, sulphites, sulphide and enhanced by heavy metal binding agents such as ethylene diamine tetra acetic acid (EDTA) (Rathi and Gadekar, 2007).

Papain consists of a single polypeptide chain made up of 212 amino acid residues stabilized by three disulfide bridges and a sulfhydryl necessary for the activity of the enzyme (Shaimi, 2010; Sigma-Aldrich, 2014). Papain has a three dimensional ribbon shaped molecular structure (Plate 2.4) that is divided into the L and R domains and separated by a deep cleft which contains the active site (Shaimi, 2010).



**Plate 2.4: Molecular structure of papain**

([http://www.en.wikipedia.org/wiki/File:Papain\\_cartoon.png](http://www.en.wikipedia.org/wiki/File:Papain_cartoon.png))

### **2.6.2 Uses of Papain**

Papain is characterized by its ability to hydrolyse large proteins into smaller peptides and amino acids which can be readily absorbed by the body (Ming *et al.*, 2002). The enzyme has applications in food processing, medicine, textile and agriculture (Nitsawang *et al.*, 2006; Puig *et al.*, 2008). In food processing, papain is used extensively for tenderizing meat, clotting milk for cheese production, in the production of chewing gum, in brewery for removing cloudiness in beer; for the reduction of viscosity in the beer and increasing the palatability of pet food among others (Puig *et al.*, 2008; Royal Tropics, 2014). In medicine, papain is usually used in topical formulations for treatment of burns, wounds, scars and peeling, used for the treatment of insect stings, treating indigestion, treating inflammatory processes and cleaning soft contact lenses (Royal Tropics, 2014). Papain is also used in the textile industry for shrink proofing wool and tanning leather (Royal Tropics, 2014).

### **2.7 Effects of irradiation on papain**

Papain is a proteolytic enzyme and like all enzymes it shows appreciable destruction under ionizing radiation (Mounter, 1960). The efficiency of inactivating an enzyme by radiation is calculated based on the complete destruction of enzymatic activity in an individual molecule (Mounter, 1960). Irradiation of proteins with gamma rays generates specific irreversible structural and chemical changes at the molecular level (Lee and Song, 2002). These changes are as a result of the breakage of the polypeptide chains. Among the many changes of proteins that results from radiation damage are molecular

fragmentation, cross-linking, aggregation, decarboxylation, breakage of disulphide bands, formation of disulphide radicals, release of aromatic amino acids, loss of helicity, dephosphorylation, chain cleavage and oxidation by oxygen radicals generated from the radiolysis (Lee and Song, 2002; Wang *et al.*, 2010). In effect, radiation damage of enzymes can be attributed to a disorder in the macromolecule leading to a reduction in enzymatic activity (Mounter, 1960). Despite this, the effect of an irradiation process on protein conformation is dependent on other factors such as protein concentrations, the presence of oxygen and the quaternary structure of the proteins (Lee and Song, 2002).

Furthermore, the effects of irradiation on a biologically active material such as papain enzyme differ from dry enzyme samples and when enzyme is in solution. When a dry enzyme sample is inactivated by irradiation, the inactivation is attributable to the direct receipt of the energy from the incident ray, while enzymes in aqueous solutions can further be inactivated by the indirect effect of the radiolysis of their aqueous environment (Furuta *et al.*, 2000). The process of inactivation of papain in its functional hydrated state is due to the generation of hydrogen peroxide ( $H_2O_2$ ) produced from the radiolysis of water leading to the oxidation of the active SH-group to Sulfenic acid (Lin *et al.* 1975). In aqueous solutions, enzymes are destroyed by the smallest radiation dose due to direct and indirect effects of ionizing radiation (Mounter, 1960). However, frozen suspension of papain responds to radiation effects in a manner similar to dry and immobilized papain samples (Furuta *et al.*, 2000).

### **2.7.1 Radiation for increasing virulence of papain**

The polymeric nature of papain makes it susceptible to physical and chemical changes especially at the active centre under high energy electron beam radiation (Muthulakshmi *et al.*, 2012). The irradiation power of ultrasound for instance, inhibits the proteolytic activity of papain (Yu *et al.*, 2014). Ultrasonic treatment of papain results in a modification of papain's proteolytic activity due to the variations in the secondary and tertiary structures (Yu *et al.*, 2014). On the other hand, papain is radioresistant to high gamma radiation doses such as 30 KGy (Furuta *et al.*, 2000). Though proteolytic activity of papain may decrease with increasing radiation dose, the direct effects of high radiation doses on proteolytic activity is not significantly affected (Singh and Singh, 2012; Furuta *et al.*, 2000; Zulli *et al.*, 2010). More so, the electrical conductivity of papain has been found to increase with exposure to radiation and this electrical conductivity has a good correlation with proteolytic activity (Muthulakshmi *et al.*, 2012). Exposure of papain to electron beam increases proteolytic activity of papain two folds for doses between 1 and 10 KGy mainly due to the influence of the electron beam radiation on the active centres of the papain (Muthulakshmi *et al.*, 2012).

### **2.7.2 Radiation for sterilization of papain**

Papain has significant bactericidal effects on gram positive and negative bacteria and thus it has several applications in the medical field (Muthulakshmi *et al.*, 2012; Singh and Singh, 2012). Despite this, the low stability nature of papain has made it unsuitable for commercialization in defined pharmaceutical products (Singh and Singh, 2012).

In a study to evaluate the sensitivity of papain irradiated with Cobalt-60 gamma rays for the purpose of sterilization, dry papain powder and frozen aqueous papain were found to be radioresistant (Furuta *et al.*, 1999). The two specimens showed significant resistance to changes in proteolytic activity after 30 KGy irradiation. In contrast, liquid papain (10 mg/ml), showed significant drop of proteolytic activity at lower doses of irradiation (ca. 0.5 KGy) but 40 % of the activity was recovered at ca. 3 KGy before total inactivation at 15 KGy (Furuta *et al.*, 1999). Despite the radiosensitivity of liquid papain, dry papain can show radioresistance at the optimal irradiation conditions and therefore, gamma radiation can be easily used as sterilizing agent without affecting the papain release profile or activity (Zulli *et al.*, 2010).

Furthermore, irradiating pharmaceutical products such as papain-incorporated chitin meant for wound dressing does not affect the release of papain in the membrane, suggesting a significant resistance to irradiation (Singh and Singh, 2012).

## **2.8 Impact of purification on the quality of papain**

Pawpaw latex can be directly sun-dried or oven-dried to obtain crude papain flakes, but, a purification step is necessary to reduce any contaminating substances (Saha and Swati, 2013). Reducing the contaminating substances (other proteases) increases the proteolytic activity of the papain. Purification and separation techniques of papain have increased greatly since papain was first crystallized in 1968 (Worthington Biochemical Corporation, 2014). Precipitation method is the conventional method for papain purification (Burke *et al.*, 1974). Ammonium sulphate  $[(\text{NH}_4)_2\text{SO}_4]$  is the salt commonly

used for the precipitation and it usually results in papain in three forms; active papain, activatable papain and non-activatable papain (Burke *et al.*, 1974). However, ammonium sulphate,  $[(\text{NH}_4)_2\text{SO}_4]$  precipitation yields enzymes contaminated with other proteases (Nitsawang *et al.*, 2006).

Further purification of crude papain extracts may involve the use chromatographic techniques and gel filtrations. Ion exchange chromatography purifies based on the molecular charges of the enzyme solution, while, bioaffinity chromatography is based on the molecular interaction of the enzyme (Shaimi, 2010). Though the advance purification methods are effective, their efficiency is dependent on the initial extraction process (Nitsawang *et al.*, 2006). As such recent advancement in the extraction of papain combines a salt phase and a polymer phase or two polymer phases in an aqueous two-phase system (Nitsawang *et al.*, 2006). This technique provides a suitable procedure of obtaining pure papain that is free of any contaminating proteases.

## **2.9 Attractance and suitability of protein baits for fruit fly control**

Food baits impregnated with insecticides are among the common fruit fly control methods the world over (Ekesi and Mohamed, 2010). The main component of these baits is protein and they were previously manufactured by hydrolysing a plant protein with hydrochloric acid (acid hydrolysis). Baits prepared from protein are complex mixtures of hydrolysed proteins from sources such as soybean, yeast, millet, among others (Yeboah, 2012). Today, protein baits produced locally by yeast autolysis facilitated by enzymatic proteolysis is being used with significant success (Gopaul and Price, 1999). These locally

produced baits are generally as attractive as the imported protein hydrolysate in McPhail traps (Yeboah, 2012). In contrast, other workers have found baits produced by yeast autolysates more attractive to fruit flies than baits from acid hydrolysis due to the possible presence of salts in the latter (Allwood, 1997).

The attraction of fruit flies to bait depends on the nutritional composition of the bait especially because these protein baits act as food source (Gopaul and Price, 1999). It is also dependent on factors such as bait pH, bait concentration, and release of volatiles such as ammonia ( $\text{NH}_3$ ) among others (Sookar *et al.*, 2003). Protein baits with higher crude protein content results in the release of more essential nutrients which are also responsible for attracting fruit flies to feed (Sookar *et al.*, 2003). There are several assays for evaluating the attractance and feeding preference of a fruit fly to an attractant (Barry *et al.*, 2006). In laboratory and field bioassays, experiments involving attractance and feeding behaviour provide conclusive evidence for the performance of a protein bait treatment in field applications (Barry *et al.*, 2006).

Though protein bait lures may not be as attractive as the parapheromones, they should be attractive enough to be detected by the fruit flies in the field (Vargas *et al.*, 2002). Attractance measurement of protein baits odour is usually established with the attractant (odour) released on one side of an olfactometer and clean air released from the other arm (Stelinski and Tiwari, 2013). In olfactometer tests, a choice is made when the organism crosses into either regions (Stelinski and Tiwari, 2013).

Feeding experiments like attractance measurement have an increased importance in bioassays as most of the insecticides used in protein baits treatments are stomach poisons rather than contact poisons (Barry *et al.*, 2006). Measurement of feeding duration in bioassays has more frequently been based on labella contact with the substrate though this may vary from scientist, genus of study and cohort (Barry *et al.*, 2006). Multiple odour fields from choice assays can result in flies selecting baits they did not detect first, thus the need to rotate cage position during assay. Though applying such precautionary measures in bio-assay would provide the needed results, there is enough evidence of the variation in the attractance behaviour of laboratory reared flies used in bioassay and wild flies used in field bioassay often due to the artificial selection in laboratory (Cayol, 2000). As such, same protein bait treatments used in the laboratory and field bioassay may perform differently from each other (Sookar *et al.*, 2002).

Assessing the efficacy of protein baits is essential to an effective control of the fruit flies in the field. However, a successful control of the fruit flies would be achieved when the protein baits and bait ingredients are readily available to the major stakeholders. Thus for a sustainable fruit fly control, it is essential to focus on local ingredients for protein bait production.

## 2.10 REFERENCES

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## CHAPTER 3

### 3.0 TO EVALUATE A STANDARD PROTOCOL FOR EXTRACTING PURE PAPAINE FROM PAPAINE FRUIT LATEX AND SKIN PEELS.

#### 3.1 Background

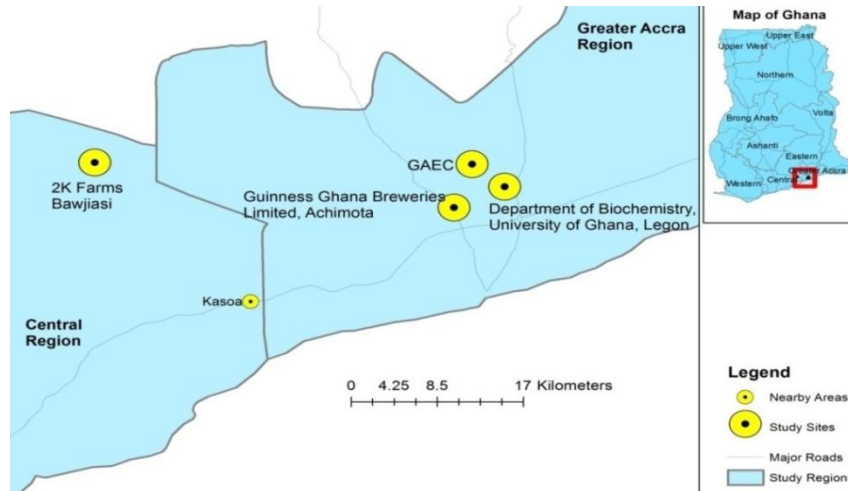
Papain is a minor constituent among four major proteolytic enzymes obtained from pawpaw fruit latex, skin peel and pawpaw leaves (Worthington Biochemical Corporation, 2014). It accounts for less than ten (10) % of the total proteolytic enzymes in pawpaw and therefore, an efficient purification of papain should consider a process that achieves the highest purification in few steps (Chaiwut *et al.*, 2007; Shaimi, 2010). Isolating the target protein is an essential first step in purification. Traditional extraction of papain involves precipitation of the pawpaw latex with ammonium sulphate which results in an enzyme contaminated with other proteases (Nitsawang *et al.*, 2006). Alternative extraction procedures, such as, the aqueous two-phase extraction protocol have proven to be more efficient than the traditional Ammonium sulphate extraction (Li *et al.*, 2010; Chaman Mehta *et al.*, 2013). This system of extracting papain is even more significant as the enzyme is extracted in a single unit and it further integrates clarification, concentration and purification into a one step process (Nitsawang *et al.*, 2006; Li *et al.*, 2010). Thus, the aqueous two phase extraction method can be used for commercial production (Li *et al.*, 2010).

Papain has diverse applications but its application is significant in fruit fly control during the proteolysis of proteins meant for protein bait production. In Ghana, scientists have found that protein bait can be produced from brewery yeast waste but the papain component can still influence the production cost. Papain is not produced in Ghana; therefore the cost of importation, clearance duties and the delays of importation can affect the production of the local protein baits. Currently, no known standard protocol has been evaluated for producing pure papain from local pawpaw varieties in Ghana. Hence, identifying and evaluating an efficient extraction process would be a significant first step to producing pure papain from local pawpaw varieties. This would aid in the timely and cost effective production of protein bait.

The aspect of the study reported in this Chapter focused on the evaluation of a standard protocol for extracting pure papain from pawpaw fruit latex and skin peel.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Study sites



**Plate 3.1** Map of Southern Ghana showing study sites

A study was conducted on a “Red lady” pawpaw variety obtained from a one (1) acre Research and Development plot of 2-K Farms Limited located at Bawjiase in the Central Region of Ghana (Plate 3.1). Bawjiase is a farming community within the Awutu-Senya District Assembly about 58 km from the Ghana Atomic Energy Commission and 24.9 km from Kasoa.

The vegetation of the Awutu Senya District is semi-deciduous forest in townships such as Bawjiase, Bontrase, Nyarkokwaa and Osae-Krodua and coastal savannah grassland along the southern coastal areas of Senya and its environs (Ministry of Food and Agriculture, 2013). Bawjiase is therefore a semi-deciduous forest zone characterised by isolated undulating highlands (Ministry of Food and Agriculture, 2013). It experiences the dry

season from November to March followed by a rainy season which starts from April and ends in October (Ministry of Food and Agriculture, 2013). During this period, the moist south-west monsoon blow across the area (Ministry of Food and Agriculture, 2013). The mean annual rainfall ranges between 50 cm and 70 cm and mean annual minimum and maximum temperatures of 22 °C and 28 °C respectively (Ministry of Food and Agriculture, 2013).

The elevation of the area is 147 metres and lies between latitude 05.65764 °N and longitude 00.54970 °W.

The rainfall pattern coupled with the loamy soil type support cassava, cocoa, maize, oil palm, pawpaw, pineapple, plantain, and yam cultivation among others (Ministry of Food and Agriculture, 2013).

Papain extraction and analysis were conducted at the Department of Biochemistry Cell and Molecular Biology, University of Ghana, Legon, Accra, Ghana about six (6) kilometres from GAEC (Plate 3.1).

### **3.2.2 Extraction of crude papain from pawpaw fruit latex and skin peel**

Latex was tapped from matured green pawpaw fruits on the pawpaw tree by first wiping with cotton wool soaked with distilled water to rid it off any dust or debris. Longitudinal incisions about 2 mm deep were made on matured green pawpaw fruits using a stainless steel knife (Plate 3.2 a). Latex from the fruits was collected into a plastic bowl till flow ceases, usually within 2 - 3 minutes. Samples of latex collected from all the fruits were

stored in a Thermos™ cooler with icepacks and transported to the laboratory. A minimum of four fruits each were tapped from a total of ten (10) trees. When a pawpaw fruit was tapped the first time, two incisions were made and the incisions were increased on subsequent extractions. In the laboratory, latex was frozen at  $-20^{\circ}\text{C}$  to prevent enzyme degradation till ready to be used.



Plate 3.2 a. Papain raw material extraction; Fruit latex extraction



Plate 3.2 b. Papain raw material extraction; Fruit skin peel extraction

Papain was also extracted from pawpaw skin peel dissolved in water (Chaiwut et al., 2007). Five (5) green pawpaw fruits, between 90 – 100 days old were plucked, washed with distilled water and peeled with a multi-purpose fruit and vegetable slicer into thin slices 1 mm thick (Plate 3.2 b). Pawpaw fruit skin peels were spread on plastic trays and oven dried at 55 °C for 24 hours. The dried peels were ground in a blender to produce a fine powder of the peels. Ten (10) grams of the ground pawpaw skin peels were soaked in 100 ml of distilled water for 30 minutes. The solution was filtered through a funnel padded with cotton wool to rid the pawpaw solution of any debris and divided into 2 portions. One portion was further processed by centrifugation at 5000 x g for 30 minutes at 4 °C to obtain crude extract which typically contains: papain, chymopapain and other constituents. The pawpaw skin peel solution was prepared fresh on the day of extraction and stored on ice in Thermos™ cooler till ready to be used. The second portion of the skin peel solution was used for aqueous-two-phase extraction of pawpaw skin peel.

### **3.2.3 Aqueous two-phase extraction**

The aqueous two-phase extraction protocol used in this study was based on the method described by Nitsawang *et al.*, (2006) with slight modifications. In their studies on papain extraction, Nitsawang *et al.*, (2006) concluded that the ideal conditions of extracting papain from fresh pawpaw latex are that the latex should have initial protein concentration of 40 mg/ml, pH of 5 and 8 % Polyethylene Glycol (PEG) – 15 % (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> phase concentration. However, preliminary extraction of papain from the “Red lady” pawpaw fruit latex with 40 mg/ml initial protein concentration yielded no visible bands of protein on Sodium Dodecyl Sulphate-Polyacrylamide Gel

Electrophoresis (SDS-PAGE) analysis. Therefore, in this study, the aqueous two-phase extraction of pawpaw fruit latex was performed with latex of initial protein of concentration  $4443.30 \pm 30.93$  mg/ml while the purity of extracts and the presence of papain were confirmed on Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

A solution of 1.25 ml of the pawpaw fruit latex and 15 ml skin peel were used for the papain extraction. Each sample was prepared in a 50 ml Perspex glass beaker of predetermined weight. The pawpaw fruit latex extraction was performed independent of the skin peel extraction. For each extraction, the glass beaker was placed on an electronic weighing balance and the sample poured into it. The fruit latex and skin peel solutions were diluted with distilled water to a weight of 30 g, while on the balance. A 2.55 g of Ammonium sulphate  $(\text{NH}_4)_2\text{SO}_4$  and 2.4 g Polyethylene glycol 6000 were dissolved in each of the different samples and further diluted with distilled water to obtain a final weight of 50 g.

The different sample mixtures were transferred into 50 ml falcon tubes and shaken gently for 15 minutes on a shaker. The pH of each mixture was adjusted to 5.00 using drop wise 6 M NaOH or 1 M HCl where necessary. The mixtures were centrifuged at  $5000 \times g$  for 30 minutes at  $4^\circ\text{C}$  to separate the liquid phase from the solid phase.

Aliquots of the two phases from both fruit latex and skin peel samples were taken for the proteolytic activity and protein concentration determination. A five microlitres (5  $\mu\text{l}$ ) of the bottom phases of both skin peel and latex were diluted to 5 ml with distilled water

due to the phase concentration and these were used for the proteolytic activity and protein determination assays. The total proteolytic activity and total protein content of the different phases were calculated using the volume of the different phases after centrifugation. The pawpaw skin peel supernatant solution from the aqueous two-phase extraction was compared with the pawpaw skin peel crude extracts based on the differences in proteolytic activity, protein concentration and specific activity.

### **3.2.4 Protein content determination**

The protein concentrations of the seven (7) different papain samples consisting of; crude latex, latex supernatant solution, latex crystals on supernatant, latex bottom phase, crude extract of pawpaw skin peel, skin peel supernatant and skin peel residue (bottom phase) were determined based on the Folin-Lowry assay (Lowry *et al.*, 1951) following the protocol outlined in the Department of Biochemistry, Cell and Molecular Biology of the University of Ghana laboratory manual. This assay produces a blue chromophore which is the result of 2 reactions. The first reaction is the formation of the coordination bond between peptide bond nitrogens and a copper ion, and the second reaction is the reduction of the Folin-Ciocalteu reagent by tyrosine when the phosphomolybdic and phosphotungstic acids of the reagent react with phenol. Details of the preparation of reagents and solutions preparation can be seen in Appendix 1.

Sixteen (16) graduated falcon tubes each of volume fifteen millilitres (15 ml) were set for the Bovine Serum Albumin (BSA) standard curve. In this method, 5 ml of the alkaline test solution was pipetted into the clean and dry tubes and 0.5 ml each of the different BSA

concentrations was added to each sample. The resulting mixtures were incubated at room temperature for 15 minutes on the laboratory bench.

The diluted Folin - Ciocalteau reagent (0.5 ml) was added to the resultant mixture of the alkaline test solution and the BSA in each falcon tube. The mixture was incubated for a further 30 minutes at room temperature after which the mixture was centrifuged at 5000 x g for 5 minutes. The absorbance value of the mixture was read at 750 nm using 3 ml plastic cuvettes in the Genesys UV/VIS spectrophotometer. Each treatment was replicated three times and a blank for the assay in which BSA was replaced with distilled water was also prepared and tested following the same procedure.

The absorbance value of the blank was deducted from the absorbance value of the different concentrations of the BSA-alkaline test solution mixtures to obtain the actual absorbance of the sample. The three (3) absorbance readings of each concentration were summed up and averaged to obtain the mean absorbance per concentration. A scatter graph was plotted using Microsoft excel with the absorbance values on the y-axis and concentration on the x-axis. A line of best fit and its corresponding regression equation were determined in Excel. The BSA standard curve and its regression equation were used in the protein concentration determination.

Three (3) graduated falcon tubes (three replicates) each of volume 15 ml were set for each of the protein samples to be tested. In this method, five (5) ml of the alkaline test solution was pipetted into the clean and dry tubes and 0.5 ml each of the different papain

samples were added to each sample. The resulting mixtures were incubated at room temperature for fifteen (15) minutes on the laboratory bench.

The diluted Folin - Ciocalteu reagent (0.5 ml) was added to the resultant mixture of alkaline test solution and papain sample in each falcon tube. The mixture was incubated for a further 30 minutes at room temperature. Within the period of incubation, the pH of the solution was adjusted using drops 1 M HCl or 6 M NaOH where necessary to a pH value between 10 - 10.5. The mixture was centrifuged at 5000 x g for 5 minutes to rid the solution of any solid suspensions before absorbance readings of the supernatant were taken at  $A_{750}$  nm using 3 ml plastic cuvettes in the Genesys UV/VIS spectrophotometer. Each treatment was replicated three times and a blank for the assay in which the papain sample was replaced with distilled water was also prepared and tested following the same procedure.

The absorbance value of the blank was deducted from the absorbance value of the test papain sample to obtain the actual absorbance due to the protein sample.

### **3.2.5 Evaluation of the proteolytic activity**

The proteolytic activity of the papain was determined using the casein hydrolysis method where casein acts as the substrate on which the enzyme acts (Nitsawang *et al.*, 2006; Sigma Aldrich, 2014). When papain hydrolyses a protein substrate, the amino acid tyrosine is released, and its absorbance can be measured at 275 nm. The assay was prepared in a fifteen millilitre (15 ml) graduated falcon tube, three (3) tubes for each

papain sample including a blank. Details of the reagents and solutions prepared for the proteolytic activity of papain can be seen in Appendix 2.

A tyrosine standard curve was generated by measuring the absorbance value of different concentrations of tyrosine solution. Eleven (11) graduated 15 ml falcon tubes were filled each with 0.02, 0.04, 0.06, 0.08, 0.10, 0.12, 0.14, 0.16, 0.18, 0.20, 0.22 mls of 1.1 mM tyrosine standard stock solution. Each falcon tube and content was topped up with distilled water to the 2 ml mark, while the blank was set with 2 ml distilled water only. The absorbance of the different tyrosine solutions and the blank were measured in a Genesys UV /VIS spectrophotometer at 275 nm using a 3 ml glass cuvette.

The absorbance value of the blank was deducted from the absorbance value of the different concentrations of the tyrosine solution to obtain the actual absorbance due to the papain sample. A scatter graph was plotted using Microsoft Excel with the absorbance values on the y-axis and amount of tyrosine in  $\mu\text{mols}$  on the x-axis. A line of best and its corresponding regression equation was determined in Excel. The tyrosine standard curve and its regression equation ( $y = 4.72x + 0.02$ ) were used to determine the amount of tyrosine released by each papain sample.

A seven hundred (700)  $\mu\text{l}$  of the Tris-HCl buffer was mixed with 200  $\mu\text{l}$  of the reaction solution; (a mixture of Cysteine and EDTA) and 100  $\mu\text{l}$  papain solutions. The mixture was incubated in a water bath at 37  $^{\circ}\text{C}$  for 5 minutes. After the period, the reaction of papain on casein substrate was initiated with the addition of 1 ml milk casein. The mixture was incubated at room temperature for 10 minutes on the laboratory bench.

Three (3) ml of 5 % TCA was added to the mixture to stop the reaction. The mixture was cooled for 1 hour on ice and then centrifuged at 5 000 x g for 10 minutes. For each papain solution, a blank was prepared by adding the enzyme solution after the addition of the TCA in order to account for the absorbance due to the enzyme only. The absorbance of the supernatant solution was measured in Genesys UV/VIS spectrophometer at 275 nm using a 3 ml glass cuvette.

The amount of tyrosine ( $\mu\text{mols}$ ) liberated by each solution (extrapolated from the tyrosine standard curve) was used to calculate the proteolytic activity of the papain sample. The  $\mu\text{mols}$  of tyrosine were substituted in a standard equation (Appendix 3.1) to obtain the proteolytic activity in tyrosine units/ml (Sigma Aldrich, 2014).

The proteolytic activity calculated in Tyrosine units/ml for each sample was multiplied by its corresponding volume in ml to obtain total proteolytic activity per phase extract (Appendix 3.2).

Data obtained from the proteolytic activity and protein determination of the different phases were used to calculate the specific activity, purification fold, percentage yield and, partition coefficient of the aqueous two-phase extraction.

Phase volume ratio was defined as the ratio of volume in the top phase and the volume in the bottom phase (Ratanapogleka, 2010) (Appendix 3.3).

The partition coefficient of the aqueous two-phase extraction was calculated as the ratio of the protein concentration in the top phase to the protein concentration in the bottom phase (Senthilkumar and Kumaresan, 2013) (Appendix 3.4).

The yield recovery of each phase extracts (fruit latex extracts and skin peel extracts) were calculated using the calculated phase volume ratio and partition coefficient (Ratanapogloeko, 2010) (Appendix 3.5).

The specific activity of each papain extract was calculated as a ratio of the proteolytic activity to the protein concentration of the same phase (National Science Foundation, 2006) (Appendix 3.6).

The purification fold was calculated as the ratio of the specific activity of the phase extract to the specific activity of the crude sample (National Science Foundation, 2006) (Appendix 3.7).

Percentage yield was calculated as the ratio of the proteolytic activity in the phase extract to the proteolytic activity of the crude sample expressed as a percentage (National Science Foundation, 2006) (Appendix 3.8).

### **3.2.6 Test for the presence and purity of papain on Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS – PAGE)**

The papain samples were prepared by mixing each papain sample with the sample buffer in a ratio of 3:1 (30  $\mu$ l papain sample: 10  $\mu$ l of sample buffer). Details of the gel assembly, reagents and buffer prepared for SDS-PAGE are provided in Appendix 4.

Thirty (30)  $\mu$ l of the protein samples were loaded into wells in the gel (within each well) using a 50  $\mu$ l pipette. The electrophoresis cell was connected to the power source and run at a constant current of 100 volts for three (3) hours until the solvent front was within one (1) cm of the gel base. The gel was stained with 10 ml of 0.1 % (w/v) Coomassie Blue over night. The dye was drained off the gel in the morning and washed in three changes of the destaining buffer to obtain clear and visible bands of the different papain samples where necessary.

The presence of papain was confirmed with visible bands obtained from commercial papain and the broad range molecular weight marker.

### **3.3 DATA ANALYSIS**

The absorbance readings of amino acid tyrosine obtained from the proteolytic activity determination, the data from protein concentration determination, specific activity, phase recovery, percentage activity yield, and partition coefficient were analysed using SPSS version 16.

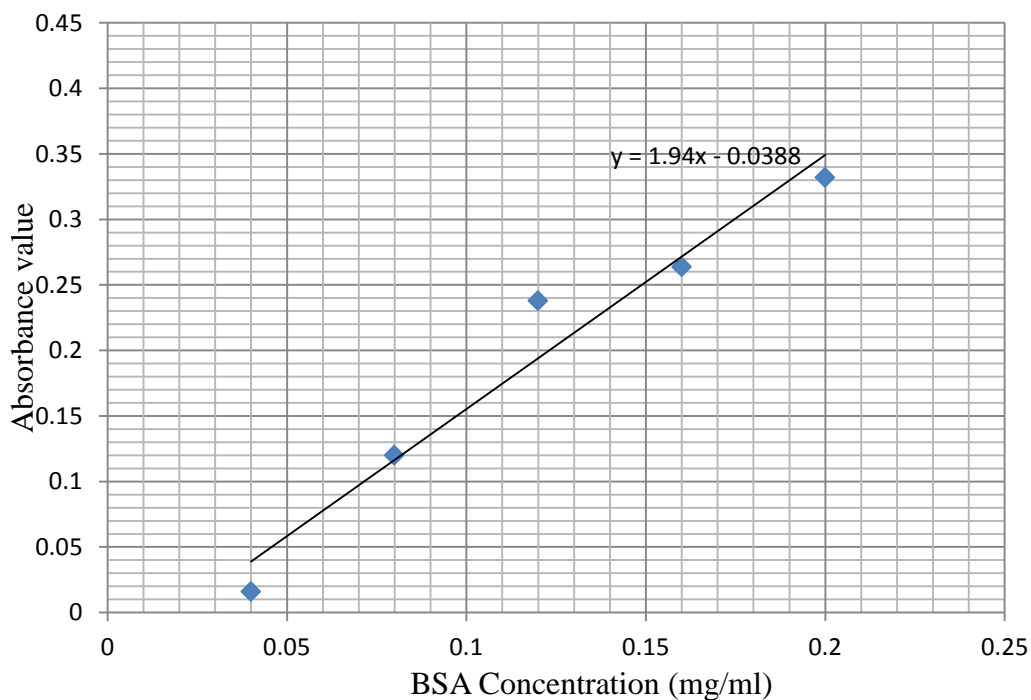
Data were further subjected to a one-way analysis of variance for the determination of differences between means, and when significant differences were observed, a Tukey HSD test was used for mean separation. A p value of 0.05 or less was considered for statistical significance level. The analysed results were presented in tables and figures.

Regression equation was used to generate the equations for the tyrosine standard curve and BSA standard curve.

### 3.4 RESULTS

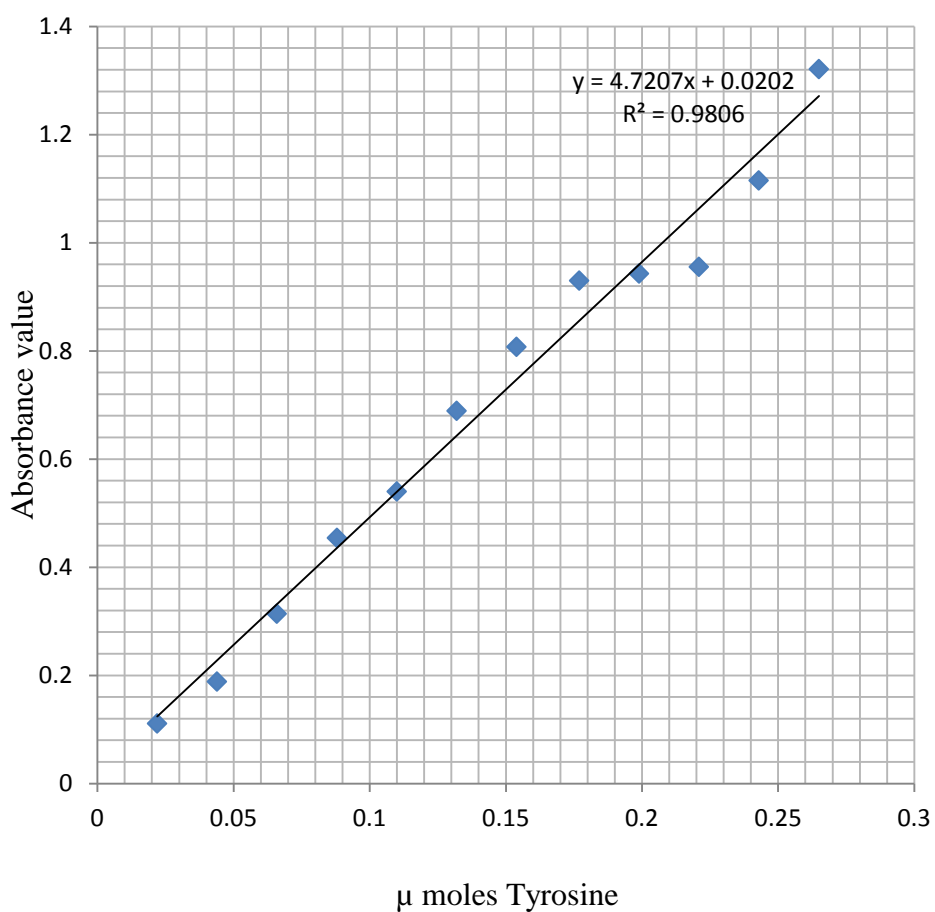
#### 3.4.1 Standard curves

The protein concentration of the different test protein solutions were calculated by substituting their resulting absorbance readings into the regression equation of the BSA standard curve (Figure 3.1). The BSA standard curve had a negative intercept of -0.038 and a gradient of 1.94 (Figure 3.1). Absorbance values of the different concentration of BSA samples increased linearly across the gradient with a mean minimum absorbance of 0.016 nm (0.04 mg/ml BSA) and mean maximum absorbance of 0.332 nm (02 mg/ml BSA) (Figure 3.1).



**Figure 3.1** Bovine Serum Albumin Standard curve for protein concentration determination.

The amount of tyrosine measured from the proteolytic activity was extrapolated from the tyrosine standard curve using the regression equation (Figure 3.2). The tyrosine standard curve had a positive gradient and intercept of 4.720 and 0.020 respectively (Figure 3.2). The absorbance value of the tyrosine increased with increasing amount ( $\mu$  mols) of tyrosine between 0.111 nm (0.022  $\mu$  mols) and 1.321 (0.265  $\mu$  mols).



**Figure 3.2 Tyrosine standard curve for proteolytic activity determination**

### **3.4.2 The efficiency of the aqueous-two-phase extraction of PEG-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> for extracting pure papain from pawpaw fruit latex and skin peel.**

Pawpaw fruit latex sample subjected to aqueous two-phase extraction separated into two distinct phases; a white milky precipitate at the bottom and a clear supernatant. However, some of the proteins within the supernatant crystallised out of the solution to form a white film above the supernatant phase after centrifugation. As such, the white crystals within the supernatant were picked along with some of supernatant and this accounted for 14.33 % (6.98 ml) of the total volume of the extracts. The clear supernatant accounted for 82.78 % (40.31 ml) while, the bottom phase accounted for 2.89 % (1.41 ml) of the total volume. The calculated phase-volume ratio of the aqueous two-phase extraction of latex was 33.54 (Table 3.1).

The skin peel subjected to aqueous two-phase extraction also separated into two distinct phases; the supernatant phase accounted for 98.56 % (47.99 ml), while, the residue accounted for 1.44 % (0.7 ml) of the total volume of the extraction mixture (Table 3.1).

The proteolytic activity and protein concentration of the crystallised supernatant of the fruit latex ( $2.25 \pm 0.18$  U;  $1.70 \pm 0.14$  mg) and supernatant solution ( $14.71 \pm 0.30$  U;  $9.48 \pm 0.06$  mg) differed significantly ( $p = 0.00$ ) from the proteolytic activity and protein concentration of the bottom phase ( $214.57 \pm 6.97$  U;  $210.77 \pm 1.16$  mg) and the crude pawpaw latex ( $231.53 \pm 4.86$  U;  $5554.13 \pm 30.93$  mg).

**Table 3.1. Protein concentration and proteolytic activity of pawpaw latex and skin peel extracts before and after the aqueous two-phase extraction.**

Sample	Total volume (ml)	Percentage phase volume of the total (%)	Phase volume ratio	Total proteolytic activity / Tyrosine units (U)	Total protein concentration (mg)
<u>Latex</u>					
Crude latex	1.25	-	-	231.53 ± 4.86 <sup>a</sup>	5554.13 ± 30.93 <sup>a</sup>
Crystallised supernatant	6.98	14.33	33.54 <sup>*</sup>	2.25 ± 0.18 <sup>c</sup>	1.70 ± 0.14 <sup>f</sup>
Supernatant solution	40.31	82.78	-	14.71 ± 0.30 <sup>b</sup>	9.48 ± 0.06 <sup>f</sup>
Bottom phase	1.41	2.89	-	214.57 ± 6.97 <sup>a</sup>	210.77 ± 1.16 <sup>b</sup>
<u>Skin peel</u>					
Crude extract	14.50	-	-	6.38 ± 0.07 <sup>c</sup>	12.11 ± 0.22 <sup>e</sup>
Supernatant solution	47.99	98.56	68.56 <sup>*</sup>	19.89 ± 1.26 <sup>b</sup>	23.59 ± 0.36 <sup>d</sup>
Bottom phase	0.70	1.44	-	217.53 ± 1.48 <sup>a</sup>	94.18 ± 4.33 <sup>c</sup>

Means in the column with the figure \* as superscript reflects the result for both phases of the extraction.

Means in the same column with the same lower case alphabet as superscript are not significantly different at the 0.05 confidence level.

There was no significant difference ( $p = 0.654$ ) between the proteolytic activity of the latex bottom phase and the crude latex, though, protein concentration of the crude latex ( $5554.13 \pm 30.93$  mg) was higher ( $p = 0.00$ ) than the bottom phase ( $210.77 \pm 1.16$  mg) (Table 3.1).

Aqueous two phase-extract of pawpaw fruit skin peel bottom phase had a significantly higher ( $p = 0.05$ ) proteolytic activity ( $217.53 \pm 1.48$  U) and protein concentration ( $94.18 \pm 4.33$  mg) than the supernatant solution [proteolytic activity ( $19.89 \pm 1.26$  U) and protein concentration ( $23.59 \pm 0.36$  mg)] (Table 3.1). Comparing the aqueous two-phase extract of skin peel (supernatant) with the crude skin extract, the proteolytic activity of crude extract ( $6.38 \pm 0.07$  U) was lower ( $p = 0.01$ ) than the skin peel supernatant solution. Similarly, the protein concentration of the supernatant solution was significantly higher ( $p = 0.00$ ) than crude extract ( $12.11 \pm 0.22$  mg) (Table 3.1).

Crude fruit latex had a specific activity of  $0.04 \pm 0.00$  U/mg which was significantly lower ( $p = 0.00$ ) than the specific activities of supernatant crystals ( $1.30 \pm 0.03$  U/mg), latex supernatant solution ( $1.55 \pm 0.04$  U/mg) and the bottom phase ( $1.22 \pm 0.10$  U/mg) (Table 3.2.). There was no significant difference ( $p = 0.73$ ) between the specific activities of the different latex supernatant solution, crystal and residue extracts (Table 3.2.).

**Table 3.2. Efficiency of the aqueous two-phase extraction based on specific activity, partition coefficient and phase yield recovery.**

Sample	Specific activity ( $\pm$ SE,) U/mg	Partition coefficient ( $\pm$ SE)	Phase yield recovery ( $\pm$ SE) %
<u>Latex</u>			
Crude latex	$0.04 \pm 0.00^d$	-	-
Crystallised supernatant	$1.30 \pm 0.03^b$	-	-
Supernatant solution	$1.55 \pm 0.04^b$	$0.06 \pm 0.01^{b*}$	$64.72 \pm 2.08^b$
Bottom phase	$1.22 \pm 0.10^b$	-	$35.28 \pm 2.08^c$
<u>Skin peel</u>			
Crude extract	$0.53 \pm 0.02^c$	-	-
Supernatant solution	$0.84 \pm 0.06^c$	$0.25 \pm 0.01^{a*}$	$94.18 \pm 0.14^a$
Bottom phase	$2.31 \pm 0.27^a$	-	$5.82 \pm 0.21^d$

Means in the column with the figure \*as superscript reflects the result for both phases of the extraction.

Means in the same column with the same lower case alphabets as a superscript are not significantly different at the 0.05 confidence level.

The partition coefficient of the latex extraction was calculated as  $0.06 \pm 0.01$ , hence enzyme recovery from the latex supernatant extracts (crystallised supernatant and supernatant solution) was  $64.72 \pm 2.08$  %, while,  $35.28 \pm 2.08$  % remained in the bottom phase (Table 3.2).

The specific activity of the supernatant of the skin peel extract was low;  $0.84 \pm 0.06$  U/mg and this value was significantly lower ( $p = 0.00$ ) than the bottom phase ( $2.31 \pm$

0.27 U/mg) (Table 3.2). Crude extract (skin peel) had a specific activity of  $0.53 \pm 0.02$  U/mg and this value was not significantly different ( $p = 0.52$ ) from the specific activity of the supernatant (skin peel) (Table 3.2). Skin peel extraction had a better partition of phases ( $p = 0.03$ ) than the latex extraction. With partition coefficient of  $0.25 \pm 0.01$  the skin peel supernatant solution recovered  $94.18 \pm 0.14$  % of the skin peel proteases with only  $5.82 \pm 0.21$  % remaining in the bottom phase (Table.3.2).

The percentage activity yield of the crystallised supernatant and supernatant solution were 0.97 % and 6.36 % while the 92.67 % of the activity in the crude fruit latex was accounted for by the bottom phase (Table 3.3). Purification fold of the fruit latex extracts; supernatant solution ( $31.76 \pm 0.86$ ), crystallised supernatant ( $26.35 \pm 0.81$ ) and bottom phase ( $24.89 \pm 2.12$ ) were not statistically different ( $p = 0.07$ ) (Table 3.3).

The percentage activity yield of the skin peel in supernatant was 8.38 % while 91.62 % remained in the bottom phase (Table 3.2).

**Table 3.3 Efficiency of the aqueous two-phase extraction based on activity yield and purification fold**

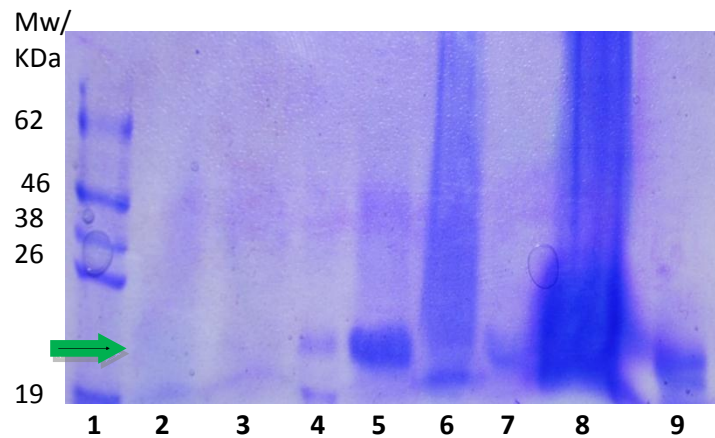
Sample	Percentage activity yield (%)	Purification fold ( $\pm$ SE)
<u>Latex</u>		
Crude latex	-	-
Crystallised supernatant	0.97	26.35 $\pm$ 0.81 <sup>a</sup>
Latex supernatant solution	6.36	31.76 $\pm$ 0.86 <sup>a</sup>
Latex bottom phase	92.67	24.89 $\pm$ 2.12 <sup>a</sup>
<u>Skin peel</u>		
Crude skin peel extract	-	-
Aq. Skin peel supernatant	8.38	-
Aq. Skin peel bottom phase	91.62	-

Means in the same column with the same lower case alphabets as a superscript are not significantly different at the 0.05 confidence level.

### 3.4.3 The presence and purity of papain on SDS – PAGE

Commercial pre-stained broad range protein molecular weight marker was run alongside the different papain extracts in order to confirm the molecular weight of the different extracts on Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). The presence of papain was further confirmed with bands obtained from commercial papain. The band of the commercial papain (Lane 9) was found within the molecular weight range of 19 and 26 kilo Daltons (KDa).

SDS-PAGE profile of the supernatant solution and bottom phase from the aqueous two-phase skin peel extract (Lanes 2 and 3) showed no visible protein bands (Plate 3.3). However, there were faint smears of their mobility tracks along the lanes (Plate 3.3).



**Plate 3.3: 10 % Acrylamide SDS-PAGE profile of aqueous two-phase extracts of latex and pawpaw skin peel.** Lane (1) Molecular weight marker (KDa) (2) skin peel bottom phase; (3) skin peel supernatant solution; (4) crude skin peel extract; (5) crystallised supernatant; (6) latex bottom phase; (7) latex supernatant solution; (8) crude latex sample; (9) commercial papain.(Merck)

➡ Expected molecular weight range of papain confirmed with the commercial papain.

The crude skin peel extract (Lane 4) on the other hand showed two bands; though the lower band was an aggregated band of proteins (Plate 3.3). The latex crystallised supernatant (Lane 5) formed a single band with molecular weight equivalent to that of the commercial papain (Plate 3.3). The first clear band of the crude skin peel also

corresponded with the band of the commercial papain as well as the crystal from the latex extract supernatant (Plate 3.3). The supernatant of the latex extraction also separated into a single faint band though it was almost smeared by the crude latex sample (Lane 7). The bottom phase of the latex extraction separated into an aggregate of protein band directly beneath the band of Lane 5 though there were smears of proteins along its mobility track. The crude latex (Lane 8) sample showed no clear visible bands.

### 3.5 DISCUSSIONS

Purifying a target protein from its natural substrate requires an extraction process that prevents denaturation of the protein (Silva *et al.*, 1997). The aqueous two-phase extraction enables papain to bind preferentially to the PEG-rich top phase owing to the higher salting out effect of ammonium sulphate and the higher hydrophobicity of papain compared to the other pawpaw proteolytic enzymes (Nitsawang *et al.*, 2006; Chaman Mehta *et al.*, 2013). Thus, phase separation observed from fruit latex and skin peel extraction is suggestive that most of the papain may have separated into the PEG-rich top phase. Papain therefore separated into a more dilute phase since in total the entire supernatant phase of the fruit latex and skin peel extracts constituted 97.10 % (crystallised supernatant and supernatant solution) and 98.56 % respectively of the total volume (48.70 ml). Despite the supernatant phase volume of the latex extract, the enzyme extracted still crystallised out of the solution and this can be attributed to a high initial protein concentration of the latex.

The protein concentration and proteolytic activity appeared to follow the same trend in both fruit latex and skin peel extracts; protein concentration and proteolytic activities were extremely lower in the supernatant than the bottom phase. In nature, papain accounts for less than 10 % of the total proteolytic enzymes in the pawpaw latex (Chaiwut *et al.*, 2006), therefore, the total proteolytic activity of the latex supernatant phase (crystallised and supernatant solution) of 7.33 % suggests that papain separated into the supernatant phase as confirmed by the SDS-PAGE. This denotes the total percentage activity yield of papain in this extraction process which is similar to findings of other workers (Nitsawang *et al.*, 2006; Chaman Mehta *et al.*, 2013). For instance, Nitsawang *et al.*, (2006) obtained papain extract which constituted approximately 8 % of the total proteolytic activity of the fresh pawpaw latex.

Similarly, the total percentage proteolytic activity of the skin peels supernatant solution was less than 10 % of the total proteolytic activity, and hence could be attributed to papain. However, no viable conclusion can be drawn from this value since skin peel supernatant showed no visible bands on SDS-PAGE. More so, separating the chaff of the skin peel with only distilled water usually yields crude extract containing different proteolytic enzymes (Chaiwut *et al.*, 2006). Therefore, the calculated percentage activity yield could also be as a result of the presence of other proteolytic enzymes other than papain. Crude extract of pawpaw skin peel had comparable specific activity as the aqueous two-phase extracts of the skin peel supernatant. Therefore, it is likely that aqueous two-phase extraction of pawpaw skin peels is not selective on the papain enzyme.

Pawpaw skin peel supernatant solution yielded a higher protein concentration and proteolytic activity than the crude extracts. A crude enzyme extract with a high enzyme concentration is essential during further purification (National Science Foundation, 2006). Therefore, aqueous two-phase extraction of pawpaw fruit skin peels maybe employed as a first step to the purification of the proteolytic enzymes in skin peels due to its significantly higher proteolytic activity and protein concentration

The separation of molecules between two phases among other factors results from an interaction between the molecules and the phase forming components (Ratanapongleka, 2010). Distribution of molecules between the phases is characterised by the partition coefficient (K) (Senthilkumar and Kumaresan, 2013). Partition coefficient (K) differs for different enzymes though in all cases a “K”-value below 1 indicates the enzyme is enriched in the bottom phase while the vice versa occurs when “K” is above 1. In both fruit latex and skin peel extractions, the partition coefficient were lower than 1, a clear indication that a significant proportion of the papain still remained in the bottom phase. A low partition coefficient of a target protein is attributable to a number of factors including molecular weight of PEG, concentration of PEG, concentration of salts and the presence of a specific ligand in the aqueous two-phase extraction system (Silva *et al.*, 1997). However, PEG 6000 as used in this experiment is suitable for extracting papain due to its high molecular weight. A high molecular weight of the polymer in an aqueous-two-phase-system enhances partitioning (Silva *et al.*, 1997). It is therefore, possible that other factors apart from the molecular weight of the PEG caused the low partitioning. In this experiment, apart from the protein concentration which was manipulated, all other conditions were maintained as recommended by Nitsawang *et al.*, (2006). A high initial

protein concentration limits the effect of PEG (Chaman Mehta *et al.*, 2013). The initial protein concentration of fruit latex is likely to have influenced the low partition coefficient. The fruit skin peel extract had comparatively lower protein concentration and a better partition coefficient than the fruit latex. Therefore, adjusting the initial protein concentration may improve the partition coefficient of the extraction process.

Whereas the partition coefficient of the fruit latex extraction depicts the enzyme separated into the bottom phase, the phase recovery depicts that a significant proportion of the enzyme separate into the supernatant. Despite the  $64.72 \pm 2.08$  % papain recovered from the top phase, the  $35.28 \pm 2.08$  % recovered from the bottom phase is significant for advanced purification procedure, since protein purification may lead to loss of some enzymes.

The SDS-PAGE profile of the fruit latex and skin peel phase extracts indicated the presence of papain in some of the phases. Samples loaded into Lanes 4, 5 and 7 showed protein bands with sizes similar to that of the commercial papain. The protein band in Lane 5 appeared thicker than that of Lane 4. The band thickness confirms the high concentration of papain in fruit latex than the skin peel as was also observed by Islam and Molinar-Toribio (2013). Despite the high loading concentrations of the fruit latex samples, the protein band in the Lanes 5 and 7 separated as single bands with faint smears. This coupled with the comparatively high specific activity of the latex supernatant solution ( $1.55 \pm 0.04$  U/mg) and crystallised supernatant solution ( $1.30 \pm 0.03$  U/mg) show the purity level of the extracts. The SDS-PAGE profile confirms the earlier assertion that papain is separated into the PEG-rich top phase of the aqueous two-

phase extraction (Nitsawang *et al.*, 2006). In addition, the protein bands from the aqueous two-phase extraction of pawpaw latex suggest that most of the papain separated into the supernatant (64.72 %) though most of the papain crystallised out of the solution.

### 3.6 CONCLUSIONS

The aqueous two-phase extraction of papain from fruit latex was used to isolate  $64.72 \pm 2.08$  % of the papain into the supernatant with 7.33 % proteolytic activity yield and a fold purification of  $58.11 \pm 1.67$  (when the results of the supernatant solution and crystallised supernatant solution were put together). The aqueous-two-phase extraction was effective for fruit latex papain extraction, though the protocol needs to be optimised in order improve the extraction efficiency; papain recovery in the supernatant, partition coefficient and to make all the papain either crystallise out of the supernatant solution or remain in the solution.

Aqueous two-phase extraction of skin peel supernatant yielded a higher proteolytic activity and crude protein concentration than the crude skin peel extracts. However, aqueous two-phase extraction of skin peel needs to be optimised since the skin peel extracts showed no visible presence of papain on SDS-PAGE.

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## CHAPTER 4

### 4.0 TO ASSESS THE EFFECTS OF GAMMA IRRADIATION ON DRIED CRUDE PAPAIN

#### 4.1 Background

Papain like all other proteins is polymeric and therefore would undergo certain physical and chemical changes when exposed to ionizing radiations (Lee and Song, 2002). The effects of irradiation on a biologically active material such as papain enzyme differ for dry papain and liquid papain (Furuta *et al.*, 2000). Whereas, dry papain is radioresistant to a larger extent, liquid papain is highly radiosensitive owing to the generation of free radicals from the aqueous environment (Audette-Stuart *et al.*, 2005; Von Sonntag, 1995). Though papain in aqueous solution is radiosensitive, cysteine offers protection to the papain by repairing a higher percentage of the inactivation caused by radiation damage (Gaucher *et al.*, 1971).

Generally, irradiation of any material is done for the purpose of sterilization, phytosanitation, and shelf-life extension among others (Markovic and Lapidot, 1995). Gamma irradiation for instance may not only represent an effective sterilising method but an alternative tool towards the enhancement of a biomolecule's intrinsic characteristics (Lugão *et al.*, 2010). Papain may also be irradiated for a similar purpose of enhancing its intrinsic characteristics such as Proteolytic activity. Irradiation has a significant effect on the proteolytic activity of papain which is a direct measure of the quality of papain.

Exposure of papain to electron beam increases proteolytic activity of papain two folds for doses between 1 and 10 KGy mainly due to the influence of the electron beam radiation on the active centres of the papain (Muthulakshmi *et al.*, 2012). In addition to the above, under suitable conditions of gamma irradiation (including purging with nitrogen gas to minimize radiolysis), it is possible to maintain the proteolytic activity of papain at more than 50 % of the initial activity at doses above 30 KGy (Furuta *et al.*, 1999).

The proteolytic activity of papain is usually enhanced by purification as observed in chapter 3 of this study. Enzyme purification method is a third step in papain production (Senthilkumar and Kumaresan, 2013). However, purification methods usually involve a series of steps and therefore require time, resources and the technical-know-how (Senthilkumar and Kumaresan, 2013). Therefore, it is essential that adequate investigation is made into other treatment processes which would serve the same purpose of enhancing the proteolytic activity of papain but require less time and resources. The sterilizing property of radiation provides an advantage over the traditional purification processes since the proteolytic activity of the papain is not affected (Zulli *et al.*, 2010)

This experiment was aimed at assessing the effect of gamma irradiation on proteolytic activity, protein concentration and specific activity of dried crude pawpaw fruit latex papain.

## **4.2 Material and Methods**

### **4.2.1 Study sites**

“Red lady” pawpaw variety used for the experiment was obtained from the 2-K Farms Limited in Bawjiase and irradiated at the Radiation Technology Centre (RTC) of BNARI, GAEC (Plate 3.1).

The biochemical and molecular analysis of the irradiated papain was conducted at the Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon, (Plate 3.1). Details of the study sites have been described in section 3.2.1.

### **4.2.2 Pawpaw fruit latex**

Pawpaw fruit latex was obtained as described in Section 3.2.2. The pawpaw fruit latex samples were oven dried at 40 °C for twenty-four (24) hours, powdered in a laboratory blender and packed in small black polyethylene bags each weighing ten (10) grams.

### **4.2.3 Papain irradiation**

Six (6) packets of dried papain (sealed in black polyethylene bags) each weighing ten (10) grams were irradiated at six (6) different doses: 2, 4, 6, 8, 10 and 12 KGy. The papain samples were irradiated using a wet storage category IV with plug source cobalt-60 gamma irradiator at a dose rate of 1.73KGy/hr. An ethanol chlorobenzene (ECB) dosimeter was used to measure the dose received by each batch irradiated. The irradiation

process was performed at ambient conditions and the entire process lasted for 24 hours and 28 minutes.

#### **4.2.4 Determination of protein contents of irradiated papain**

Protein concentrations of irradiated papain samples were determined using the Folin-Lowry assay (Lowry *et al.*, 1951) as described in section 3.2.4. The samples tested were the non-irradiated papain (control), commercial papain and the locally-produced crude papain from pawpaw fruit latex irradiated at doses of, 2, 4, 6, 8, 10 and 12 (KGy). The commercial papain (Merck's laboratory grade papain- 3000 USP-U/mg) was dissolved in distilled water while the crude papain samples were diluted in enzyme diluents to a final concentration of 0.1mg/ml (Appendix 5). The protein concentration of the different papain samples were extrapolated from the generated BSA standard curve shown in Figure 3.1.

#### **4.2.5 Determination of the proteolytic activity of irradiated samples**

The proteolytic activities of the commercial papain, non-irradiated and irradiated papain samples were determined using the casein hydrolysis (Nitsawang *et al.*, 2006; Sigma Aldrich, 2014) method as described in Section 3.2.5. Amount of tyrosine released by each papain sample was extrapolated from the tyrosine standard curve (Figure 3.2). All the papain samples used were in solution prepared as described in Section 4.2.4 above.

#### **4.2.6 Test for the presence and purity of irradiated papain samples on SDS-PAGE**

The presence of papain in the irradiated papain samples was evaluated on SDS-PAGE as described in Section 3.2.6.

#### **4.3 Data analysis**

Regression analysis was used to arrive at the equation for the tyrosine standard curve and BSA standard curve.

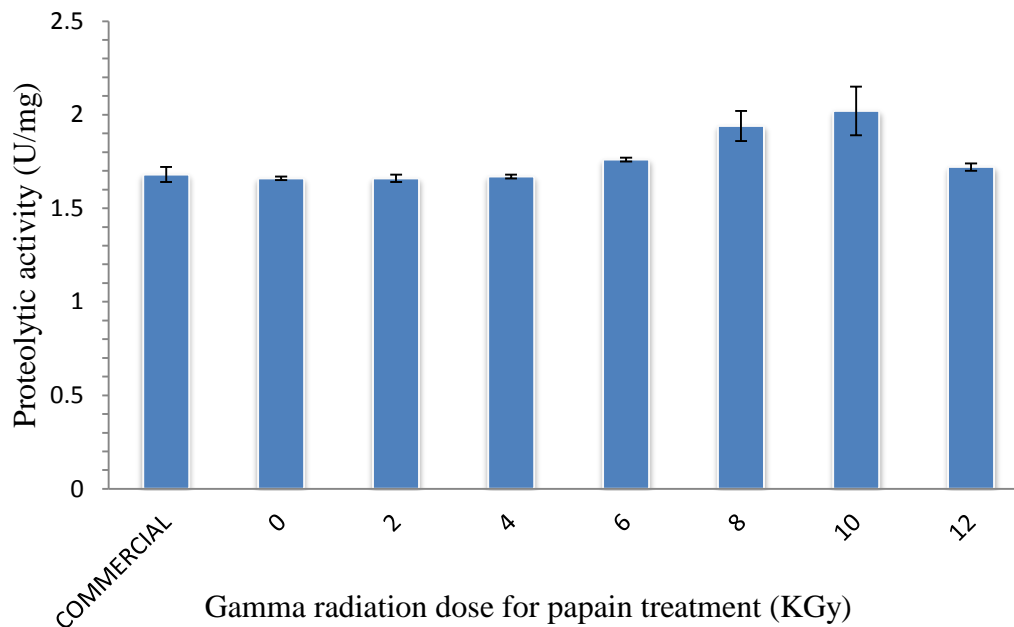
The absorbance readings of amino acid tyrosine obtained from the proteolytic activity determination of irradiated latex papain, the data from protein concentration determination and specific activity were analysed using SPSS version 16.

Data were further subjected to a one-way analysis of variance for the determination of differences between means, and when significant differences were observed, a Tukey HSD test was applied for mean separation. A p value of 0.05 or less was considered for statistical significance level. The analysed results were presented in tables and figures.

## 4.4 RESULTS

### 4.4.1 Effect of irradiation on the proteolytic activity

The papain irradiated at 2 KGy had a proteolytic activity of  $1.66 \pm 0.03$  U/mg and this was similar to the proteolytic activity of the non-irradiated papain. The proteolytic activity increased steadily from  $1.66 \pm 0.03$  U/mg for the batch irradiated at 2 KGy to peak proteolytic activity of  $2.02 \pm 0.18$  U/mg for those irradiated at 10 KGy (Figure 4.1). The peak proteolytic activity ( $2.02 \pm 0.18$  U/mg) and this accounted for a 21.69 % increase in the proteolytic activity of the non-irradiated papain (Table 4.1). However, there was no significant difference ( $p = 0.08$ ) between the proteolytic activities of papain irradiated at doses between 6 KGy and 12 KGy.



**Figure 4.1 Proteolytic activities of irradiated of crude fruit latex papain**

#### 4.4.2 Effect of irradiation on the protein concentration of the crude fruit latex papain and specific enzyme activity

Table 4.1 shows that protein concentration of crude fruit latex papain irradiated at 4 KGy ( $2.07 \pm 0.04$  mg/ml) did not vary significantly ( $p = 0.06$ ) from the protein concentrations of the commercial papain ( $1.65 \pm 0.18$  mg/ml) and the non-irradiated papain ( $2.05 \pm 0.06$  mg/ml). There was also no significant difference between the protein concentrations of all irradiated papain including the control (Table 4.1).

**Table 4.1. Effect of gamma radiation on the protein concentration and specific activity**

Radiation treatment (KGy)	Percent increase in proteolytic activity relative to control (%)	Protein concentration (mg/ml $\pm$ SE)	Specific Activity, (U/mg per ml protein)
0 (Control)	-	$2.05 \pm 0.06^{ab}$	0.81 <sup>d</sup>
2	0.00	$2.09 \pm 0.01^a$	0.79 <sup>c</sup>
4	0.00	$2.07 \pm 0.04^{ab}$	0.81 <sup>d</sup>
6	6.02	$2.14 \pm 0.05^a$	0.82 <sup>d</sup>
8	16.87	$2.08 \pm 0.01^a$	0.93 <sup>c</sup>
10	21.69	$2.10 \pm 0.08^a$	0.96 <sup>b</sup>
12	3.61	$2.22 \pm 0.01^a$	0.77 <sup>f</sup>
Commercial papain	-	$1.65 \pm 0.18^b$	1.02 <sup>a</sup>

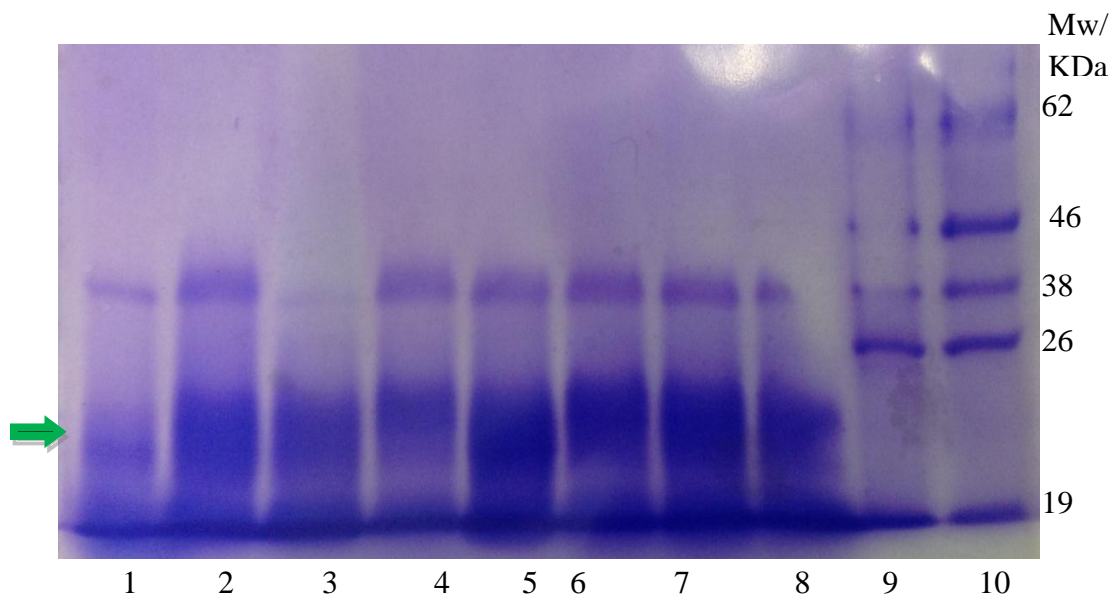
Means in the same column with same lower case alphabet as superscript are not significantly different at the 0.05 confidence level.

Specific activity appeared to follow a similar trend as the proteolytic activity; radiation dose between 4 to 10 KGy caused significant increase in specific activity (Table 4.1). The commercial papain had a significantly higher ( $p= 0.00$ ) specific activity of 1.02 U/mg compared with the crude papain. Papain irradiated at 10 KGy had a specific activity (0.96 U/mg) which was higher ( $p = 0.00$ ) than all other crude pawpaw fruit latex papain samples including the non-irradiated sample. The 12 KGy radiation dose yielded the papain with the least specific enzyme activity of 0.77 U/mg papain. Furthermore, the specific activities of all the irradiated papain were significantly different from each other except the non-irradiated (control), and papain irradiated at 4 and 6 KGy ( $p = 0.126$ ).

#### **4.4.3. The deleterious effect of irradiation on papain using SDS-PAGE**

The molecular weight of the irradiated papain was determined from bands generated by a commercial pre-stained broad range protein molecular weight marker and confirmed by bands from commercial papain and non-irradiated papain run along the irradiated samples (Plate 4.1).

The non-irradiated papain sample, commercial papain and the irradiated papain samples all separated into 2 bands with suspected impurities (Plate.4.1). The molecular weight of the irradiated papain samples were equivalent to the non-irradiated and the commercial papain as confirmed by their SDS-PAGE profile (Plate 4.1). Irradiated crude papain samples under the same loading volumes in the different lanes showed no smears of low molecular weight proteins.



**Plate 4.1.A 10 % Acrylamide SDS-PAGE profile of crude papain from fruit latex irradiated at six (6) different doses. Lanes:(1) Commercial papain; (2) 0 KGy; (3) 2 KGy; (4) 4 KGy; (5) 6 KGy; (6) 8 KGy; (7) 10 KGy; (8) 12 KGy; (9 and 10) Molecular weight markers.**

➡ Expected molecular weight range of the irradiated papain confirmed with the commercial papain

#### 4.5 Discussions

Radiation damage to enzyme molecules in general may result in the impairment of the catalytic efficiency without complete destruction of proteolytic activities (Mounter, 1960). This type of damage may be described as partial denaturation resulting from the creation of disorder in the macromolecule (Mounter, 1960). Under high energy electron beam radiation and gamma radiation, the proteolytic activity of papain increases (Furuta *et al.*, 1999; Muthulakshmi *et al.*, 2012). Gamma irradiation doses between 4 and 10 KGy

significantly increased the proteolytic activity of the crude papain samples. This is probably as a result of the direct action of the radiation energy on the active centre of the papain due to its polymeric nature as reported by Muthulakshmi *et al.*, (2012). Gamma irradiation up to 30 KGy and above can maintain the proteolytic activity of dried and frozen papain at more than 50 % of the initial proteolytic activity only under suitable irradiation conditions (Furuta *et al.*, 1999). The percentage increase in proteolytic activity observed for radiation dose between 6 and 10 KGy (6.02 – 21.69 %) indicates that gamma radiation may be effective in increasing proteolytic activity under ambient conditions for doses up to 10 KGy. Percentage increases of proteolytic activity above 21.69 % may only be achieved under optimum irradiation conditions, such as, purging with nitrogen gas to minimise the formation of free radicals (Furuta *et al.*, 1999).

In contrast to dried papain, aqueous papain has been shown to exhibit a high radiosensitivity due to the formation of free radicals and the subsequent oxidation of its sulfhydryl group to sulfenic acid (Lin *et al* 1975). It has also been reported that the effect of irradiation on the proteolytic activity of aqueous papain can be minimised when the papain concentration is increased 10 - 100 mg/ml and purged with N<sub>2</sub> gas (Furuta *et al.*, 2000). Thus, papain like all other enzymes requires specific environments to maintain their bioactivity lest they are inactivated by denaturation.

The arrangement of the amino acid residues of papain has been shown to confer some level of radioresistance to the enzyme by Von Sonntag, (1995). More so, tyrosine, one of the major amino acid residues of papain also shows significant resistant to radiation damage and can offer protection to proteins even at high radiation doses beyond 10 KGy

(Assemand *et al.*, 2003; Hatano *et al.*, 1962). In animals, L-tyrosine incorporated into therapeutic protein caeruloplasm and haemoglobin during irradiation protects the proteins from aggregation after the irradiation procedure (Assemand *et al.*, 2003).

According to Mounter (1960), gamma radiation generally causes the removal of peptides from their protein chain, though their proteolytic activity may not be affected. Thus an increase in protein concentration of an irradiated papain sample could be as a result of the increase in the amount of free protein resulting from action of the radiation energy on the peptide bonds. Protein concentration of papain was not affected by all gamma doses of radiation and can be inferred that damage on the protein structure could be superficial since radiation doses could not cause significant differences in protein concentration.

Gamma irradiation also appeared to affect overall enzyme purity. Specific activity provides a means to measure the amount of enzyme within all contaminants; therefore, it increases only with purity (National Science Foundation, 2006; Phillips, 2014). A low specific activity is suggestive of a contaminated sample. Increasing radiation doses between 4 to 10 KGy resulted in a corresponding increase in specific activity. The increase in specific activity appeared to follow the trend that an increase in proteolytic activity caused a corresponding increase in specific activity probably because protein concentration was not affected by radiation. It is evident that specific activity is sensitive to even the negligible changes in the proteolytic activity and protein concentration. Though the proteolytic activity and protein concentration of papain irradiated at 12 KGy were statistically similar to papain irradiated at 10 KGy, the resultant specific activity due to the 12 KGy irradiated papain was low. High proteolytic activity and low protein

concentration results in high specific activity values as observed in the papain irradiated at 10 KGy and commercial papain. The increase in specific activity of the crude papain enzyme could also be as a result of the removal of some inhibitors as gamma irradiation is effective for decontamination (Monti *et al.*, 2000).

Major radiation damages to proteins are fragmentation and aggregation and these are often observed as new bands below the major band (fragmentation) and a degraded pattern of the protein molecules (aggregation) (Lee and Song 2002). The SDS-PAGE profile of irradiated crude papain resulted in bands with equivalent molecular weight as the commercial papain and the non-irradiated papain indicating the presence of papain at all levels of radiation. The intensity of the bands generated by the different radiation doses was consistent with the non-irradiated papain. Band intensity decreases among irradiated samples when there is a breakdown of the polypeptide chain which yields smeared smaller molecular weight bands. The 38 KDa protein as observed in the SDS-PAGE profile was consistent in the non-irradiated and commercial papain samples and therefore cannot be due to irradiation. More so, the protein bands generated by all irradiated papain samples indicate that Cobalt-60 radiation did not induce protein aggregation, hence, no irradiated papain sample showed degraded pattern of protein molecules. A degraded pattern of protein molecules on SDS-PAGE profile results from aggregated protein molecules which cannot penetrate the separating gel matrix, resulting in no visible band formation (Lee and Song, 2002).

Gamma radiation is effective for product decontamination but cannot be a substitute to purification. Usually, a pure protein sample separates into a single band after

electrophoresis. SDS-PAGE profile of the irradiated papain samples showed two bands with several suspected contaminants beneath the lower band protein suspected to be papain.

#### **4.6 Conclusions**

Gamma irradiation at doses between 4 and 10 KGy increased the proteolytic activity by 21.62 % and specific enzyme activity by 18.51 % of the crude papain though protein concentrations was not affected. Irradiating crude papain at doses up to 10 KGy under ambient conditions can increase proteolytic activity by 21.62 % of the proteolytic activity of the non-irradiated. However, Cobalt-60 gamma radiation does not improve the purity of the crude papain, though, it can be used as a treatment to maintain or enhance the proteolytic activity of dried crude papain.

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## CHAPTER FIVE

### 5. TO ASSESS THE EFFICACY OF PROTEIN BAITs PRODUCED WITH LOCALLY-PRODUCED CRUDE PAPAIn

#### 5.1 Background

Proteins are essential to fruit fly development and maturity. Low levels of protein in diets of developmental immature stages of several species of fruit flies can result in increased developmental time, decreased percentage emergence, and reduction in adult size (Taylor *et al.*, 2011). Newly emerged male and female fruit flies actively search for proteins for sexual maturation, including the development of their gonads, synthesis of sex pheromones and other aspects of development that can influence sexual performance (Lyon, 2014).

In the wild, adult fruit flies feed on faecal droppings of birds, honey dew, bat guano, pollen, and other low protein diets to meet their protein requirements (Vargas *et al.*, 2002; Weldon and Taylor, 2011). The concept of protein bait sprays is therefore based on this fact that sexually immature male and female fruit flies actively search and feed on proteinaceous substances. As such, the introduction of poisoned protein baits as substitutes to the low protein food has become an important aspect of fruit fly control.

Brewery yeast waste is a rich source of B complex vitamins, proteins (essential amino acids) and minerals, and as such, a good raw material for producing cheap local substitutes of commercial protein baits (Sookar *et al.*, 2003). However, the papain grade

used for proteolysis can influence the cost of local protein bait production (Gopaul and Price, 1999). Thus, to cheaply produce protein baits locally, there is the need to source for cheaper sources of this proteolytic enzyme.

Papain, ficin, and bromelain are among the major plant proteolytic enzymes with great economic values (Bala *et al.*, 2012; Ortega, 2011). Whereas crude papain and crude ficin can be obtained directly by drying pawpaw fruit latex and latex of fig fruits respectively, crude extraction of bromelain requires the process of cooling the crushed pineapple stem or peels, centrifugation, ultrafiltration and lyophilisation (Bala *et al.*, 2012). Fig tree has a disadvantage of long maturation and fruiting period of two (2) years minimum, whereas pawpaw matures and fruits within the first year of cultivation (Heather, 2012). Papain is comparatively easier to obtain than ficin or bromelain. Thus, the extraction process of bromelain and the maturation period of fig fruits make papain from pawpaw a more suitable proteolytic enzyme for the production of local protein baits for *B. invadens* control in Ghana.

This experiment was therefore aimed at assessing the efficacy of protein baits produced with crude papain from pawpaw fruit latex and skin peel using biological indicators.

## 5.2 Materials and methods

### 5.2.1 Study sites

Protein bait preparation and laboratory bioassays of prepared protein baits were performed at the Fruit Fly Insectary and the Experimentation Room of the Mosquito Insectary, Radiation Entomology and Pest Management Centre (REPMC) under the Biotechnology and Nuclear Agricultural Research Institute (BNARI) of the Ghana Atomic Energy Commission (GAEC), Kwabenya – Accra, Ghana (Plate 3.1).

Crude protein concentration, pH and dry matter content of prepared protein baits, brewery yeast waste and local laboratory diet formulation were determined at the Department of Biochemistry, Cell and Molecular Biology and REMPC (Plate 3.1).

### 5.2.2 Source of adult *Bactrocera invadens*

*Bactrocera invadens* used for the bioassay were obtained from wild populations in infested mango fruits collected from mango orchards on the BNARI farms located at the Ghana Atomic Energy Commission, Kwabenya. The infested fruits were transported to the laboratory in plastic buckets for incubation.

In the laboratory, infested mangoes were transferred into plastic storage containers (1.7 litres) covered with thin mesh cloth and incubated for three (3) days. Third instar larvae which were distinguished by their relatively big size and almost cream colour were

picked from the fruits using stainless steel forceps into sterilized sand (sterilization of sand in the oven at 160 °C for one hour) for pupation.

Pupae were sifted out of sand after two (2) days and transferred into 150 ml glass jars covered with wire mesh cut to fit within the lid. Pupae were transferred into five (5) litre plastic bucket cages after 7 days for emergence (Plate 5.1).



**Plate 5.1 Plastic bucket cages for rearing adult *B. invadens***

Newly emerged adult flies were separated according to sex using manual aspirator and maintained in the laboratory for ten (10) days after which they were used for the experiments. During that period, food and water were provided *ad libitum* to the flies; however, adult feeding regimes differed with experiment.

All developing larvae, pupae and adults were kept on the laboratory bench at a temperature and relative humidity of  $28 \pm 2$  °C and  $78 \pm 5$  % respectively under natural photoperiod (LD 12: 12).

### **5.2.3 Brewery yeast waste**

Brewery yeast waste (beer) was obtained from Guinness Ghana Limited, Achimota-Accra, Ghana which is located about 5.8 km from Ghana Atomic Energy Commission. The brewery yeast waste was stored in 4.5 litre gallons and frozen at -20 °C until needed. The freezing of the brewery yeast waste was to reduce contamination and also to stop further multiplication of yeast cells. This is to ensure compositional uniformity of protein baits produced with the different batches of brewery yeast waste.

### **5.2.4 Protein bait preparation**

Protein bait was prepared based on the method of Gopaul and Price (1999). In the method by Gopaul and Price, brewery wastes were prepared in 2.5 litres Winchester bottles and autolysed in the oven at 70 °C. This was followed by proteolysis of the autolysed cells with commercial papain after twenty four (24) hours and placed in the oven for a further nine (9) hours at 70 °C. Three different types of protein baits were prepared in this study using three different papain types. The papain types were commercial papain, crude papain from fruit latex and crude papain from skin peel. The control was autolysed brewery yeast waste without papain.

Four (4) dispenser bottles each of volume one litre (1 L) were set up and one litre (1 L) of brewery waste was poured into each of the dispenser bottles and autolysed in the oven at 70 °C for 9 hours. The autolysed brewery yeast waste was allowed to cool overnight at room temperature and ambient relative humidity. To prepare the protein baits, the different papain types were added to the autolysed brewery yeast waste at 4 g/L. The

bottles were agitated for a minute and returned to the oven for a further nine (9) hours at 70 °C. The control was returned to the oven though no papain was added. Each bottle and its contents were allowed to cool overnight before the addition of potassium sorbate (preservative) at a rate of 2 g/L. The resultant liquids were allowed to settle at room temperature for seven (7) days after which the supernatants representing the protein baits were decanted using Pasteur's pipette into 150 ml storage bottles. The recovered protein baits from the three different papain types were measured in milliliters.

## **5.2.5 Effect of papain type on the physicochemical properties of protein baits**

### **5.2.5.1 Dry weight determination**

Dry weight was determined using the oven drying method (University of Wisconsin Soil Testing Laboratory, 2009). Twenty millilitres (20 ml) each of the brewery waste and the three prepared baits were transferred into four (4) different glass Petri dishes of pre-determined weights (W<sub>0</sub>). Each Petri dish and content was weighed to obtain the initial weight of the sample (W<sub>1</sub>). All samples were oven dried at 100 °C to rid them of all their moisture till constant weight was attained. The final weight of the sample was determined after drying (W<sub>2</sub>). For each sample that was oven dried, one of its three replicates was selected as a test sample and its weight was checked periodically to monitor the trend of drying. The weights of the test samples were checked three times in a day till the constant weight was attained. Each treatment was replicated three (3) times. The percentage dry weight was calculated as the weight of sample after drying expressed as percentage of the weight of wet sample (Appendix 3.9).

The actual dry weight (Dw) of the protein baits were calculated as a product of the percentage dry weight and the weight of the wet sample (Appendix 3.10)

Dry weights of the different protein bait samples were compared with the dry weight of the brewery yeast waste (Dry weight retained). Dry weight retained was calculated as the dry weight of the protein bait sample (Dw) expressed as a percentage of the dry weight of the brewery yeast waste (Bw) (Appendix 3.11).

#### **5.2.5.2 Determination of pH of prepared baits**

The pH values of prepared baits were determined by immersing the bulb of the pH metre (Jenway 3510 pH metre) separately into each protein bait sample being analysed. The bulb of the pH metre was washed with distilled water before and after immersion into the protein bait solution. Three (3) pH readings were taken and the mean pH value found for all the bait solutions.

#### **5.2.5.3 Protein content determination**

Crude protein concentration of the prepared protein baits, brewery yeast waste and local laboratory diet formulation were determined using the Folin-Lowry assay (Lowry et al., 1951) as described in Section 3.2.4.

## **5.2.6 To assess the acceptability of the prepared protein baits as a good protein source for adult *Bactrocera invadens* in laboratory bioassay.**

Ten (10) days old adult flies were used in bioassays to evaluate the efficacy of the protein baits. The parameters used to assess the bioefficacy of the baits include: attractance, feeding preference, survival and fecundity studies of adult flies.

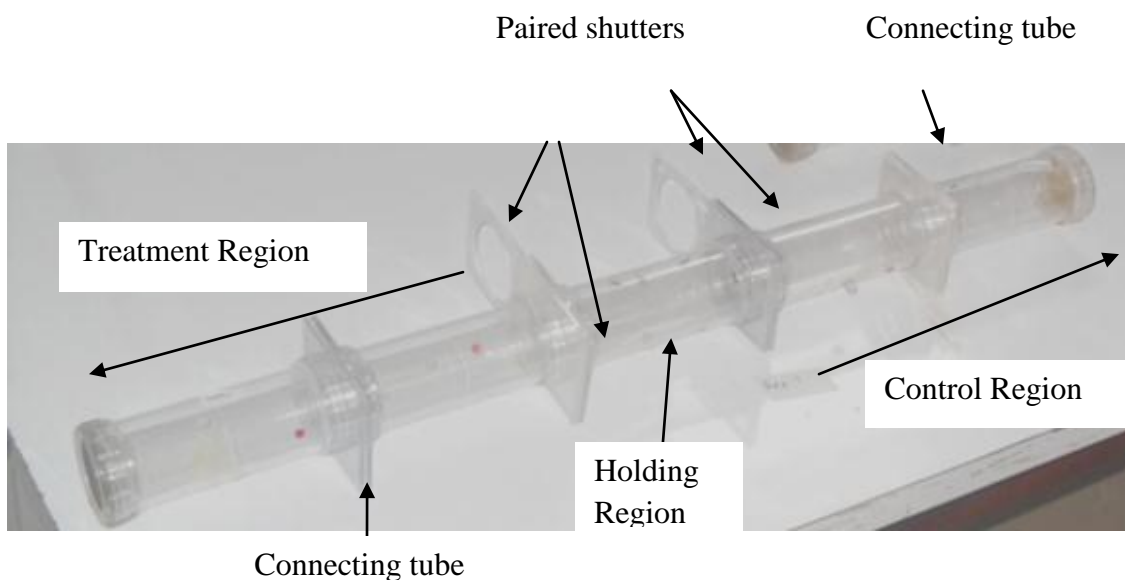
### **5.2.6.1 Attractance test**

The adult flies used for the experiment were initially fed on a ten percent (10 %) sucrose solution and distilled water from emergence to ten (10) days after emergence when they were used for the test. This was to ensure that the flies were protein deprived. However, the 10 % sugar solution and the water were taken out of the cage twenty four (24) hours prior to the experiment. This was aimed at increasing the urge of the test flies to search for food.

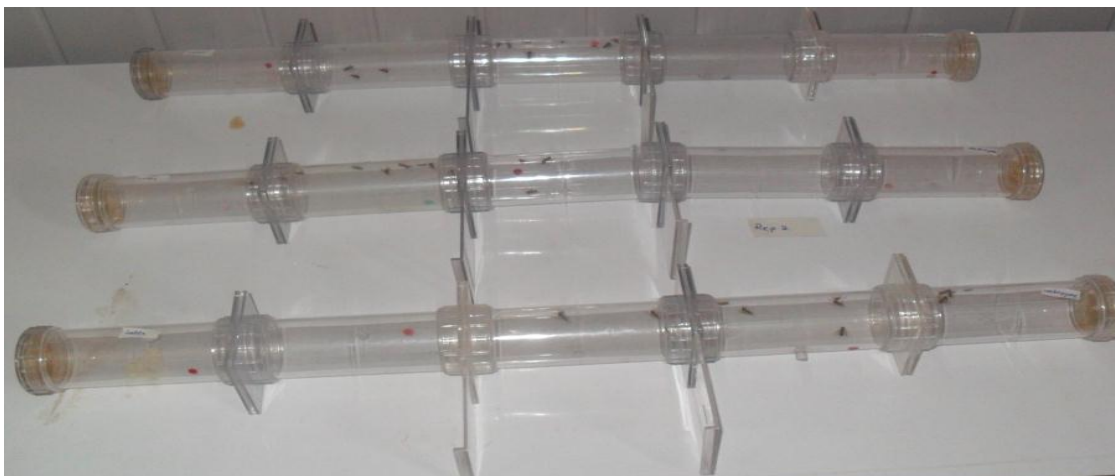
The attractance test was performed between the hours of 8:00 am to 12:00 pm, a period when fruit flies actively search for food (Christenson and Foote, 1960). It was conducted under uniform artificial light intensity of 90 lux, a temperature of  $26 \pm 2$  °C and a relative humidity of  $72 \pm 4$  %.

Attractance of the baits prepared with the different papain types to *B. invadens* of both sexes were evaluated in the laboratory using the WHO insecticide resistance test tubes. A single attractance test setup consisted of five individual tubes each of length 12.5 cm and

diameter of 4.4 cm linked to each other by connecting tubes (Plate 5.2a). The third test tube within the set up was used as the holding region while the remaining two tubes on either side of the holding region were labelled as test region and control region. The entire experimental set up was on a 72 cm x 53.5 cm table surface, 78 cm from the ground and 123 cm from the light source.



**Plate 5.2a. Parts of WHO insecticide resistance test tubes used for attractance test.**



**Plate 5.2b. Experimental setup for evaluating the attractance of the protein baits**

Three (3) attractance test set ups were used at a time so that all three treatments were evaluated at the same time for a single replicate (Plate 5.2b). Treatments were tested independent of each other against the control. Depending on the sex of the *B. invadens* being tested for attractance to protein bait, either 20 males or 20 females were aspirated into the holding region of the test tube and held there for 2 minutes before the experiment. This was to enable the flies to adjust to the conditions of the new environment, to minimize agitation and eliminate bias of treatment response that may arise from the agitated flies.

Each protein bait type and control was soaked in cotton wool cut to fit the wire mesh linings of the lid and placed at the end of the test region and control region respectively. When the two plastic shutters that separated the three regions of the resistance test tubes were removed, the flies were observed for one (1) hour. The number of flies on each side

of the test tube was recorded every five (5) minutes for the one (1) hour observation period.

The positions of the baits and control were interchanged after each replicate to prevent or minimise any possible positional effect that may influence the choice of the insects. Clean tubes and new cotton pads were used for each bait type and each replicate to prevent test flies from being attracted to regurgitated fluid which might be disposed by the flies during feeding. Furthermore, flies were tested only once and discarded to ensure that flies used were completely protein deprived.

The attractance of the protein bait types to the control was expressed as a percentage of the ratio of the difference between the number of flies attracted to the bait and the control for the same assay period to the total number of flies used for the assay (Appendix 3.12).

#### **5.2.6.2 Feeding preference test**

Flies used for the feeding preference were preconditioned as described in Section 5.2.6.1 above. Feeding preference test was conducted under similar laboratory conditions as described in Section 5.2.6.1

A 30 x30 x 30 (cm<sup>3</sup>) Bug dorm™ insect rearing cage lined with two (2) white A4 sheets was set on a 72 cm x 53.5 cm table surface, 78 cm from the ground and 123 cm from the light source and used for the experiment (Plate 5.3a). The different protein baits were tested for their acceptability to the flies in a competitive environment, that is, the three

baits were tested at once together with autolysed brewery yeast waste without papain as control (Choice test).



a. Bug dorm insect rearing cage



b. Positions of the protein bait samples within the insect cage

**Plate 5:3: 30 x 30 x 30 (cm<sup>3</sup>) Bug dorm insect rearing cage used for feeding preference test**

Twenty (20) ml of each protein bait type was soaked in a cotton wool put in a 12.5 ml sugar cup. One soaked cotton ball was transferred from the sugar cup unto a square-cut parafilm and placed in each corner of the cage (Plate 5.3b).

A single fly was released into the centre of the cage and observed for the type of bait it selected, the duration of feeding and the total time spent not feeding on a particular bait type. Each test fly was observed for a maximum of one (1) minute within which it had to make a choice. A choice was said to have been made when a fly settles on protein bait, while feeding was measured in relation to the labella contact with the protein bait. A fly

was allowed to remain on a diet till it moves away after feeding. A fly that made no choice after one (1) minute was considered as agitated or confused and was subsequently removed from the cage.

The experimental setup was rotated through an angle of  $90^{\circ}$  after five (5) tests, while the positions of the protein baits within the cage were interchanged after ten (10) tests to minimize the possibility of bait being most preferred because of its location within the cage. All flies were tested only once and discarded to ensure uniformity of the test samples. A total of fifty (50) flies were tested and replicated three (3) times.

The feeding response of the flies per bait was expressed as a percentage of the total test flies per replicate. Average time spent feeding was expressed as a ratio of the sum of individual feeding time to the total number of flies per treatment per replicate.

### **5.2.6.3 Survival, fecundity and eggs hatchability studies**

Sixty (60) ten-day old adult flies of *B. invadens* were set in five (5) litres plastic bucket cages in a ratio of 1:1 that is thirty (30) males to thirty (30) females (Plate 5.1). This experiment was aimed at establishing the suitability of the protein baits to meet the protein requirements of the fruit flies. Prepared protein baits replaced the laboratory adult diet in a study to determine the survival, fecundity and hatchability of eggs of adult *Bactrocera invadens* relative to the protein bait consumption. Flies were fed *ad libitum* based on the type of protein bait being evaluated by mixing protein bait solution with 10 % sucrose solution at a ratio of 1:1 and observed for 92 days.

The experiment was conducted in the laboratory at temperature and relative humidity of  $26 \pm 2$  °C and  $72 \pm 4$  % respectively under natural photoperiod (LD 12:12). The flies were mated on day ten (10) post emergence.

Parameters monitored in the laboratory included total eggs laid, fecundity (eggs per female), hatchability of eggs and survival of the adult over an observation period of ninety-two (92) days post mating.

Total number of eggs laid within the period was estimated by counting the number of eggs harvested twice a week from the perforated plastic oviposition cups (Plate 5.4).



**Plate 5.4: Perforated plastic oviposition cup smeared with mango juice**

A perforated plastic oviposition cup smeared with mango juice (Plate 5.4) was placed in each cage for a period of six (6) hours, from 8:00 am to 2:00pm. All the eggs laid within the oviposition cups were picked with artists brush into plastic Petri dishes. The freshly

harvested eggs were disinfected with 0.025 % Sodium hypochlorite solution followed by three washes with distilled water. The inner surface of the Petri dish was divided into four sectors and the number of eggs per dish estimated by counting the total number of eggs in one sector of the Petri dish and multiplying the number by four (4) to obtain the total eggs per cage.

The number of eggs laid per female (fecundity) was obtained by dividing the total number of eggs collected from a cage by the number of females alive in the cage at the time.

Hatchability of eggs was determined by placing hundred (100) eggs on a moist black cloth-lined Petri dish and incubating for two (2) days within which the eggs hatched (Plate 5.5). Egg hatchability was expressed as a percentage of the number of eggs hatched divided by the total eggs set up. In instances where the total eggs laid was below hundred (100), all the collected eggs were used for the hatchability test. However, if the number of eggs collected were above hundred (100), the remaining eggs were used as replicates. Hatched eggs were distinguished on the black cloth by the transparent and distorted egg shell that remains after the 1st instar larvae had eclosed.



**Plate 5.5: Moist black cloth used for evaluating hatchability of the eggs**

Survival of flies was evaluated by counting and recording the number of dead flies hand-picked from the experimental cages twice a week. The number of dead flies was deducted from the total number of flies set to obtain the number of flies alive at the time.

### **5.3 Data analysis**

The volume of the different protein baits were analysed with chi-square test at  $p = 0.05$  significance level.

Protein concentration determined, measured pH, dry weight of each sample, percentage dry weight, percentage dry weight retained, count data obtained from survival studies, attractance test and feeding preference test were analysed for means using SPSS version 16.

Data were further subjected to a one-way analysis of variance for the determination of differences between means, and when significant differences were observed, a Tukey HSD test was used for mean separation. A p value of 0.05 or less was considered for statistical significance level. The analysed results were presented in tables and figures.

## 5.4 Results

### 5.4.1 Protein bait recovery

The different protein baits produced with the different papain types showed significant variation in colour (Plate 5.6). Whereas baits produced with crude latex and commercial papain had a dark brown colour, protein baits produced with skin peel and the control (No papain) were light-brown in colour (Table 5.1).



a. Imported papain bait

b. Crude fruit latex papain bait

c. Crude skin peel papain bait

d. No papain (control)

Plate 5.6: **Autolysed brewery yeast waste with arrowed portions showing the protein baits produced from the different papain types immediately after proteolysis.**

When protein baits produced were allowed to stand for 96 days in ambient conditions, the control bait yielded a total of 366 ml (36.6 %) protein bait, while the protein bait produced with crude papain from latex yielded 390 ml (39 %) (Table 5.1). However, there was no significant difference in volumes of protein baits produced with the different papain types when subjected to chi-square test at  $p = 0.05$  significance level (Chi-square=0.814, d.f=3,  $p=0.846$ ).

**Table 5.1. Effects of papain on the colour and yield of protein bait.**

Treatment	Colour of protein bait	Yield of protein bait from 1 litre brewery yeast waste (ml)	Percentage protein bait recovery (%)
Fruit Latex	Dark brown	390.00 <sup>a</sup>	39.00
Fruit Skin peel	Light brown	373.00 <sup>a</sup>	37.30
Commercial papain	Dark brown	375.00 <sup>a</sup>	37.50
Control	Light brown	366.00 <sup>a</sup>	36.60

Means with same lower case alphabet as subscript are not significantly different at the 0.05 confidence interval.

#### 5.4.2 Physicochemical properties of prepared baits

Protein baits prepared with the different papain types had significantly different dry matter contents,  $p = 0.00$  (Table 5.2). However, there was no significant difference between the mean percentage dry weights ( $p = 0.26$ ) and mean actual dry weight ( $p =$

0.56) of protein baits produced with crude fruit latex papain ( $12.32 \pm 0.64$  %;  $0.12 \pm 0.01$ g/ml) and commercial papain ( $11.22 \pm 0.24$  %;  $0.11 \pm 0.00$  g/ml), similarly, there was no significant difference between the mean percentage dry weight ( $p = 0.67$ ) and actual dry weight ( $p = 0.78$ ) of baits produced with skin peel papain ( $10.00 \pm 0.09$  %;  $0.10 \pm 0.00$  g/ml) and control ( $9.38 \pm 0.34$  %;  $0.09 \pm 0.00$  g/ml) (Table 5.2). Despite these observations, there was no significant difference between the percentage dry weight ( $p = 0.19$ ) and actual dry weight ( $p = 0.09$ ) of baits produced with skin peel papain and commercial papain (Table 5.2).

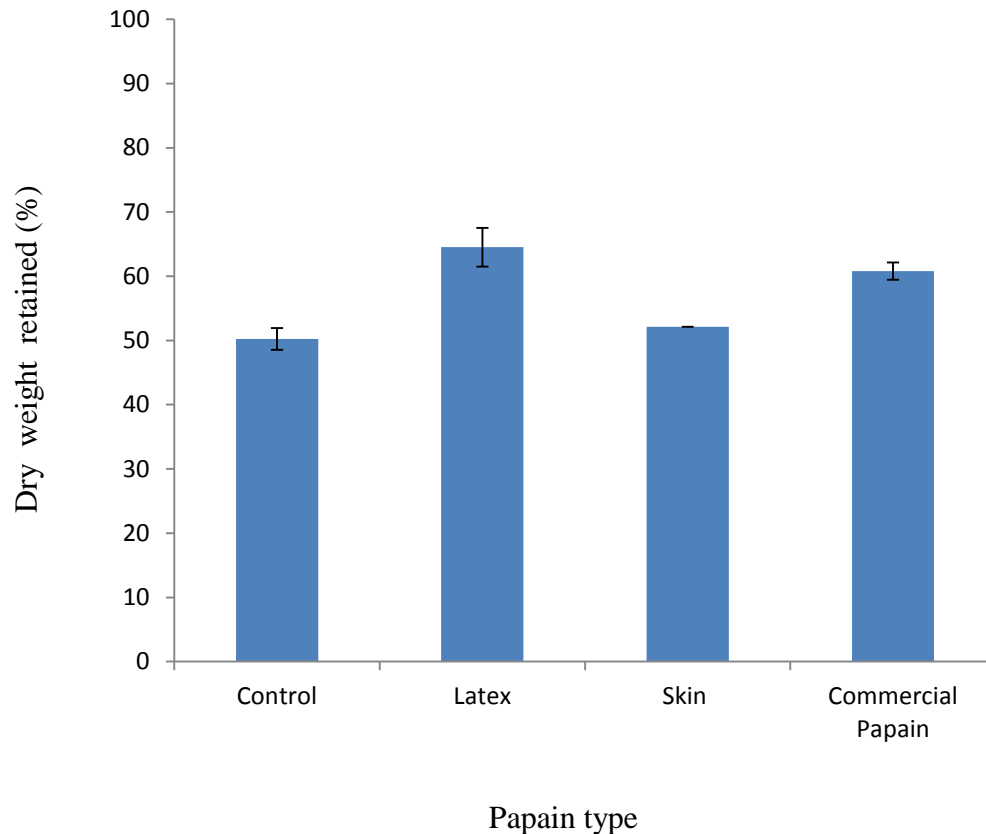
**Table 5.2. Mean dry weight and protein bait recovery of the prepared protein baits**

Treatment	Percentage dry weight (%)	Mean Dry Weight ( $\pm$ SE) g/ml
Control	$9.38 \pm 0.34^c$	$0.09 \pm 0.00^f$
Skin peel	$10.00 \pm 0.09^{bc}$	$0.10 \pm 0.00^{ef}$
Commercial	$11.22 \pm 0.24^{ab}$	$0.11 \pm 0.00^{de}$
Latex	$12.32 \pm 0.64^a$	$0.12 \pm 0.01^d$

Means in the same column with the same lower case alphabets as superscript are not significantly different at the 0.05 confidence interval.

Relative to the dry weight of the brewery yeast waste, crude fruit latex papain bait ( $64.52 \pm 3.02$  %) and commercial papain ( $60.80 \pm 1.35$  %), retained a significant ( $p = 0.00$ ) proportion of the dry weight present in brewery yeast waste than the control ( $50.21 \pm 1.71$  %). There was however no significant difference between the mean percentage dry

weight of the brewery yeast waste retained by skin peel papain bait ( $52.11 \pm 0.00$  %) and the control ( $50.21 \pm 1.71$  %) (Figure 5.2).



**Figure 5.1. Mean percentage dry weight of protein bait relative to the dry weight of the brewery yeast waste.**

The protein concentration of the different protein baits including the control was extrapolated from the BSA standard curve (Figure 3.1). Table 5.3 shows the protein concentrations and pH values of the prepared baits, brewery yeast waste and the local laboratory diet formulation. Protein concentration of bait produced with crude papain latex was  $12.94 \pm 0.1$  mg/ml and this accounted for 47.05 % of the total protein from the brewery yeast waste while, the control protein bait had a protein concentration of  $11.91 \pm$

0.1 mg /ml and this accounted for 43.30 % of the proteins in the brewery yeast waste (Table 5.3). However, there was no significant difference between the protein concentration of the baits produced with the different papain types including the control ( $p = 0.392$ ). Though crude fruit latex papain bait had a pH value of  $5.17 \pm 0.01$  and the control had a pH of  $5.12 \pm 0.04$ , there was no significant difference between the pH of the protein baits produced with the different papain types including the control ( $p= 0.324$ ) (Table 5.3).

**Table 5.3. Protein concentration and pH of prepared baits, brewery yeast waste and local laboratory diet formulation.**

Treatment	Crude Protein content $\pm$ (S.E) (mg/ml)	Percentage crude protein relative to the brewery yeast waste / %	Bait pH $\pm$ (S.E)
Control	$11.91 \pm 0.10^c$	43.31	$5.12 \pm 0.04^d$
Skin Peel	$12.16 \pm 0.10^c$	44.22	$5.15 \pm 0.00^d$
Fruit Latex	$12.94 \pm 0.10^c$	47.05	$5.17 \pm 0.01^d$
Commercial	$12.33 \pm 0.00^c$	44.84	$5.17 \pm 0.02^d$
Brewery Yeast Waste	$27.50 \pm 0.10^a$	-	-
Local laboratory diet formulation	$17.40 \pm 0.80^b$	-	-

Means with the same letters as superscript are not significantly different at the 0.05 confidence interval.

#### 5.4.4 Attractance of the different protein baits

Females of *B. invadens* were equally attracted ( $p = 0.808$ ) to protein baits prepared with commercial papain ( $32.86 \pm 8.23$  %), crude fruit latex ( $31.67 \pm 4.59$  %) and skin peel papain ( $25.00 \pm 13.13$  %) (Table 5.4). Similarly, males showed equal attraction to all the baits when subjected to statistical analysis ( $p=0.482$ ), with mean attractance of commercial papain, crude latex and skin peel baits being:  $25.00 \pm 10.00$  %,  $47.50 \pm 11.09$  % and  $25.00 \pm 7.56$  % respectively (Table 5.4). There was no significant difference ( $p > 0.05$ ) between the attractance behaviour of male *B. invadens* and female *B. invadens* to the different protein baits.

**Table 5.4. Attractance of the prepared protein baits relative to the control**

Papain source	Mean Attractance ( $\pm$ SE)/ %	
	Males	Females
Latex	$47.50 \pm 11.09^{a*}$	$31.67 \pm 4.59^{b*}$
Skin peel	$25.00 \pm 7.56^{a*}$	$25.00 \pm 13.13^{b*}$
Commercial Papain	$25.00 \pm 10.00^{a*}$	$32.86 \pm 8.23^{b*}$

Means in the same column with the same lower case alphabet as superscript are not significantly different at the 0.05 confidence interval.

Means in the same row with \* as superscript are not significantly different at the 0.05 confidence interval.

### 5.4.5 Feeding preference

The type of protein bait influenced the duration of feeding (Table 5.5). Protein bait produced with skin peel papain had the mean percentage response of  $34.67 \pm 4.90$  % and average feeding time of  $46.29 \pm 20.04$  seconds (sec.), while the control had a response  $21.03 \pm 8.21$  % and feeding time of  $15.72 \pm 2.90$  sec. (Table 5.5). There was no significant difference between the feeding responses of the flies ( $p = 0.292$ ) and duration of feeding ( $p = 0.356$ ) for all four protein bait types.

**Table 5.5. Feeding response and feeding times per female *B. invadens***

Treatment	Mean feeding response ( $\pm$ SE)/ %	Time spent on Bait (sec.)	
		Feeding Time ( $\pm$ SE)	No feeding time ( $\pm$ SE)
Latex	$22.95 \pm 4.60^a$	$23.41 \pm 7.98^b$	$101.14 \pm 93.57^c$
Skin Peels	$34.67 \pm 4.90^a$	$46.29 \pm 20.04^b$	$27.92 \pm 20.04^c$
Commercial	$21.35 \pm 1.32^a$	$20.50 \pm 7.08^b$	$10.94 \pm 4.99^c$
Control	$21.03 \pm 8.21^a$	$15.72 \pm 2.90^b$	$36.48 \pm 23.24^c$

Means in the same column with the same lower case alphabet as superscript are not significantly different at the 0.05 confidence interval.

## 5.4.6 Survival, fecundity and egg hatchability

### 5.4.6.1 Survival of *B. invadens* fed on the different protein baits and the local laboratory diet formulation

Adult flies fed on the different protein baits and the local laboratory diet formulation showed differences in survival at all stages of the study with the male flies surviving longer in all cases (Figure 5.2 a; Figure 5.2b ). Apart from adult flies fed on the local laboratory diet formulation that survived the 92 days post-mating period, all other flies died completely before the 92nd day elapsed (Figure 5.2a; Figure 5.2b).

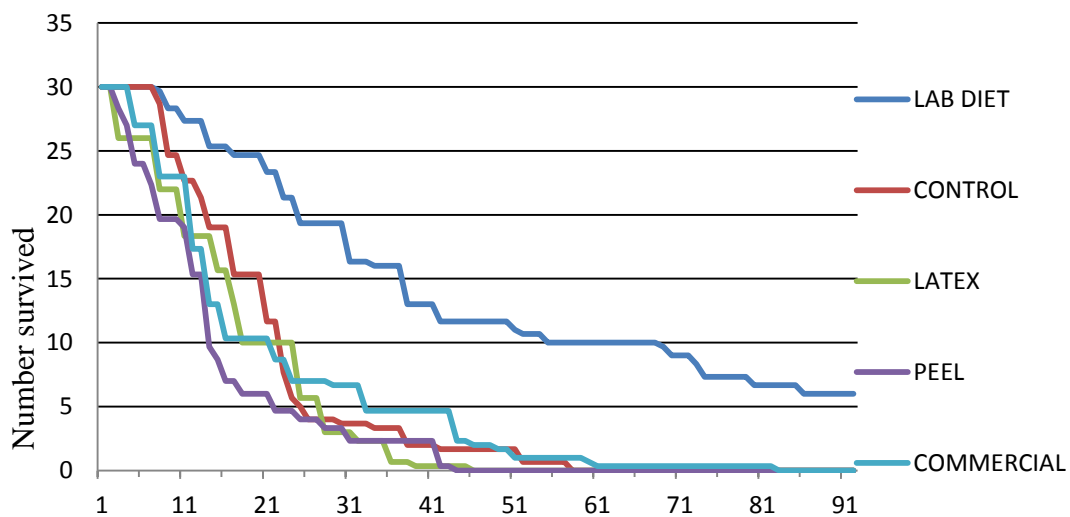
The thirty female flies fed on the local laboratory diet formulation started to decline in number from 30 flies to 10 flies between day eight (8) and day fifty five (55), and to six (6) flies on day 92 (Figure 5.2a). Males on the other hand declined in number to 12 flies between day 9 and day fifty eight (58) and to 8 flies on day 92 (Figure 5.2b).

The rates of decline of flies fed on the different bait types were rather sharp. On the average, flies fed on bait prepared with crude fruit latex papain survived for the least number of days (44 – 51 days), while flies fed with bait prepared with commercial papain survived the longest (83 – 85 days).

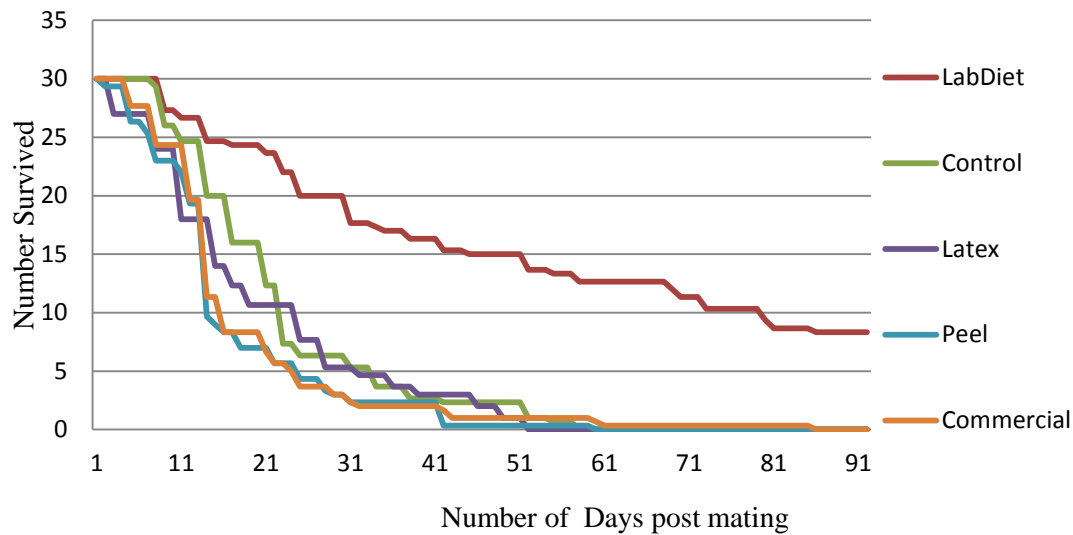
Female flies fed on the control declined in number between day seven (7) and day fifty six (56) when they all died (Figure 5.2a). A similar trend was observed with the male flies, which survived between day 8 and day 57 (Figure 5.2b). Adult flies fed on protein

bait prepared with crude fruit latex papain on the other hand survived between day 2 and day 44 for females, and males between day 3 and day 51(Figure 5.2a;Figure 5.2b).

Female flies that fed on protein bait prepared with skin peels survived between the 3rd and 44th days while males survived between day 2 and day 59. Female flies fed on protein bait prepared with the commercial papain survived between day 5 and 83rd day, while male survived between day 8 and the 85th day (Figure 5.2a; Figure 5.2b).



**Figure 5.2 a. Female survival curve over a period of 92 days post mating**



**Figure 5.2b. Male survival curve over a period of 92 days post mating**

#### **5.4.6.2 Fecundity of *B. invadens* fed on different protein baits and local laboratory diet formulation and mean egg hatchability.**

Adult female *B. invadens* flies fed on the control laid significantly higher ( $p = 0.00$ ) number of eggs ( $1965.30 \pm 8.77$ ) than flies fed on the local laboratory diet formulation ( $646.67 \pm 3.33$ ) (Table 5.6). There was no significant difference ( $p = 0.142$ ) between the number of eggs laid by flies fed on the local laboratory diet formulation and the skin peel papain bait ( $709.33 \pm 3.58$ ), commercial papain bait ( $1189.00 \pm 1.40$ ) and the crude fruit latex papain bait ( $1630.30 \pm 1.14$ ) (Table 5.6). Similarly, there was no significant difference ( $p = 0.307$ ) between the total number of eggs laid by flies fed on the control, commercial papain bait and the crude fruit latex papain bait.

**Table 5.6. Fecundity of female *B. invadens* fed on the different protein baits and local laboratory diet formulation**

Treatment	Total eggs ( $\pm$ SE)	Eggs per female ( $\pm$ SE)	% Hatched ( $\pm$ SE)
Latex	1630.30 $\pm$ 1.14 <sup>ab</sup>	13.28 $\pm$ 0.88 <sup>ab</sup>	73.62 $\pm$ 5.26 <sup>a</sup>
Skin peel	709.33 $\pm$ 3.58 <sup>b</sup>	13.96 $\pm$ 4.78 <sup>a</sup>	78.25 $\pm$ 4.87 <sup>a</sup>
Commercial	1189.00 $\pm$ 1.40 <sup>ab</sup>	11.39 $\pm$ 0.78 <sup>ab</sup>	64.25 $\pm$ 10.45 <sup>a</sup>
Control	1965.30 $\pm$ 8.77 <sup>a</sup>	17.55 $\pm$ 1.22 <sup>a</sup>	59.65 $\pm$ 7.13 <sup>a</sup>
Lab diet	646.67 $\pm$ 3.33 <sup>b</sup>	3.03 $\pm$ 1.01 <sup>b</sup>	84.71 $\pm$ 1.57 <sup>a</sup>

Means in the same column with the same lower case alphabets as superscript are not significantly different at the 0.05 confidence interval.

Whereas flies fed on control had a mean eggs per female of 17.55  $\pm$  1.22, flies fed on the local diet formulation had mean eggs per female of 3.03  $\pm$  1.01 (Table 5.6). However, there was no significant difference ( $p = 0.385$ ) between eggs per female of flies fed on the different protein bait types including the control.

Despite the 84.71  $\pm$  1.57 % hatchability of eggs recorded for females fed on local laboratory diet formulation against 59.65  $\pm$  7.13 % for females fed on control, there was no significant difference when subjected to statistical analysis ( $p = 0.122$ ) (Table 5.6).

## 5.5 DISCUSSIONS

Managing fruit flies with poisoned protein baits is an effective control strategy suitable for many tephritid fruit flies (Balagawi *et al.*, 2014). Hence, it is important to determine protein bait efficiency before field applications to ensure maximum control.

Autolysed brewery yeast waste is increasingly gaining grounds as a good substitute to commercial protein hydrolysate baits in poisoned protein bait sprays, for the control of Tephritid species of fruit flies (Gopaul and Price, 1999). Furthermore, replacing the proteolytic enzyme with local substitutes can minimise production cost of protein baits, and still make the protein bait effective and attractive to fruit flies (Sookar *et al.*, 2003). The proteolytic enzyme is responsible for breaking down the complex peptide bonds into simple amino acids. The difference in the colour of the resulting protein bait solution is a likely attribute the enzymes action. Protein baits prepared in this experiment were “dark-brown” when crude papain from latex fruit and commercial papain were used but “light-brown” for skin peel and control. It is possible that the crude papain from fruit latex and commercial papain were more effective in breaking down the peptide bonds than the skin peel, resulting in the colour variation.

Protein bait recovery for the different papain types did not vary significantly from each other. This confirms the fact that protein bait recovery is essentially dependent on the amount of the supernatant of the non-treated brewer’s yeast waste (Yeboah, 2012). Without any further treatment to concentrate the bait, protein baits produced with different papain types had higher dry weights than the control. Again, with respect to the

dry weight of the brewery yeast waste, the crude papain from fruit latex and the commercial papain had significantly higher dry weight retained relative to the dry weight of brewery yeast waste which may be as a result of the efficiency of their proteolysis of the yeast cell contents.

Protein baits with high percentage solids or dry weight are more attractive in laboratory and field bioassays (Sookar *et al.*, 2003). It is likely the percentage solids or dry weight increase as a result of proteolysis of the yeast cell contents. In a similar study by Sookar *et al.*, 2003, protein hydrolysate, was observed to have a significantly higher attractance than the yeast autolysed protein bait due to its high dry matter content (52 %). The dry weight of bait is essential in field application or bait sprays because flies feed on the liquids that adhere to the leaf surfaces (Secretariat of the Pacific Community, 2002). Protein bait with a low dry weight may not be suitable for protein bait sprays in the field because they can easily drip off the leaves after spraying.

Among other factors crude protein concentration is essential to attractive protein bait (Secretariat of the Pacific Community, 2002). Generally, protein bait with higher crude protein content results in the release of more essential amino acids and this makes it more attractive (Sookar *et al.*, 2003). The observations made from this experiment confirm a relationship between protein concentration and attractance of baits. The different protein baits had same protein concentrations and hence they were equally attractive to the test flies. Furthermore, the addition of the papain to the protein bait preparation may result in an increase of the protein bait pH (Sookar *et al.*, 2003). However, the pH of the different protein baits produced including the control did not differ significantly from each other;

thus, suggesting that bait pH is not solely dependent on the addition of the proteolytic enzyme. Therefore acidity of protein bait can be manipulated by the addition of other chemical enhancers. Basic bait is more attractive than acidic bait, but since all the protein baits prepared were slightly acidic attractance was equal for all baits.

The attractance of the different protein baits was not significantly different for male flies and female flies. This further supports the principle behind protein bait sprays that it is effective for the control of both male and female flies. Moreover, it is clear that males like females require protein for sexual development and therefore actively search for protein like their female counterparts.

In the non-choice feeding preference assay of only female flies, there was no significant difference between the preferred baits and the duration of feeding. These findings could be as a result of the non-variation in the properties of the protein bait (pH and crude protein concentration). Apart from the non-variation in the properties of the protein bait, the assay procedure can influence the results. In choice assays, it is possible that a fly would settle on protein bait other than the protein odour plume it detected first (Rousse et al., 2005). This phenomenon is due to the alteration in odour plume morphology owing to the odour plumes from the different protein baits present (Rousse et al., 2005).

The effect of alteration of odour plumes was controlled by cage rotation during the experiment; therefore the control was equally preferred as a feeding substrate with no interference from other protein baits. This confirms the finding by Sookar *et al.*, (2003)

that autolysis of brewery yeast waste without further treatment with proteolytic enzyme causes enough lysis of the yeast cells to release enough attractive volatiles.

Though feeding preference were similar for all the test flies, the feeding times recorded for baits prepared with skin peel and crude papain from fruit latex were within the range of feeding times recorded in an assay with F1 generation of *Bactrocera cucurbitae* and *Bactrocera dorsalis* using various commercial protein bait formulations (Barry *et al.*, 2006). When *B.cucurbitae* and *B. dorsalis* were fed on protein baits poisoned with spinosad for twenty (20) seconds, 99 % *B. cucurbitae* and 89 % *B. dorsalis* died after the experiment (Barry *et al.*, 2006). The results from the feeding assay (feeding time) provides enough evidence that in the field, spraying of skin peel papain bait, crude fruit latex papain bait and commercial papain bait, would result in wild flies consuming enough poisoned bait to increase mortality than the control.

Most of the insecticides used in protein bait production are systemic in action rather than contact (Barry *et al.*, 2006). However, the time spent on the different protein baits without feeding suggests that it is possible to broaden the application of protein baits with the use of contact insecticides. Most of the flies spent significant proportion of the time grooming on the bait after feeding and therefore particles from the feeding substrate can adhere to their integument when they fly off. Therefore, contact insecticides in protein baits may have effect on the fly that lands on it even without feeding.

Yeast-based baits are an excellent source of proteins and vitamins required for growth (Taylor *et al.*, 2011). The amino acids in the diets of adult males and females facilitate mostly sexual development which leads to more fecund adult flies. The results from the

survival and fecundity experiments suggest that the amino acids released from the autolysis and proteolysis of the yeast cell protein could meet the reproductive development requirements of the flies. Though the local laboratory diet formulation had a higher protein concentration than the protein baits, flies fed on the laboratory diet formulation could not lay corresponding high number of eggs. This is likely due to the solid nature of the local laboratory diet formulation and probably because the proteins were not subjected to autolysis and proteolysis (complex nature of protein). Hence, solid or powders of protein baits may not be as effective for control as the liquid protein bait formulation due to the lapping mouthparts of fruit flies.

The results of this experiment indicate a relationship between the survival of the flies and their fecundity. *B. invadens* fed on the control were more fecund than their counterparts fed on the local laboratory diet formulation. Flies fed on the local laboratory diet formulation had significantly higher survival rates, but low fecundity throughout the experimental period. The relatively high fecundity observed for flies fed on the protein baits may be attributed to an enhanced reproductive development resulting from the protein-rich baits (Taylor *et al.*, 2011). According to Taylor *et al.*, (2011), an increase in reproductive development results in high copulatory behaviour, increased mating probability, increased sperm storage, and increased sexual inhibition by mated females.

Flies need sugars for survival; but proteinaceous food is required for reproductive development (Vargas *et al.*, 2002). Thus, protein bait is more related to reproduction than survival hence the addition of 10 % sucrose solution to all protein baits used in laboratory bioassay. As such, the low survival rate of protein bait-fed flies is a likely attribute of life

span-reproduction trade-off. Among reproductive adult insects, there is a negative relationship between survival and fecundity, such that a high fecundity results in low survival rates (Muller *et al.*, 2001). Again, age patterns in reproduction rather than the total reproductive output influence long life span (Molleman *et al.*, 2008). In the present experiment, flies fed on the local laboratory diet formulation had a delayed oviposition to two weeks post-mating (data not reported) which corresponds to 24 days old, while flies fed on the different protein baits began oviposition within the same week of mating. Female longevity is influenced directly by the rate of egg production (Muller *et al.*, 2001). Therefore, it is likely that the late oviposition behaviour of flies fed on the local laboratory diet formulation enhanced female longevity.

The fact that all flies reared on the different protein baits showed reproductive development provides enough evidence that the flies were attracted to feed on the protein baits.

## **5.6 Conclusions**

The data collected from this study indicate that, locally produced papain from “Red lady” variety of pawpaw is as effective as the commercial papain in the proteolysis of yeast autolysate used for protein bait production. Although the addition of papain into the protein bait preparation did not have any significant effect on the values of the pH, and the protein concentration, the dry weight of the bait and the colour of the bait changed significantly. The source of papain (fruit latex or skin peels) has no effect on the efficacy of the protein baits. Autolysed brewery yeast waste without further treatment with papain

may be attractive to flies, but feeding duration of flies measured in this present work suggested that it may be inefficient for field applications.

Laboratory reared flies fed on the different protein baits had high fecundity and fertility and could be exploited in mass rearing of *Bactrocera invadens*.

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## CHAPTER 6

### 6.0 GENERAL CONCLUSIONS AND RECOMMENDATIONS

#### 6.1 General Conclusions

In evaluating a protocol for producing pure papain from “Red lady” pawpaw fruit latex and skin peels, an aqueous two-phase extraction procedure composed of 15 %  $(\text{NH}_4)_2\text{SO}_4$  – 8 % PEG was chosen due to its ease of application and efficiency. The extraction of papain from pawpaw fruit latex using the aqueous two-phase extraction recovered 64.72 % of the papain into the supernatant which accounted for 7.33 % of the total proteolytic activity of the crude latex and a fold purification of  $58.11 \pm 1.67$ . The presence of papain in the supernatant of the fruit latex extract was confirmed on SDS-PAGE and it had an equivalent molecular weight as the commercial papain.

When skin peel supernatant solution was compared with crude extracts of skin peel, the skin peel supernatant solution had a higher proteolytic activity and protein concentration than the crude skin peel extract though their specific activities were statistically similar. However, SDS-PAGE analysis of aqueous two-phase extracts of pawpaw fruit skin peel generated no visible protein bands probably due to the volume of water used for extraction.

The effects of irradiation were assessed by the total residual proteolytic activity, crude protein concentration and specific activity of the papain after irradiation. Increasing radiation dose up to 10 KGy increased the proteolytic activity and specific activity of the

crude papain though protein concentration was not affected. Under ambient conditions the proteolytic activity increased by up to 21.69 % of the non-irradiated crude papain. Effects of Cobalt-60 gamma radiation on the crude papain appeared to follow the trend that whenever proteolytic activity was affected, the specific activity was affected since effect of radiation on protein concentration was not significant. SDS-PAGE profile of the irradiated papain confirmed the presence of papain at all levels of radiation dose. Irradiation had no effect on papain purity and therefore cannot be used as a substitute to purification. However, radiation dose of up to 10 KGy under ambient conditions can be used as a non-destructive treatment to maintain or enhance proteolytic activity of crude dried papain.

The effects of the papain source (fruit latex and skin peel) used for the proteolysis of brewery yeast on the physicochemical properties of produced protein baits were assessed by the protein concentration, pH, dry matter and protein bait yield. Protein concentration, pH and protein bait recovery were not affected by the type of papain used for proteolysis. Protein concentration of protein baits extrapolated from the BSA standard curve ranged between  $11.91 \pm 0.1$  mg/ml for control and  $12.94 \pm 0.1$  mg/ml for the fruit latex; pH readings were in the mild acidic region being  $5.12 \pm 0.04$  for the control and  $5.17 \pm 0.01$  for the latex. Mean bait recovery of 366 ml (control) – 390 ml (fruit latex) were achieved.

The colour of the bait was affected by the type of papain, with crude fruit latex papain and commercial papain yielding dark brown protein baits while the control and skin peel baits were light brown. This is suggestive that based on colour as attractive cue, crude

fruit latex papain may be effective as the commercial papain for proteolysis of protein baits and more effective than the crude skin peel extracts.

Moreover, crude fruit latex papain ( $12.32 \pm 0.64$  %;  $0.12 \pm 0.01$ g/ml) and commercial papain ( $11.22 \pm 0.24$  %;  $0.11 \pm 0.00$  g/ml) baits had significantly higher ( $p = 0.00$ ) dry weights than control ( $9.38 \pm 0.34$  %;  $0.09 \pm 0.00$  g/ml), but there was no significant difference between the percentage dry weight ( $p = 0.67$ ) and actual dry weight ( $p = 0.78$ ) of skin peel papain bait ( $10.00 \pm 0.09$  %;  $0.10 \pm 0.00$  g/ml) and control ( $9.38 \pm 0.34$  %;  $0.09 \pm 0.00$  g/ml). When the dry weights of the different protein baits were compared with the dry weight of the brewery yeast waste (dry weight retained), crude fruit latex and commercial papain baits retained higher proportions of the dry weight of the brewery yeast waste. Whereas on the average, baits prepared with commercial papain and fruit latex papain retained between  $60.80 \pm 1.35$  % and  $64.52 \pm 3.02$  % of brewery yeast waste dry weight, skin peel papain bait and the control retained lower proportions between  $50.21 \pm 1.71$  % and  $52.11 \pm 0.00$  %.

Laboratory bioassays of prepared protein baits covered attractance test, feeding preference test, and survival and fecundity tests. The results showed that protein bait pH and crude protein concentration influence the attractance of protein bait to adult flies. As such, in a non-choice assay conducted under uniform artificial light of 90 lux intensity, temperature of  $26 \pm 2$  °C and relative humidity of  $72 \pm 4$  %, the different protein baits had equal attractance properties to the flies tested. It was evident from the attractance test that males like females actively search and feed on proteins since there was no difference between the percentage attractance of males and females to the different protein baits.

In a choice feeding preference test of only female flies, the statistical analysis of the data shows that the test flies chose and fed on the different protein baits at the same frequency and durations. Though test flies had equal preference for all the protein baits, the feeding duration observed between  $20.50 \pm 7.08$  sec. and  $46.29 \pm 20.04$  sec. may be adequate for wild fruit fly to take up enough poisoned protein bait from field sprays that could lead to lethality in field population.

The results obtained from the fecundity and survival studies suggested that flies actually were attracted to feed on the local protein baits. A relationship between survival and fecundity was observed where increasing fecundity resulted in decreased survival duration. The flies fed on the control laid significantly higher eggs of  $1965.30 \pm 8.77$  eggs (17.55 eggs per female) than the flies fed on the local laboratory diet formulation;  $646.67 \pm 3.33$  eggs ( $3.03 \pm 1.01$  eggs per female). The short survival duration of flies fed on the different protein baits was most likely affected by high fecundity of those flies. Whereas 14 flies (8 males and 6 females) fed on the local laboratory diet survived to 92 days post mating, fewer more fecund flies (males and females) fed on control survived only within 57 days post mating.

## **6.2 Recommendations**

The results of the biochemical and bioassay could not draw major distinctions between the efficacy of the papain extracted from the pawpaw fruit latex and the skin peels.

Therefore, future work could look at some of the following recommendations in order to improve the efficacy assessment:

1. Aqueous two-phase extraction may be applicable to latex extraction; yet the protocol needs to be optimised in order improve papain recovery in the supernatant, the partition coefficient and to make all the papain either crystallise out of the supernatant solution or remain in the solution.
2. The “Red lady” pawpaw variety used in this experiment had a high protein concentration; future work on papain extraction from this pawpaw variety should focus on the suitable initial protein concentration for efficient papain extraction.
3. The phase concentration of the aqueous two-phase extraction system should be evaluated to improve especially the papain extraction from the pawpaw fruit skin peel.
4. The effect of the irradiation on the papain should be assessed on the individual molecules that make up the enzyme to provide enough evidence of the influence of gamma radiation on papain that can be further manipulated to improve the efficacy of the enzyme in proteolysis.
5. Alternative radiation sources such as ultraviolet radiation, x-rays and electron beam ought to be exploited in order to enhance proteolytic activity and increase protein concentrations of protein baits produced from irradiated papain.

6. Prepare protein baits with papain extracted from the fruit latex and skin peel using the aqueous two-phase extraction procedure.
  
7. Conduct shelf life studies of protein baits produced with the different papain types based on food analysis and bioassay to evaluate the efficacy of the local papain stored over a period of time.

## APPENDICES

### APPENDIX 1

#### 1.0 REAGENTS AND SOLUTIONS FOR PROTEIN CONCENTRATION DETERMINATION

##### 1.1. Alkaline test solution

Alkaline test solution was prepared fresh on the day of use by mixing 50 ml of alkaline solution (1) and 1 ml of alkaline solution (2) together. The alkaline solution is added to the assay to regulate any drop in pH that would occur after the addition of the Folin–Ciocalteu reagent.

Hundred (100) ml of alkaline solution (1) was prepared by dissolving 2 g of Sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) in 100 ml of 0.1 M Sodium hydroxide (NaOH) while five (5) ml alkaline solution 2 was prepared by dissolving 0.025 g Copper (II) sulphate pentahydrate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) in 5 ml of Sodium potassium tartate at a concentration of 10 g/litre.

##### 1.2 Folin-Ciocalteu reagent

Folin-Ciocalteu reagent used for all assays was a commercial formulation which consists of Sodium tungstate and Sodium molybdate in Phosphoric and Hydrochloric acid. It was diluted with distilled water in a ratio of 1:2 on the day of use.

### **1.3 Bovine Serum Albumin solution**

A Bovine Serum Albumin (BSA) standard curve was generated using a stock solution of 10 mg/ml. Fifty (50) ml BSA stock solution was prepared by dissolving 0.5 g of Bovine Serum Albumin (Sigma Aldrich) into 50 ml of distilled water. The solution was stirred on a Stuart stirrer hot plate at 0 °C for 15 minutes to increase dispersion of the solid BSA. From the stock solution, five (5) different dilutions resulting in five (5) concentrations of 0.04 mg/ml, 0.08 mg/ml, 0.12 mg/ml, 0.16 mg/ml and 0.2 mg/ml BSA were prepared and their protein concentration determined. Their absorbance readings were used to generate a concentration-absorbance reading standard curve which was later used for protein concentration determination.

## **APPENDIX 2**

### **2.0 REAGENTS AND SOLUTIONS FOR PROTEOLYTIC ACTIVITY DETERMINATION**

#### **2.1 1.1 mM Tyrosine stock solution**

A 0.01g of L-tyrosine powder was dissolved in 50 ml distilled water and heated gently at 80 °C on a Stuart stirrer hot plate for 10 minutes. The tyrosine stock solution was allowed to cool to room temperature before the dilutions were prepared.

#### **2.2 Tris-HCl Buffer**

A 50 mM Tris-HCl buffer was prepared by dissolving 0.3939 g of Trizma (w/v) in 45 ml of distilled water. The pH of the solution was adjusted to 8 with drops of 1M HCl and then topped up with distilled water to the 50 ml mark of the glass beaker. The buffer solution was poured into a 50 ml graduated falcon tube and stored in the refrigerator at 0 °C till it was ready to be used.

#### **2.3 Cysteine – Ethylene diamine tetraacetic acid reaction mixture.**

To prepare the reaction mixture, 0.372 g of 20mM EDTA (w/v) was dissolved in 30 ml of distilled water after which 0.303 g of 50mM cysteine was dissolved in the EDTA solution. The mixture was topped up with distilled water to obtain a final volume of 50 ml. The final pH of the cysteine–EDTA mixture was adjusted to 8 using 6 M NaOH and 1 M HCl where necessary. The reaction mixture was prepared fresh on the day of use.

#### **2.4 5% Trichloroacetic acid (TCA)**

To prepare 5 % TCA, 0.408 g (w/v) of trichloroacetic acid was dissolved in distilled water to the 50 ml mark in a 50 ml falcon tube.

#### **2.5 1 % Casein solution**

Casein solution was prepared by dissolving 0.5 g casein from bovine milk in 50 ml of distilled water to obtain 1 % (w/v) casein. The casein solution was heated gradually to 80 °C for ten (10) minutes at a constant stirring speed on a Stuart stirrer hotplate. This was done to obtain a homogenous dispersion of the solid casein. Casein solution was prepared fresh on the day of use.

### APPENDIX 3

#### 3.0 STANDARD EQUATIONS

##### 3.1 PROTEOLYTIC ACTIVITY

$$PA, (\text{unit/ml}) = \frac{(\mu\text{mol Tyrosine})(V_t)}{(V_e)(T)(V_c)}$$

Where:

PA = Proteolytic activity, (tyrosine units/ml)

V<sub>t</sub> = Total assay volume, (ml)

V<sub>e</sub> = Volume of enzyme used in assay (ml)

T = Time of assay (minutes)

V<sub>c</sub> = Volume of final protein assay mixture used in calorimetric reaction

##### 3.2 TOTAL PROTEOLYTIC ACTIVITY

Total PA, (Tyrosine Units) = Units/ml enzyme x phase volume

Where PA = Total proteolytic activity per phase

##### 3.3 PHASE VOLUME RATIO

$$R_v = \frac{V_t}{V_b}$$

Where:

R<sub>v</sub> = Phase volume ratio

V<sub>t</sub> = Volume of supernatant

V<sub>b</sub> = Volume of bottom phase

### 3.4 PARTITION COEFFICIENT

$$K = \frac{PCs}{PCb}$$

Where:

K = Partition coefficient

PCs = Protein concentration in supernatant

PCb = Protein concentration in bottom phase

### 3.5 PAPAN YIELD RECOVERY

$$R_s = \frac{100}{1 + \frac{1}{RvK}}$$

$$R_b = \frac{100}{1 + RvK}$$

Where:

R<sub>s</sub> = Yield recovery in the supernatant

R<sub>b</sub> = Yield recovery in the bottom phase

R<sub>v</sub> = Phase volume ratio

K = Partition coefficient

### 3.6 SPECIFIC ACTIVITY

$$SA = \frac{PA}{PC}$$

Where:

SA = Specific activity

PA = Proteolytic activity

PC = Protein concentration

### 3.7 PURIFICATION FOLD

$$Pf = \frac{SAe}{SAc}$$

Where:

Pf = Purification fold

Sae = Specific activity of phase extract

Sac = Specific activity of the crude sample

### 3.8 PERCENTAGE YIELD

$$Y = \frac{PAe}{PAc} \times 100$$

Where:

Y = Percentage yield

PAe = Proteolytic activity of the phase extract

Pac = Proteolytic activity of the crude sample

### 3.9 PERCENTAGE DRY MATTER CONTENT

$$\% Dw = \frac{(W2 - W0)}{(W1 - W0)} \times 100$$

Where:

% Dw = Percent dry matter content

W0 = Weight of empty Petri dish

W1 = Initial weight of Petri dish and content before drying

W2 = Final weight of Petri dish and contents after drying

### 5.10 ACTUAL DRY WEIGHT

$$Dw = \frac{\% Dw \times (W1 - W0)}{100}$$

Where:

Dw = Actual dry weight

% Dw = Percentage dry weight

W0 = Weight of empty Petri dish

W1 = Initial weight of Petri dish and content before drying

### 5.11 PERCENTAGE DRY WEIGHT RETAINED

$$\% Wr = \frac{Dw}{Bw} \times 100$$

Where:

% Wr = Percentage dry weight retained

Dw = Actual dry weight of sample

Bw = Dry weight of brewery yeast waste

### 5.12 PERCENTAGE ATTRACTANCE

$$\% A = \frac{Tn - Cn}{To} \times 100$$

Where:

% A = percentage attractance

Tn = Number of test flies in the treatment region

Cn = Number of test flies in the control region

To = Total number of flies used in assay

## **APPENDIX 4**

### **4.0 SOLUTIONS, REAGENT AND 10 % ACRYLAMIDE GEL ELECTROPHORESIS**

#### **4.1 Running buffer**

The running buffer used in the upper and lower chambers was prepared by dissolving 30.30 g of Tris base, 144 g Glycine and 10.00 g Sodium Dodecyl Sulphate (SDS) in distilled water to a final volume of one (1) Litre. The pH was adjusted to 8.3 using drops of 1M HCl or 6M NaOH where necessary.

#### **4.2 Sample buffer**

An eight (8) ml sample buffer was prepared to a concentration of 2X by mixing one (1) ml 0.5 mM Tris-HCl (pH=6.8), two (2) ml of 25 % Glycerol, 0.08 ml of one (1) % Coomassie Blue dye, 1.6 ml of ten (10) % SDS and three (3) % B-Mercaptoethanol. The sample buffer was prepared fresh on the day of use

#### **4.3 Gel preparation and casting**

##### **4.3.1 Preparation of 10 % Resolving Gel**

A twenty (20) ml resolving gel was prepared by mixing 6.7 ml of thirty (30) % acrylamide mix, five (5) ml of 1.5 M Tris (pH=8.8), 0.2 ml of ten (10) % SDS, 0.2 ml of ten (10) % Ammonium per sulphate and 0.008 ml of N,N,N,N'-Tetramethylenediamine (TEMED). The mixture was diluted with 7.9 ml of distilled water and poured into the cassette (a thin space between two glass plates within the set up).

#### **4.3.2 Preparation of 5 % Stacking Gel**

A ten (10) ml stacking buffer was prepared by mixing 1.7 ml of thirty (30) % acrylamide mix, 1.25 ml of 1.0 M Tris (pH=6.8), 0.1 ml of ten (10) % SDS, 0.1 ml of ten (10) % Ammonium per sulphate and 0.01 ml of TEMED. The mixture was further diluted with 6.8 ml of distilled water and poured into the same cassette containing the resolving gel so that the stacking gel is set on the resolving gel. The gels were combed and the gel assembly taped together with a masking tape to set.

#### **4.4 Gel assembly**

The electrophoresis cell was assembled as follows: the comb and masking tape holding the gel cassette were removed from the gels. The gel was mounted vertically into the electrophoresis apparatus such that the upper and lower surfaces of the gel were all in contact with the chambers that contain anode and cathode. The inner (upper) chamber was filled with 200 ml of the running buffer and outer (lower) chamber was filled with 550 ml of the running buffer.

The different papain samples were prepared by mixing each sample with the sample buffer in a ratio of 3:1, that is 30  $\mu$ l papain sample to 10  $\mu$ l of sample buffer in 1 ml Eppendorf tubes. The mixture was vortex to ensure complete mixture before heating. The sample was heated in a water bath of temperature 100 °C for 5 minutes and centrifuged in an Eppendorf centrifuge at 1000 x g for twenty (20) seconds (quick spin) to remove any insoluble materials that would clog the pores of the acrylamide gel.

#### **4.5 Staining dye**

Fifty millilitres (50ml) of 0.1 % (w/v) Coomassie Blue solution was prepared by dissolving 0.125 g of Coomassie Blue dye in a mixture of 22.5 ml of methanol and 5 ml of glacial acetic acid. The solution is further diluted with 22.5 ml of distilled water to obtain the final volume of 50 ml.

#### **4.6 Destaining Buffer**

Seventy millilitres (70 ml) of 100 % (v/v) Acetic acid was mixed with 100 ml of methanol and the volume topped up with distilled water to the one (1) litre mark in a one (1) litre conical flask.

## **APPENDIX 5**

### **5.0 PREPARATION OF AQUEOUS PAPAIN**

#### **5.1 Enzyme diluent**

An enzyme diluent for dried papain samples was prepared by mixing 10 ml of 0.01 M EDTA, 0.1 ml of 0.06 M Mercaptoethanol and 10 ml of 0.05 M Cysteine with 70 ml of distilled water.

#### **5.2 Sample preparation**

0.1 g of the different irradiated samples were weighed into 50 ml graduated falcon tubes and dissolved with the enzyme diluent to the ten (10) ml mark of the falcon tube. A 20  $\mu$ l of each enzyme solution was further diluted to 2ml with the enzyme diluent to obtain a final concentration of 0.1 mg/ml. The enzyme solutions were prepared fresh on the day of use and stored in an ice box with ice cubes till ready to use.