

**MAKERERE**



**UNIVERSITY**

**SAFETY ASSESSMENT OF CYSTATINS USED TO CONTROL  
PESTS IN TRANSGENIC BANANAS IN UGANDA**

**BY**

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**A THESIS SUBMITTED TO THE SCHOOL OF GRADUATE  
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## DECLARATION

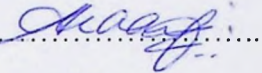
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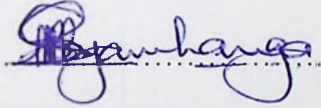
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## DEDICATION

This work is dedicated to my family, parents, brothers, sisters and friends.

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## ABSTRACT

In this study the safety of the maize and papaya cystatins used to control nematodes and banana weevil borer was assessed by determining the allergenicity and toxicity of the proteins using both *in vitro* as well as the *in vivo* method of oral gavage using albino rats. The maize and papaya cystatins were expressed in *Escherichia coli* strain M15 since this organism is easily cultured. Determination of *in vitro* digestive assays for both cystatins as well oral feeding of the animals with the cystatins in various concentrations of 10mg, 100mg and 1000mg/kg body weight was carried out followed by sacrificing the animals on the fourteenth day for clinical and histopathological examination of the blood and animal organs.

The results indicate that the maize and papaya cystatins were fully expressed in the *E.coli* M15 and *E.coli* M15 culture grew well to produce enough protein. The two proteins rapidly degraded in the simulated gastric fluid taking less than 2 minutes to degrade in the pepsin. The maize and papaya cystatins had amino acid sequence similarity with *Actinidia deliciosa* a kiwi fruit of 48.3% and 40.4% respectively over their full length, suggesting that both cystatins are not potential allergens. The results of the amino acid sequence homology comparison with the known toxins in the database did not indicate any relatives, suggesting that these cystatins are not potential toxins. The feed intake data showed that the maize and papaya cystatins did not affect feed intake of the animals. The results of urinalysis, haematology, serum chemistry and organ weights showed that feeding the rats with acute doses of maize and papaya cystatins did not affect the liver and kidneys which play a big role in various metabolic processes. This study shows that the maize and papaya cystatins used to control pests in transgenic bananas in Uganda do not have allergenic and toxic effects. These cystatins may therefore be safely used in transgenic bananas to control nematodes and weevils.

## CHAPTER ONE

### INTRODUCTION

#### 1. Background

Bananas (*Musa spp*) are the fourth most important food crop after rice, wheat and maize in terms of gross value of production (Picq, 2000). World banana production is most recently reported to be 97 million tonnes annually (FAOSTAT, 2008). In Africa, bananas are a major staple food, supplying upto 25% of the carbohydrates for approximately 70 million people. They are in comparison with other starchy staples, rich in a number of minerals and vitamins, namely Vitamin A, C, B6, potassium, phosphorus and calcium (FAO, 1990; Ploetz, 2001; BARNESA, 2008).

The East and Central Africa sub-region produces nearly 20 million tons of bananas annually of which approximately 10 million tons produced are from Uganda (BARNESA, 2008). This contributes 10 percent of the worlds' plantain and banana production (FAOSTAT, 2008). Bananas occupy a high position in the socioeconomic set up of Uganda. A culture based on their cultivation and utilization in various ways is well established, making them highly cherished crops (Tushemereirwe *et al.*, 2001). In the East African Highlands, cooking (AAA),and juice (ABB) bananas dominate. In Uganda more than 131 different banana varieties have been identified of which 75 are cooking types while 35 are for juice, dessert and roasting (Rubaihayo, 1991).

Bananas in Uganda face a number of constraints. These include pests like nematodes and weevils (Gold *et al.*, 1998), diseases and degenerating soil fertility. Nematodes and banana weevils have constrained banana production in the East African Highlands for a long time (Gold *et al.*, 1998). Nematodes attack and damage banana roots causing stunting, delayed maturation, slow ratooning, reduced root weight, reduced bunch size and toppling (Speijer *et al.*, 1994; Robinson, 1996; Barekye *et al.*, 2003). The pest also causes lesions on the roots thereby reducing water and nutrient uptake to the upper parts of the plant as well

as paving the way for other pathogenic microorganisms (Tushemereirwe *et al.*, 2001). All these effects result in reduced yields and other forms of production loss. The most damaging nematode species on bananas in Uganda include: *Radopholus similis*, *Pratylenchus coffeae*, *P. goodeyi* and *Helicotylenchus multicinctus* (Kashaija *et al.*, 1994; Namaganda *et al.*, 2000).

In addition to nematodes, the banana weevil borer, *Cosmopolites sordidus* has been identified amongst the most devastating pests of banana rhizomes (Gold, 1998; Robinson, 1996). Although the banana weevil is not considered an important pest in established commercial Cavendish plantations, the pest appears to be economically important in East African Highland banana plantations. The East African Highland bananas (AAA) and the plantain (AAB) are more susceptible to banana weevil attack than the juice and beer bananas, like *Kisubi* (AB) and *Kayinja* (ABB) cultivars (Gold *et al.*, 1993). *Bogoya* (AAA) suffers peripheral damage, but is not easily penetrated into the corm. In older plantations with higher weevil infestations, snapping can be quite extensive, leading to yield losses of between 50 to 100% and reduced plantation life span (Gold *et al.*, 1998).

## 1.2. Problem statement

Banana production still depends on a limited number of landraces selected by farmers from the natural germplasm. This practice has maintained a small genetic pool and has ultimately put the banana germplasm at risk of pest and disease outbreak (Tushemereirwe *et al.*, 2001). Additionally, breeding for disease and pest resistance is not easy because of the long generation times, various levels of ploidy, sterility of most edible cultivars and limited genetic variability (Ortiz *et al.*, 1995).

Conversely, pest control is currently based on application of cultural practises such as the use of clean planting materials, systematic trapping of adult weevils, as well as field sanitation to remove plant residues (Gold, 1998). Cultural methods contribute to pest management, but the high labour input and material

requirements are limiting factors for adoption (Gold, 1998). Application of effective pesticides would have been a suitable alternative but it is economically unfeasible for subsistence producers. Unfortunately, the banana weevil has developed resistance to a range pesticides (Collins *et al.*, 1991). Improvement of bananas to overcome these problems therefore, relies heavily on biotechnology to find cost effective strategies from which African farmers can benefit in the future (Jones, 2000).

Cystatins are plant protease inhibitors that reduce the ability of nematodes and insects to utilise dietary protein resulting in delayed development and reduced ability to reach their egg laying size (Urwin *et al.*, 1997; Atkinson *et al.*, 2004). Use of novel defences against plant parasitic nematodes and banana weevil borer through genetic engineering provides a breeding option for bananas. Maize cystatins are the proposed proteins to induce resistance against parasitic nematodes while the papaya cystatins will induce resistance against the banana weevil borer (Kiggundu, 2008).

Various researchers demonstrated the effectiveness of cystatins against nematodes in plants such as *Musa spp* (Atkinson *et al.*, 2004), *Oryzae sativa* (Vain *et al.*, 1998), *Arabidopsis thaliana* (Urwin *et al.*, 1997), *Solanum lycopersum* (Urwin, *et al.*, 1995), and, *Solanum tuberosum* (Urwin, *et al.*, 2001), Abel *et al.* (1987), and, Dunavesky, (2005) reported the use of cystatins in plants to control insects. Research on use of cystatins has led to the expression of genes for inhibitors in various transgenic banana lines to provide resistance to nematodes and banana weevil borer in Uganda (Atkinson *et al.*, 2004). However, safety information pertaining to toxicity and allergenicity of the maize and papaya cystatins expressed in the transformed bananas is scanty. Phytocystatins from the cortex and seeds of kiwi fruit (*Actinidia deliciosa*; Rassam and Laing, 2004) and actinidin (Pastorello *et al.*, 1998) from kiwi fruit as well as papain from papaya (Metcalf *et al.*, 1996) have been identified as allergens. Therefore this study aims to determine the toxicity and allergenicity of cystatins used in the transgenic

bananas to induce resistance against nematodes and banana weevil borer respectively before release for field trials in Uganda.

### **1.3. Objectives**

#### **1.3.1. Overall objective**

The overall objective of this study was to assess the safety of the maize and papaya cystatins used in the transgenic bananas to induce resistance against nematodes and banana weevil borer in Uganda.

#### **1.3.2. Specific objectives**

1. To establish the stability of the maize and papaya cystatins in simulated gastric and intestinal tract conditions
2. To determine amino acid sequence homology of the maize and papaya cystatins in comparison to known toxins and allergens in the databases
3. To determine the toxic effects of the cystatins when fed to albino rats

### **1.4. Hypotheses**

1. Maize and papaya cystatins readily degrade in the simulated gastric and intestinal tract conditions
2. Amino acid sequences of maize and papaya cystatins are identical to some of the known toxins and allergens in the databases
3. Maize and papaya cystatin will induce toxic and allergenic effects in albino rats

### **1.5. Significance of the study**

Breeding for resistance against pests and diseases is very important for the Ugandan economy and the farmers at large since bananas are both a food and cash crop, as well as The Cartagena Protocol on Biosafety to the Convention on Biological Diversity an international treaty governing the use of modern biotechnology to which Uganda is a signatory, and in which specific requirements for safety assessment of genetically engineered organisms and

products are required are requirements that signify these studies. However, there are concerns that the proteins used to induce resistance against nematodes and weevils may be toxic or allergenic to humans after consumption of bananas. This study will generate data that will be used by the breeders and policy makers in decision making about the safety of the developed bananas. In case the proteins are found to be toxic or allergenic alternative measures will be sought for pest control in bananas. However, if the cystatins are proven to be safe for use, the results will be used to further the breeding process for nematode and weevil resistance in bananas. The small scale farmers will benefit through the acquisition of clean, safe and pest resistant planting materials for better food security as well as improved household incomes.

## CHAPTER TWO

### Literature Review

#### 2.1. Bananas and their origin

Bananas belong to the family *Musaceae*, the order Zingiberales, subclass Liopsida division *Magnoliophyta* with two genera; *Musa* and *Ensete* (Stover and Simmonds, 1987). The genus *Musa* is comprised of all the edible bananas and plantains with over 50 species in five sections namely, *Eumusa*, *AustraliMusa*, *Rhodachlamys*, *Callimusa* and *Ingentimusa*. Amongst these, *Eumusa* is the biggest and geographically most widely distributed section of the genus giving rise to the great majority of the edible bananas world wide (Stover and Simmonds, 1987). Almost all the 300 or more cultivars arose from two seeded diploid species, *Musa acuminata* Colla and *M. balbisiana* Colla; these are diploid, triploid and tetraploid hybrids among subspecies of *M. acuminata*, and between crosses of *M. acuminata* and *M. balbisiana* (Robinson, 1996; Ploetz, 2001).

#### 2.2. Importance of bananas to Uganda's economy

In Uganda, bananas rank first as a major food source. The majority of banana producers are small scale farmers growing the crop for both home consumption and for local markets (Tushemereirwe *et al.*, 2001). The locally consumed bananas are of vital importance to food security for millions of people (Frison and Sharrock, 1998). In Africa, bananas provide food for about 70 million people, while in the East African Highlands, bananas are a staple food for about 20 million people. This region alone produces nearly 15 million tonnes annually. It is in the East African Highlands that bananas reach their greatest importance as a staple food crop (Frison and Sharrock, 1998).

Uganda has highest per capita consumption of bananas in the world with a rate of 243 kg per capita per year (BARNESA, 2008). In Uganda, bananas locally called 'matooke', are eaten daily. In addition, the crop has great cultural and social significance (Frison and Sharrock, 1998).

### 2.2.1. Utilization of bananas

Banana utilisation in Uganda is currently largely limited to the fresh form with only rudimentary efforts to process the crop for storage. Four uses are recognised according to use namely:- cooking, dessert, juice/ beer and roasting types

Cooking bananas traditionally called 'matooke' are harvested green, peeled, boiled or wrapped in banana leaves, steamed, mashed or unmashed and then eaten with a sauce (Karugaba and Kimeru, 1999; Tushemereirwe *et al.*, 2001). These constitute the majority of the East African highland bananas and belong to the AAA genome group (Karugaba and Kimaru, 1999).

Dessert bananas comprise those varieties eaten raw when ripe and belong to genome AB, AAA. These bananas have a very good aroma and flavour and they remain relatively firm when ripe. The most popular cultivars are *Bogoya* and *Sukali ndizi* (Karugaba and Kimenju, 1999).

Juice and beer bananas are harvested green, ripened and processed into juice, which can further be processed into a local beer popularly known as *tonto* (Gensi and Kyamuhangire, 1994; Aked and Kyamuhangire, 1996; Karugaba and Kimaru, 1999). These varieties include the East African highland bananas which are mutants of the cooking bananas, *Kayinja*, *Kivuvu* and *Kisubi* varieties with the ABB, AB, AAA- EA genome group (Rubaihayo, 1990; Karugaba and Kimenju, 1999).

Roasting banana varieties are from the AAB genotype, which is popularly referred to as plantains. In Uganda, they are often eaten when roasted after ripening but they may also be boiled or deep fried (Aked and Kyamuhangire, 1996). According to Nowakunda and Muranga, (1996) plantain bananas have been established to be the most promising variety for banana crisps because of firmness of the fruit when ripe. In addition to traditional processing methods, bananas can be made into a wide range of products with longer shelf life (Nowakunda and Muranga, 1996; Frison and Sharrock, 1998). The products range from convenient food items such as bakery

products, flour, crisps, to non food products such as fiber, starches and biogas.  
Nutrient composition of bananas

Bananas provide a good source of nutrients for both human and animal consumption as shown in Table 1. Bananas have a high carbohydrate and low fat content, making them particularly useful in low fat diets (Chandler, 1995). As a staple food crop, the protein level is low compared to major cereal crops. Bananas are also a good source of vitamins and minerals, particularly vitamins A, B6, thiamine, riboflavin, C and Potassium (Morton, 1987).

**Table 1: Nutritional composition of cooking bananas**

| Nutrient      | Content (%) (Wet basis) |
|---------------|-------------------------|
| Water         | 70 %                    |
| Carbohydrates | 27 %                    |
| Proteins      | 1 %                     |
| Fats          | 0.3 %                   |
| Fibre         | 0.5 %                   |
| Ash           | 0.9 %                   |
| Vitamins      | 0.3%                    |

Source: Chandler (1995)

## **2.3. Banana pests**

### **2.3.1. Nematodes**

Nematodes are small worm-like organisms which are present in almost all agro-ecosystems where they interact directly and indirectly with plants and other micro fauna, regulating decomposition and release of nutrients to plants (Kimenju *et al.*, 2005).

In Uganda, bananas are attacked by a complex of nematode species. Eight nematode species have been identified as the major causes of losses in bananas namely; *Radopholus similis*, *Pratylenchus goodeyi*, *Pratylenchus coffeae*, *Pratylenchus zaeae*, *Helicotylenchus multicinctus*, *Helicotylenchus dihystra*, *Helicotylenchus pseudorobustus* and *Meloidogyne spp.* These attack banana roots and were encountered during a survey in the major banana growing areas of western, south-western, central and eastern Uganda (Kashaija *et al.*, 1994). Of these, the species *Pratylenchus goodeyi*, *Helicotylenchus multicinctus* and *Radopholus similis* are wide spread and abundant thus considered the most important in Uganda (Kashaija *et al.*, 1994). *Radopholus similis*, *Pratylenchus goodeyi*, and *Helicotylenchus multicinctus* are migratory endoparasites, feeding and completing their life cycle within the root, in the root cortex. Nematodes enter the banana roots from soil, at any point along the entire root (Tushemereirwe *et al.*, 2001). They feed on the cytoplasm of the parenchyma cells leading to their destruction and formation of cavities or lesions (Kashaija *et al.*, 1994).

During their process of feeding, nematodes tunnel through the root cortex, leading to expansion of lesions. The lesions coalesce into large necrotic patches, reddish – purple in colour; a symptom known as root necrosis (Robinson, 1996). Lesions provide entry sites for secondary plant pathogens a combination of which results in purplish to reddish black necrosis on the root tissue and eventually, complete root decay. Root necrosis may result in premature root death or fracture at points where necrosis girdles across. The necrosis further, interferes with the water and nutrient exchange passage. This affects plant growth and vigour which, in turn, leads to reduced bunch size and lengthening of the vegetative life cycle (Gowen and Queneherve, 1990). Severe root destruction deprives the plant of its nutrients and anchorage subjecting the plant to toppling.

Losses to nematodes in banana plantations in Uganda average 20% and reach 40% or more where frequent tropical storms topple plants with rotten nematode infested root systems (Speijer *et al.*, 1999). Yield loss trials carried out on-station showed 51% loss

caused by a combined population of *Radopholus similis* and *Helicotylenchus multicinctus* (Tushemereirwe *et al.*, 2001; Namaganda *et al.*, 2003).

### 2.3.2. Banana weevil

The banana weevil *Cosmopolites sordidus* (Germar) is the most important insect pest on bananas and plantains (*Musa* spp). The adults are free living and have a nocturnal habit. They rarely fly but are most often found at the base of the banana mats or associated with crop residues (Gold *et al.*, 2004). Their eggs are deposited inside the plant tissue at the base of the pseudo stem or on an exposed corm (Kiggundu *et al.*, 2003; Dubois *et al.*, 2004; Tinzaara *et al.*, 2005). On hatching, the larvae tunnel through the corm and lower pseudostem for feeding and development (Robinson, 1996). The larvae are often found beneath the soil surface deep within the plant, making them largely inaccessible to their natural enemies (Tumuhaise *et al.*, 2004). Ruzambuga (1996) and Gold *et al.* (2004) reported that due to the slow weevil population build up, problems increase extremely high in ratoon crops where damage is caused entirely by larval feeding. Weevil attack can prevent crop establishment followed by significant yield reductions in ratoon cycles which contributes to shortened plantation life.

Tunnelling reduces the water and mineral transport, thereby weakening the plant, reducing the bunch weight (yield) and causing plant toppling during windstorms (Kiggundu *et al.*, 2003; Tinzaara *et al.*, 2005). Attack in newly established banana stands may lead to crop failure (Tinzaara *et al.*, 2005). Heavy infestation in established fields may result in mat disappearance and shortened life span of the plants (Tinzaara *et al.*, 2005). Ruzambuga *et al.* (1998) and Gold *et al.* (2004) estimated yield losses attributable to banana weevil at 40 to 60% in ratoon cycles, while Sengooba (1986) observed total crop failure on banana plantations during banana weevil outbreaks in the country.

#### 2.4. Strategies for control and management of pests and diseases

The combination of the perennial nature of the banana crop plus variation in nature of the pests that invade the banana plant makes pest control difficult to achieve (Tushemereirwe *et al.*, 2001). Farmers in Uganda, therefore, have limited alternative means of controlling the pests.

Chemical control measures do exist but are the least recommended practices because they are unaffordable to the resource poor small farmers who grow the bulk of the crops in Uganda (Collins *et al.*, 1991; Gold *et al.*, 1999; Tushemereirwe, *et al.*, 2001). Furthermore, chemicals used in plantation crops like bananas involve risk to agricultural workers, and their abuse may lead to development of resistant pests.

Cultural control methods contribute to the reduction of banana weevils and nematodes. Most promising is the use of clean planting material such as tissue culture plants (Gold *et al.*, 2001). Destruction of crop residues eliminates adult weevil refuges, food sources and breeding sites, hence, lowering the overall banana weevil population. This brings about a reduction in damage on standing plants in susceptible clones (Gold *et al.*, 2001; Dubois *et al.*, 2004; Masanza *et al.*, 2004). Crop sanitation as a means of banana weevil control continues to be widely recommended, good crop husbandry (Ruzambuga, 1996) and trapping in case of weevils (Tinzaara *et al.*, 2005). However, re-infestation remains a primary concern where banana weevils readily invade from neighbouring fields, while the nematodes are often already in the soil fields. Furthermore, a farmer participatory appraisal carried out by Gold *et al.* (1994), revealed that the most recommended cultural control practices are considered to be highly labour demanding and expensive by the farmers.

According to the works of Gold *et al.* (2001), no single strategy can provide complete control of pests. An integrated pest management involving these methods; cultural control, biological control, host plant resistance, and judicious use of chemical control might offer some success in controlling banana pests.

#### 2.4.1. Banana breeding for resistance

Another approach to control pests is breeding for resistance in the host plant. However, breeding for disease and pest resistance and for environmental extremes, is not easy (Rowe and Rosales, 2000). Despite considerable efforts of *Musa* improvement programmes, banana production still depends upon a limited number of landraces selected by man from natural germplasm (Ortiz *et al.*, 1995). Inability to replace landraces by cultivars obtained through cross breeding has created the increased risk of epidemic diseases among genetically similar cultivars (Ortiz *et al.*, 1995).

Banana genetic improvement has been severely hampered by a lack of useful genetic variability and low levels of female fertility (Ortiz *et al.*, 1995). The commercial *Musa* cultivars are mainly triploid cultivars and triploidy leads to problems during meiosis due to uneven numbers of chromosomes. Consequently, *Musa* species exhibit low seed set and poor seed germination.

Successful plant cross-breeding requires the production of true seed through sexual hybridisation as the first step. However, the majority of the edible *Musa* species are seedless due to the high inherent reproductive sterility (Rowe, 1984). Sterility may be due to failure of pollination and /or fertilisation; failure of the embryo sac and zygote development (Vuylsteke, 2000). Plant multiplication is by vegetative means, which is inherently slow. According to Stover and Buddenhagen (1986), recreating an existing cultivar with added beneficial traits such as disease resistance is an extremely unlikely event, because of the enormous number of crosses that would be required for success. *Musa* breeding is further limited at the practical level in terms of time, plant material and land management resources. At least 2 years are required to complete a seed to seed crop cycle, very few viable seedlings are obtained from extensive numbers of crosses and each plant occupies 6 m<sup>2</sup> in the field for evaluation (Ortiz *et al.*, 1995; Crouch *et al.*, 1999).

The banana breeding programmes in Africa, namely the International Institute of Tropical Agriculture (IITA) in Nigeria and Uganda, and the Centre Africain de

Rescherches sur Bananier et Plantain (CARBAP) in Cameroon, have developed improved diploid males that can be crossed with desired triploid as the female parent. Another breeding strategy used at CARBAP is where the chromosome number of desired diploids is doubled through the use of colchicine. The resulting tetraploids are then used in tetraploid x diploid crosses to obtain triploids. A number of new plantain type hybrids have been registered from IITA breeding programme, while several new highland banana selections are currently in preliminary evaluation trials in Uganda (Viljoen *et al.*, 2003).

#### **2.4.2. Genetic transformation in banana breeding**

Classical breeding of banana and plantain is hampered by long generation times, triploidy and sterility of most edible cultivars (Vuylsteke, 2000; Bosque-Pérez *et al.*, 2000; Sági, 2000). These factors impede successful breeding for resistance to major diseases and pests of banana and plantains (Sági, 2000).

Engineering plants to express novel defences offers a simple, short generation time, effective basis for overcoming current limitations to wide spread use of crop resistance against important pests and diseases (Atkinson *et al.*, 1995; Urwin *et al.*, 1995; Sági, 2000). These require no change to agronomic practices if appropriate range cultivars are transformed.

Genetic engineering of crops is a fast process, particularly since the development of enabling techniques for the faster transfer of foreign genetic material into plant cells (Vuylsteke, 2000). In banana, transformation progress has been slow, mainly due to problems with the *in vitro* system for plant regeneration from single cells and lack of useful genes (Vuylsteke, 2000). Sági, (2000) reported that three systems appear applicable to transformation of banana and plantain; particle bombardment using a biolistic gun device has been optimised for transformation of embryonic cell suspension; introduction of DNA into regenerable, embryonic cell suspension derived protoplasts by electroporation and *Agrobacterium* mediated transformation of *in vitro* meristems. The above techniques have been found to have a great potential in

molecular breeding in banana and plantain, especially to control the major diseases and pests of this crop (Sági, 2000). Apart from the controlling of pests and diseases, FAO considers genetic transformation of banana as an option in the reduced use of pesticides. FAO identifies proteinase inhibitors in roots for nematode control as one of the key approaches (Atkinson *et al.*, 2004). For instance, Cavendish banana was transformed using *Agrobacterium tumefaciens* to express a protein engineered rice cystatin (OclD86) resulting in plants that displayed nematode resistance (Atkinson *et al.*, 2004).

## **2.5. Protease and protease inhibitor systems**

### **2.5.1. Proteases**

Enzymes constitute a specialized and diverse group of proteins that have several roles in many physiological processes. Proteolytic enzymes such as proteinases which are involved in digestive processes, proenzyme activation, liberation of physiologically active peptides, complement activation, inflammation processes and others are part of this protein group (Oliveira *et al.*, 2003).

Proteases are grouped into four categories according to the essential amino acid residues at their active sites, the optimum pH range of activity, amino acid sequence similarities, similarity to inhibitors. They are classified as serine, cysteine, aspartic and metallo-proteases (Bode and Huber, 1992). Proteases are classified as endopeptidases when they hydrolyse internal peptide bonds and exopeptidases when they hydrolyse only the N-terminal or C-terminal bonds of proteins (Filho, 1992). Proteases of all these four classes have been found in different plant organs or tissues (Filho, 1992). Examples of proteases have been identified in plants, insects, microorganisms and are like those found in mammals (Table 2).

**Table 2: Classes of proteases**

| Protease class    | Amino acid in pH active site | Optimum (Range) | Proteins                                                                |
|-------------------|------------------------------|-----------------|-------------------------------------------------------------------------|
| Serine protease   | Ser; His, Asp                | 7 – 9           | Trypsin, Chymotrypsin, Elastase, Cathepsin (+) G                        |
| Cysteine protease | Cys, His, Asn                | 4 – 7           | Papain*, Ficin, Bromelain, Ananain, Cathepsin (+)B,C, H, K, L,O,S and W |
| Aspartic protease | (2) Asp, Try                 | Below 5         | Cathepsin (+) D and E, Renin, Pepsin                                    |
| Metallo-protease  | Metal ion                    | 7 - 9           | Carboxypeptidases A and B, aminopeptidases                              |

\*Papain isolated from the latex and fruit of *Carica papaya* has been used to identify cysteine proteinase inhibitors of plant origin; (+) The term “Cathepsin” is generally used for the lysosomal cysteine protease (Oliviera *et al.*, 2003).

### 2.5.2. Cysteine proteases

The cysteine class of enzymes has been known in plants, micro-organisms and even much longer in animal tissues (Ryan, 1990; Lawrence and Nielsen, 2001; Oliviera *et al.*, 2003). The cysteine proteases depend on a reduced thiol group of a cysteine residue as the nucleophile involved in peptide bond hydrolysis (Rawlings and Barrett, 1994). The cysteine proteases of the papain family comprise a single polypeptide chain of ~25 kDa, which is folded to form a globular protein with two domains. There is a deep cleft in between the two domains where the substrates can bind, and where the active site cysteine (Cys-25) and histidine (His-159) residues form a thiolate imidazolium ion pair for catalytic activity. According to Stepek *et al.* (2004) the structures of these enzymes are very similar and the catalytic mechanism is identical in all those studied. They can differ, however in the amino acids lining the substrate

binding pocket, which are responsible for binding amino acid side chains of the substrate. They could therefore differ in substrate specificity, cleaving different peptide bonds of the substrate protein (Figure 1).

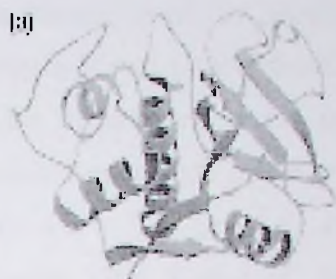


Figure 1 The structure of cysteine proteinases (a) Ribbon diagram of the structure of papain. The structure is similar between the different papain family cysteine proteinases, and the catalytic site cysteine and histidine residue (Stepek *et al.*, 2003)

The cysteine proteases require processing such as removal of the amino terminal fragment to produce the active enzyme. The amino terminal region, also called proregion, plays an important role not only as an inhibitor of enzyme activity but for the correct folding of the newly synthesized protein and to protect it against denaturing effects in sudden changes in pH conditions (Oliviera *et al.*, 2003). Cathepsin L and Cathepsin B like enzymes are subfamilies of the papain family that are found at the proregion. These form two distinct group enzymes: the proregion of Cathepsin B is significantly shorter (~60 amino acid residues) than the proregion of Cathepsin L (~100 amino acid residues). In the three dimensional structures of the members of the papain family two distinct domains are commonly observed, the amino terminal domain is mostly  $\alpha$  helical while the carboxyl terminal domain contains predominantly  $\beta$  sheets (Oliviera *et al.*, 2003). The active site of Cathepsin B and C consists of Cys 25 and His 159 and is supplemented by Asn 175 hydrogen bonded to His 159, and Gln 19. The active site of Cathepsin B differs from other Cathepsins due to the presence of the occluding loop, which is responsible for exopeptidase activity of the protein. According to Karrer *et al.* (1993) several members of the papain family show preferences for bulky hydrophobic residues

occupying the S2 sub site, due to presence of the Ser 205 residue in this sub site, whereas Cathepsin B prefers arginine.

Several enzymes in the papain family with various activities have been found, including endopeptidases (such as papain and Cathepsin B, H, L and C), amino peptidases (Cathepsin H and C) and those with carboxypeptidase activity (Cathepsin C). There are also differences in the proteolytic activities of the Cathepsins in relationship to inhibition and pH optimum: the Cathepsin B (pI=4.5 – 5.5; pH 6.0), Cathepsin H (pI= 6.0 – 7.1; pH 6.8) and Cathepsin L (pI= 5.5 – 6.1; pH 5.5) (Rawlings and Barrett, 1994; Turk *et al.*, 1983).

In plants, cysteine proteases are widely distributed in various tissues and are involved in various physiological events (Yamada *et al.*, 2001). They are particularly found in vacuoles and are responsible for the mobilization of endosperm storage proteins during seed germination (Oliviera *et al.*, 2003). According to Hao *et al.* (2006), cysteine proteases have been reported to be involved in many processes in plants, including pro-protein processing, protein turnover, programmed cell death, and degradation of stress damaged proteins to fuel synthesis of enzymes associated with stress adaptations. Further-more, plant stresses associated with changes in expression of cysteine proteinase genes include low or high temperature, drought, salt, wounding and diseases.

### 2.5.3. Aspartic proteases

Aspartic proteases found in animals, plants, fungi, yeast, some bacteria and viruses, constitute one of the four distinct superfamilies of proteolytic enzymes (Kervinen *et al.*, 1999). These enzymes include pepsins, chymosin, renin, cathepsin D and human immunodeficiency virus type 1 (HIV-1) proteinase (Kervinen *et al.*, 1999).

Aspartic proteases use the acid base mechanism of substrate hydrolysis. The water molecule is believed to be located between the aspartic residues in the active site, and when that molecule is partly activated by the deprotonated aspartic acid, it makes a nucleophilic attack at the carbonyl carbon in the substrate (A). The protonated

aspartic acid donates a proton to the nitrogen and forms a zwitterionic tetrahedral intermediate (B) that collapses into two products: the acid (N- terminal cleavage product) and the amine (C-terminal cleavage product) (Figure 2) (Lueng *et al.*, 2000).

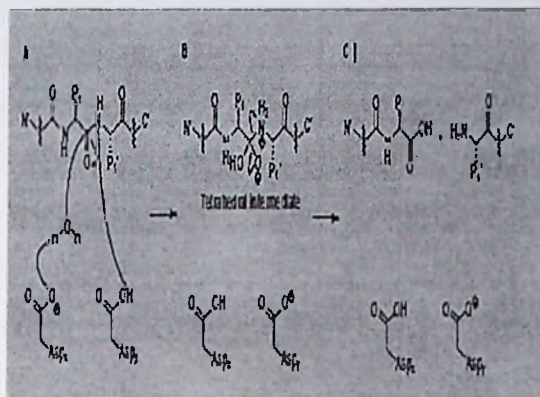


Figure 2 Catalytic mechanism of substrate hydrolysis by aspartic proteases. (Lueng *et al.*, 2000).

#### 2.5.4. Serine proteases

Serine proteases possess a serine residue in the active site (Antão and Malcata, 2005). Three classes of serine proteases are known today, which are classified as trypsin, elastase and chymotrypsin like serine proteases according to the amino acid found in the P1 position of the substrate (Rawling and Barret, 1994). Most works relating to plant serine proteases have focused on cucumisin-like proteases, a family of endopeptidases found in *Cucurbitaceae* (Yagamati *et al.*, 1994; Chen *et al.*, 2004). Other plant serine proteases that have been found include pathogen induced PR-P69 (P69A and P69B) from tomato (Tonero *et al.*, 1996); the root starvation induced protease RSIP from maize roots (*Zea mays* L. cv. DEA) (Brouquisse *et al.*, 2001); and a trypsin-like protease from soybean seeds (Guo *et al.*, 1998).

Serine and cysteine proteases form covalent enzyme substrate complexes, which are not formed by aspartic and metalloproteases. This is because they have strong nucleophilic amino acid residues in their catalytic site, thereby performing a nucleophilic attack on the carbonyl group of the peptide bond (Antão and Malcata, 2005). Proteolytic enzymes that depend on a serine residue for activity are widespread in nature, this is on a basis of their three dimensional structures (Rawling and

Barrett, 1994). The serine protease mechanism consists of a catalytic triad that is composed of Ser195 (a nucleophile), His57 (base), and Asp102 (an electrophile) and also an oxyanion hole (Gly193 and Ser195). The basic mechanism of action of serine proteases involves transfer of the acyl portion of a substrate to a functional group of the enzyme. This involves two basic steps of catalysis by this group of enzymes; First, the formation of an ester between the oxygen atom of serine and the acyl portion of the substrate, which produces a tetrahedral intermediate and releases the amino part of the substrate. Second, the attack of water on the acyl enzyme intermediate, which breaks it down and releases the acidic product while regenerating the original enzyme form (Antão and Malcata, 2005). The mechanism is depicted in detail in Figure 3.

Plant serine proteases, namely subtilases are involved in many physiological processes. These include microsporogenesis, symbiosis, hypersensitive response, signal transduction and differentiation, senescence and protein degradation (Antão and Malcata, 2005).

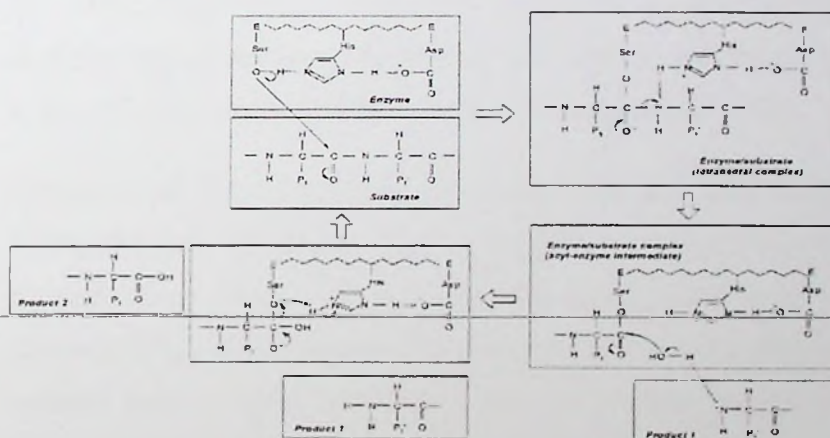


Figure 3 Mechanism of reactions catalysed by serine proteases. Source: Antão *et al.*, 2005

### 2.5.5. Insect/ nematode proteases

There are generally two major proteinase classes in the digestive system of phytophagous insects i.e. serine and cysteine proteinase classes. Serine proteases have been identified in extracts from the digestive tracts of insects from many families

such as Lepidoptera, Dictyoptera and Hymenoptera (Houseman *et al.*, 1989), while the cysteine protease activity is characteristic for Odoptera and Hemiptera. Coleopteran insects mainly use cysteine proteinases (Gatehouse *et al.*, 1985; Lawrence and Nielsen, 2001), but studies indicate a combination of both serine and cysteine proteinase inhibitors have been effective in controlling coleopteran insects (Gerald *et al.*, 1997).

In non vertebrates such as nematodes and mites, cysteine proteinases are among the digestive enzymes (Oliviera *et al.*, 2003). In arthropods such as lobsters, they play a digestive role, but are also related in the nervous system. In insects, they are utilized in the digestive processes (Rawlings and Barrett, 1994) but are found in several other tissues, indicating that they may also play other roles (Yamamoto *et al.*, 1994). The effect of class specific inhibitors on the pest digestive enzymes is not always a simple inhibition of proteolytic activity. Studies have indicated that there are often two or more populations of digestive enzymes in target pests, some with susceptibility to inhibition and other insensitive to specific inhibitors (Bown *et al.*, 1997; Michaud *et al.*, 1996). Some insects respond to ingestion of plant proteinase inhibitors such as soybean trypsin inhibitor (Broadway and Duffey, 1986) and oryzacystatin (Michaud *et al.*, 1996), by hyper producing inhibitor resistant enzymes.

Isolation and characterisation of midgut proteases from larvae of Cowpea weevil, *Callosobruchus maculatus* (Kitch and Murdock, 1986; Campos *et al.*, 1989) and the Mexican bean weevil *Zabrotes subfasciatus* (Lemos *et al.*, 1987) confirmed the presence of a cysteine class of protease in such insects. Similar proteases have been isolated from midguts of the confused flour beetle *Tribolium castaneum*, Mexican bean beetle *Epilacha varivestis* (Murdock *et al.*, 1987) and the Common bean weevil *Acanthoscelides obtectus* (Wieman and Nielsen, 1988).

In a study of the proteases from the midgut of several members of the order Coleoptera, 10 of 11 species representing 11 different families had gut proteases that were inhibited by *p*-chloromercuribenzenesulphonic acid (PCMBs), a potent sulphhydryl reagent (Murdock *et al.*, 1988) indicating that the proteases were of the

cysteine class. The optimum activity of cysteine proteases is usually in pH range 5-7, which is the pH of the guts of insects which use cysteine proteases (Murdock *et al.*, 1987).

## 2.6. Protease inhibitors

Proteins that form complexes with proteases and inhibit their proteolytic activity are widespread in nature, in addition to their role of regulating proteolytic activities, they are important for protecting fluids or tissues from degradation by foreign proteolytic activities (Ryan, 1990; Carozi and Kozeiel, 1997; Sharma *et al.*, 2001). Amongst these are protease inhibitors that apparently contribute to host plant resistance against pests and pathogens (Ryan, 1990; Filho, 1992). Protease inhibitor proteins are the defensive chemicals in plant tissues that are developmentally regulated and induced in response to insect and pathogen attacks (Ryan, 1990).

Inhibitor families have been found that are specific for each of the four classes of proteolytic enzymes, i.e. serine, cysteine, aspartic and metallo proteases (Ryan, 1990; Oliviera *et al.*, 2003; Dunaevsky *et al.*, 2004). Most inhibitor proteins in these families are small, and usually exhibit molecular weights from 5 to 25 kDa (Ryan, 1990, Dunaevsky *et al.*, 2005). Their content in seeds reaches 4 – 10% of the total water soluble protein (Dunaevsky *et al.*, 2005). The protective role of such inhibitors is supported by their preferential association with seeds. Most of these are most attractive to various pests and most vulnerable due to inactive metabolism and consequent lack of an active defence system (Ryan, 1990; Pernas *et al.*, 2000; Dunaevsky *et al.*, 2005). A further role of plant protease inhibitors is in programmed cell death in plants. Programmed cell death (PCD), also referred to as apoptosis, is a physiological process by which cells or organs that have reached a certain age are spontaneously killed to preserve the integrity of the whole organism. Cysteine proteases are involved in a key step in animal PCD and have recently also been found to be important in plants (Solomon *et al.*, 1999). Evidence of protease inhibitors active against insect pests has been reported. These include both *in-vitro* assays

against insect gut proteases (Koiwa *et al.*, 1997) and *in-vivo* artificial diet bioassays (Urwin *et al.*, 1997; Vain *et al.*, 1998).

The majority of protease inhibitors studied from the plant kingdom originate from three main plant families, namely the Leguminosae, Solanaceae and Gramineae (Richardson, 1991). Many of these protease inhibitors are rich in cysteine and lysine, contributing to better and enhanced nutritional quality (Ryan, 1998). Protease inhibitors also exhibit a very broad spectrum of activity including suppression of nematodes like the tobacco cyst nematode; *Globodera tabaccum* (Lownsbery and Lownsbery, 1989), inhibition of spore germination and mycelium growth of *Alternaria alternate* by buckwheat trypsin/chymotrypsin (Dunaevski *et al.*, 1997), and cysteine protease inhibitors from pearl millet inhibiting growth of many pathogenic fungi including *Trichoderma reesei* (Joshi *et al.*, 1998). These inhibitor families that have been found are specific for each of the four classes of proteolytic enzymes.

#### 2.6.1. Serine proteinase inhibitors

Serine protease inhibitors are highly varied and have been extensively studied in both animals and plants. They are mostly small proteins, 6 -25kDa in molecular weight, they are reversible inhibitors of serine proteases mainly trypsin and chymotrypsin. Their functions seem to range from regulation of endogenous protease activity to storage proteins, as they tend to accumulate in large amount in storage organs like tubers and seeds reaching concentrations of about 2% of total protein (Gatehouse *et al.*, 1997).

The role of serine protease inhibitors as defensive compounds against predators is particularly well established. The main proteinases used in plants for processes such as protein mobilization in storage tissues belong to the cysteine class (Lawrence and Koundal, 2002) so serine PIs are less likely to have a fundamental role in the plant metabolism (Reeck *et al.*, 1997).

In contrast, a major role for serine PIs in animals is to block the activity of endogenous proteinases in tissues where this activity would be harmful, as in the case of pancreatic trypsin inhibitors found in mammals (Lawrence and Koundal, 2002). Serine proteases such as trypsin, chymotrypsin and elastase, which belong to a common family, are responsible for the initial digestion of proteins in the gut of higher animals (Lawrence and Koundal, 2002). *In vivo* they are used to cleave long, essentially intact polypeptide chains into short peptides which are then acted upon by exopeptidases to generate amino acids, the end products of protein digestion. These three types of digestive serine proteinases are distinguished based on their specificity. Trypsin specifically cleaves after residues carrying a basic side chain (Lys, Arg), chymotrypsin shows a preference for cleavage and after residues carrying a hydrophobic side chain (Phe, Tyr, Leu), and elastase shows a preference for cleaving C-terminal to residues carrying a small neutral side chain (Ala, Gly) (Ryan, 1990). Lawrence and Koundal (2002) reported that all serine protease inhibitor families from plants are competitive inhibitors and all of them inhibit proteinases with a similar mechanism. The enzyme reacts with the inhibitor molecule as if it were a proper substrate. Instead of dissociation of the cleaved molecule the partners remain bound in a strong covalent complex through the amino acid residue at the N-terminal side of a so called reactive site (Filho, 1992). Substrate like mechanism of inhibition of a trypsin like serine proteinase by a canonically binding protein inhibitor (Figure 4). Basically a binding loop sticking out of the inhibitor contains an active site and a peptide bond. The inhibitor active site loop fits into protease active site and the inhibitor peptide bond may or may not be cleaved. However, the cleavage and hydrolysis of the inhibitor does not affect the interaction. The inhibitor therefore mimics a normal substrate but does not allow to be completely hydrolysed. In this case as productively bound peptide substrate, the inhibitor provides a reactive site loop forming several inter main chain hydrogen bonds with proteinase binding sites, with its scissile peptide bond partially added to the active site Ser 195 O $\gamma$ , and with the PI side chain as the principal cleavage site determinant projecting into the SI specificity pocket, making numerous substrate like interactions (Bode and Huber, 2000)

Serine PIs have also been classified in families according to their molecular size and primary structures in such a way that inhibitors belonging to the same family are thought to be evolutionarily related (Filho, 1992). Seven families of serine proteinase inhibitors are known. These are Bowman-Birk, Kunitz, Potato II, Curcubit, Cereal super family, Ragi AI, and Thaumatin PR like families (Richardson, 1991).

Several researchers have reported various transgenic plants expressing serine protease inhibitor genes; Cowpea trypsin inhibitor (CpTi) in tobacco (Hilder *et al.*, 1987), CpTi in rice (Xu *et al.*, 1996), CpTi in Wheat (Lawrence *et al.*, 2001), Tomato inhibitor I and II in tobacco (Johnson *et al.*, 1989), Soybean Kunitz TI in rice (Lee *et al.*, 1999).

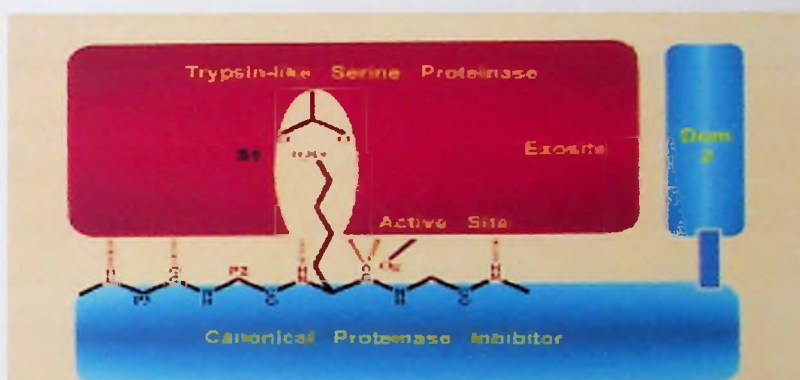


Figure 4 Schematic interactions between Trypsin like Serine proteinase and its inhibitor. (Bode and Huber, 2000).

### 2.6.2. Aspartic and metallo-protease inhibitors

There is far less knowledge on aspartic protease inhibitors and their inhibition of insect digestion. Aspartic proteases (cathepsin D like proteases) together with cysteine proteases have been reported in species of six families of the order Hemiptera (Houseman and Down, 1983). The low pH of midguts of many members of Coleoptera and Hemiptera provides more favourable environments for aspartic proteases (pH optima ~3 -5) than the high pH of most insect guts (pH optima ~ 8 –

11) (Houseman *et al.*, 1987) where the aspartic and cysteine proteases would not be active. Therefore these inhibitors would be expected in Coleopteran insects. Aspartic protease inhibitors have been isolated from sunflower (Park *et al.*, 2000), barley (Kervinen *et al.*, 1999).

At least two families of metallo protease inhibitors, the metallo carboxypeptidase inhibitor family in potato (Rancour and Ryan, 1968) and tomato plants (Graham and Ryan, 1981) and a cathepsin D inhibitor family in potatoes (Keilova and Tomasek, 1976), have been identified in plants. The cathepsin D inhibitor (27kDa) is unusual as it inhibits trypsin and chymotrypsin as well as cathepsin D, but does not inhibit aspartyl proteases such as pepsin, rennin or cathepsin E. The inhibitors of metallo carboxypeptidase from tissue of tomato and potato are polypeptides (4kDa). They strongly and competitively inhibit a broad spectrum of carboxypeptidases from both animals and microorganisms, but not the serine carboxypeptidases from yeast and plants (Havkoija and Neuvonen, 1985). This type of inhibitor is found in tissues of potato tubers where it accumulates during tuber development along with the potato inhibitor I and I families belonging to the serine protease inhibitor type. The inhibitor is also induced and accumulates in potato leaf tissues in response to wounding (Graham and Ryan, 1981). Thus, the inhibitors accumulated in the wounded leaf tissues of potato have the capacity to inhibit all the five major digestive enzymes i.e. trypsin, chymotrypsin, elastase, carboxypeptidase A and carboxypeptidase B of many insects (Hollander- Czytko *et al.*, 1985).

### **2.6.3. Cysteine proteinase inhibitors (cystatins)**

Cystatins refer to proteins that specifically inhibit the activity of papain and related cysteine proteases such as cathepsin B, H and L, ficin and bromelain. A number of cystatins exist in nature forming the "cystatins super family". Proteinaceous cysteine proteinase inhibitors of animal origin have been extensively investigated to clarify their chemical structures and physiological functions (Abe *et al.*, 1987). These inhibitors are considered to have evolved from a cognate ancestral gene and form the cystatins super family which is further classified into four different families on the

basis of their molecular structures (Barret, 1987; Abe *et al.*, 1987). Family 1 cystatins, commonly known as stefins, contain no disulphide bonds or carbohydrate groups. With molecular mass of around 11 kDa, the molecules consist of 100 amino acid residues i.e. human cystatins A, B and rat cystatins (Sato *et al.*, 1990).

Family 2 cystatins, are proteins that contain two intra-chain disulfide bonds near the carboxyl terminus and are glycosylated. They have a molecular mass of about 13 – 24 kDa with approximately 115 amino acid residues. Human cystatin S (Isemura *et al.*, 1984), human cystatin C (Grubb and Lofberg, 1982), bovine colostrum cystatins (Hirado *et al.*, 1985), and chicken cystatins (Turk *et al.*, 1983) are examples of family 2 cystatins.

The family 3 cystatin or kininogen family comprise the blood plasma kininogens which are larger than the members of the two other families and are the most complex cystatin molecules with a high molecular mass of 60 – 120 kDa. These proteins have composite sequences comprising about 355 amino acids. The kininogens play an important role in the blood coagulation process (Salvesen *et al.*, 1986). Some family 2 and 3 cystatins have been reported to exhibit sequence homology with the active site sequences of Bowman-birk type trypsin inhibitor (Saito *et al.*, 1991).

Family 4 cystatins, the phytocystatins, include almost all plant cysteine proteinase inhibitors. This family of proteins are known to have characteristics that are found commonly in mostly family 1 and 2 cystatins (Oliviera *et al.*, 2003). Among the plant cystatins, the oryza-cystatin from rice seeds was the first inhibitor of plant origin to be considered a member of the cystatin super family that is related by structure to chicken egg white cystatin (Barrett *et al.*, 1986; Colella *et al.*, 1989). Other cysteine proteinase inhibitors have been isolated from plant species as well, including from potato (Rodis and Hoff, 1984), corn (Abe and Whitaker, 1988; Abe *et al.*, 1992), rice (Abe *et al.*, 1987), wheat, barley and rye (Fossom, 1970) and millet (Tashiro and Maki, 1986). The classification in families is based on primary sequence similarities, molecular masses, number of disulphide bonds and sub cellular localization (Oliviera *et al.*, 2003).

#### Stefin family

hca                    ---G-----QVVAG-----

#### Cystatin family

cc                    ---G-----FAM-----QLVSG-----C---C-----C---PW---C-----

hcc                   ---G-----FAV-----QIVAG-----C---C-----C---PW---C-----

bcc                   ---G-----FAV-----QVVSG-----C---C-----C---PW---C-----

#### Kininogen family

hk1                   ---G-----FAV-----TVGSD-----C---C-----C---PW---C-----

hk2                   ---G-----FAV-----QVVAG-----C---C-----C---PW---C-----

#### Phytocystatin family

OC-I                   ---G-----FAVTEHNKKAN-----QVVAG-----PW-----

OC-II                   ---G-----FAVTEHNKKAN-----QVVAG-----PW-----

Figure 5 Alignment of selected members of the four cystatin families illustrating the sequence conservation regions within the family members. The sequences are human cystatin-A (hca), human cystatin- B (hcb), chicken cystatin (cc), human cystatin -C (hcc), beef colostrum (bcc), human kininogen segment 1 (hk1) and segment 2 (hk2), oryzacystatinI and II (OC-I and OC-II) (Adapted from Oliviera *et al.*, 2003)

Various biological functions have been attributed to phytocystatins, these include; physiological functions of regulation of the endogenous proteinase activities in seeds during seed maturation; participation of inhibitors as an important element against attack by insects and nematodes that generally contain cysteine proteinases in their guts (Oliviera *et al.*, 2003; Dunaevsky, 2005). Research works have proven the effect of cystatins on pathogens and insects; purified Oryzacystatin-I (OC-I) (Abe *et al.*, 1987), papaya cystatins (Kiggundu, 2008) against banana weevil borer; Ocl-Δ86 (Urwin *et al.*, 1995; Urwin *et al.*, 1997; Urwin *et al.*, 2001; Vain *et al.*, 1998 and Atkinson *et al.*, 2004) against nematodes.

The cystatins (cysteine protease inhibitors) and in particular some thrombin (serine proteinase) inhibitors are examples of exosite binding inhibitors, which mainly bind

to surface sites adjacent to the active centres of their target proteinase. In this way they prevent access of peptide bonds from larger substrate molecules to the active site, without direct blockage of the catalytic residues (Figure 6). The mechanism of interaction between the cystatins with a papain like cysteine proteinase is shown in Figure 6. The inhibitor mainly interacts through its edge loops with proteinase surface subsites adjacent to the active centre, with its N terminal segment bridging over the active centre of the cognate proteinase and the P2 and P1 (Gly) like residues placed in the S2 and S1 subsites of the proteinase like P2 and P1 peptide substrate side chains (Bode and Huber, 2000).

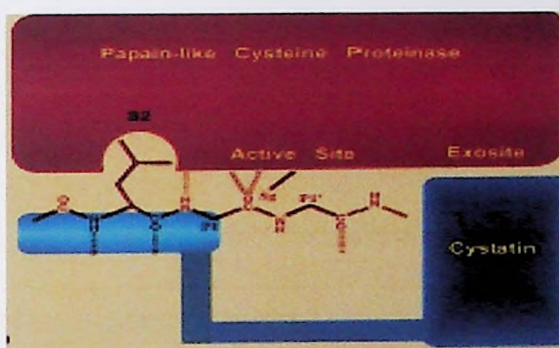


Figure 6 Schematic representation of cystatin interaction with papain like cysteine proteinase. (Bode and Huber, 2000).

#### 2.6.4. Protease inhibitors in the control of insects and nematodes

Protease inhibitor genes have advantages over genes encoding components of complex pathways i.e. single defence genes can be transferred from one plant species to another and expressed either from wound inducible or constitutive promoters. It thereby imparts resistance against insect pests (Boulter, 1993) and may not interfere with other plant functions as pathway related proteins would. This was first demonstrated by Hilder *et al.* (1987) by transferring the trypsin inhibitor gene from *Vigna unguiculata* to tobacco. This conferred resistance to a wide range of insect pests including Lepidopterans, such as *Heliothis* and *Spodoptera*, Coleopterans, such as *Diabrotica*, *Anthonomus*, and Orthoptera such as the locust. Further, there is no evidence that it had toxic or deleterious effects on mammals.

Serine protease inhibitors have anti nutritional effects against several Lepidopteran insect species (Applebaum, 1985). Broad way and Duffey (1986) compared the effects of purified soybean trypsin inhibitor (SBTI) and potato inhibitor II (an inhibitor of both trypsin and chymotrypsin) on the growth and digestive physiology of larvae of *Heliothis zea* (Boddie) (Lepidoptera: Noctuidae) and *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae). They demonstrated that growth of larvae was inhibited at levels of 10% of the proteins in their diet. Trypsin inhibitors at 10% of the diet were toxic to larvae of *Callosobruchus maculatus* (Fabricius) (Coleoptera: Bruchidae) (Gatehouse and Boulter, 1983). However the mechanism of action of these protease inhibitors towards insect digestive enzymes seems rather complicated and has been a subject of investigation (Barrett, 1986). Knowledge on mechanisms of protease action and their regulation *in vitro* and *in vivo* in animals, plants, microorganisms and more recently in viruses has contributed to many practical applications for inhibitor proteins in agriculture.

Several members of the Coleoptera order are tuber, seed, root and leaf eating insects. The presence of cysteine proteases in the gut of these insects to hydrolyze ingested proteins, have led to suggestions that phytocystatins are involved in the exogenous plant defence mechanisms (Waldron *et al.*, 1993; Gruden *et al.*, 1997). Studies have shown that the cysteine proteinases isolated from insect larvae can be inhibited by both non proteinaceous and proteinaceous cysteine proteinase inhibitors (Oliviera *et al.*, 2003).

The secretion of proteases in insect guts seems to depend upon midgut protein content rather than food volume (Baker *et al.*, 1984). The secretion of proteases has been attributed to two mechanisms. This involves either a direct effect of food components (proteins) on the midgut epithelial cells, or a hormonal effect triggered by food consumption (Applebaum, 1985). Protease inhibitors then directly inhibit the protease activity of these enzymes and reduce the quantity of proteins that can be digested. These also cause hyper production of the digestive enzymes which enhances the loss of sulphur amino acids (Shulke and Murdock, 1983). As a result, the insects become weak resulting in stunted growth and ultimate death.

Isolation of the midgut proteases from the larvae of cowpea weevil, (Kitch and Murdock, 1986) and bruchid, (Lemos *et al.*, 1987) confirmed the presence of the cysteine class of protease inhibitors. Similar proteases have been isolated from midguts of the flour beetle and Mexican beetle (Murdock *et al.*, 1987).

Oryzacystatin-I (OC-I, wild type) and a protein engineered variant form, named OC-I $\Delta$ 86 in which residue Asp86 was deleted, were both expressed in tomato hairy roots, and, showed a detrimental effect on growth and development of *Globodera pallida* (plant parasitic nematode) (Urwin *et al.*, 1995). Both oryzacystatins were also expressed in *Arabidopsis thaliana* and affected the size and fecundity of *Heterodera schachtii* and *Meloidogyne incognita* females (Urwin *et al.*, 1997). The growth of both nematode species on the transgenic plants was suppressed with the cystatin having two distinct effects. Few female nematodes reached egg laying size and those that did were much smaller than the controls and they had a reduced fecundity. These two effects were probably caused by inhibition of digestive enzymes leading to nutritional stress since female nematodes taken from cystatin expressing plants had a reduced level of intestinal cysteine proteinase activity (Urwin *et al.*, 1997). The fact that OC-I $\Delta$ 86 is able to control species from three different nematode genera (*Globodera*, *Heterodera* and *Meloidogyne*) suggests value for proteinase inhibitors as a broad range resistance strategy (Lilley *et al.*, 1999).

## **2.7. Safety of the proteins**

### **2.7.1. Allergenic potential of proteins**

A food allergy is an aberrant fast immunological reaction to normal harmless food components, usually proteins (Bernstein *et al.*, 2003). A food allergy is distinguished from food intolerance and other disorders by the production of antibodies (IgE) and the release of histamine and similar substances (Ladics *et al.*, 2003; Kuiper and Kleter, 2003; Bernstein *et al.*, 2003). These reactions can have effects on gastrointestinal tract, skin and respiratory tract (Ladic *et al.*, 2003).

Food allergies are associated with a small number of specific proteins represented by thirteen major allergens; peanuts, tree nuts, cow's milk, hen's eggs, fish, shrimp, barley, kiwi fruit, cod, salmon, celery, wheat and soybeans (Pastorello *et al.*, 1998). According to Huby *et al.* (2000), the intrinsic potential of a protein to induce sensitisation will be manifest only in susceptible individuals. This occurs only if the allergen is encountered in sufficient quantities and via a relevant route of exposure.

Several researchers have reported that most common allergenic food proteins are relatively abundant in food, stable in simulated gastric fluids, heat, and denaturants (Astwood *et al.*, 1996; Sen *et al.*, 2002). Since labile proteins are unlikely to persist in the gastrointestinal tract for sufficient time to provoke an immune response, it is predictable that they are typically nonallergenic when administered orally. This suggests that the stability of a protein in simulated gastric fluid may predict not only its allergenicity when administered orally, but also its inherent allergenicity regardless of route of exposure (Huby *et al.*, 2000). In this case, stability in simulated gastric fluid is presumably acting as a surrogate marker of other properties of proteins that contribute directly to their allergenicity. Ramachandra *et al.* (1999); Sen *et al.* (2002) reported that stability of proteins in simulated gastric fluid reflects stability in the acidic proteolytic vesicles of antigen presenting cells (APCs) wherein proteins are processed and T cell epitopes released. This may lead to the differential processing of proteins, which in turn can affect both antigenicity and allergenicity (Vijh *et al.*, 1998).

Allergy describes the adverse health effects that result from the stimulation of a specific immune response (Huby *et al.*, 2000). As a result, allergic diseases develop from immune responses of various types e.g. allergic contact dermatitis results from cell-mediated immunity and the elicitation in the skin of a delayed type hypersensitivity reaction. Other forms of allergic disease are associated with IgG antibodies and formation of local or circulating immune complexes. In the context of food allergy and sensitisation to ingested proteins, it is immunoglobulin E (IgE) antibody responses that are of greatest relevance in the vast majority of instances (Huby *et al.*, 2000). IgE can bind specifically via its F (ab) arms to the protein antigen

that induces its production. Antibodies of the IgE isotype, however, are also able to bind via their constant (Fc) regions to specific IgE receptors (Fc $\epsilon$ R) found on the surface of mast cells and basophils (Dean, 1997). When two or more of these captive IgE molecules bind to their specific antigen, the Fc $\epsilon$ R can become cross linked on the surface of the cell, initiating intracellular events (Costello *et al.*, 1996). These signals activate the cells, leading to degranulation with the release of histamine, and the production of prostaglandins, leukotrienes, and other inflammatory mediators (Figure 7) (Aalberse, 2000). Together, these agents mediate the symptoms of immediate-type hypersensitivity reactions. Particular subclasses of IgE may be induced preferentially by allergens, perhaps lending further specificity to this process (Huby *et al.*, 2000).

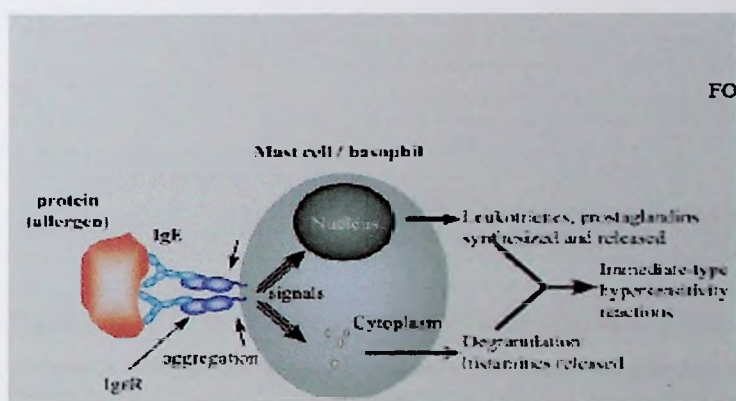


Figure 7 Immediate type hypersensitivity is mediated by factors released by mast cells and basophils as a response to intracellular signals generated by Ig $\epsilon$ R on the surface of such cells. (Huby *et al.*, 2000).

The crosslinking of Fc $\epsilon$ R requires that at least two antibody molecules bind to the inducing allergen. An allergen must therefore contain at least two IgE binding sites (epitopes), each of which will be a minimum of approximately 15 amino acid residues long, in order that antibody binding can occur. This implies a lower size limit for protein allergens of approximately 30 amino acid residues (M.W of 3kD) (Huby *et al.*, 2000).

Allergic sensitisation to proteins involves the induction of an IgE response of sufficient magnitude to facilitate elicitation of an inflammatory reaction following

subsequent exposure to the same allergen. Sensitisation occurs when these IgE antibodies are distributed systematically and bind to Fc $\epsilon$ R on mast cells and basophils. Figure 8 shows the reaction. For a B cell to differentiate into a plasma cell and produce antibodies, the clonotypic B-cell receptor expressed on its surface must bind to specific B epitopes on the surface of a protein antigen. The antibodies subsequently produced by the plasma cells, into which the B cell differentiates, have the same specificity as the B cell receptor, and are therefore able to bind specifically to the same B epitopes on the surface of the protein antigen.

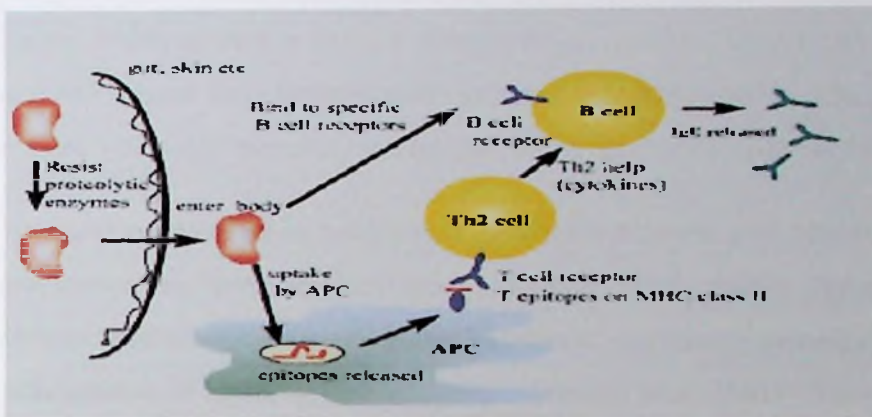


Figure 8 In order for immunoglobulin (Ig) antibodies to be released from a B cell, two signals must be delivered: first, the clonotypic B cell receptor must bind specifically to B epitopes on the surface of the antigen. Second, help from T cells in the form of cytokines must be delivered by specific T cells. For B cells to respond by releasing IgE, this help must be predominantly of type 2. (Huby *et al.*, 2000).

Efficient secretion of antibodies normally requires that B cells receive help from T-helper (Th) cells that specifically recognise separate epitopes on the same protein antigen. Such recognition is mediated by the clonotypic T-cell antigen receptor, which delivers stimulatory intracellular signals to the T cell.

These signals are able to activate resting, immature Th cells (designated Th0), causing their differentiation into mature, effector Th cells, which can be either type 1 (Th1) or type 2 (Th2). According to O' Rourke *et al.* (1997), the mature T cells can help B cells to secrete immunoglobulin in two ways. First, they can stimulate accessory molecules on the B cell surface, an effect mediated by cell/cell contact.

Second, they can release cytokines into the milieu that can bind to, and hence activate, specific cytokine receptors on the surface of the B cells. The identity of cytokines that stimulate the B cell in this way can affect both the strength and quality of the antibody response. Typically, Th2 cells produce a cocktail of cytokines including interleukin (IL)-4, IL-5, IL-6, IL-10 and IL-13 that, among other actions, encourage plasma cells to switch to synthesis of IgE (Romagnani *et al.*, 1991).

Cysteine protease inhibitors (cystatins) are not known sources of allergens although some from kiwi fruit (Pastorello *et al.*, 1998), kiwi fruit cortex and seeds (Rassam and Laing, 2004) as well as Anisakis 4 allergen from *Anisakis simplex* (Rodriguez-Mahillo *et al.*, 2007) have been isolated and characterised. Although the kiwi allergen actinidin evokes 100% IgE reactivity in populations its mechanism has not been outlined.

*Anisakis simplex* is a nematode that parasitizes humans who eat raw or under cooked fish containing live L3s (Rodriguez – Mahillo *et al.*, 2007). The L3 can invade the gastrointestinal mucosa releasing excretory/ secretory proteins involved in the pathogenesis of anisakiasis (Rodriguez – Mahillo *et al.*, 2007). This tissue penetrating parasite can induce IgE mediated reactions with several clinical manifestations ranging from urticaria, conjunctivitis, asthma, angioedema to anaphylaxis (Scala *et al.*, 2001; Gonzalez-Quijada *et al.*, 2005).

### 2.7.2. Protein toxicity

A broad range of neurodegenerative disorders are characterised by neuronal damage that may be caused by toxic, aggregation-prone proteins (Taylor *et al.*, 2002). Several toxic proteins have been identified by researchers. These include canatoxin from jack bean (*Canavalia ensiformis*) (Follmer *et al.*, 2001), abrin and ricin from seeds of *Abrus precatorius* L and *Ricinus communis*, modeccin from roots of *Adenia digitata* (Sandvig and Olsnes, 1982), and diphtheria toxin (Sandvig and Olsnes, 1982).

The toxicity of abrin, modeccin and ricin proteins is maximal at neutral and slightly alkaline pH while diphtheria toxin is most toxic at low pH (Sandvig and Olsnes,

1982). According to Lord *et al.* (1994), abrin and ricin exert cancerostatic properties in eukaryotic systems by inhibiting protein synthesis. Thus, they strongly reduce incorporation of labelled amino acids into protein in intact cells as well as in cell free systems. This cancerous effect has been shown to be due to inactivation of the ribosomes, apparently by an enzymatic process. Canatoxin, a toxic protein from jack beans (*Canavalia ensiformis*), has a native form of 184kDa, comprising a non covalently linked dimer of a 95 kDa polypeptide with zinc and nickel. Canatoxin is lethal to rats and mice ( $LD_{50} = 2\text{mg/kg}$ ) and insects. This is effective through intraperitoneal injection, but in-active given orally (Carlini and Guimaraes, 1991). It induces exocytosis in several cell types, including platelets, synaptosomes, pancreatic islets, macrophages, neutrophils and mast cells (Barja-Fidalgo *et al.*, 1991). According to Benjamin *et al.* (1992), lipoxygenase metabolites are involved in most pharmacological effects of canatoxin and the protein disrupts  $\text{Ca}^{2+}$  transport across the membrane (Ghazaleh *et al.*, 1997).

However, based on the toxicity amino acid sequence homology search, in addition to a search of the available literature, there is no evidence on the likely hood of the maize and papaya cystatins being associated with any form of toxicity.

## CHAPTER THREE

### Materials and Methods

#### 3.1. Determination of the level of expression of the maize and papaya cystatins in the *Escherichia coli* M15 (pRep4) construct

Cystatins were expressed using the QiaExpress system (Qiagen). Cystatin coding regions were cloned in the pQE30 vector under control of an inducible promoter and *Escherichia coli* strain plasmid. The cystatins could therefore be easily cultured and purified as recommended by Qiagen (2003). The form of cystatins expressed from (pQE30 vector) contain 6 N-terminal vector encoded histidine residues allowing one step nickel-chelate affinity purification. Cystatins were purified from 1 litre cultures of *E. coli* M15 [pREP4] harbouring the appropriate pQE30 derived expression plasmid.

Essentially, 20 ml of an over-night *E. coli* culture containing the constructs were inoculated into 1 litre of Luria-Bertani (LB) media supplemented with 100mg/l ampicillin and 25 mg/l kanamycin and grown at 37°C. The concentration of the cultures were determined at an absorbance of 600nm using Biomate 3 spectrophotometer (Thermo Electron Corporation, UK) so as to ascertain the cell growth rate of between 0.7 – 0.9 optical density (Qiagen, 2003). Isopropyl  $\beta$  thiogalactoside (IPTG) that induces the expression of the protein was added to a final concentration of 2mM and growth was allowed to continue for a further 2 hours. The cells were harvested by centrifugation (10, 000 x g for 10 min at 20° C) re-suspended in 2mls of sonication buffer (50mM Na<sub>2</sub>HPO<sub>4</sub>, 300mM NaCl) stored at -20°C overnight, thawed and divided into three 15 ml tubes and sonicated on ice (3 x 1 minute) per tube. Cell debris was removed by centrifugation (9,000 x g for 15 minutes at 4°C), 0.5 ml of Ni-NTA resin slurry (Qiagen) was added to each tube containing the supernatant and mixed gently on ice for 1 hour. This enabled the proteins of interest to bind closely with the Ni-NTA resin. The resin was collected by centrifuging (5,000 x g for 3 minutes) and washed five times with 5ml aliquots of wash buffer (50mM Na<sub>2</sub>HPO<sub>4</sub> pH 6.0, 500mM NaCl, 40mM Imidazole) at 4°C for

0.5h). This was aimed at removing unwanted proteins that had been expressed during culturing and do not bind well with the Ni-NTA resin slurry. Proteins were eluted with 1ml of elution buffer (50mM Na<sub>2</sub>HPO<sub>4</sub>, 300mM NaCl, 100mM EDTA). The resin was repelleted by centrifugation (1,500 x g for 1 minute at 4°C) and the elution was repeated once to maximise the protein extraction process.

### **3.2. Determination of purity of the expressed proteins**

The purity, and molecular weight of the protein was determined using SDS-PAGE (Hames, 1981). Purified protein samples (15 µl) were subjected to SDS-PAGE in reducing conditions, according to the method used by Laemmli (1970), using 10 well, 0.75 mm thick, 15% polyacrylamide gels. Standard markers (10748 -010 Invitrogen prestained protein ladder, UK) containing 10 proteins ranging in size from 6 – 180 kDa were included on all the gels. The gels were stained with Coomassie brilliant blue R-250 (B0149-5G Sigma Aldrich, UK) for 1 hour and destained in 40% methanol with 10% glacial acetic acid for 30 minutes on a shaker at 70 rpm in order to obtain a visibly clear gel.

### **3.3. Protein determination assay**

The total protein content of all samples was determined using the Bradford (1976) method as adapted to the 96 well micro-titre plate format of Bio-Rad Laboratories Ltd., (Richmond, CA). Known concentrations (0, 0.2, 0.4, 0.6, 0.8 and 1mg/ml) respectively of Bovine Serum Albumin (BSA) were used to develop a standard curve.

The protein samples were diluted in a ratio of 1:10 using dialysis buffer (0.5M Na H<sub>2</sub>PO<sub>4</sub>, 0.5M Na<sub>2</sub>PO<sub>4</sub> pH 7.0). As a result, 30 µl of diluted sample was obtained, 25 µl of the diluted protein sample or standard solution were put in into 1.5 ml eppendorf tubes and 125 µl (78675A Biorad reagent A, UK ) as well as 1ml of (79463A Biorad reagent B, UK) were added. Aliquot volumes of 200 µl of each sample were loaded onto a 96 well microtitre plate and the reaction was allowed to proceed for 15 minutes to enable the development of the blue colour as the reaction end point. The absorbance of the samples was measured at 650nm using 680 Biorad model

microplate reader (UK), the protein concentrations were determined from the plotted graph.

#### **3.4. Determination of the simulated gastric fluid digestion stability assay conditions**

The procedure described by Thomas *et al.* (2004) was followed with some modifications in time. For each protein, a single tube containing 1.52ml of SGF (0.084N HCL, 35mM NaCL, pH 1.2, and 4000 U of pepsin (P-6887) Sigma Aldrich UK) was preheated to 37°C prior to the addition of 0.08 ml of test protein solution (5 mg/ml). The tube contents were mixed by mild vortexing and the same tube was immediately placed in a water bath. Samples of 200 µl were removed at 0, 2s, 5s, 10s, 15s, 30s, 2, 5, 15, 30, 60 mins after initiation of the incubation. Each 200 µl sample was quenched by addition of 70 µl of 200mM NaHCO<sub>3</sub>, pH 11, and 70 µl 5 x Laemmli (1970) buffer (40% glycerol, 5% β-mercaptomethanol, 10% SDS, 0.33M Tris, 0.05% bromophenol blue, pH 6.8). Quenched samples were immediately heated to 95°C for 10 min then analyzed directly, or stored at -20°C. The zero time point protein digestion samples were prepared by quenching the pepsin in the solution before adding the test protein. Control samples for pepsin auto-digestion (pepsin without test protein) and test protein stability (reaction buffer with test protein but without pepsin) were included. Protein samples were then blotted onto Immobilon P membrane (Armstrong), using standard equipment and procedures (Mini Protean II, Biorad). Maize and papaya cystatins were detected using primary rabbit polyclonal antibody against these proteins at a 1:2000 dilution. A second antibody of anti rabbit IgG/alkaline phosphatase conjugate (Sigma) was used at 1:2000 dilution. The binding of the latter antibody was detected using BCPIP/NBT substrate (Sigma).

#### **3.5. Determination of simulated intestinal fluid digestion stability assay.**

The procedure described by Fu *et al.* (2002) was followed. Simulated intestinal fluid was prepared with 10mg/ml pancreatin (P8096 Sigma Aldrich, UK) dissolved in intestinal control solution (0.05M KH<sub>2</sub>SO<sub>4</sub>, pH 7.5). Aliquots (64 µl) of SIF were placed in 1.5 ml microcentrifuge tubes and incubated at 37°C for 10 minutes in a water bath. The test protein (10 µl) at concentration of 5 mg/ml was added to each of

the microcentrifuge tubes to start the reaction. The ratio of pancreatin to test protein used was 13:1 (w:w). At time durations of 0, 2, 5, 10, 20, 30, 60, 90, 120 min, 15µl of 5 x Laemmli buffer was immediately added and the reaction stopped by heating the tubes at 100°C for 10 min to denature the remaining proteins. For the 0 min sample, test protein solutions (10µl) were added to 64µl of SIF, heat inactivated at 100°C for 5 minutes and 15µl 5x Laemmli buffer were added and the solution boiled at 100°C for 10 min.

Samples (15 µl) from each time point and control reactions were subjected to SDS-PAGE electrophoresis in reducing conditions, according to the method used by Laemmli (1970), using 10 well, 0.75 mm thick, 15% polyacrylamide Tris-glycine gels. Standard markers (Invitrogen prestained protein ladder 10748 -010) containing 10 proteins ranging in size from 6 – 180 kDa were included on all the gels. The gels were stained with Coomassie brilliant blue R-250 (B0149-5G Sigma, UK) for 1 hour and destained in 40% methanol with 10% glacial acetic acid for 30 minutes on a shaker at 70 rpm. Protein samples were then blotted onto Immobilon P membrane (Armstrong), using standard equipment and procedures (Mini Protean II, Biorad). Maize and papaya cystatins were detected using primary rabbit polyclonal antibody against these proteins at a 1:2000 dilution. A second antibody of anti rabbit IgG/alkaline phosphatase conjugate (Sigma) was used at 1:2000 dilution. The binding of the latter antibody was detected using BCPIP/NBT substrate (Sigma).

### **3.6. Determination of database amino acid sequence homology**

The AllergenOnline version 8.0 (updated January, 2008) database was used for the primary comparisons to allergens. The data is available at <http://www.allergenonline.com/>. The database contains 1313 known and/putative allergens associated with food, airway, contact or injected (biting or stinging insects) allergenic sources. This curated database is maintained by the Food Allergy Research and Resource Program of the University of Nebraska. All database entries are linked to sequences in the National Centre for Biotechnology Information (NCBI) of the National Institute of Health (NIH). The identification of relatively short regions of

high identity shared by a query sequence and an allergen may indicate similarities that could also share IgE binding, or cross reactivity (Goodman *et al.*, 2007). Based on the recommendation of Codex (2003), the FASTA8 algorithm was used to compare all the contiguous amino acid segments of maize and papaya cystatins proteins with all sequences listed in AllergenOnline. Every possible contiguous 80 amino acid sequence of each query protein was searched, beginning with amino acids 1-80, and continuing until the last 80 amino acid segment of each protein was compared with the database on AllergenOnline, using the FASTA8 algorithm.

### **3.7. Determination of toxicity of maize and papaya cystatins.**

The toxicity of the proteins was determined by dosing the rats with maize and papaya cystatins. In this study a total of 64 male albino rats aged 5 weeks were obtained from a barrier maintained colony from Makerere University Department of Veterinary Parasitology. They were housed in polypropylene cages with stainless steel grid tops and floors. Each cage housed four rats and five treatment groups were considered. The animals were kept in the study room for 14 days prior to the start of treatment to allow them to acclimatize to environmental conditions. They were the sole occupants of the room with 12 hour light per 24 hours. The rats were fed with a nutritionally adequate maintenance diet free from papaya and papaya products as well as maize and maize by-products as ingredients (UgaChick formulation). The rats had free access to food and water throughout the study. The rats were assigned to treatment groups by use of random number techniques and proportionately to body weight.

### **3.8. Dosing of albino rats with cystatins.**

Maize and papaya cystatins fed to the rats were kept at -20° C in 25mg aliquots. The prepared dosing solutions were administered by oral gavage at a dose volume of 10ml/kg body weight to give acute administered dosage of 0 (control), 10, 100 and 1000 mg/kg body weight) based on the rat weight. The dosing was based on the level margin of exposure (MOE) of the previously studied OclΔD86 cystatin and provided a range of > 200 - 2000 fold when 10 mg of protein was fed to the rats (Atkinson *et*

*al.*, 2004). The test substance dosing solutions were administered using disposable plastic syringes and separate syringes were used for each treatment group.

### 3.8.1 Clinical examination

Throughout the study, the rats were observed daily for mortality, morbidity and overt signs of toxicity. Once a week at the time of weighing, a more detailed examination was performed. Food and water consumption for each cage of rats was recorded daily until sacrifice. Urine was collected over ice from all the rats on the day before sacrifice. Food, but not water was removed from rats overnight before necropsy. The urine collected was examined for volume, refractive index, pH, protein, blood, ketones, bilirubin, glucose, urobilinogen and blood pigments. A microscopic examination of the urine sediment was done. The chemical testing of urine was carried out using commercial disposable test strips from Bayer's Multistix 19 SG and the result of this testing was semi quantitative.

At necropsy, the rats were sacrificed by exsanguinations under a barbiturate overdose. Blood samples from the dorsal aorta were collected into tubes containing EDTA anticoagulant and analysed using a Technicon HI haematology analyzer for total erythrocytes, red blood cell count (RBC), total and differential leukocytes, platelet count, haemoglobin concentration and mean cell volume. Additionally, hematocrit, mean cell haemoglobin and mean cell haemoglobin concentration were determined by calculation. Another blood sample collected into Trisodium citrate anticoagulant was used to measure prothrombin time, activated partial thromboplastin time. These tests were aimed at determining the general level of clottability of a plasma sample.

Serum separated from the blood was analyzed with a chemical analyzer (400 Roche Integra, USA) for glucose, blood urea nitrogen, total protein, albumin, globulin, creatinine, total bilirubin, albumin/ globulin ratio, calcium, phosphate, chloride, plus sodium and potassium concentrations. In addition, activities of alkaline phosphatase, alanine aminotransferase, aspartate amino transferase and gamma glutamyltransferase was determined using a Hitachi 717 clinical analyzer.

### 3.8.2 Anatomic pathology examination

During necropsy procedures, a macroscopic examination of the internal viscera and cavities was made, the heart, brain, kidneys, liver, spleen, testes, adrenals and thymus were removed and their weights recorded. Additionally, mesenteric lymph node and pancreas weights were obtained after fixation.

Samples of the following tissues were retained in 10% neutral buffered formalin from all animals i.e. adrenal glands, brain, heart, small intestine (duodenum, jejunum, ileum), large intestine (colon, rectum), kidneys, liver, mesenteric lymph node, pancreas, spleen, stomach, thyroids, parathyroid and testes to allow follow up of any indications of toxic effects associated with those organs. The following organs; caecum, heart, kidneys, pancreas, spleen, sternum and thymus of each rat were processed, embedded in wax. Sections were stained with haematoxylin and eosin using standard histological methods. Pathological screening of the tissues was done using a light microscope so as to examine them for defects.

### 3.9 Statistical analysis

The continuous variable data from the control and test groups was tested for normality using the Kolmogorov-Smirnov (K.S) test and homogeneity of variance using Bartlett's test. Statistical significance was determined at  $p \leq 0.05$  in K.S. test and at  $p \leq 0.01$  in Bartlett's test. Both the control and test groups were compared using Genstat 7<sup>th</sup> edition statistical package for Analysis of Variance (ANOVA) and the means were separated by the least significant difference (L.S.D.) test.

## CHAPTER FOUR

### RESULTS AND DISCUSSION

#### 4.1 Expression of maize and papaya cystatins in *E.coli* M15.

Figure 9 shows the protein extracts from *E.coli* M15. Before elution, the crude protein extract (track L) showed several protein bands ranging from 6 to 180kDa. After elution in imidazole most of the protein bands were no longer visible except for the bands in 64 to 49 kDa and 35 to 26 kDa regions. The thick bands appearing at 15kDa regions through the elutions correspond to cystatins. Misaka *et al* (2005) reported that cystatins have kDa 11 to 16. The results show that in this experiment, both maize and papaya cystatins were successfully expressed in *E coli* M15. Urwin *et al* (1995) and Song *et al* (1995) also reported expression of maize and papaya cystatins in the *E-coli* M15 under similar conditions. The elutions signify that the proteins were able to be released from the *Ecoli* construct while the washes are indicative of how much protein could have been lost during the extraction process before it was eluted.

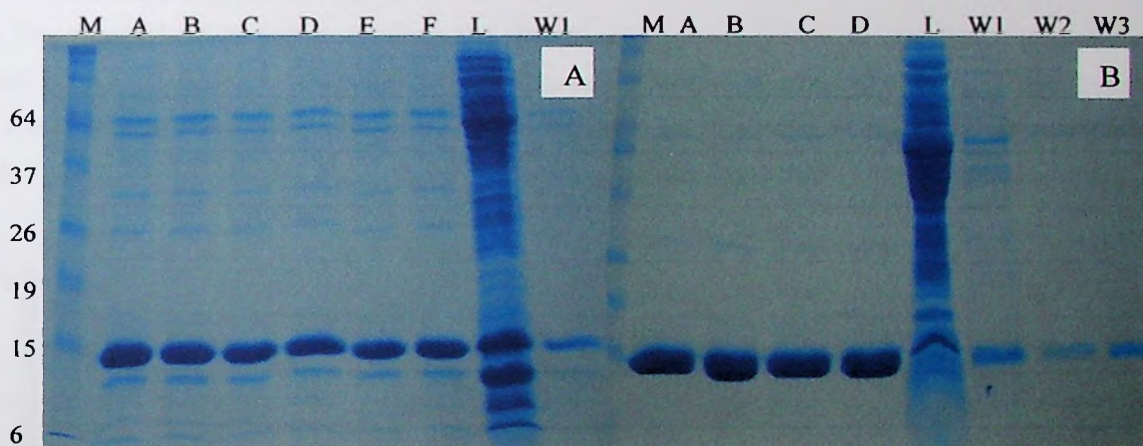
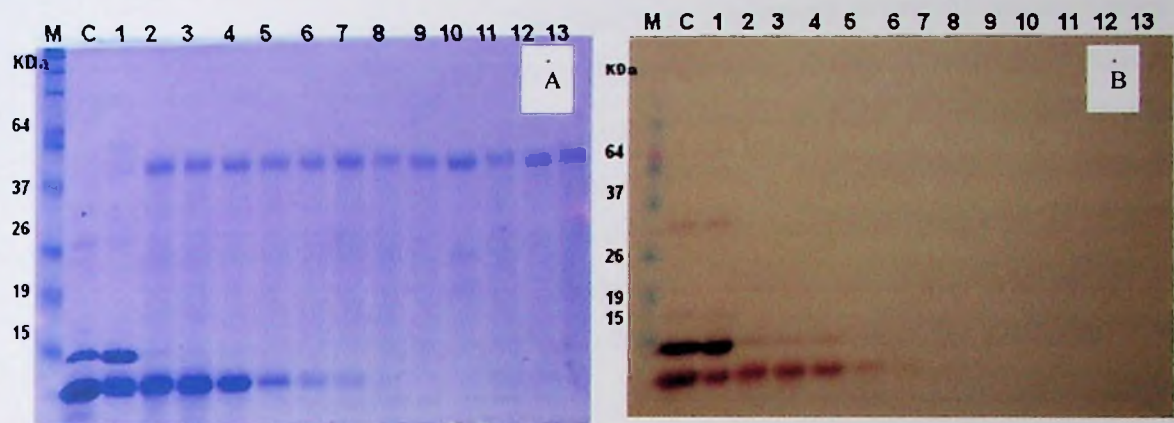


Figure 9 SDS -PAGE (A) maize cystatin, (B) papaya cystatin expressed from *E-coli*M15. Track M, molecular marker, A-F are expressed proteins, L is the lysate, W1 -W5 are washes

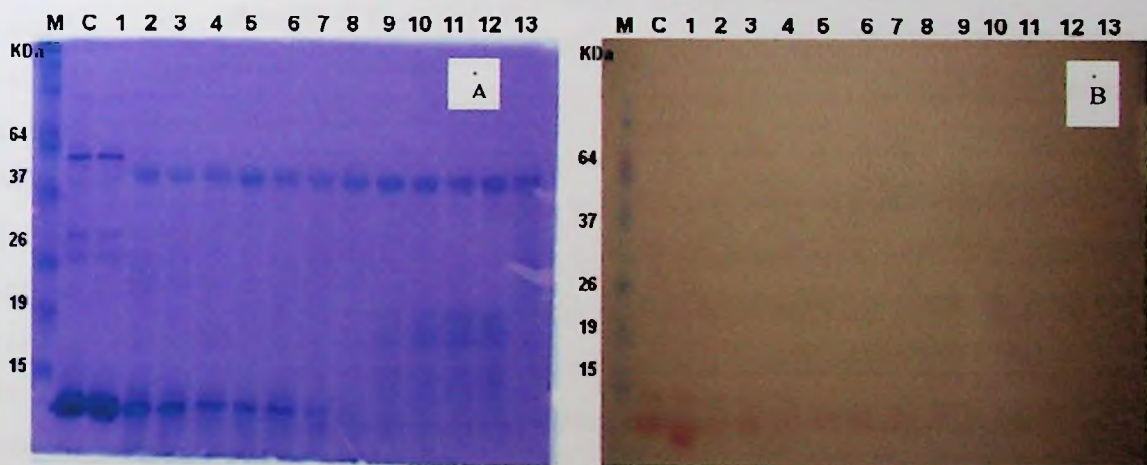
#### 4.2. Maize and papaya cystatins protein stability in pepsin

Figures 10 and 11 show the SDS-PAGE of the maize and papaya cystatins digested at different stages of pepsin digestion. The maize cystatin digested rapidly, gradually disappearing under the *in vitro* conditions used in the assay. The strong band representing

maize cystatin in pepsin (Figure 10A) was no longer visible after 5 seconds, indicating rapid degradation. A very small amount of a residual 7 kDa fragment stainable maize cystatin was seen at 2 seconds, but after that, only a faint, band was visible at 15 seconds, and this staining disappeared after 30 seconds. The western blot (Figure 10B) confirmed that by 30 seconds the fragment from maize cystatins bands had been degraded and where nolonger detectable by the alkaline phosphatase immuno conjugate from the polyclonal antibodies.



**Fig 10.** SDS-PAGE (A) and Western blot (B) of Maize cystatin protein. Protein was incubated in SGF containing pepsin for 0-60 min at 37°C. M is molecular weight markers; C is maize cystatin protein; Lane 1 contains maize cystatin protein at “time 0”; lanes 2 – 12 contain maize cystatin following SGF digestion of 2 s, 5 s, 7 s, 15 s, 30 s, 2, 5, 10, 30, and 60 min, respectively; lane 13 contains pepsin control.



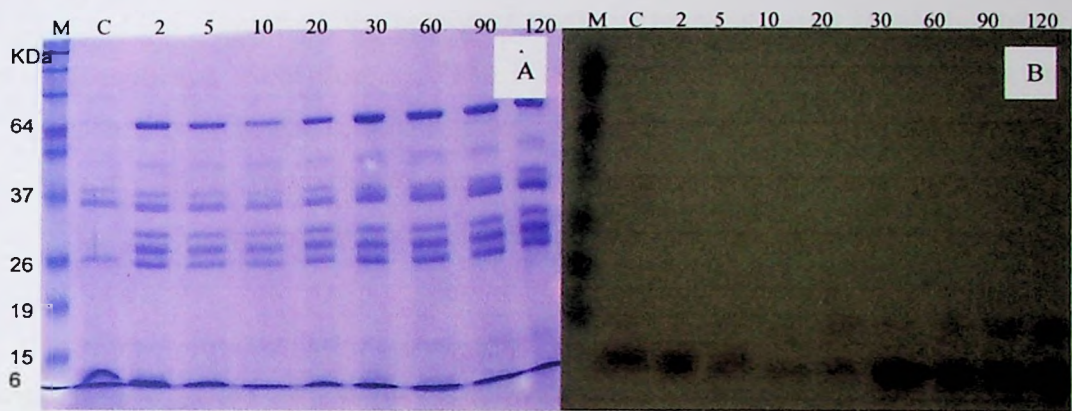
**Fig 11.** SDS-PAGE (A) and Western blot (B) of Maize cystatin protein. Protein was incubated in SGF containing pepsin for 0-60 min at 37°C. M is molecular weight markers; C is maize cystatin protein; Lane 1 contains maize cystatin protein at “time 0”; lanes 2 – 12 contain maize cystatin following SGF digestion of 2 s, 5 s, 7 s, 15 s, 30 s, 2, 5, 10, 30, and 60 min, respectively; lane 13 contains pepsin control

The papaya cystatin also degraded gradually disappearing under the *in vitro* conditions used in the assay. The strong band representing papaya cystatin (Figure 11A) started disintegrating into fragments by 10 seconds with the strong band disappearing within 2 minutes. A very small amount of a residual 7 kDa fragment papaya cystatin was seen at 30 seconds, but after that, only a faint band was visible at 2 minutes and this fragment band disappeared after 5 minutes. According to Astwood *et al.* (1996) an indicator that a protein is unlikely to be an allergen is its rapid partial digestion by pepsin in simulated gastric fluid. This is because stability to proteolytic and acidic conditions of the digestive tract imparts an increased probability of a protein reaching the intestinal mucosa, where absorption occurs. This has been implicated as one of the properties shared by allergens (Fu *et al.*, 2002; Sen *et al.*, 2002; Goodman *et al.*, 2007). The gastrointestinal tract is specifically designed to digest dietary proteins by conversion to small peptides and amino acids, which are absorbed by the intestinal tract (Fuchs *et al.*, 1993). Based on that most proteins rapidly degrade upon consumption and exposure to the mammalian tract (Fuchs *et al.*, 1993; Bannon *et al.*, 2003), while many major food allergens are stable to pepsin, this stability is assumed to be a risk factor for allergenic sensitization through the gastrointestinal tract (Astwood *et al.*, 1996; Fu *et al.*, 2002; Thomas *et al.*, 2004).

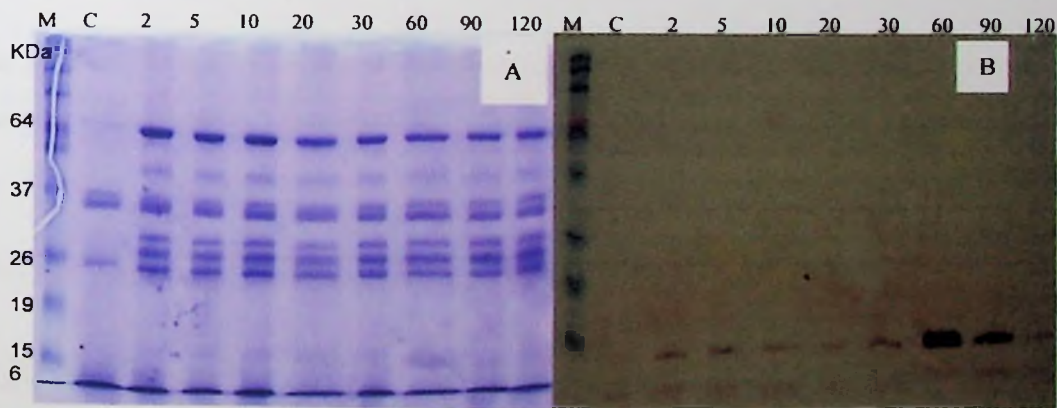
Digestion of the maize and papaya cystatins therefore, means that these proteins are not potential allergens, since they did not withstand the digestive conditions that simulated those in the stomach. Similar findings were reported on lack of stability of several Bt proteins (Betz *et al.*, 2000) and Ocl $\Delta$ D86 (Atkinson *et al.*, 2004) that were no longer detectable after 30 - 60 seconds in the simulated gastric fluid. Herman *et al.* (2005) also measured the digestion of allergenic and non allergenic proteins under simulated gastric conditions and reported that non allergens have a relatively short half life compared to the allergens, with a relatively longer half life due their stability in simulated gastric fluid. He concluded that this was a confirmation that digestibility of proteins in simulated gastric fluid is considered to be an indicator of reduced risk of allergenic potential in food. Therefore, since all the maize and papaya cystatins fragments disappeared within 5 minutes compared to the 8 to 60 minutes of allergens (Bannon *et al.*, 2002) it indicates that these proteins can be readily degraded in mammalian stomach.

4.3. Maize and papaya stability in the pancreatin digest

Figures 12A and 13A show the maize and papaya cystatins digestibility results in pancreatin. The original band of intact maize cystatin decreased substantially in pancreatin (Figure 12A) for 20 minutes and the band intensity was visible upto 120 minutes. Residual amounts of 6kDa stainable protein were seen and these persisted through out the 120 minutes of digestion. This was proved further by the immunoassay of the digested proteins by western blot (Figure 13B).



**Fig. 12.** SDS-PAGE (A) and (B) western blot of maize cystatin at different rates in a standardized in vitro pancreatin digestion assay. Track M is molecular marker, 2 – 120 are minutes during the digestive assay



**Fig.13.** SDS-PAGE (A) and (B) western blot of papaya cystatin at different rates in a standardized in vitro pancreatin digestion assay. Track M is molecular marker, 2 – 120 are minutes during the digestive assay

The pancreatin digestion of the papaya cystatin lasted for 120 minutes as faint bands as shown in Figure 13 A. These bands appeared pronounced as shown in the western blot (Figure 13B) with substantially little digestibility. Pancreatin, a major component of simulated intestinal fluid (SIF), is a mixture of five enzymes; amylase, trypsin, lipase, ribonuclease, and protease (Takagi *et al.*, 2003; Fu *et al.*, 2002). These enzymes are usually found in the duodenum whose conditions are usually close to neutral ranging from pH 6.8 and 7.5 (Fu *et al.*, 2002; Takagi *et al.*, 2003). The lack of complete digestibility of maize and papaya cystatins may be attributed to the assay conditions. This agrees with the findings of Murdock *et al.* (1987) that cystatins are active at acidic pH ranges of 1.2 – 5. Therefore, the persistent track of fragment bands should not be of any health consequence. According to Ryan (1990) protease inhibitors to which cystatins belong do not pose problems for humans, since foods that have high levels of these proteins are cooked, which inactivates the inhibitors. To this effect since they were rapidly digested in the pepsin assay conditions, both maize and papaya are proven to be safe.

#### 4.4. Comparison of amino acid sequence homology between maize and papaya cystatins and allergens in the database

Tables 3 and 4 show the amino acid sequence similarities of maize and papaya cystatins with known allergens in the database.

**Table 3:** Overall FASTA3.5 search of AllergenOnline version 8.0 with maize cystatin

| Sequence<br>GI # | Organism                       | Description         | Length | E-score  | %<br>Identity | aa<br>alignment |
|------------------|--------------------------------|---------------------|--------|----------|---------------|-----------------|
| 40807635         | <i>Actinidia<br/>deliciosa</i> | phytocystatin       | 116    | 2.1e-016 | 48            | 89              |
| 110346534        | <i>Anisakis<br/>simplex</i>    | ani s 4<br>allergen | 115    | 6.0e-010 | 40.2          | 97              |

Table 4: Overall FASTA35.5 search of AllergenOnline version 8.0 with papaya cystatin

| Sequence<br>GI # | Organism                       | Description         | Length | E-score  | %<br>Identity | aa<br>alignment |  |
|------------------|--------------------------------|---------------------|--------|----------|---------------|-----------------|--|
| 40807635         | <i>Actinidia<br/>deliciosa</i> | phytocystatin       | 116    | 5.5e-013 | 40.4          | 89              |  |
| 110346534        | <i>Anisakis<br/>simplex</i>    | ani s 4<br>allergen | 115    | 5.1e-007 | 33.7          | 97              |  |

The best sequence similarity of maize cystatin (GI: 809608/emb/X87126.1) and papaya cystatin (GI/311504/emb/X71124.1) amino acids with the known and putative allergens listed in AllergenOnline.com version 8 revealed some matches. The maize and papaya cystatins amino acid sequence were compared to 1313 amino acid sequences encoding for allergens in order to ascertain whether they had homology with known allergens in the database. The closest match to an allergen by maize and papaya cystatins was kiwi fruit (*Actinidia deliciosa*), a phytocystatin Actinid (GI:40807635) (Rassam and Laing, 2004) with an E score of 2.1e-016 and 48.3% identity over an alignment of 89 amino acids (Table 3) and E score of 6.0e-010 and 40.2% identity over an alignment of 89 amino acids (Table 3). Another allergen was from a herring worm known as *Anisakis simplex* containing ani s 4 allergen as a cysteine proteinase inhibitor (GI: 110346543) (Rodriguez-Mahillo *et al.*, 2007) with an E score of 5.5e-013 and 40.4% identity over an alignment of 99 amino acids (Table 4) and an E score of 5.1e- 007 and 33.7% identity over an alignment of 106 amino acids. While these are evolutionary homologues, the low degree of identity indicates a very low probability of any shared IgE binding epitopes.

However, much as there exists 48.3% and 40.4% homology of maize and papaya cystatins respectively with phytocystatin Actinid and 40.20% and 32.1% of maize and papaya cystatins respectively with ani s 4 allergen in the allergen database. The best predictor of cross reactivity would be a match of greater than 50% identity over the full length of the query protein and allergen (Goodman *et al.*, 2007). Aalberse (2000) reported that a protein sharing greater than 70% identity over its length, relative to an allergen is

likely to be cross reactive, or share IgE binding. Those that have less than 50% identity are not very likely to be cross reactive implying therefore that the maize and papaya cystatins are unlikely to be potential allergens.

Maize and papaya cystatins were also compared to the amino acid sequences of 2632 toxins collected from public domain genetic databases (GenBank, EMBL, PIR and Swiss Prot) for homology. The results confirm that maize and papaya cystatins had no biologically significant similarity nor immunological significant sequences were found to known toxins. These results further confirm that maize and papaya cystatins are unlikely to be toxic protein to humans when used in transformation.

#### 4.5. Feed intake of albino rats and clinical pathology observations

During the course of the feeding experiments with maize and papaya cystatins no mortality or moribundity was observed in any control or treatment groups throughout the study. No treatment related signs of adverse effects in clinical appearance of the animals were observed. All animals were healthy and appeared normal.

##### 4.5.1. Feed intake of the albino rats fed with maize cystatin

Results in Table 4 show that feed intake did not differ significantly between groups with animals that were fed with maize cystatins ( $P>0.01$ ). These findings therefore show that acute doses of maize cystatin generally did not affect the feed intake.

**Table 4. Total feed intake mean values of the albino rats following administration of maize cystatin(Mcy)**

| Parameter        | 0 (control)             | 10mg/kg Mcy             | 100mg/kg Mcy            | 1000mg/kg Mcy           | LSD  |
|------------------|-------------------------|-------------------------|-------------------------|-------------------------|------|
| Feed intake, (g) | 55.11±4.57 <sup>a</sup> | 61.41±3.97 <sup>a</sup> | 60.68±7.89 <sup>a</sup> | 63.43±3.46 <sup>a</sup> | 9.73 |

<sup>a</sup> Values in the row with same superscript are not significantly different between groups at  $P>0.01$ .

##### 4.5.2. Feed intake of animals feed with papaya cystatins

The results of the feed intake are presented in Table 5, and it was observed that the feed intake did not differ on period of admission. These findings therefore show that the

papaya cystatin generally did not affect the feed intake especially when administered at a higher dosage. This shows that papaya cystatin maintains feed intake and according to Ryan (1990), protease inhibitors contribute to better and enhanced nutritional quality.

**Table 5. Feed intake mean values of albino rats fed with papaya cystatin(Pcy) at various concentrations**

| Parameter          | 0 control               | 10mg/kg Pcy             | 100mg/kg Pcy            | 1000mg/kg Pcy           | LSD   |
|--------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------|
| Feed intake<br>(g) | 55.11±4.57 <sup>a</sup> | 63.40±8.06 <sup>a</sup> | 60.25±5.97 <sup>a</sup> | 60.16±5.50 <sup>a</sup> | 12.29 |

<sup>a</sup> Values in the row with same superscript are not significantly different between groups at P>0.01.

#### 4.5.3. Urinalysis of rats fed with maize and papaya cystatins

##### 4.5.3.1 Urinalysis of rats fed with maize cystatin

The results presented in Table 6 indicate that there were no differences in the pH and specific gravity of urine from control and dosed rats (P>0.01). While other parameters such glucose, bilirubin, ketone, blood, protein, uribilinogen, nitrite and leucocytes were not observed in the urine sample analysed qualitatively.

**Table 6: Urinalysis mean results of albino rats fed with maize cystatin at various concentrations**

| Parameter        | 0 control             | 10mg/kg.wt               | 100mg/kgwt               | 1000mg/kgwt              | L.S.D |
|------------------|-----------------------|--------------------------|--------------------------|--------------------------|-------|
| Glucose          | -ve                   | -ve                      | -ve                      | -ve                      |       |
| Bilirubin        | -ve                   | -ve                      | -ve                      | -ve                      |       |
| Ketone           | -ve                   | -ve                      | -ve                      | -ve                      |       |
| Specific gravity | 1.03±0 <sup>a</sup>   | 1.029±0.002 <sup>a</sup> | 1.029±0.002 <sup>a</sup> | 1.028±0.004 <sup>a</sup> | 0.009 |
| Blood            | -ve                   | -ve                      | -ve                      | -ve                      |       |
| pH               | 6.37±0.5 <sup>a</sup> | 6.62±0.5 <sup>a</sup>    | 6.25±0.6 <sup>a</sup>    | 6.43±0.4 <sup>a</sup>    | 0.73  |
| Protein          | -ve                   | -ve                      | -ve                      | -ve                      |       |
| Uribilinogen     | N                     | N                        | N                        | N                        |       |
| Nitrite          | -ve                   | -ve                      | -ve                      | -ve                      |       |
| Leucocytes       | -ve                   | -ve                      | -ve                      | -ve                      |       |

N=, Normal urine colour - ve = parameters were not observed in urine. <sup>a</sup> Values in the row with same superscript are not significantly different between groups at P>0.01.

According to Bayer (2007) the normal specific gravity of animal urine ranges from 1.001 to 1.035, but if the specific gravity of urine specimen is  $>1.023$ , the concentration ability of the kidney is considered normal while the acceptable pH ranges are 4.6 – 8.0.

Further more, absence of blood, leucocytes, nitrite, ketone, bilirubin and urobilinogen in urine samples usually indicates normal renal concentration and this makes chronic renal insufficiency unlikely (Bidhe *et al.*, 2004). The absence of the above parameters is an indication that the maize cystatin did not have adverse effects to the body organs of the rats such as the liver and the kidney, thus, indicating normal hepatocellular and nephritic function (Bidhe *et al.*, 2004).

#### 4.5.3.2. Urinalysis of rats fed with papaya cystatin

The results presented in Table 7 show that there were no differences between the pH and specific gravity ( $P > 0.01$ ) when the rats are fed with papaya cystatin. According to Bayer (2007) the normal specific gravity of urine ranges from 1.001 to 1.035, but if the specific gravity of urine specimen is  $>1.023$ , the concentration ability of the kidney is considered normal. The values established in this study were within the normal range. The acceptable pH ranges according to Bayer (2006) are 4.6 – 8.0 and the findings of this study are within this range. Further more, absence of blood, leucocytes, nitrite, ketone, bilirubin and urobilinogen in urine samples usually indicates normal renal concentration and this makes chronic renal insufficiency unlikely.

**Table 7: Urinalysis mean values of albino rats fed with papaya cystatin at various concentrations**

| Parameter        | 0 (control)      | 10mg/kg.wt         | 100mg/kgwt          | 1000mg/kgwt         | LSD  |
|------------------|------------------|--------------------|---------------------|---------------------|------|
| Glucose          | -ve              | -ve                | -ve                 | -ve                 |      |
| Bilirubin        | -ve              | -ve                | -ve                 | -ve                 |      |
| Ketone           | -ve              | -ve                | -ve                 | -ve                 |      |
| Specific gravity | $1.03 \pm 0^a$   | $1.02 \pm 0.006^a$ | $1.023 \pm 0.004^a$ | $1.025 \pm 0.007^a$ | 0.01 |
| Blood            | -ve              | -ve                | -ve                 | -ve                 |      |
| pH               | $6.37 \pm 0.5^a$ | $6.68 \pm 0.4^a$   | $6.64 \pm 0.4^a$    | $6.75 \pm 0.4^a$    | 0.58 |
| Protein          | -ve              | -ve                | -ve                 | -ve                 |      |
| Uribilinogen     | N                | N                  | N                   | N                   |      |
| Nitrite          | -ve              | -ve                | -ve                 | -ve                 |      |
| Leucocytes       | -ve              | -ve                | -ve                 | -ve                 |      |

N= Normal urine, - ve = parameters were not observed in urine. <sup>a</sup> Values in the row with same superscript are not significantly different between groups at  $P > 0.01$ .

Therefore, these results show that administering an acute dosage of papaya cystatin at 1000mg/kg body weight did not have adverse effects on the renal and liver functions of the animals under the study conditions.

#### **4.5.4. Haematology results of rats fed with maize and papaya cystatins**

##### **4.5.4.1. Haematology of rats fed with maize cystatin**

Results of the haematology tests of the albino rats fed with maize cystatin on the last day of the study (day 8) are presented in Table 8. Haematology analysis showed no differences between the groups of animals fed with varying doses of maize cystatin.

The maize cystatin did not exert toxic effects to the animals since it did not affect the circulating red cells, hematopoiesis and leucopoiesis. This could otherwise have caused megaloblastic anaemia (Bidhe and Ghosh, 2004), as well as changes in packed cell volume (PCV). Research findings have reported that lack of changes in haemoglobin (HGB) as well as red blood cell (RBC) functions such as mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration that typically correlate with decreased RBC function indicate lack of toxic effects on animal (Mackenzie *et al.*, 2007; Bidhe and Ghosh, 2004).

**Table 8: Haematology mean values in albino rats following an acute dose administration of maize cystatin**

| Parameter                                                | 0 (control)                   | 10mg Mcy                     | 100mg Mcy                     | 1000mg Mcy                    | LSD   |
|----------------------------------------------------------|-------------------------------|------------------------------|-------------------------------|-------------------------------|-------|
| White blood cells (WBC) ( $10^3/\mu\text{l}$ )           | 5.29 $\pm$ 2.9 <sup>a</sup>   | 6.71 $\pm$ 3.5 <sup>a</sup>  | 8.51 $\pm$ 7.8 <sup>a</sup>   | 6.05 $\pm$ 2.6 <sup>a</sup>   | 6.34  |
| Lymphocytes (LYM) ( $10^3/\mu\text{l}$ )                 | 84.35 $\pm$ 4.0 <sup>a</sup>  | 87.51 $\pm$ 5.1 <sup>a</sup> | 87.24 $\pm$ 4.9 <sup>a</sup>  | 86.19 $\pm$ 4.2 <sup>a</sup>  | 6.45  |
| Red blood cells (RBC) ( $10^6/\mu\text{l}$ )             | 6.76 $\pm$ 0.3 <sup>a</sup>   | 6.99 $\pm$ 0.2 <sup>a</sup>  | 7.15 $\pm$ 0.3 <sup>a</sup>   | 7.05 $\pm$ 0.2 <sup>a</sup>   | 0.44  |
| Haemoglobin (HG) (g/dl)                                  | 12.78 $\pm$ 0.3 <sup>a</sup>  | 12.9 $\pm$ 0.4 <sup>a</sup>  | 13.11 $\pm$ 0.3 <sup>a</sup>  | 12.85 $\pm$ 0.3 <sup>a</sup>  | 0.55  |
| Haematocrit (HC) %                                       | 42.25 $\pm$ 1.70 <sup>a</sup> | 42.71 $\pm$ 1.9 <sup>a</sup> | 43.61 $\pm$ 1.9 <sup>a</sup>  | 44.14 $\pm$ 0.9 <sup>a</sup>  | 2.37  |
| Mean corpuscular volume (MCV) (fl)                       | 62.53 $\pm$ 1.8 <sup>a</sup>  | 61.02 $\pm$ 0.9 <sup>a</sup> | 61.0 $\pm$ 1.2 <sup>a</sup>   | 62.70 $\pm$ 2.1 <sup>a</sup>  | 2.23  |
| Mean corpuscular haemoglobin (MCH) (pg)                  | 18.93 $\pm$ 0.7 <sup>a</sup>  | 18.44 $\pm$ 0.3 <sup>a</sup> | 18.36 $\pm$ 0.6 <sup>a</sup>  | 18.15 $\pm$ 0.7 <sup>a</sup>  | 0.82  |
| Mean corpuscular haemoglobin concentration (MCHC) (g/dl) | 30.26 $\pm$ 0.6 <sup>a</sup>  | 30.21 $\pm$ 0.6 <sup>a</sup> | 30.08 $\pm$ 1.0 <sup>a</sup>  | 29.11 $\pm$ 0.4 <sup>a</sup>  | 0.98  |
| Platelets (PLT) ( $10^3/\mu\text{l}$ )                   | 839.5 $\pm$ 55.5 <sup>a</sup> | 896 $\pm$ 29.9 <sup>a</sup>  | 784.4 $\pm$ 37.1 <sup>a</sup> | 806.8 $\pm$ 35.8 <sup>a</sup> | 158.7 |

<sup>a</sup> Values in the row with same superscript are not significantly different between groups at  $P>0.01$ .

Research findings have reported that lack of changes in haemoglobin (HGB) as well as red blood cell (RBC) functions such as mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration that typically correlate with decreased RBC function indicate lack of toxic effects on animal (Mackenzie *et al.*, 2007; Bidhe and Ghosh, 2004). Therefore, the acute dose of 1000mg/kg.body weight maize cystatin administered does not in any way cause toxic effects on the albino rat cell blood counts.

In addition, there were no significant differences between the different groups of animals with the control in the white blood cell counts. According to Ugochukwu (2008), the increase in the white blood cell counts indicates a pathological condition. This therefore, affirms that maize cystatin when fed to animals to a high dose of 1000mg/kgbody weight does not affect the immune and blood circulatory system of the animals.

#### 4.6.4.2. Haematology of rats fed with papaya cystatin

Results of the haematology tests of rats fed with papaya cystatins are presented in Table 9. There were no statistically significant differences observed ( $P>0.01$ ) in all of the haematology parameters determined when animals were fed with papaya cystatin at various doses. According to Mackenzie *et al.*(2007), Bidhe and Ghosh, (2004), lack of changes in haemoglobin (HGB) as well as red blood cell (RBC) functions such as mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration that typically correlate with decreased RBC function indicates lack of toxic effects on animals.

This indicates that the papaya cystatin may not be toxic since it did not affect the circulating red blood cells, hematopoiesis and leucopoiesis (Bidhe and Ghosh, 2004). Furthermore, according to Ugochukwu (2008), the increase in the white blood cell counts indicates a pathological condition. This condition signifies toxic effects on the bone marrow of the animals. However, this condition was not observed in the rats that were fed with papaya cystatins to a high dose of 1000mg/kg body weight. Therefore, dosing albino rats with 1000mg/kg body weight of papaya cystatin did not have adverse effect on the cell blood counts of the animals, hence it is not likely to be a toxic protein.

**Table 9: Haematology mean values in albino rats following acute dose administration to papaya cystatin**

| Parameter                                                | 0 (control)                  | 10mg pcy                     | 100mg pcy                     | 1000mg pcy                   | LSD  |
|----------------------------------------------------------|------------------------------|------------------------------|-------------------------------|------------------------------|------|
| White blood cells (WBC) ( $10^3/\mu\text{l}$ )           | 5.29 $\pm$ 2.9 <sup>a</sup>  | 5.93 $\pm$ 1.1 <sup>a</sup>  | 5.16 $\pm$ 1.6 <sup>a</sup>   | 6.10 $\pm$ 3.9 <sup>a</sup>  | 3.62 |
| Lymphocytes ( $10^3/\mu\text{l}$ )                       | 84.35 $\pm$ 4.0 <sup>a</sup> | 83.36 $\pm$ 6.7 <sup>a</sup> | 84.65 $\pm$ 5.46 <sup>a</sup> | 85.66 $\pm$ 3.98             | 7.40 |
| Red blood cells (RBC) ( $10^6/\mu\text{l}$ )             | 6.76 $\pm$ 0.4 <sup>a</sup>  | 6.93 $\pm$ 0.4 <sup>a</sup>  | 7.13 $\pm$ 0.3 <sup>a</sup>   | 7.25 $\pm$ 0.5 <sup>a</sup>  | 0.58 |
| Haemoglobin (HG) (g/dl)                                  | 12.78 $\pm$ 0.4 <sup>a</sup> | 12.81 $\pm$ 0.7 <sup>a</sup> | 13.11 $\pm$ 0.3 <sup>a</sup>  | 13.11 $\pm$ 0.7 <sup>a</sup> | 0.76 |
| Haematocrit (HC) %                                       | 42.25 $\pm$ 1.7 <sup>a</sup> | 43.14 $\pm$ 2.3 <sup>a</sup> | 44.03 $\pm$ 2.0 <sup>a</sup>  | 43.40 $\pm$ 2.2 <sup>a</sup> | 2.89 |
| Mean corpuscular volume (MCV) (fl)                       | 62.53 $\pm$ 1.8              | 62.29 $\pm$ 1.5              | 61.75 $\pm$ 2.27              | 59.90 $\pm$ 1.88             | 2.65 |
| Mean corpuscular haemoglobin (MCH)(pg)                   | 18.93 $\pm$ 0.7 <sup>a</sup> | 18.51 $\pm$ 0.5 <sup>a</sup> | 18.43 $\pm$ 0.4 <sup>a</sup>  | 18.08 $\pm$ 0.7 <sup>a</sup> | 0.84 |
| Mean corpuscular haemoglobin concentration (MCHC) (g/dl) | 30.26 $\pm$ 0.6 <sup>a</sup> | 29.71 $\pm$ 0.6 <sup>a</sup> | 29.91 $\pm$ 0.9 <sup>a</sup>  | 30.23 $\pm$ 0.8 <sup>a</sup> | 1.07 |
| Plateletes (PLT) ( $10^3/\mu\text{l}$ )                  | 824 $\pm$ 65.5 <sup>a</sup>  | 806 $\pm$ 80.5 <sup>a</sup>  | 808 $\pm$ 70.5 <sup>a</sup>   | 838 $\pm$ 70.0 <sup>a</sup>  | 80.2 |

<sup>a</sup> Values in the row with same superscript are not significantly different between groups at  $P>0.01$ .

#### 4.5.5. Serum chemistry of rats fed with maize and papaya cystatins

##### 4.5.5.1. Serum chemistry of rats fed with maize cystatin

The effects of maize cystatin on the liver and kidney function indices are presented in Table 10. Serum analysis showed that there were no differences in the analysed parameters of serum from rats dosed with different maize cystatin doses. Researchers have reported that liver cell damage is characterized by a rise in plasma enzymes such as AST and ALT (Aniagu *et al.*, 2005; Joshi *et al.*, 2007; Ugochukwu *et al.*, 2008).

Therefore, the maize cystatin can not induce hepatocellular changes in the liver based on the low alanine amino transferase (ALT) levels hence is not potentially toxic to the animals when administered at 1000mg/kg body weight.

Plasma levels of total proteins, albumin, blood urea nitrogen, cholesterol, creatine, glucose and potassium ions were not affected by feeding albino rats on maize cystatin.

This indicates that the protein did not interfere with the animals' normal renal capacity to excrete these metabolites (Aniagu *et al* 2005; Kaneku, 1989). Therefore, the renal integrity of the treated rats was preserved.

**Table 10: Serum analysis mean values in albino rats fed on different levels of maize cystatin**

| Parameter                              | 0 (control)             | 10mg<br>Mcy             | 100mg<br>Mcy            | 1000mg<br>Mcy           | LSD   |
|----------------------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------|
| Alkaline Phosphatase (ALP)(U/L)        | 319±29.3 <sup>a</sup>   | 297.8±17.6 <sup>a</sup> | 271.9±26.1 <sup>a</sup> | 326.0±44.8 <sup>a</sup> | 90    |
| Alanine amino transferase(ALT) (U/L)   | 145.7±66.4 <sup>a</sup> | 101.8±30.8 <sup>a</sup> | 100.2±31.9 <sup>a</sup> | 112.2±34.8 <sup>a</sup> | 60.23 |
| Aspartate amino transferase(AST) (U/L) | 343.9±35.8 <sup>a</sup> | 322.4±18.9 <sup>a</sup> | 312.1±20.7 <sup>a</sup> | 291.9±31.1 <sup>a</sup> | 79.7  |
| Blood Urea Nitrogen(BUN) (mg/dl)       | 3.55±0.6 <sup>a</sup>   | 3.01±0.4 <sup>a</sup>   | 3.21±0.6 <sup>a</sup>   | 3.77±0.2 <sup>a</sup>   | 0.64  |
| Creatinine (mg/dl)                     | 14.51±2.7 <sup>a</sup>  | 13.88±4.7 <sup>a</sup>  | 14.65±2.4 <sup>a</sup>  | 13.93±1.3 <sup>a</sup>  | 4.24  |
| Total Bilirubin (TB) (mg/dl)           | 2.17±0.4 <sup>a</sup>   | 2.86±0.5 <sup>a</sup>   | 2.86±0.8 <sup>a</sup>   | 3.69±0.6 <sup>a</sup>   | 3.25  |
| Total Protein (TP) (g/dl)              | 5.53±0.5 <sup>a</sup>   | 5.70±0.1 <sup>a</sup>   | 5.66±0.5 <sup>a</sup>   | 5.90±0.3 <sup>a</sup>   | 0.53  |
| Albumin(g/dl)                          | 3.31±0.4 <sup>a</sup>   | 3.22±0.1 <sup>a</sup>   | 3.25±0.2 <sup>a</sup>   | 3.30±0.1 <sup>a</sup>   | 0.32  |
| Cholesterol (mg/dl)                    | 2.13±0.2 <sup>a</sup>   | 2.23±0.4 <sup>a</sup>   | 2.11±0.3 <sup>a</sup>   | 2.08±0.3 <sup>a</sup>   | 0.39  |
| Triglycerides (mg/dl)                  | 0.83±0.2 <sup>a</sup>   | 1.00±0.2 <sup>a</sup>   | 0.82±0.3 <sup>a</sup>   | 0.79±0.2 <sup>a</sup>   | 0.30  |
| Albumin/Globulin                       | 1.51±0.3 <sup>a</sup>   | 1.30±0.1 <sup>a</sup>   | 1.64±0.7 <sup>a</sup>   | 1.28±0.1 <sup>a</sup>   | 0.51  |
| Globulin (g/dl)                        | 2.22±0.3 <sup>a</sup>   | 2.48±0.1 <sup>a</sup>   | 2.41±0.5 <sup>a</sup>   | 2.50±0.2 <sup>a</sup>   | 0.43  |
| Glucose (mg/dl)                        | 5.88±1.7 <sup>a</sup>   | 5.10±3.2 <sup>a</sup>   | 5.03±2.0 <sup>a</sup>   | 8.12±2.0 <sup>a</sup>   | 3.18  |
| Calcium (mg/dl)                        | 2.48±0.1 <sup>a</sup>   | 2.55±0.2 <sup>a</sup>   | 2.66±0.1 <sup>a</sup>   | 2.89±0.3 <sup>a</sup>   | 0.29  |
| Phosphorus(mg/dl)                      | 5.37±1.3 <sup>a</sup>   | 4.65±2.4 <sup>a</sup>   | 4.35±1.2 <sup>a</sup>   | 3.43±1.9 <sup>a</sup>   | 2.47  |

<sup>a</sup> Values in the row with same superscript are not significantly different between groups at P>0.01.

#### 4.5.5.2. Serum chemistry of rats fed with papaya cystatin

Results showing the effect of papaya cystatin on the liver and kidney are presented in Table 11. Serum chemistry analysis showed that there were no statistical differences in majority of the analyzed parameters except for the total bilirubin at 10mg/kgbody weight.

The AST and ALT values were not statistically different ( $P>0.01$ ). Liver cell damage is characterized by a rise in plasma enzymes such as AST and ALT (Aniagu *et al.*, 2005; Joshi *et al.*, 2007; Ugochukwu *et al.*, 2008). According to Mayne (1996) AST values are higher than ALT levels since body cells contain more AST than ALT. This was the same observation with serum chemistry results on rats fed with papaya cystatins. This is because about 80% of AST is found in the mitochondria whereas ALT is a purely cytosolic enzyme (Aniagu *et al.*, 2005). Therefore, AST appears in higher concentrations in a number of tissues (liver, kidneys, heart and pancreas) and is released slowly in comparison to ALT (Aniagu *et al.*, 2005). But, since ALT is localised primarily in the cytosol of hepatocytes, this enzyme is considered a more sensitive marker of hepatocellular damage than AST and can provide a quantitative assessment of damage sustained by the liver (Al-Mamary *et al.*, 2002; Ugochukwu *et al.*, 2008). Therefore, results from the liver function tests show that the papaya cystatin is unlikely to induce hepatocellular changes in the liver based on the low ALT levels hence it is not potentially toxic to the animals when administered at 1000mg/kg body weight.

On serum lipid profile, there was no significant difference between the dose levels and the control in serum triglyceride and total cholesterol concentrations. This shows that the proteins when fed to the animals may not cause Ischaemic and coronary heart diseases such as atherosclerosis (Dixter *et al.*, 2000).

Plasma levels such as total proteins, albumin, glucose and blood urea nitrogen were not significantly affected by feeding papaya cystatin. This indicates that the normal metabolism of the rats might have not been affected. Hence, the protein did not interfere with renal function (Kaneko, 1989) and therefore, the renal integrity of the treated rats was preserved. Therefore, the findings show that feeding papaya cystatin to rats at a higher dose is unlikely to cause toxicological effects as observed from the serum results (Table 11).

**Table 11. Serum chemistry mean values in albino rats fed with papaya cystatin at various concentrations**

| Parameter                               | 0 (control)             | 10mg Pcy                | 100mg Pcy                | 1000mg Pcy               | LSD   |
|-----------------------------------------|-------------------------|-------------------------|--------------------------|--------------------------|-------|
| Alkaline phosphatases (ALP) (U/L)       | 279.2±26.6 <sup>a</sup> | 385±18.4 <sup>a</sup>   | 357±22.9 <sup>a</sup>    | 251±35.5 <sup>a</sup>    | 115.7 |
| Alanine amino transferases (ALT) (U/L)  | 175±20.42 <sup>a</sup>  | 158.8±13.9 <sup>a</sup> | 164.2±26.57 <sup>a</sup> | 137.3±18.05 <sup>a</sup> | 81.3  |
| Aspartate amino transferase (AST) (U/L) | 393±42.8 <sup>a</sup>   | 392±44.3 <sup>a</sup>   | 344±30.3 <sup>a</sup>    | 395± 48.1 <sup>a</sup>   | 148.6 |
| Blood Urea Nitrogen (BUN) (mg/dl)       | 3.55±0.6 <sup>a</sup>   | 3.53±0.3 <sup>a</sup>   | 3.22±0.3 <sup>a</sup>    | 3.44±0.9 <sup>a</sup>    | 0.55  |
| Creatinine(mg/dl)                       | 15.21±1.9 <sup>a</sup>  | 15.05±2.3 <sup>a</sup>  | 13.03±1.2 <sup>a</sup>   | 13.20±2.5 <sup>a</sup>   | 3.11  |
| Total bilirubin (TBIL) (mg/dl)          | 5.87±1.5 <sup>a</sup>   | 10.49±3.9 <sup>a</sup>  | 9.46±1.5 <sup>a</sup>    | 8.46±3.1 <sup>a</sup>    | 4.4   |
| Total protein (g/dl)                    | 5.53±0.5 <sup>a</sup>   | 5.87±0.4 <sup>a</sup>   | 5.76±0.3 <sup>a</sup>    | 5.81±0.5 <sup>a</sup>    | 0.62  |
| Albumin(g/dl)                           | 3.31±0.4 <sup>a</sup>   | 3.28±0.2 <sup>a</sup>   | 3.29±0.3 <sup>a</sup>    | 3.66±0.7 <sup>a</sup>    | 0.64  |
| Cholesterol(mg/dl)                      | 2.13±0.21 <sup>a</sup>  | 1.98±0.20 <sup>a</sup>  | 2.09±2.03 <sup>a</sup>   | 2.45±1.1 <sup>a</sup>    | 0.85  |
| Triglycerides (mg/dl)                   | 0.83±0.2 <sup>a</sup>   | 0.89±0.3 <sup>a</sup>   | 1.02±0.2 <sup>a</sup>    | 1.11±0.2 <sup>a</sup>    | 0.35  |
| Albumin/Globulin                        | 1.51±0.2 <sup>a</sup>   | 1.30±0.1 <sup>a</sup>   | 1.39±0.3 <sup>a</sup>    | 1.50±0.3 <sup>a</sup>    | 0.38  |
| Globulin(g/dl)                          | 2.22±0.3 <sup>a</sup>   | 2.58±0.3 <sup>a</sup>   | 2.46±0.4 <sup>a</sup>    | 2.15±0.7 <sup>a</sup>    | 0.65  |
| Glucose(mg/dl)                          | 5.88±0.5 <sup>a</sup>   | 7.11±1.4 <sup>a</sup>   | 8.32±1.1 <sup>a</sup>    | 8.40±1.7 <sup>a</sup>    | 3.31  |
| Calcium(mg/dl)                          | 2.48±0.2 <sup>a</sup>   | 2.28±0.2 <sup>a</sup>   | 2.39±0.2 <sup>a</sup>    | 2.43±0.2 <sup>a</sup>    | 0.29  |
| Phosphorus(mg/dl)                       | 5.37±1.3 <sup>a</sup>   | 4.05±0.5 <sup>a</sup>   | 4.28±0.7 <sup>a</sup>    | 4.75±1.0 <sup>a</sup>    | 2.08  |

<sup>a</sup> Values in the row with same superscript are not significantly different between groups at P>0.01.

#### 4.5.6. Organ weights of rats fed with maize and papaya cystatins

##### 4.5.6.1. Organ weights of rats fed with maize cystatins

The results of organ/body weights of the albino rats fed on different levels of maize cystatin are presented in Table 12. Generally, there were no statistically significant changes in the organ weights (P>0.01) of maize cystatin fed rats compared to controls.

This therefore shows that feeding the rats with maize cystatin at a high dose of 1000mg/kg body weight did not affect the integrity of the animal organs in any way. Therefore, maize cystatin when fed to humans are unlikely to be toxic to their body organs.

Table 12. Shows organ weight mean values of albino rats fed with maize cystatin at various concentrations

| Parameter  | 0 (control)            | 10mg Mcy               | 100mg Mcy              | 1000mg Mcy             | LSD  |
|------------|------------------------|------------------------|------------------------|------------------------|------|
| Heart      | 0.45±0.03 <sup>a</sup> | 0.45±0.1 <sup>a</sup>  | 0.44±0.1 <sup>a</sup>  | 0.43±0.1 <sup>a</sup>  | 0.09 |
| Kidney     | 1.04±0.1 <sup>a</sup>  | 0.96±0.1 <sup>a</sup>  | 0.99±0.2 <sup>a</sup>  | 0.92±0.1 <sup>a</sup>  | 0.18 |
| Liver      | 4.40±0.4 <sup>a</sup>  | 4.20±0.7 <sup>a</sup>  | 4.47±0.9 <sup>a</sup>  | 4.89±0.2 <sup>a</sup>  | 0.88 |
| Lymphnodes | 0.10±0.02 <sup>a</sup> | 0.10±0.06 <sup>a</sup> | 0.08±0.04 <sup>a</sup> | 0.14±0.06 <sup>a</sup> | 0.06 |
| Pancreas   | 0.28±0.1 <sup>a</sup>  | 0.24±0.1 <sup>a</sup>  | 0.32±0.1 <sup>a</sup>  | 0.37±0.04 <sup>a</sup> | 0.12 |
| Spleen     | 0.34±0.1 <sup>a</sup>  | 0.34±0.2 <sup>a</sup>  | 0.35±0.2 <sup>a</sup>  | 0.39±0.1 <sup>a</sup>  | 0.19 |
| Testes     | 1.27±0.2 <sup>a</sup>  | 1.37±0.2 <sup>a</sup>  | 1.38±0.2 <sup>a</sup>  | 1.21±0.2 <sup>a</sup>  | 0.55 |
| Thymus     | 0.34±0.1 <sup>a</sup>  | 0.30±0.01 <sup>a</sup> | 0.32±0.1 <sup>a</sup>  | 0.30±0.1 <sup>a</sup>  | 0.11 |
| Empty      | 0.51±0.2 <sup>a</sup>  | 0.55±0.1 <sup>a</sup>  | 0.51±0.2 <sup>a</sup>  | 0.55±0.1 <sup>a</sup>  | 0.17 |
| Caecum     |                        |                        |                        |                        |      |

<sup>a</sup> Values in the row with same superscript are not significantly different between groups at P>0.01.

#### 4.5.6.2. Organ weights of rats fed with papaya cystatin

Table 13. Organ weight mean values of albino rats fed with papaya cystatin at various concentrations

| Parameter  | 0 (control)            | 10mg Pcy               | 100mg Pcy              | 1000mg Pcy             | LSD  |
|------------|------------------------|------------------------|------------------------|------------------------|------|
| Heart      | 0.45±0.3 <sup>a</sup>  | 0.42±0.0 <sup>a</sup>  | 0.43±0.0 <sup>a</sup>  | 0.38±0.1 <sup>a</sup>  | 0.07 |
| Kidney     | 1.04±0.1 <sup>b</sup>  | 0.87±0.1 <sup>a</sup>  | 0.87±1.0 <sup>a</sup>  | 0.89±0.1 <sup>a</sup>  | 0.12 |
| Liver      | 4.40±0.4 <sup>a</sup>  | 4.80±0.3 <sup>a</sup>  | 4.94±0.5 <sup>a</sup>  | 5.30±1.1 <sup>a</sup>  | 0.90 |
| Lymphnodes | 0.10±0.0 <sup>a</sup>  | 0.15±0.1 <sup>a</sup>  | 0.14±0.0 <sup>a</sup>  | 0.12±0.0 <sup>a</sup>  | 0.05 |
| Pancreas   | 0.28±0.1 <sup>a</sup>  | 0.38±0.1               | 0.29±0.1 <sup>a</sup>  | 0.32±0.1 <sup>a</sup>  | 0.08 |
| Spleen     | 0.34±0.1 <sup>a</sup>  | 0.35±0.1 <sup>a</sup>  | 0.29±0.1 <sup>a</sup>  | 0.39±0.1 <sup>a</sup>  | 0.15 |
| Testes     | 1.27±0.2 <sup>a</sup>  | 1.34±0.1 <sup>a</sup>  | 1.28±0.2 <sup>a</sup>  | 1.25±0.2 <sup>a</sup>  | 0.24 |
| Thymus     | 0.34±0.06 <sup>a</sup> | 0.29±0.10 <sup>a</sup> | 0.31±0.07 <sup>a</sup> | 0.29±0.10 <sup>a</sup> | 0.12 |
| Empty      | 0.51±0.2 <sup>a</sup>  | 0.54±0.1 <sup>a</sup>  | 0.45±0.1 <sup>a</sup>  | 0.52±0.1 <sup>a</sup>  | 0.15 |
| Caecum     |                        |                        |                        |                        |      |

<sup>a</sup> Values in the row with same superscript are not significantly different between groups at P>0.01.

Generally, there were no dose dependent reductions in the organ sizes of the animals administered with various doses of papaya cystatin. These observations were statistically significant (P>0.01). According to Joshi *et al.* (2007) increase in size of organs such the liver, kidney and the heart is an indication of hepatocyte damage. However, this was not the case with the rat organs. Histosto-pathological section of these organs i.e. liver, kidney, heart, lymphnode and pancreas revealed normal architecture in comparison with the control. This, therefore, suggests that feeding rats to a high dose with papaya cystatin doses does not cause toxic effects to body organs.

## CHAPTER 5

### CONCLUSIONS AND RECOMMENDATIONS

#### 5.1 Conclusions

Based on the rapid degradation of the maize and papaya cystatins in the simulated gastric conditions, is an indication that these cystatins may not be potential sources allergens. Therefore cystatins may safely be used in banana transformation.

Based on the comparison of amino acid sequence homology data for allergenicity and toxicity in the database with the cystatins, the results have shown that they are not a potential source of allergens since their homology identity is less than 50% for related allergens reported while there were close homologs of the cystatins in relation to known toxins in the database.

Feeding the albino rats with acute doses of maize and papaya cystatins did not affect their feed intake but just maintained the animal weights. Further more, urinalysis, haematology, serum chemistry and well as histopathological examinations showed that the cystatins did not in any way interfere with the blood circulation, renal functions and liver functions. Therefore, suggesting that both the maize and papaya cystatins are safe for use in transgenic banana lines to control pests.

#### 5.2 Recommendations

This assessment demonstrates that the cystatins do not present a risk for adverse effects in humans when used in the context of agricultural biotechnology therefore their use should be continued with.

Incase the cystatins are found to be effective against the nematodes and banana weevil borers, the public should be made aware that they do not pose any health effects but protect them from adverse use of toxic pesticides.

Since maize and papaya cystatins target root specific pests like weevils and nematodes, the extent of expression in various parts of the transgenic banana plant needs to be determined.

In future banana lines carrying maize and papaya cystatins should be analysed for proximate and nutritional composition to ascertain the effect of the cystatins on the banana composition compared to the non-transformed banana varieties.

## References

- Aalberse, R. C. 2000. Structural biology of allergens. *Journal of Allergy Clinical Immunology* 106: 227 -258.
- Abe, K., Emore, Y., Kondo, H., Suzuki, K., and Arai, S. 1987. Molecular cloning of cysteine proteinase inhibitor of rice (*oryzacystatin*). Homology with animal cystatins and transient expression in ripening process of rice seeds. *Journal of Biological Chemistry* 262: 16793 -97.
- Abe, K., Kondo, H., and Arai, S. 1987. Purification and characterization of a rice cysteine proteinase inhibitor. *Agricultural Biological Chemistry* 51: 2763 - 68.
- Abe, M. and Whitaker, J. R. 1988. Purification and characterization of a cysteine proteinase inhibitor from endosperm of corn. *Agricultural Biological Chemistry* 52: 1583 -84.
- Abe, M., Abe, K., Kuroda, M., and Arai, S. 1992. Corn kernel cysteine proteinase inhibitor as a novel cystatins super family member of plant origin. Molecular cloning and expression studies. *European Journal of Biochemistry* 209: 933- 937.
- Abrahamson, M., Ritonja, A., Brown, M. A., Grubb, A., Machleidt, W. and Barrett, A. J. 1987. Identification of the probable inhibitory reactive sites of the proteinase inhibitors, human cystatins C and chicken cystatins. *Journal of Biological Chemistry* 262: 9688 – 9694.
- Aked, J. and Kyamuhangire, W. 1996. Postharvest aspects of highland bananas in Uganda. *Tropical Science* 36: 50 – 60.
- Al-mamary, M., Al-Habori, M., Al-Aghbari, A. M., and Baker, M. M. 2002. Investigation into toxicological effects of *Catha edulis* leaves: a short term study in animals. *Phytotherapy Research* 16: 127 – 132.
- Aniagu, O. S., Nwinyi, C. F., Akumba, D. D., Ajoku, A. G., Dzarma, S., Izebe, S. K., Ditse, M., Nwaneri, C. E. P., Wambembe, C. and Gamaniel, K. 2005. Toxicity studies in rats fed nature cure bitters. *African Journal of Biotechnology* 4 (1): 72 – 78.
- Antão, M. C. and Malcata, X. F. 2005. Plant serine proteases: biochemical, physiological and molecular features. *Plant Physiology and Biochemistry* 43: 637 – 650.
- Applebaum, S. W. 1985. Biochemistry of digestion. *Comprehensive Insect Physiology Biochemistry and Pharmacology* 4: 279 – 311.
- Atkinson, H. J. 1995. Pathogenesis and Host Specificity in plant diseases. Pages 355 -370. In Kohmoto, K., Singh, U.S. and Singh, R. P. (eds). *Histopathological, Biochemical, Genetic and Molecular Bases Diseases*. Volume II: Eukaryotes Oxford: Pergamon Press, pp. 355 – 370.
- Atkinson, H. J., Green, J., Cowgill, S., and Levesley, A. 2001. The case for genetically modified crops with poverty focus. *Trends in Biotechnology* 19 (3): 91 – 96.

Benjamin, C. F., Carlin, C. R and Barja-Fidalgo, C. 1992. Pharmacological characterization of rat paw edema induced by canatoxin, the toxic protein from *Canavalia ensiformis* (jack bean) seeds. *Toxicon* 30: 879 -885.

Bernstein, A. J., Bernstein, L., Bucchini, L, Goldman, R. L., Hamilton, G. R., Lehrer, S., Rubin, C. and Sampson, A. H. 2003. Clinical and laboratory investigation of allergy to genetically modified foods. *Environmental Health Perspectives* 111 (8): 1114 – 1121.

Betz, B., S. F., Hammond, G. B. and Fuchs, L. R. 2000. Safety and advantages of *Bacillus thuringiensis* protected plants to control insect pests. *Regulatory Toxicology and Pharmacology* 32: 156 - 173

Bidhe, M. R. and Ghosh, S. 2004. Acute and subchronic (28 day) Oral toxicity study in rats with novel surfactants. *AAPS Pharmacology Science* 6 (2): 1 – 10.

Birk, Y. and Applebaum, Y. 1960. Effect of soybean trypsin inhibitors on the development and midgut proteolytic activity of *Tribolium castaneum* larvae. *Enzymologia Acta Biocatalytica* 22 (5): 318 – 326.

Bode, W. and Huber, R. 1992. Natural protein proteinase inhibitors and their interaction with proteinases. *Journal of Biochemistry* 204: 433 – 451.

Bosque- Pérez, A. N., May, D. G., and Arntzen, J. C. 2000. Applicability of an agrobacterium-based system for the transformation of *Musa* species with diverse genomic constitution and ploidy level. In: Craenen, K. Ortiz, R. Karamura, E.B., Vuylsteke, D. R (eds) *Proceedings of the First International Conference on Banana and Plantain for Africa*. 540: 193 – 201.

Bown, D. P., Wilkinson, H. S. and Gatehouse, J. A. 1997. Differentially regulated inhibitor sensitive and insensitive protease genes from the phytophagous insect pest, *Helicovera armigera*, are members of complex multigene families. *Insect Biochemistry and Molecular Biology* 27 (7): 625 – 638.

Bradford, M, M. 1976. A rapid and sensitive method for the quantification of microgram quantities of proteins utilising the principle of protein dye binding. *Analytical Biochemistry* 72: 248 – 254.

Broadway, R. M. and Duffey, S. S. 1986. The effect of dietary protein on the growth and digestive physiology of larval *Heliothis zea* and *Spodoptera exigua*. *Journal of Insect Physiology* 32: 673 – 680.

Brouquisse, R., Evrand, A., Rolin, D., Raymond, P. and Roby, C. 2001. Regulation of protein degradation and protease expression by mannose in maize root tips. Pi seqPi sequestration by mannose may hinder the study of its signalling properties. *Plant Physiology* 125: 1485 – 1498.

Brovosky, D. 1986. Proteolytic enzymes and blood digestion in the mosquito *Culex nigripalpus*. *Archives of Insect Biochemistry and Physiology* 3: 147 – 160.

Campos, F. A. P., Xavier- Filho, J., Silva, C. P. and Ary, M. B. 1989. Resolution and partial characterization of proteinases and  $\alpha$  amylases from midguts of larvae of bruchid beetle *Callosobruchus maculatus* (F). *Comparative Biochemistry and Physiology-B* 92: 51 – 57.

Carlini, C.R. and Guinaraes, J.A. 1991. Plant and microbial toxic proteins as hemilectins; emphasis on canatoxin. *Toxicon* 29: 791 – 806.

Carozi, N and Koziel, M. 1997. Advances in insect control the role of transgenic plants. Taylor and Francis London. Pages 100 -120.

Chandler, S. 1995. The nutritional value of bananas. Pages 468 – 480. In: Bananas and Plantains. Gowen S (ed). Chapman and Hall, London.

Chen, T., Huang, D., and Lin, Y. 2004. Isolation and characterization of a serine protease from the storage roots of sweet potato (*Ipomea batatas* L.). *Plant Science* 166: 1019 – 1026.

Chen, Y. L., Chow, L.P., Tsugita, A., Lin, J.Y. 1992. The complete primary structure of Abrin a B chain. *FEBS Letters* 308: 115 – 118.

Codex Alimentarius Committee. 2003. Guidelines for the conduct of food safety assessment of foods derived from recombinant – DNA plants. *CAC/GL* 45; 1 -13.

Collins, P. J., Treverrow, N. L., Lambkin, T. M. 1991. Organophosphorus insecticide resistance and its management in the banana weevil borer, *Cosmopolites sordidus* Germar (Coleoptera: Curculionidae) in Australia. *Crop Protection* 10: 215 – 221.

Costello, P. S., Turner, M., Walters, A. E., Cunningham, C. N., Bauer, P. H., Downward, J., and Tybulewicz, V. L. 1996. Critical role for tyrosine kinase SyK in signaling through the high affinity IgE receptor of mast cells. *Oncogene* 13: 2595 – 2605.

Crouch, J. H., Crouch, H. K., Constandt, H., Van Gysel, A., Breyne, P., Van Montagu, M., Jarret, R. L., Ortiz, R. 1999. Comparison of PCR-based molecular marker analyses of *Musa* breeding populations. *Molecular Breeding* 5: 233 – 244.

Dean, T. 1997. Factors predicting food allergy. *Environmental Toxicology and Pharmacology* 4: 85 -89.

Dixit, V. P., Varma, M., Marthur, N. T., Marthur, R., Sharma, S. 1992. Hypocholesterolaemic and anti-arterosclerotic effects of solasodine in cholesterol fed rabbits. *Phytotherapy Research* 6: 270 – 273.

Dubois. T., Gold. C. S., Coyne. D., Paparu. P., Mukwaba. E., Athman. S. Kapinduand. S. and Adipala. E. 2004. Merging biotechnology with biological control: Banana *Musa* tissue

culture plants enhanced by endophytic fungi. *Uganda Journal of Agricultural Sciences* 9 (2): 445 – 451.

Dunaevsky, E. Y., Elpidina, N. E., Vinokurov, S. K., and Belozersky, A. M. 2005. Protease inhibitors in improvement of plants resistance to pathogens and insects. *Molecular Biology* 39 (4): 608 – 613.

Eguchi, M., Lwamoto, A. and Yamaguchi, K. 1982. Interaction of proteases from the midgut lumen, epithelial and peritrophic membrane of the silkworm *Bombyx mori* L. *Comparative Biochemistry and Physiology- A* 72: 359 – 363

FAOSTAT, 2008. FAOSTAT data. Available online. <http://www.faostat.fao.org>, last accessed June, 4<sup>th</sup> 2008.

FAO. 1990. Roots, Tubers, Plantain and Bananas in Human Nutrition. Food and Agriculture Organisation. Rome pp 20- 30.

FAO/WHO. 2001. Evaluation of allergenicity of genetically modified foods. Report of a joint FAO/WHO expert consultation of allergenicity of foods derived from biotechnology. Available: <http://www.fao.org/esn/gm/allergygm.pdf>. Last accessed 21 January 2008.

FDA, 2000. Toxicological principles for the safety assessment of food ingredients Red book. Pages 1-20.

Fiebig, H., Nandy, A., Suck, R., Cromwell, O., Petersen, A. and Becker, W. M. 2003. DNA sequence and recombinant production of the grass pollen allergen phl p4. Patent: WO 2004000881-A 31-DEC-2003; MERCK PATENT GmbH (DE).

Filho, J. 1992. The biological roles of serine and cysteine proteinase inhibitors in plants. *Research Brassica. Fisiologie. Vegetative* 4 (1): 1 – 6.

Follmer, C., Barcellos, S. B. G., Zingali, B. R., Machado, T. L., Alves, W. E., Barja-Fidalgo, C., Guimaraes, A. J. and Carlini, R. C. 2001. Canatoxin, a toxic protein from jack beans (*Canavalia ensiformis*), is a variant form of urease (EC 3.5.1.5): biological effects of urease independent of its ureolytic activity. *Biochemistry Journal* 360: 217 – 224.

Frazao, C., Bento, I., Costa, J., Soares, C. M., Verissimo, P., Faro, C., Pires, E., Cooper, J and Carrondo, M. A. 1999. Crystal structure of cardosin A, a glycosylated and Arg-Gly-Asp containing aspartic proteinase from the flowers of *ICyanara cardunculus* L. *Journal of Biological Chemistry* 274 (39): 27694 – 27701.

Fossom, K. 1970. Proteolytic enzymes and biological inhibitors, III. Naturally occurring inhibitors in some animal and plant materials and their effect upon enzymes of various origin. *Acta Pathology, Microbiology, Scandanavia Section B Microbiology* 78: 741 – 54.

Frison, E and Sharroek, S. 1998. The economic, social and nutritional importance of banana in the world. Pages 17 – 21. In: Picq, C, Fouré, E. and Frison, E.A (eds). Bananas and Food Security. *International symposium*, Doula, Cameroon.

Fu, T., Abbott, R. U. and Hatzos, C. 2002. Digestibility of food allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluid – A comparative study. *Journal of Agricultural and Food Chemistry* 50: 7154 – 7160.

Fu, L. R., Ream, E. J., Hammond, G. B., Naylor, W. M., Leimgruber, M. R. and Berberich, A. S. 1993. Safety assessment of the Neomycin Phosphotransferase II (NPTII) protein. *Biotechnology* 11: 1543 – 1547.

Gatehouse, A. M. R., Butler, K. J., Fenton, K. A., Gatehouse, J. A. 1985. Presence and partial characterization of a major proteolytic enzyme in the gut of larval *Callosobruchus maculatus*. *Entomologia experimentia et applicata* 39: 279 -286.

Gatehouse, A. M. R., Davidson, G. M., Newell, C. A., Merrywaether, A., Hamilton, W. D. O., Burgess, E. P. J., Gilbert, R. J. C. and Gatehouse, J. A. 1997. Transgenic potato plants with enhanced resistance to the tomato moth, *Lacanobia oleracea*: growth room trials. *Molecular Breeding* 3: 49 – 63.

Gensi, R. M., and Kyamuhangire, W. 1994. Traditional production of and characterisation of banana juice in Uganda. In Adipala, E., Bekunda, M. A., Tenwya, J.S., Ogenga – Latigo, M. W. and Mugah. J.O (eds). *African Crop Science Conference Proceedings*, 1: 200 - 214

Gerald, R.R., Kramer, K. J., Baker, J. E., Kanost, J. F., Behke, C. A. 1997. Proteinase inhibitors and resistance of transgenic plants to insects. In Carozi, N., Koziel, M., (eds), *Advances in Insect Control the role of transgenic plants*. Taylor and Francis, London, pp. 157 - 183.

Ghazaleh, F. A., Franscischetti, I. M., Gombarovitis, M. E. and Carlini, C. R. 1997. Stimulation of calcium influx and platelet activation by canatoxin: methoxy erapamil inhibition and downward regulation by cGMP. *Archives of Biochemistry and Biophysics* 339: 362 – 367.

Gold, C. S. 1998. Banana weevil: Ecology, pests status and prospects of intergrated control with emphasis on East Africa. Pages 49 – 74. In: *Proceedings of a Symposium on Biological Control in Tropical Crop Habitat*. Third International Conference on Tropical Entomology. 30 Oct – 4 Nov., 1994. Nairobi, Kenya.

Gold, C.S., Kagezi, G. H., Night, G. and Ragama, P.E. 2004. The effects of banana weevil, *Cosmopolites* (Germar), damage on Highland banana growth, yield and stand duration in Uganda. *Annals of Applied Biology* 30: 112 – 130.

Gold, C. S., Ogenga – Latigo, M, W.; Tushemerweirwe, W. K.; Kashiija, I. and Nankinga, C. 1993. Farmer perceptions of banana pest constraints in Uganda: Results from a rapid rural appraisal. In: C.S. Gold and B. Gemmil (editors), *Proceedings of a Research Coordination*

Gold, C. S., Pena, J. E., and Karamura, E. B. 2001. Biology and integrated pest management for the banana weevil, *Cosmopolites sordidus* (Germar) (Coleoptera: Curculionidae). *Integrated Pest Management Reviews* 6: 79 – 155.

Gold, C.S., Ragama, P.E., Coe, R. and Ruzambuga, N.D.T.M. 2004. Selection of assessment methods for evaluating banana weevil damage on highland cooking banana. In. C. Ebong and Bekunda- Mateete(editors), *Uganda Journal of Agricultural Science* 9 (1): 274 - 280.

Gonzalez-Quijada, S., Gonzalez Escudero, R., Arias Garcia, L., Gil Martin, A. R., Vicente Serrano, J., Corral-Fernandez, E. 2005. *Anisakiasis* gastrointestinal manifestations: description of 42 cases. *Review Clinical Epidemiology* 205: 311 – 315.

Goodman, E. R., Taylor, L. S., Yamamura, J., Kobayashi, T., Kawakami, H., Kruger, L. C and Thompson, P. G. 2007. Assessment of the potential allergenicity of a milk basic protein fraction. *Food Chemical Toxicology* 45: 1787 – 1794.

Gowen, S. and Queneherve, P., 1990. Nematode parasites of bananas, plantains and Abaca. Pages 100 -120. In: M. Luc, R. A. Sikora and J. Bridge (Eds). *Plant parasitic Nematodes in Subtropical and Tropical Agriculture*. CAB International, Wallingford. Grubb, A.. and Lofberg, H. 1982. *Proceedings of Natural. Academy of Science, U.S.A.* 79; 3024 – 3027.

Graham, J. S. and Ryan, C. A. 1981. Accumulation of metalocarboxy-peptidase inhibitor in leaves of wounded potato plants. *Biochemical and Biophysics Research Communication* 101: 1164 -1170.

Gruden, K., Strukel, J. B., RaRavnikar, M., Poljsak-Prijatelj, M., Mavric, I., Brzin, J., Pungercar, J. and Kregar, I. 1997. Potato cysteine proteinase inhibitor gene family: molecular cloning, characterization and immunocytochemical localisation studies. *Plant Molecular Biology* 34: 317 -323.

Guo, J. Z., Lamb, C. and Dixon, A. R. 1998. A serine protease from suspension cultured soybean cells. *Pytochemistry* 47: 547 – 553.

Hames, B. D. 1981. An introduction to polyacrylamide gel electrophoresis. Pages 1- 86. In *Gel Electrophoresis of proteins; A Practical Approach*. (Hames, B.D and Rick wood, D.C eds). Oxford; IRL Press.

Hao, L., Hsiang, T., and Goodwin, H. P. 2006. Role of two cysteine proteinases in the susceptible response of *Nicotiana benthamiana* to *Colletotrichum destructivum* and the hypersensitive response to *Pseudomonas syringae* pv. tomato. *Plant Science* 170: 1001 – 1009.

Havkioja, E. and Neuvonen, L. 1985. Induced long term resistance to birch foliage against defoliators: defense or incidental. *Ecology* 66: 1303 -1308.

- Herman, A. R., Korjagin, A. V., Schafer, W. B. 2005. Qualitative measurement of protein digestion in simulated gastric fluid. *Regulatory Toxicology and Pharmacology* 41: 175 – 184.
- Hilder, V. A., Gatehouse, A. M. R., Sheerman, S. E., Baker, R. F., and Boulter, D. 1987. A novel mechanism of insect engineered into tobacco. *Nature* 330: 160 – 163.
- Hirado, M., Tsunasawa, S., Sakiyama, F., Niinobe, M., and Fuji, S. 1985. *FEBS Letters* 186: 41 – 45.
- Huby, J. D. R., Dearman, J. R. and Kimber, I. 2000. Why are some proteins allergens? *Toxicological Sciences* 55: 235 – 246.
- Hollander-Cztko, H., Andersen, J. L. and Ryan, C. A. 1985. Vacuolar localization of wound induced carboxy peptidase inhibitor in potato leaves. *Plant Physiology* 78: 76 – 79.
- Houseman, J. G., Campbell, F. C and Morrison, P. E. 1987. A preliminary characterization of digestive proteases in the posterior midgut of the fly *Stomoxys calcitrans* (L) (Diptera: Muscidae). *Insect Biochemistry* 17: 213 – 218.
- Houseman, J. G. and Downe, A. E. R. 1983. Cathepsin D like activity in the posterior midgut of Hemiptera insects. *Comparative Biochemistry and Physiology-B* 28: 8951 -8966.
- Houseman, J. G., Down, A. E. R. and Philogene, B. J. R. 1989. Partial characterization of proteinase activity in larval midgut of the European corn borer *Ostrinia nubilalis* Hubner (Lepidoptera: Pyralidae). *Canadian Journal of Zoology* 78: 76 – 79.
- INIBAP. 2001. News Releases; Global Consortium announces plans to sequence banana genome. Available online. <http://www.inibap.org>, last accessed April, 4<sup>th</sup> 2006.
- Isemura, S., Saitoh, E., Sanada, K., Isemura, M., and Ito, S. 1984. *Journal of Biochemistry* 96: 41 -45.
- Johnson, R., Narvaez, J., An, G., and Ryan, C. 1989. Expression of proteinase inhibitor I and II in transgenic tobacco plants: effects on natural defence against *Manduca sexta* larvae. *Proceedings of the National Academy of Sciences* 86: 9871 – 9875.
- Jonas, D. A., Antignac, E., Antoine, J. M., Classen, H. G., Huggett, A. Knudsen, I., Mahler, J., Ockhuizen, T., Smith, M., Teuber, M., Walker, R., and Vogel, D. P. 1996. The safety assessment of novel foods. *Food and Chemical Toxicology* 34: 931 - 940.
- Jones, D. R. 2000. Introduction to banana, abaca and enset. In: Jones D.R. (ed) Diseases of banana, Abaca and Enset. CABI publications, Oxon, UK. Pages 1 – 36.
- Joshi, S. C., Priya, S. E. and Venkataraman, S. 2007. Acute and subacute toxicity studies on the polyherbal antidiabetic formulation Diakyur in experimental animal models. *Journal of Health Sciences* 53 (2): 245 – 249.

Kamande, M. G., Baah, J., Cheng, J. K., McAllister, A. T. and Shelford. 1999. Effects of Tween 60 and Tween 80 on Protease activity, thiol group reactivity, protein adsorption, and cellulose degradation by rumen microbial enzymes. *Journal of Dairy Science* 83: 536 – 542.

Kaneko, J. J. 1989. Clinical Biochemistry of Domestic Animals. Academic press, San Diego. Pages 250.

Karugaba, A. and Kimaru, G. 1999. Banana Production in Uganda. An essential food and cash crop. Regional Land Management Unit, Relma/Sida, ICRAF house, Gigiri Nairobi, Kenya. Pages 200.

Kashaija, I. N., Speijer, P. R., Gold, C. S. and Gowen, S. R. 1994. Occurrence, distribution and abundance of plant parasitic Nematodes of bananas in Uganda. Preliminary results of a diagnostic survey. In Adipala, E., Bekunda, M. A., Tenywa, J. S., Ogenga, Latigo, M. W., and Mugah, J. O. (eds). *Proceeding of the African Crop Science Society*. 2. Pages 309 – 321.

Keilova, H. and Tomasek, V. 1976. Isolation and properties of cathepsin D inhibitor from potatoes. *Collection of Czechoslovak Chemical Communication* 41: 489 – 497.

Kervinen, J., Tobin, G. J., Costa, J., Waugh, D. S., Wlodawer, A and Zdanov, A. 1999. Crystal structure of plant aspartic proteinase prophytepsin: inactivation and vacuolar targeting. *Journal of European Molecular Biology Organization* 18 (14) 3947 – 3955.

Kessler, D. A., Taylor, M. R., Maryanski, J. H., Flamm, E. L. and Kahl. L. S. 1992. The safety of foods developed by biotechnology. *Science* 256: 1747 -1749.

Kimenju, J. W., Karanja, N. K., Mutua, G. K., Rimberia, B. M. and Nyongesa, M. W. 2005. Impact of land use changes on nematode diversity and abundance. In Bekunda, M., Tenywa, J. S (eds) *Proceedings of the African Crop Science Society* 7: 433 -436

Kiggundu, A. 2008. Engineering plant cysteine protease inhibitors for transgenic control of banana weevil, *Cosmopolites sordidus* (Germar) (Coleoptera; Curculionidae) and other coleopteran insects in transgenic plants. Phd thesis University of Pretoria. Pages 151.

Kiggundu, A., Kunert, K., Vijoien, A., and Muchwezi, J. M. 2003. Strategies for enhancing resistance to weevils. *African Journal of Biotechnology* 2 (12): 563 – 569.

Kitch, L. W. and Murdock, L. L. 1986. Partial characterization of a major gut thiol proteinase from larvae of *Callosobruchus maculatus* (F). *Archives of insect Biochemistry and Physiology* 3: 561 – 575.

Klein, B. I and Kirsch, F. J. 1969. The activation of papain the inhibition of the active enzyme by carbonyl reagents. *The Journal of Biological Chemistry* 244: 5928 – 5935.

Koiwa, H., Bressan, R. A. and Hasegawa, P. M. 1997. Regulation of protease inhibitors and plant defense. *Trends Plant Science* 2: 379 – 384.

Kuiper, A. H. and Kleter, A. G. 2003. The scientific basis for risk assessment and regulation of genetically modified foods. *Trends in Food Science and Technology* 14: 277 – 293.

Ladies, S. G., Holsapple, P. M., Astwood, D. J., Kimber, I., Knippels, J. M. L., Helm, M. R., and Dong, W. 2003. Forum Workshop overview: Approaches to the assessment of the allergenic potential of food from Genetically Modified Crops. *Toxicological sciences* 73: 8 – 16.

Laemmli, U.K., 1970. Cleavage of structural proteins during assembly of the head bacteriophageT4. *Nature* 227 (259): 680 – 685.

Lawrence, P. K., and Koundal, K. R. 2001. Plant protease inhibitors in control of phytophagus insects. *Journal of Biotechnology* 5 (1): 1 – 17.

Lawrence, C. J. and Nielsen, S. 2001. Partial isolation and characterisation of Cysteine proteinase inhibitor from Lima bean (*Phaseolus lunatus*). *Journal of Agriculture Food Chemistry* 49: 1020 – 1025.

Lee, S. I., Lee, S. H., Koo, J. C., Chun, H. J., Lim, C. O., Mun, J. H., Song, Y. H., and Cho, M. J. 1999. Soybean Kunitz trypsin inhibitor (SKTI) confers resistance to the brown planthopper (*Nilaparvata lugens* Stal) in transgenic rice. *Molecular Breeding* 5: 1 – 9.

Lemos, F. J. A., Xavier-Filho, J. and Campos, F. A. P. 1987. Proteinases of the midgut of *Savrotes subfasciatus* larvae. *Agricultural Biology Technology* 80: 46.

Lilley, C. J., Urwin, P. E. and Atkinson, H. J. 1999. Characterization of plant nematode genes: identifying targets for transgenic defence. *Parasitology* 118: S63 – S72.

Lueng, C. J., Kitch, L. W. and Murdock, L. L. 2000. Proteases Inhibitors: Current status and future prospects. *Journal of Medical Chemistry* 43 (3): 305 341.

Lord, J. M., Roberts, L. M., Roberts, J. D. 1994. *FASEB Journal* 8 (2): 201 – 208.

MacKenzie, A. S., Lamb, I., Schmidt, J., Deege, L., Morriey, J. M., Harper, M., Layton, J. R., Prochaska, M. L., Sanders, C., Locke, M., Mattsson, L. J., Fuentes, A. and Delany, B. 2007. Thirteen week feeding study with transgenic maize grain containing event DAS-Ø15Ø7-1 in Sprague – Dawley rats. *Food and Chemical Toxicology* 45: 551 – 562.

Masanza, M., Gold, C. S., Van Huis, A., and Ragama, P.E. 2004. Use of crop sanitation for the management of the banana weevil in Uganda. *Uganda Journal of Agricultural Sciences* 9: 636 – 644.

Mayne, P. D. 1996. Clinical chemistry in diagnosis and treatment. 6<sup>th</sup> ed (International Students Edition). Arnold London/Oxford University Press Inc. New York. Pages 150.

Metcalf, D. D., Astwood, J. D., Townsend, R., Sampson, H. A., Taylor, S. L. and Fuchs, R. L. 1996. Assessment of the allergenic potential of foods derived from genetically engineered crop plants. *Critical Review Food Science Nutrition* 36: S165 - S186.

Michaud, D., Nguyen-Quoc, B., Vrain, T. C., Fong, D. and Yelle, S. 1996. Response of digestive cysteine proteinases from the Colorado potato beetle (*Leptinotarsa decemlineata*) and the black vine weevil (*Otiorhynchus sulcatus*) to a recombinant form of human stefin A. *Archives of Insect Biochemistry and Physiology* 31: 451 - 464.

Morton, J. 1987. Banana. Pages. 29 - 46. In: Fruits of warm climates. J. F. Morton, Miami, FL.

Murdock, L. L., Brookhart, G., Dunn, P. E., Foard, D. E. and Kelley, S. 1987. Cysteine digestive proteinases in Coleoptera. *Comparative Biochemistry and Physiology-B* 87: 783 - 787.

Murdock, L. L., Shade, R. E. and Pomeroy, M. A. 1988. Effects of E 64 a cysteine proteinase inhibitor on cowpea weevil growth, development and fecundity. *Environmental Entomology* 17: 467 - 469.

Namaganda, J. M., Gowen, S.R., Namanya, P., Kashaia, I.N., and Bagamba, F. 2003. Management of nematodes in banana systems in Uganda. *Uganda Journal of Agricultural Sciences* 8: 101 - 106.

Namaganda, J. M., Karamura, E., Namanya, P., Gowen, S. R., and Bridge, J. 2000. Studies on host range of the banana lesion nematode, *Pratylenchus goodeyi*. *Proceeding on International Symposium On Banana and Plantain for Africa*. Creaenen *et al.* (eds). *Acta Horticulturae* 540: 419 - 425

Nowakunda, K. and Muranga, I. F., 1996. Alternative utilisation of bananas and plantains in Uganda. *MUSAFRICA* 10: 10 -15.

Oliviera, S. A., Filho, J.X., and Sales, P. M. 2003. Cysteine proteinases and cystatins. *Brazilian Archives of Biology and Technology*. 46: 96 - 104.

O'Rourke, L., Tooze, R., and Fearon, D.T. 1997. Coreceptors of B lymphocytes. *Current Opinions in Immunology* 9: 324 - 329.

Ortiz, R., Ferris, R. S. B and Vuylsteke, D.R. 1995. Banana and Plantain breeding. In (ed) S. Gowen. *Banana and Plantains*. Chapman and Hall London. pp 110 - 145.

Otto, H and Schirmeister, T. 1997. Cysteine protease and their inhibitors. *Chemical Reviews* 97: 133-171.

Park, H., Yamanaka, N., Mikkonen, A., Kusakabe, I. and Kobayashi, H. 2000. Purification and characterization of aspartic proteinase from sunflower seeds. *Bioscience Biotechnology and Biochemistry* 64: 931 - 939.

- Pastorello, A. E., Conti, A., Pravettoni, V., Farioli, L., Rivolta, F., Ansaloni, R., Ispano, M., Incorvaia, C., Guiffrida, G. M. and Ortolani, C. 1998. Identification of actinidin as the major allergen of kiwi fruit. *Journal of Allergy Clinical Immunology* 101 (4): 531 – 537.
- Patočka, J. 1998. Toxicological characteristics of ricin. *Voj.Zdrav. Listy*. 67: 166 – 168.
- Pernas, M., Sanchez-Mong R., Salcedo G. 2000. Biotic and abiotic stress can induce cystatin expression in chestnut. *FEBS Letters* 467: 206 -210.
- Picq. C. 2000. Bananas. INBAP/IPGRI Publication, International Network for the Improvement of Banana and Plantain. Page 14.
- Ploetz, R.C. 2001. Black Sigatoka of Banana. *The Plant Health Instructor* 10: 1094 – 1096.
- Ploetz, R.C. and Pegg, K. G. 2000. Fungal diseases of the root, corm and pseudostem. Pages 143 – 172. In: Jones D. R. (ed) Diseases of banana and plantain, Abaca and Ensete. CABI Publications, Oxon, United Kingdom.
- Qiagen, 2003. The QIA expressionist. A handbook for high level expression and purification of 6X His tagged protein. USA. Pages 15 – 20.
- Ramachandra, L., Song, R., and Harding, C. V. 1999. Phagosomes are fully competent antigen processing organelles that mediate the formation of peptide: class II MHC complexes. *Journal of Immunology* 162: 3263 – 3272.
- Rancour, J. M. and Ryan, C. A. 1968. Isolation of a carboxypeptidase B inhibitor from potatoes. *Archives of Biochemistry and Biophysics* 125: 380 -382.
- Rassam, M. and Liang, W. 2004. Purification and characterization of phytocystatins from kiwifruit cortex and seeds. *Phytochemistry* 65 (1): 19 -30.
- Rawlings, N. D. and Barrett, A. J. 1994. Families of cysteine peptidases. *Methods Enzymology* 244: 461 – 486.
- Richardson, M. J. 1991. Seed storage proteins: The enzyme inhibitors. In: Richardson, M. J. (ed) *Methods in Plant Biochemistry*, New York, Academic Press. Pages 259 – 305.
- Robinson, J.C. 1996. Banana and Plantains. CABI Publishing, Wallingford, UK. Pages 15 –30.
- Rodis, P., Hoff, J.E., 1984. Naturally occurring protein crystals in the potato. *Plant Physiology* 74: 907 - 911.
- Rodriguez-Mahillo, I. A., Gonzalez-Munoz, M., Gomez-Aguado, F., Rodriguez-Perez, R., Corcuera, T. M., Caballero, L. M. and Monco, Ignacio. 2007. Cloning and characterisation of the *Anisakis simplex* allergen Ani s 4 as a cysteine protease inhibitor. *International Journal For Parasitology* 37: 907 – 917.

- Romagnani, S., Maggi, E., Parronchi, P., Macchia, D., and Piccinni, M. P. 1991. Regulation of IgE synthesis by CD4<sup>+</sup> human T cell subsets. *Research Immunology* 142: 63 – 67.
- Rowe, P.R., 1984. Breeding bananas and plantains. *Plant Breeding Review* 2: 135-155.
- Rowe, P. R., and Rosales, F. E. 2000. Conventional banana breeding in Honduras. Pages 435 - 449. In: Jones, D. R. (ed) Diseases of Banana, Abaca and Ensete. CABI Publications, Oxon, UK.
- Rubaihayo P.R. 1991. Banana Based cropping Systems Research. A report on rapid rural appraisal survey of banana production. *Research Bulletin* 2, Makerere University, Kampala, Uganda. Pages 126- 130.
- Rukazambuga, N.D. T. M. 1996. The effect of banana weevil (*Cosmopolites sordidus* Germar) on the growth and productivity of bananas (Musa AAA) and the influence of host vigour on weevil attack. Ph.D Thesis. University of Reading.
- Ruzambunga, N.D.T.M., Gold C.S. and Gowen, S. R. 1998. Yield loss in East African Highland banana (*Musa* spp., AAA-EA group) caused by the banana weevil, *Cosmopolites sordidus* Germar. *Crop Protection* 17: 581 – 589.
- Ryan, C.A. 1990. Protease inhibitors in plants: Genes for improving defence against insects and pathogens. *Annual Reviews of Phytopathology* 28: 425 – 429.
- Saito, F., Isemura, S., Sanadah, K. and Ohnishi, K. 1991. Cystatins of family II are harbouring two domains which retain inhibitory activities against proteinase. *Biological Ciophysics Reseacrh Communication* 175: 1070 – 1075.
- Sagi, L. 2000. Genetic engineering of banana for disease resistance. Future possibilities. Pages 465 – 515. In: Jones D. R. (ed) Diseases of banana and plantain, Abaca and Ensete. CABI Publications, Oxon, United Kingdom.
- Salvesen, G., Parkes, C., Abrahamson, M., Grubb, A. and Barrett, A. J. 1986. Human low-Mr kininogen contain three copies of a cystatins sequence that are divergent in structure and inhibitory activity for cysteine proteases. *Biochemistry Journal* 234: 429 – 434.
- Sandvig, K. and Olsnes, S. 1982. Entry of the toxic proteins abrin, modeccin, ricin and diphtheria toxins into cells. *The Journal Of Biological Chemistry* 257 (13): 7504 – 7513.
- Sato, N., Ishidoh, K., Uchiyama, Y and Kominami, E. 1990. Molecular cloning and sequencing of cDNA for rat cystatins  $\beta$ . *Nucleic Acids Research* 18 (22): 6698.
- Sen, M., Kopper, R., Pons, L., Abraham, C. E., Burks, W. A. and Bannon, A. G. 2002. Protein structure plays a critical role in peanut allergen stability and may determine immunodominant IgE binding epitopes. *The Journal of Immunology* 169: 882 – 887.

Sengooba, T. 1986. Survey of banana pests problems complex in Rakai and Masaka districts of in Uganda. August 1986: Preliminary trip report. Namulonge Research Station, Namulonge Uganda.

Sharma, C. H., Sharma, K. K., Seetharama, N. and Ortiz, R. 2001. Prospects for using transgenic resistance to insects in crop improvement. *Journal of Biotechnology* 3 (2): 1-20.

Shulke, R. H. and Murdock, L. L. 1983. Lipxygenase trypsin inhibitor and lectin from soybeans: effects on larval growth of *Manduca sexta* (Lepidoptera: Sphingidae). *Environmenta Entomology* 12: 789 – 791.

Solomon, M., Belenghi, B., Delledonne, M., Menachem, E. and Levine, A. 1999. The involvement of cysteine proteases and protease inhibitor genes in the regulation of programmed cell death in plants. *Plant cell* 11: 431 – 443.

Song, I., Taylor, M., Baker, K., and Baterman, R. C. 1995. Inhibition of cysteine proteinases by *Carica papaya* cystatin. *Genes* 62 (2): 222 – 224.

Speijer, P.R., Gold, C. S., Karamura, E. B., Kashaija, I. N. 1994. Banana weevil and nematodes distribution patterns in highland banana systems in Uganda: Preliminary results of a diagnostic survey. In: Adipala, E., Bekunda, M. A., Tenywa, J. S., Ogenga, Latigo, M. W., and Mugah, J. O. (eds). *Proceedings of the African Crop Science Society* 2: 285 – 289.

Speijer, P. R., Kajumba, C. and Ssango, F. 1999. East African highland banana production as influenced by nematodes and crop management in Uganda. *International Journal of Pest Management* 45: 41 – 49.

Steppek, G., Behnke, M. J., Buttle, J. D. and Duce, R. I. 2004. Natural plant cysteine proteinases as anthelmintics? *Trends in Parasitology* 20 (7): 321 -327.

Stephen, G. A., Powls, R. and Beynon, J. R. 1993. Activation of oxidized cysteine proteinases by thioredoxin mediated reduction *in vitro*. *Biochemistry Journal* 291: 345 – 347.

Stover, R. H. and Buddenhagen, I. W. 1986. Banana breeding, polyploidy, disease resistance and productivity. *Fruits* 41: 175 – 191.

Stover, R. H and Simmonds. N. W. 1987. Bananas: Tropical Agricultural Series. Third edition. Longmans, UK. Pages 10 – 48.

Takagi, K., Teshima, R., Okunuki, H. and Sawada, J. 2003. Comparative study of *in vitro* digestibility of food proteins and effects of preheating on the digestion. *Biological Pharmacology Bulletin* 26 (7) 969 – 973.

Tashiro, M., Maki, Z. 1986. Isolation of protein inhibitors of papain, trypsin, and alpha amylase in the grain of foxtail millet. *Agricultural Biological Chemistry* 50: 2955 -2957.

Taylor, P. J., Hardy, J. and Fischbeck, H. K. 2002. Toxic proteins in neurodegenerative disease. *Biomedicine* 296: 1991 – 1995.

Tezenas du Montcel, H. 1987. Plantain bananas. Macmillan Publishers, London and CTA, France. Pages 15 - 20.

Thomas, K., Aalbers, M., Bannon, A. G., Bartels, M., Dearman, J. R., Esdaile, J. D., Fu, J. T., Glatt, M. C., Hadfield, N., Hatzos, C., Helfe, L. S., Heylings, R. J., Goodman, E. R., Henry, B., Herouet, C., Holsapple, M., Ladic, S. G., Landry, D. T., MacIntosh, C. S., Rice, A. E., Privalle, S. L., Steiner, Y. H., Teshima, R., van Ree, R., Woolhiser, M., and Zawodny, J. 2004. A multi-laboratory evaluation of a common in vitro pepsin digestion assay protocol used in assessing the safety of novel proteins. *Regulatory Toxicology and Pharmacology* 39: 87 – 98.

Tinzaara, W., Gold, C. S., Dicke, M., Van Huis, A., Kagezi, H. G., Nakinga, K. C. and Tushemereirwe, W. 2005. Infochemicals and management of the banana weevil *Cosmopolites sordidus*: An overview. In Tenywa, J.S., Adipala, E., Nampala, P., Tusiime, G., Okori, P. and Kyamuhangire, W. (Eds). *Proceedings of African Crop Science Society* 7: 403 – 412.

Tonero, P., Conejero, V. and Vera, P. 1996. Primary structure and expression of a pathogen induced protease (PR-P69) in tomato plants: similarity of functional domains to subtilisin like endoprotease. *Proceedings of the National Academy Of Science* 93: 6332 – 6337.

Tumuhaise, V., Nankinga, C.M., Gold, C.S, Kyamanywa, S., Ragama, P., Tushemereirwe, W. 2004. Kairomone trapping system for delivering of *Beauveria bassiana* to control the banana weevil. *Uganda Journal of Agricultural Sciences* 9 (2): 630 – 635.

Turk, V., Brzin, J., Longer, M., Ritonja, A., Eropkin, M., Borchart, U., and Machleidt, W. 1983. *Hoppe Seyler's Zoology, Physiological Chemistry*, 364: 1487 – 1496.

Tushemereirwe, W. 1996. Factors influencing expression of leaf spot diseases of highland bananas in Uganda. Ph.D Thesis, Reading University. Pages 40 – 50.

Tushemereirwe, W.K., Karamura, D., Ssali, H., Bwamiki, D., Kashaija, C., Nakinga, C., Bagamba, F., Kangire, A. and Ssebuliba, R. 2001. Banana (*Musa* spp).Pages 340 – 450. In: Mukiibi, J.K (ed.) *Agriculture in Uganda Vol. II Crops*. National Agricultural Research Organisation.

Tushemereirwe, W.K., and Waller, J. M. 1993. Black leaf streak (*Mycosphaerella fijiensis* and associated fungal diseases of banana in Uganda. *Journal of Plant Pathology* 42: 471 – 472.

Ugochukwu, C. N. C., Ebong, E. P. and Eyong, U. E. 2008. Biochemical implication of long term administration of Halofantrine Hydrochloride (Halfan) on Estradiol levels of female wistar rats. *Pakistan Journal of Nutrition* 7 (2): 227 – 230.

Urwin, E. P., Atkinson, H. J., Waller, D. A., and McPherson, M. J. 1995. Engineered *oryzacystatin-I* expressed in transgenic hairy roots confers resistance to *Globodera pallida*. *The Plant Journal* 8 (1): 121 – 131.

- Urwin, E. P., Lilley, C. J., McPherson, M. J., and Atkinson, H. J. 1997. Resistance to both cyst and root-knot nematodes conferred by transgenic *Arabidopsis* expressing a modified plant cystatin. *The Plant Journal* 12 (2): 455 – 461.
- Urwin, E. P., Troth, M.K., Zubko, I.E., and Atkinson, H. J. 2001. Effective transgenic resistance to *Globodera pallida* in potato field trials. *Molecular Breeding* 8: 95 – 101.
- Vain, P., Worland, B., Clarke, M. C., Richard, G., Beavis, M., Lui, H., Kohli, A., Leech, M., Snape, J., Christou, P. and Atkinson, J. H. 1998. Expression of an engineered cysteine proteinase inhibitor (*Oryzacystatin* -IΔD86) for nematode resistance in transgenic rice plants. *Theoretical and Applied Genetics* 96: 266 – 271.
- Vijh, S., Pilip, I. M., and Pamer, E. G. (1998). Effect of antigen processing efficiency on *in vivo* T cell response magnitude. *Journal of Immunology* 160: 3971 – 3977.
- Viljoen, A., Kunert, K., Kiggundu, A., and Escalant, J. V. 2003. Biotechnology for sustainable banana and plantain production in Africa: the South African contribution. *South African Journal of Botany* 70 (1): 67 – 74.
- Vuylsteke, D. 2000. Breeding bananas and plantains: from intractability to feasibility. In Craenen, K. Ortiz, R. Karamura, E.B., Vuylsteke, D. R (eds) Proceedings of the First International Conference on Banana and Plantain for Africa. *Acta Horticulturae* 540: 149 – 156.
- Waldron, C., Lynett, M., Wegrich, P., Merlo, A. O. and Walsh, T. A. 1993. Characterization of a genomic sequence coding for potato multicystatin, an eight domain cysteine proteinase inhibitor. *Plant Molecular Biology* 23: 801 – 812.
- Wegrzyn, N. A. 2003. Future approaches to food allergy. *Pediatrics* 111 (6): 1672 – 1680.
- Wieman, K. F. and Nielsen, S. S. 1988. Isolation and partial characterization of a major gut proteinase from larval *Acanthoscelides obtectus* Say (Coleoptera: bruchidae). *Comparative Biochemistry and Physiology-B* 89: 419 -426.
- Wolfson, J. L. and Murdock, L. L. 1990. Diversity in digestive proteinase activity among insects. *Journal of Chemical Ecology* 16: 1089 – 1102.
- Xu, D. P., Xue, Q. Z., McElroy, D., Mawal, Y., Hilder, V. A., and Wu, R. 1996. Constitutive expression of a cowpea trypsin inhibitor gene, Cpti, in transgenic rice plants confers resistance to two major rice insect pests. *Molecular Breeding* 2: 167 – 173.
- Yagamati, H., Masuzawa, T., Nagaoka, Y., Ohnishi, T., and Iwasaki, T. 1994. Cucumisin, a serine protease from melon fruits, shares structural homology with subtilisin and is generated from a large precursor. *The Journal of Biological Chemistry* 269 (52): 32726 – 32731.

Yagami, T., Haishima, Y., Nakamura, A., Osuna, H. and Ikezawa, Z. 2000. Digestibility of allergens extracted from natural rubber latex and vegetable foods. *Journal of Allergy Clinical Immunology* 106 (4): 752 – 762.

Yamada, T., Kondo, A., Ohta, H., Masuda, T., Shimada, H., and Takamiya, K. 2001. Isolation of the protease component of maize cysteine protease- cystatins complex: Release of cystatins is not crucial for the activation of the cysteine protease. *Plant Cell Physiology* 42 (7): 710 – 716.

Yamamoto, Y., Takimoto, K., Izumi, S., Toriyama- Sakurai, M., Kageyama, T. and Takahashi, S. Y. 1994. Molecular cloning and sequencing of cDNA that encodes cysteine proteinase in the eggs of silkworm, *Bombyx mori*. *Journal of Biochemistry* 116: 1330 – 1335.

## APPENDIX 1

GenStat Release 7.1 (PC/Windows XP)

01 May 2008 00:55:37

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GenStat Seventh Edition

GenStat Procedure Library Release PL15

### Analysis of variance

Variate: FEED INTAKE

| Source of variation | d.f. | s.s.   | m.s.  | v.r. | F pr. |
|---------------------|------|--------|-------|------|-------|
| Dosage              | 3    | 190.03 | 63.34 | 2.28 | 0.118 |
| Residual            | 16   | 444.00 | 27.75 |      |       |
| Total               | 19   | 634.03 |       |      |       |

### Tables of means

Variate: FEED INTAKE

Grand mean 60.2

| Dosage | 0mg  | 1000mgMcy | 100mg Mcy | 10mg Mcy |
|--------|------|-----------|-----------|----------|
|        | 55.1 | 63.4      | 60.7      | 61.4     |

### Least significant differences of means (1% level)

| Table  | Dosage |
|--------|--------|
| rep.   | 5      |
| d.f.   | 16     |
| l.s.d. | 7.06   |

### Analysis of variance

Variate: FEED INTAKE

| Source of variation | d.f. | s.s.    | m.s.  | v.r. | F pr. |
|---------------------|------|---------|-------|------|-------|
| Dosage              | 4    | 188.46  | 47.11 | 1.01 | 0.426 |
| Residual            | 20   | 933.13  | 46.66 |      |       |
| Total               | 24   | 1121.59 |       |      |       |

### Tables of means

Variate: FEED\_INTAKE

Grand mean 60.1

| Dosage | 0mg  | 1000mgPcy | 100mgPcy | 10mgPcy |
|--------|------|-----------|----------|---------|
|        | 55.1 | 60.2      | 60.3     | 63.4    |

### Least significant differences of means (1% level)

| Table | Dosage |
|-------|--------|
| rep.  | 5      |

d.f. 20  
 l.s.d. 12.29  
 Analysis of variance

Variate: pH

| Source of variation | d.f. | s.s.    | m.s.   | v.r. | F pr. |
|---------------------|------|---------|--------|------|-------|
| Protn_dose          | 4    | 0.7750  | 0.1937 | 0.62 | 0.652 |
| Residual            | 35   | 10.9688 | 0.3134 |      |       |
| Total               | 39   | 11.7437 |        |      |       |

Tables of means

Variate: pH

Grand mean 6.388

| Protn_dose | 0mg   | 1000mg | 100mgMcy | 10mgMcy |
|------------|-------|--------|----------|---------|
|            | 6.375 | 6.438  | 6.250    | 6.625   |

Least significant differences of means (1% level)

| Table  | Protn_dose |
|--------|------------|
| rep.   | 8          |
| d.f.   | 35         |
| l.s.d. | 0.737      |

Analysis of variance

Variate: SG

| Source of variation | d.f. | s.s.       | m.s.       | v.r. | F pr. |
|---------------------|------|------------|------------|------|-------|
| Protn_dose          | 4    | 0.00001615 | 0.00000404 | 0.09 | 0.986 |
| Residual            | 35   | 0.00161975 | 0.00004628 |      |       |
| Total               | 39   | 0.00163590 |            |      |       |

Tables of means

Variate: SG

Grand mean 1.0246

| Protn_dose | 0mg    | 1000mg | 100mgMcy | 10mgMcy |
|------------|--------|--------|----------|---------|
|            | 1.0250 | 1.0250 | 1.0250   | 1.0244  |

Least significant differences of means (1% level)

| Table  | Protn_dose |
|--------|------------|
| rep.   | 8          |
| d.f.   | 35         |
| l.s.d. | 0.00937    |

Analysis of variance

Variate: pH

| Source of variation | d.f.(m.v.) | s.s.   | m.s.   | v.r. | F pr. |
|---------------------|------------|--------|--------|------|-------|
| Protn_dose          | 4          | 1.5020 | 0.3755 | 1.66 | 0.183 |
| Residual            | 34(1)      | 7.7009 | 0.2265 |      |       |
| Total               | 38(1)      | 9.1923 |        |      |       |

# Tables of means

Variate: pH

Grand mean 6.541

| Protn_dose | 0mg   | 1000mg Pcy | 100mg pcy | 10mg pcy |
|------------|-------|------------|-----------|----------|
|            | 6.375 | 6.750      | 6.643     | 6.688    |

Least significant differences of means (1% level)

| Table  | Protn_dose |
|--------|------------|
| rep.   | 8          |
| d.f.   | 34         |
| l.s.d. | 0.578      |

Analysis of variance

Variate: SG

| Source of variation | d.f.(m.v.) | s.s.       | m.s.       | v.r. | F     | pr. |
|---------------------|------------|------------|------------|------|-------|-----|
| Protn_dose          | 4          | 0.00025340 | 0.00006335 | 1.34 | 0.275 |     |
| Residual            | 33(2)      | 0.00155559 | 0.00004714 |      |       |     |
| Total               | 37(2)      | 0.00178600 |            |      |       |     |

# Tables of means

Variate: SG

Grand mean 1.0229

| Protn_dose | 0mg    | 1000mg Pcy | 100mg pcy | 10mg pcy |
|------------|--------|------------|-----------|----------|
|            | 1.0250 | 1.0250     | 1.0229    | 1.0181   |

Least significant differences of means (1% level)

| Table  | Protn_dose |
|--------|------------|
| rep.   | 8          |
| d.f.   | 33         |
| l.s.d. | 0.00927    |

Analysis of variance

Variate: HCT

| Source of variation | d.f.(m.v.) | s.s.   | m.s.  | v.r. | F     | pr. |
|---------------------|------------|--------|-------|------|-------|-----|
| Prot_dose           | 3          | 17.500 | 5.833 | 2.02 | 0.138 |     |
| Residual            | 25(3)      | 72.371 | 2.895 |      |       |     |
| Total               | 28(3)      | 87.898 |       |      |       |     |

# Tables of means

Variate: HCT

Grand mean 43.18

| Prot_dose | 0mg   | 1000mg mcy | 100mg mcy | 10mg mcy |
|-----------|-------|------------|-----------|----------|
|           | 42.25 | 44.14      | 43.61     | 42.71    |

Least significant differences of means (1% level)

| Table | Prot_dose |
|-------|-----------|
|-------|-----------|

|        |       |
|--------|-------|
| rep.   | 8     |
| d.f.   | 25    |
| l.s.d. | 2.371 |

#### Analysis of variance

Variate: HGB

| Source of variation | d.f.(m.v.) | s.s.   | m.s.   | v.r. | F pr. |
|---------------------|------------|--------|--------|------|-------|
| Prot_dose           | 3          | 0.1999 | 0.0666 | 0.37 | 0.776 |
| Residual            | 26(2)      | 4.6883 | 0.1803 |      |       |
| Total               | 29(2)      | 4.8670 |        |      |       |

#### Tables of means

Variate: HGB

Grand mean 12.883

| Prot_dose | 0mg    | 1000mg mcy | 100mg mcy | 10mg mcy |
|-----------|--------|------------|-----------|----------|
|           | 12.783 | 12.850     | 13.000    | 12.900   |

#### Least significant differences of means (1% level)

| Table  | Prot_dose |
|--------|-----------|
| rep.   | 8         |
| d.f.   | 26        |
| l.s.d. | 0.554     |

#### Analysis of variance

Variate: LYM

| Source of variation | d.f.(m.v.) | s.s.   | m.s.  | v.r. | F pr. |
|---------------------|------------|--------|-------|------|-------|
| Prot_dose           | 3          | 40.98  | 13.66 | 0.77 | 0.522 |
| Residual            | 26(2)      | 461.70 | 17.76 |      |       |
| Total               | 29(2)      | 496.12 |       |      |       |

#### Tables of means

Variate: LYM

Grand mean 86.11

| Prot_dose | 0mg   | 1000mg mcy | 100mg mcy | 10mg mcy |
|-----------|-------|------------|-----------|----------|
|           | 84.35 | 86.29      | 86.29     | 87.51    |

#### Least significant differences of means (1% level)

| Table  | Prot_dose |
|--------|-----------|
| rep.   | 8         |
| d.f.   | 26        |
| l.s.d. | 6.47      |

#### Analysis of variance

Variate: Mch

| Source of variation | d.f.(m.v.) | s.s.    | m.s.   | v.r. | F pr. |
|---------------------|------------|---------|--------|------|-------|
| Prot_dose           | 3          | 2.4997  | 0.8332 | 2.01 | 0.138 |
| Residual            | 26(2)      | 10.7921 | 0.4151 |      |       |
| Total               | 29(2)      | 12.8297 |        |      |       |

#### Tables of means

Variate: Mch

Grand mean 18.47

|           |       |            |           |          |
|-----------|-------|------------|-----------|----------|
| Prot_dose | 0mg   | 1000mg mcy | 100mg mcy | 10mg mcy |
|           | 18.93 | 18.25      | 18.25     | 18.44    |

Least significant differences of means (1% level)

|        |           |
|--------|-----------|
| Table  | Prot_dose |
| rep.   | 8         |
| d.f.   | 26        |
| l.s.d. | 0.826     |

Analysis of variance

Variate: Mchc

|                     |            |         |        |       |       |
|---------------------|------------|---------|--------|-------|-------|
| Source of variation | d.f.(m.v.) | s.s.    | m.s.   | v.r.  | F pr. |
| Prot_dose           | 3          | 10.1737 | 3.3912 | 11.39 | <.001 |
| Residual            | 26(2)      | 7.7396  | 0.2977 |       |       |
| Total               | 29(2)      | 17.1697 |        |       |       |

Tables of means

Variate: Mchc

Grand mean 29.676

|           |        |            |           |          |
|-----------|--------|------------|-----------|----------|
| Prot_dose | 0mg    | 1000mg mcy | 100mg mcy | 10mg mcy |
|           | 30.267 | 29.113     | 29.113    | 30.213   |

Least significant differences of means (1% level)

|        |           |
|--------|-----------|
| Table  | Prot_dose |
| rep.   | 8         |
| d.f.   | 26        |
| l.s.d. | 0.987     |

Analysis of variance

Variate: Mcv

|                     |            |         |       |      |       |
|---------------------|------------|---------|-------|------|-------|
| Source of variation | d.f.(m.v.) | s.s.    | m.s.  | v.r. | F pr. |
| Prot_dose           | 3          | 15.883  | 5.294 | 1.59 | 0.216 |
| Residual            | 26(2)      | 86.548  | 3.329 |      |       |
| Total               | 29(2)      | 102.248 |       |      |       |

Tables of means

Variate: Mcv

Grand mean 62.24

|           |       |            |           |          |
|-----------|-------|------------|-----------|----------|
| Prot_dose | 0mg   | 1000mg mcy | 100mg mcy | 10mg mcy |
|           | 62.53 | 62.70      | 62.70     | 61.03    |

Least significant differences of means (1% level)

|       |           |
|-------|-----------|
| Table | Prot_dose |
|-------|-----------|

|        |      |
|--------|------|
| rep.   | 8    |
| d.f.   | 26   |
| l.s.d. | 2.23 |

Analysis of variance  
Variate: Plt

| Source of variation | d.f.(m.v.) | s.s.    | m.s.   | v.r. | F pr. |
|---------------------|------------|---------|--------|------|-------|
| Prot_dose           | 3          | 38502.  | 12834. | 1.44 | 0.257 |
| Residual            | 23(5)      | 205046. | 8915.  |      |       |
| Total               | 26(5)      | 236135. |        |      |       |

Tables of means  
Variate: Plt

Grand mean 832.

| Prot_dose | 0mg  | 1000mg mcy | 100mg mcy | 10mg mcy |
|-----------|------|------------|-----------|----------|
|           | 824. | 807.       | 807.      | 891.     |

Least significant differences of means (1% level)

| Table  | Prot_dose |
|--------|-----------|
| rep.   | 8         |
| d.f.   | 23        |
| l.s.d. | 158.7     |

Analysis of variance  
Variate: RBC

| Source of variation | d.f.(m.v.) | s.s.    | m.s.    | v.r. | F pr. |
|---------------------|------------|---------|---------|------|-------|
| Prot_dose           | 3          | 0.44730 | 0.14910 | 1.57 | 0.220 |
| Residual            | 26(2)      | 2.46397 | 0.09477 |      |       |
| Total               | 29(2)      | 2.82467 |         |      |       |

Tables of means  
Variate: RBC

Grand mean 6.963

| Prot_dose | 0mg  | 1000mg mcy | 100mg mcy | 10mg mcy |
|-----------|------|------------|-----------|----------|
|           | 6.76 | 7.048      | 7.048     | 6.996    |

Least significant differences of means (1% level)

| Table  | Prot_dose |
|--------|-----------|
| rep.   | 8         |
| d.f.   | 26        |
| l.s.d. | 0.3164    |

Analysis of variance  
Variate: RBC

| Source of variation | d.f.(m.v.) | s.s.    | m.s.    | v.r. | F pr. |
|---------------------|------------|---------|---------|------|-------|
| Prot_dose           | 3          | 0.44730 | 0.14910 | 1.57 | 0.220 |
| Residual            | 26(2)      | 2.46397 | 0.09477 |      |       |
| Total               | 29(2)      | 2.82467 |         |      |       |

Tables of means  
Variate: RBC

Grand mean 6.963

|           |     |            |           |          |
|-----------|-----|------------|-----------|----------|
| Prot_dose | 0mg | 1000mg mcy | 100mg mcy | 10mg mcy |
|           | 6.7 | 7.048      | 7.048     | 6.996    |

Least significant differences of means (1% level)

|        |           |
|--------|-----------|
| Table  | Prot_dose |
| rep.   | 8         |
| d.f.   | 26        |
| l.s.d. | 0.439     |

Analysis of variance

Variate: RET

|                     |            |           |           |      |       |
|---------------------|------------|-----------|-----------|------|-------|
| Source of variation | d.f.(m.v.) | s.s.      | m.s.      | v.r. | F pr. |
| Prot_dose           | 3          | 0.0010940 | 0.0003647 | 0.75 | 0.530 |
| Residual            | 26(2)      | 0.0125705 | 0.0004835 |      |       |
| Total               | 29(2)      | 0.0136384 |           |      |       |

Tables of means

Variate: RET

Grand mean 0.0224

|           |        |            |           |          |
|-----------|--------|------------|-----------|----------|
| Prot_dose | 0      | 1000mg mcy | 100mg mcy | 10mg mcy |
|           | 0.0259 | 0.0257     | 0.0257    | 0.0123   |

Least significant differences of means (1% level)

|        |           |
|--------|-----------|
| Table  | Prot_dose |
| rep.   | 8         |
| d.f.   | 26        |
| l.s.d. | 0.0349    |

Analysis of variance

Variate: WBC

|                     |            |         |        |      |       |
|---------------------|------------|---------|--------|------|-------|
| Source of variation | d.f.(m.v.) | s.s.    | m.s.   | v.r. | F pr. |
| Prot_dose           | 3          | 36.675  | 12.225 | 1.75 | 0.181 |
| Residual            | 27(1)      | 189.147 | 7.005  |      |       |
| Total               | 30(1)      | 225.390 |        |      |       |

Tables of means

Variate: WBC

Grand mean 6.92

|           |      |            |           |          |
|-----------|------|------------|-----------|----------|
| Prot_dose | 0    | 1000mg mcy | 100mg mcy | 10mg mcy |
|           | 5.29 | 7.57       | 8.12      | 6.71     |

Least significant differences of means (1% level)

|        |           |
|--------|-----------|
| Table  | Prot_dose |
| rep.   | 8         |
| d.f.   | 27        |
| l.s.d. | 6.34      |

Analysis of variance

Variate: HCT

|                     |            |         |       |      |       |
|---------------------|------------|---------|-------|------|-------|
| Source of variation | d.f.(m.v.) | s.s.    | m.s.  | v.r. | F pr. |
| Protn_dose          | 4          | 13.005  | 3.251 | 0.88 | 0.486 |
| Residual            | 31(4)      | 114.309 | 3.687 |      |       |
| Total               | 35(4)      | 125.390 |       |      |       |

Tables of means

Variate: HCT

Grand mean 43.20

|            |       |            |           |          |
|------------|-------|------------|-----------|----------|
| Protn_dose | 0mg   | 1000mg Pcy | 100mg pcy | 10mg pcy |
|            | 42.25 | 43.40      | 44.02     | 43.14    |

Least significant differences of means (1% level)

|        |            |
|--------|------------|
| Table  | Protn_dose |
| rep.   | 8          |
| d.f.   | 31         |
| l.s.d. | 1.958      |

Analysis of variance

Variate: HGB

| Source of variation | d.f.(m.v.) | s.s.   | m.s.   | v.r. | F pr. |
|---------------------|------------|--------|--------|------|-------|
| Protn_dose          | 4          | 1.0970 | 0.2743 | 1.11 | 0.370 |
| Residual            | 31(4)      | 7.6687 | 0.2474 |      |       |
| Total               | 35(4)      | 8.6297 |        |      |       |

Tables of means

Variate: HGB

Grand mean 12.999

|            |        |            |           |          |
|------------|--------|------------|-----------|----------|
| Protn_dose | 0mg    | 1000mg Pcy | 100mg pcy | 10mg pcy |
|            | 12.783 | 13.114     | 13.113    | 12.812   |

Least significant differences of means (1% level)

|        |            |
|--------|------------|
| Table  | Protn_dose |
| rep.   | 8          |
| d.f.   | 31         |
| l.s.d. | 0.5072     |

Analysis of variance

Variate: LYM

| Source of variation | d.f.(m.v.) | s.s.    | m.s.  | v.r. | F pr. |
|---------------------|------------|---------|-------|------|-------|
| Protn_dose          | 4          | 43.02   | 10.76 | 0.32 | 0.862 |
| Residual            | 31(4)      | 1039.85 | 33.54 |      |       |
| Total               | 35(4)      | 1079.53 |       |      |       |

Tables of means

Variate: LYM

Grand mean 84.87

|            |       |            |           |          |
|------------|-------|------------|-----------|----------|
| Protn_dose | 0mg   | 1000mg Pcy | 100mg pcy | 10mg pcy |
|            | 84.35 | 85.66      | 84.65     | 83.36    |

Least significant differences of means (1% level)

|        |            |
|--------|------------|
| Table  | Protn_dose |
| rep.   | 8          |
| d.f.   | 31         |
| l.s.d. | 5.906      |

Analysis of variance

Variate: Mch

| Source of variation | d.f.(m.v.) | s.s.    | m.s.   | v.r. | F pr. |
|---------------------|------------|---------|--------|------|-------|
| Protn_dose          | 4          | 3.4552  | 0.8638 | 2.40 | 0.072 |
| Residual            | 31(4)      | 11.1780 | 0.3606 |      |       |
| Total               | 35(4)      | 14.0631 |        |      |       |

Tables of means

Variate: Mch

Grand mean 18.551

| Protn_dose | 0mg    | 1000mg Pcy | 100mg pcy | 10mg pcy |
|------------|--------|------------|-----------|----------|
|            | 18.933 | 18.086     | 18.437    | 18.512   |

Least significant differences of means (1% level)

| Table  | Protn_dose |
|--------|------------|
| rep.   | 8          |
| d.f.   | 31         |
| l.s.d. | 0.6123     |

Analysis of variance

Variate: Mch

| Source of variation | d.f.(m.v.) | s.s.    | m.s.   | v.r. | F pr. |
|---------------------|------------|---------|--------|------|-------|
| Protn_dose          | 4          | 3.4552  | 0.8638 | 2.40 | 0.072 |
| Residual            | 31(4)      | 11.1780 | 0.3606 |      |       |
| Total               | 35(4)      | 14.0631 |        |      |       |

Tables of means

Variate: Mch

Grand mean 18.551

| Protn_dose | 0mg    | 1000mg Pcy | 100mg pcy | 10mg pcy |
|------------|--------|------------|-----------|----------|
|            | 18.933 | 18.086     | 18.437    | 18.512   |

Least significant differences of means (1% level)

| Table  | Protn_dose |
|--------|------------|
| rep.   | 8          |
| d.f.   | 31         |
| l.s.d. | 0.6123     |

Analysis of variance

Variate: Mchc

| Source of variation | d.f.(m.v.) | s.s.    | m.s.   | v.r. | F pr. |
|---------------------|------------|---------|--------|------|-------|
| Protn_dose          | 4          | 3.0089  | 0.7522 | 1.24 | 0.313 |
| Residual            | 31(4)      | 18.7537 | 0.6050 |      |       |
| Total               | 35(4)      | 21.5600 |        |      |       |

Tables of means

Variate: Mchc

Grand mean 30.12

| Protn_dose | 0mg   | 1000mg Pcy | 100mg pcy | 10mg pcy |
|------------|-------|------------|-----------|----------|
|            | 30.27 | 30.23      | 29.91     | 29.71    |

Least significant differences of means (1% level)

| Table  | Protn_dose |
|--------|------------|
| rep.   | 8          |
| d.f.   | 31         |
| l.s.d. | 0.793      |

Analysis of variance

Variate: Mcv

| Source of variation | d.f.(m.v.) | s.s.    | m.s.  | v.r. | F pr. |
|---------------------|------------|---------|-------|------|-------|
| Protn_dose          | 4          | 34.037  | 8.509 | 2.72 | 0.048 |
| Residual            | 31(4)      | 97.031  | 3.130 |      |       |
| Total               | 35(4)      | 126.447 |       |      |       |

Tables of means

Variate: Mcv

Grand mean 61.61  
 Protn\_dose      0mg      1000mg Pcy      100mg pcy      10mg pcy  
                     62.53      59.90              61.75              62.29

Least significant differences of means (1% level)

Table      Protn\_dose  
 rep.              8  
 d.f.              31  
 l.s.d.            1.804

Variate: Plt

| Source of variation | d.f.(m.v.) | s.s.    | m.s.   | v.r. | F pr. |
|---------------------|------------|---------|--------|------|-------|
| Protn_dose          | 4          | 7859.   | 1965.  | 0.08 | 0.989 |
| Residual            | 30(5)      | 776579. | 25886. |      |       |
| Total               | 34(5)      | 783388. |        |      |       |

Tables of means

Variate: Plt

Grand mean 815.  
 Protn\_dose      0mg      1000mg Pcy      100mg pcy      10mg pcy      BSA  
                     824.      838.      808.      806.      799.

Least significant differences of means (1% level)

Table      Protn\_dose  
 rep.              8  
 d.f.              30  
 l.s.d.            164.3

Analysis of variance

Variate: RBC

| Source of variation | d.f.(m.v.) | s.s.   | m.s.   | v.r. | F pr. |
|---------------------|------------|--------|--------|------|-------|
| Protn_dose          | 4          | 1.1545 | 0.2886 | 1.93 | 0.131 |
| Residual            | 31(4)      | 4.6411 | 0.1497 |      |       |
| Total               | 35(4)      | 5.6038 |        |      |       |

Tables of means

Variate: RBC

Grand mean 7.020  
 Protn\_dose      0mg      1000mg Pcy      100mg pcy      10mg pcy  
                     6.762      7.259              7.132              6.930

Least significant differences of means (1% level)

Table      Protn\_dose  
 rep.              8  
 d.f.              31  
 l.s.d.            0.3946

Analysis of variance

Variate: WBC

| Source of variation | d.f.(m.v.) | s.s.    | m.s.   | v.r. | F pr. |
|---------------------|------------|---------|--------|------|-------|
| Protn_dose          | 4          | 49.835  | 12.459 | 1.25 | 0.308 |
| Residual            | 34(1)      | 338.125 | 9.945  |      |       |
| Total               | 38(1)      | 387.957 |        |      |       |

Tables of means

Variate: WBC

Grand mean 6.15

|            |      |        |     |       |     |      |     |
|------------|------|--------|-----|-------|-----|------|-----|
| Protn_dose | 0mg  | 1000mg | Pcy | 100mg | pcy | 10mg | pcy |
|            | 5.29 | 6.10   |     | 5.16  |     | 5.94 |     |

Least significant differences of means (1% level)

|        |            |
|--------|------------|
| Table  | Protn_dose |
| rep.   | 8          |
| d.f.   | 34         |
| l.s.d. | 3.204      |

Analysis of variance

Variate: ALB

|                     |      |         |         |      |       |     |
|---------------------|------|---------|---------|------|-------|-----|
| Source of variation | d.f. | s.s.    | m.s.    | v.r. | F     | pr. |
| Protn_dose          | 4    | 0.05954 | 0.01488 | 0.27 | 0.893 |     |
| Residual            | 35   | 1.90710 | 0.05449 |      |       |     |
| Total               | 39   | 1.96664 |         |      |       |     |

Tables of means

Variate: ALB

Grand mean 3.262

|            |       |        |          |         |
|------------|-------|--------|----------|---------|
| Protn_dose | 0mg   | 1000mg | 100mgMcy | 10mgMcy |
|            | 3.311 | 3.301  | 3.256    | 3.224   |

Least significant differences of means (1% level)

|        |            |
|--------|------------|
| Table  | Protn_dose |
| rep.   | 8          |
| d.f.   | 35         |
| l.s.d. | 0.323      |

Analysis of variance

Variate: ALP

|                     |      |         |        |      |       |     |
|---------------------|------|---------|--------|------|-------|-----|
| Source of variation | d.f. | s.s.    | m.s.   | v.r. | F     | pr. |
| Protn_dose          | 4    | 59559.  | 14890. | 1.12 | 0.363 |     |
| Residual            | 35   | 465830. | 13309. |      |       |     |
| Total               | 39   | 525389. |        |      |       |     |

Tables of means

Variate: ALP

Grand mean 255.

|            |      |        |          |         |
|------------|------|--------|----------|---------|
| Protn_dose | 0mg  | 1000mg | 100mgMcy | 10mgMcy |
|            | 243. | 326.   | 257.     | 209.    |

Least significant differences of means (1% level)

|        |            |
|--------|------------|
| Table  | Protn_dose |
| rep.   | 8          |
| d.f.   | 35         |
| l.s.d. | 159.9      |

Analysis of variance

Variate: ALT

|                     |      |        |       |      |       |     |
|---------------------|------|--------|-------|------|-------|-----|
| Source of variation | d.f. | s.s.   | m.s.  | v.r. | F     | pr. |
| Protn_dose          | 4    | 10771. | 2693. | 1.11 | 0.367 |     |
| Residual            | 35   | 84915. | 2426. |      |       |     |
| Total               | 39   | 95686. |       |      |       |     |

Tables of means

Variate: ALT

Grand mean 115.3

|            |       |        |          |         |
|------------|-------|--------|----------|---------|
| Protn_dose | 0mg   | 1000mg | 100mgMcy | 10mgMcy |
|            | 145.7 | 112.2  | 100.2    | 101.8   |

Least significant differences of means (1% level)

|        |            |
|--------|------------|
| Table  | Protn_dose |
| rep.   | 8          |
| d.f.   | 35         |
| l.s.d. | 60.23      |

Analysis of variance

Variate: AST

| Source of variation | d.f. | s.s.    | m.s.   | v.r. | F     | pr. |
|---------------------|------|---------|--------|------|-------|-----|
| Protn_dose          | 4    | 35110.  | 8778.  | 0.58 | 0.682 |     |
| Residual            | 35   | 533267. | 15236. |      |       |     |
| Total               | 39   | 568377. |        |      |       |     |

Tables of means

Variate: AST

Grand mean 347.

| Protn_dose | 0mg  | 1000mg | 100mgMcy | 10mgMcy |
|------------|------|--------|----------|---------|
|            | 405. | 341.   | 331.     | 322.    |

Least significant differences of means (1% level)

|        |            |
|--------|------------|
| Table  | Protn_dose |
| rep.   | 8          |
| d.f.   | 35         |
| l.s.d. | 181.5      |

Analysis of variance

Variate: A\_G

| Source of variation | d.f. | s.s.   | m.s.   | v.r. | F     | pr. |
|---------------------|------|--------|--------|------|-------|-----|
| Protn_dose          | 4    | 0.8668 | 0.2167 | 1.98 | 0.119 |     |
| Residual            | 35   | 3.8284 | 0.1094 |      |       |     |
| Total               | 39   | 4.6952 |        |      |       |     |

Tables of means

Variate: A\_G

Grand mean 1.405

| Protn_dose | 0mg   | 1000mg | 100mgMcy | 10mgMcy |
|------------|-------|--------|----------|---------|
|            | 1.512 | 1.282  | 1.643    | 1.306   |

Least significant differences of means (1% level)

|        |            |
|--------|------------|
| Table  | Protn_dose |
| rep.   | 8          |
| d.f.   | 35         |
| l.s.d. | 0.50       |

Analysis of variance

Variate: BUN

| Source of variation | d.f. | s.s.   | m.s.   | v.r. | F     | pr. |
|---------------------|------|--------|--------|------|-------|-----|
| Protn_dose          | 4    | 2.8285 | 0.7071 | 3.59 | 0.015 |     |
| Residual            | 35   | 6.9016 | 0.1972 |      |       |     |
| Total               | 39   | 9.7301 |        |      |       |     |

Tables of means

Variate: BUN

Grand mean 3.379

| Protn_dose | 0mg   | 1000mg | 100mgMcy | 10mgMcy | BSA   |
|------------|-------|--------|----------|---------|-------|
|            | 3.553 | 3.774  | 3.209    | 3.007   | 3.354 |

Least significant differences of means (1% level)

| Table  | Protn_dose |
|--------|------------|
| rep.   | 8          |
| d.f.   | 35         |
| l.s.d. | 0.64       |

Analysis of variance

Variate: CA

| Source of variation | d.f. | s.s.    | m.s.    | v.r. | F pr. |
|---------------------|------|---------|---------|------|-------|
| Protn_dose          | 4    | 0.91741 | 0.22935 | 6.00 | <.001 |
| Residual            | 35   | 1.33810 | 0.03823 |      |       |
| Total               | 39   | 2.25551 |         |      |       |

Tables of means

Variate: CA

Grand mean 2.618

| Protn_dose | 0mg  | 1000mg | 100mgMcy | 10mgMcy |
|------------|------|--------|----------|---------|
|            | 2.48 | 2.894  | 2.663    | 2.556   |

Least significant differences of means (1% level)

| Table  | Protn_dose |
|--------|------------|
| rep.   | 8          |
| d.f.   | 35         |
| l.s.d. | 0.294      |

Analysis of variance

Variate: CHOL

| Source of variation | d.f. | s.s.    | m.s.    | v.r. | F pr. |
|---------------------|------|---------|---------|------|-------|
| Protn_dose          | 4    | 0.23203 | 0.05801 | 0.80 | 0.532 |
| Residual            | 35   | 2.53046 | 0.07230 |      |       |
| Total               | 39   | 2.76250 |         |      |       |

Tables of means

Variate: CHOL

Grand mean 2.110

| Protn_dose | 0mg   | 1000mg | 100mgMcy | 10mgMcy |
|------------|-------|--------|----------|---------|
|            | 2.130 | 2.080  | 2.106    | 2.234   |

Least significant differences of means (1% level)

| Table  | Protn_dose |
|--------|------------|
| rep.   | 8          |
| d.f.   | 35         |
| l.s.d. | 0.389      |

Analysis of variance

Variate: CL

| Source of variation | d.f. | s.s.    | m.s.   | v.r. | F pr. |
|---------------------|------|---------|--------|------|-------|
| Protn_dose          | 4    | 18253.4 | 4563.3 | 4.67 | 0.004 |
| Residual            | 35   | 34214.3 | 977.6  |      |       |
| Total               | 39   | 52467.7 |        |      |       |

Tables of means

Variate: CL

Grand mean 86.1

| Protn_dose | 0mg  | 1000mg | 100mgMcy | 10mgMcy |
|------------|------|--------|----------|---------|
|            | 48.1 | 77.1   | 98.8     | 100.0   |

Standard errors of differences of means

|        |            |
|--------|------------|
| Table  | Protn_dose |
| rep.   | 8          |
| d.f.   | 35         |
| s.e.d. | 15.63      |

Least significant differences of means (1% level)

|        |            |
|--------|------------|
| Table  | Protn_dose |
| rep.   | 8          |
| d.f.   | 35         |
| l.s.d. | 47.88      |

Analysis of variance

Variate: CREA

| Source of variation | d.f. | s.s.    | m.s.  | v.r. | F pr. |
|---------------------|------|---------|-------|------|-------|
| Protn_dose          | 4    | 72.53   | 18.13 | 0.50 | 0.737 |
| Residual            | 35   | 1272.72 | 36.36 |      |       |
| Total               | 39   | 1345.25 |       |      |       |

Tables of means

Variate: CREA

Grand mean 14.90

| Protn_dose | 0mg   | 1000mg | 100mgMcy | 10mgMcy |
|------------|-------|--------|----------|---------|
|            | 14.51 | 13.94  | 14.66    | 13.89   |

Least significant differences of means (1% level)

|        |            |
|--------|------------|
| Table  | Protn_dose |
| rep.   | 8          |
| d.f.   | 35         |
| l.s.d. | 4.23       |

Analysis of variance

Variate: GLOB

| Source of variation | d.f. | s.s.    | m.s.    | v.r. | F pr. |
|---------------------|------|---------|---------|------|-------|
| Protn_dose          | 4    | 0.64899 | 0.16225 | 1.83 | 0.146 |
| Residual            | 35   | 3.10689 | 0.08877 |      |       |
| Total               | 39   | 3.75588 |         |      |       |

Tables of means

Variate: GLOB

Grand mean 2.449

| Protn_dose | 0mg   | 1000mg | 100mgMcy | 10mgMcy |
|------------|-------|--------|----------|---------|
|            | 2.228 | 2.606  | 2.413    | 2.480   |

Least significant differences of means (1% level)

|        |            |
|--------|------------|
| Table  | Protn_dose |
| rep.   | 8          |
| d.f.   | 35         |
| l.s.d. | 0.439      |

Analysis of variance

Variate: GLU

| Source of variation | d.f. | s.s.    | m.s.   | v.r. | F pr. |
|---------------------|------|---------|--------|------|-------|
| Protn_dose          | 4    | 102.998 | 25.749 | 5.44 | 0.002 |
| Residual            | 35   | 165.774 | 4.736  |      |       |
| Total               | 39   | 268.772 |        |      |       |

Tables of means

Variate: GLU

Grand mean 5.17

|            |      |        |          |         |
|------------|------|--------|----------|---------|
| Protn_dose | 0mg  | 1000mg | 100mgMcy | 10mgMcy |
|            | 3.39 | 8.13   | 5.03     | 5.11    |

Least significant differences of means (1% level)

|       |            |
|-------|------------|
| Table | Protn_dose |
|-------|------------|

|      |   |
|------|---|
| rep. | 8 |
|------|---|

|      |    |
|------|----|
| d.f. | 35 |
|------|----|

|        |      |
|--------|------|
| l.s.d. | 3.18 |
|--------|------|

Analysis of variance

Variate: K

|                     |      |         |        |       |       |
|---------------------|------|---------|--------|-------|-------|
| Source of variation | d.f. | s.s.    | m.s.   | v.r.  | F pr. |
| Protn_dose          | 4    | 354.490 | 88.623 | 59.82 | <.001 |
| Residual            | 35   | 51.850  | 1.481  |       |       |
| Total               | 39   | 406.340 |        |       |       |

Tables of means

Variate: K

Grand mean 3.60

|            |      |        |          |         |
|------------|------|--------|----------|---------|
| Protn_dose | 0mg  | 1000mg | 100mgMcy | 10mgMcy |
|            | 0.00 | 5.20   | 6.12     | 6.67    |

Least significant differences of means (1% level)

|       |            |
|-------|------------|
| Table | Protn_dose |
|-------|------------|

|      |   |
|------|---|
| rep. | 8 |
|------|---|

|      |    |
|------|----|
| d.f. | 35 |
|------|----|

|        |       |
|--------|-------|
| l.s.d. | 1.235 |
|--------|-------|

Analysis of variance

Variate: PHOS

|                     |      |         |       |      |       |
|---------------------|------|---------|-------|------|-------|
| Source of variation | d.f. | s.s.    | m.s.  | v.r. | F pr. |
| Protn_dose          | 4    | 21.697  | 5.424 | 1.31 | 0.285 |
| Residual            | 35   | 144.957 | 4.142 |      |       |
| Total               | 39   | 166.655 |       |      |       |

Tables of means

Variate: PHOS

Grand mean 4.65

|            |      |        |          |         |
|------------|------|--------|----------|---------|
| Protn_dose | 0mg  | 1000mg | 100mgMcy | 10mgMcy |
|            | 5.38 | 3.44   | 4.35     | 4.65    |

Least significant differences of means (1% level)

|       |            |
|-------|------------|
| Table | Protn_dose |
|-------|------------|

|      |   |
|------|---|
| rep. | 8 |
|------|---|

|      |    |
|------|----|
| d.f. | 35 |
|------|----|

|        |      |
|--------|------|
| l.s.d. | 2.47 |
|--------|------|

Analysis of variance

Variate: TBIL

|                     |            |        |       |      |       |
|---------------------|------------|--------|-------|------|-------|
| Source of variation | d.f.(m.v.) | s.s.   | m.s.  | v.r. | F pr. |
| Protn_dose          | 4          | 167.36 | 41.84 | 1.87 | 0.139 |
| Residual            | 34(1)      | 761.72 | 22.40 |      |       |
| Total               | 38(1)      | 929.04 |       |      |       |

Tables of means

Variate: TBIL

Grand mean 4.32

|            |     |        |          |         |
|------------|-----|--------|----------|---------|
| Protn_dose | 0mg | 1000mg | 100mgMcy | 10mgMcy |
|------------|-----|--------|----------|---------|

1.54    7.88    3.81            3.86

Least significant differences of means (1% level)

|        |            |
|--------|------------|
| Table  | Protn_dose |
| rep.   | 8          |
| d.f.   | 34         |
| l.s.d. | 5.93       |

Analysis of variance

Variate: TP

|                     |      |        |        |      |       |
|---------------------|------|--------|--------|------|-------|
| Source of variation | d.f. | s.s.   | m.s.   | v.r. | F pr. |
| Protn_dose          | 4    | 0.5666 | 0.1416 | 0.96 | 0.444 |
| Residual            | 35   | 5.1871 | 0.1482 |      |       |
| Total               | 39   | 5.7536 |        |      |       |

Tables of means

Variate: TP

Grand mean 5.711

|            |       |        |          |         |
|------------|-------|--------|----------|---------|
| Protn_dose | 0mg   | 1000mg | 100mgMcy | 10mgMcy |
|            | 5.539 | 5.907  | 5.669    | 5.704   |

Least significant differences of means (1% level)

|        |            |
|--------|------------|
| Table  | Protn_dose |
| rep.   | 8          |
| d.f.   | 35         |
| l.s.d. | 0.533      |

Analysis of variance

Variate: TRIG

|                     |      |         |         |      |       |
|---------------------|------|---------|---------|------|-------|
| Source of variation | d.f. | s.s.    | m.s.    | v.r. | F pr. |
| Protn_dose          | 4    | 0.24554 | 0.06138 | 1.41 | 0.251 |
| Residual            | 35   | 1.52437 | 0.04355 |      |       |
| Total               | 39   | 1.76991 |         |      |       |

Tables of means

Variate: TRIG

Grand mean 0.849

|            |       |        |          |         |
|------------|-------|--------|----------|---------|
| Protn_dose | 0mg   | 1000mg | 100mgMcy | 10mgMcy |
|            | 0.831 | 0.792  | 0.820    | 1.002   |

Least significant differences of means (1% level)

|        |            |
|--------|------------|
| Table  | Protn_dose |
| rep.   | 8          |
| d.f.   | 35         |
| l.s.d. | 0.641      |

Analysis of variance

Variate: ALP

|                     |      |         |        |      |       |
|---------------------|------|---------|--------|------|-------|
| Source of variation | d.f. | s.s.    | m.s.   | v.r. | F pr. |
| Prot_dose           | 3    | 97552.  | 32517. | 5.73 | 0.003 |
| Residual            | 28   | 158846. | 5673.  |      |       |
| Total               | 31   | 256398. |        |      |       |

Tables of means

Variate: ALP

Grand mean 316.

|           |   |            |           |          |
|-----------|---|------------|-----------|----------|
| Prot_dose | 0 | 1000mg pcy | 100mg pcy | 10mg pcy |
|-----------|---|------------|-----------|----------|

289. 245. 336. 393.

Least significant differences of means (1% level)

|        |           |
|--------|-----------|
| Table  | Prot_dose |
| rep.   | 8         |
| d.f.   | 28        |
| l.s.d. | 135.1     |

Analysis of variance

Variate: ALT

| Source of variation | d.f.(m.v.) | s.s.   | m.s.  | v.r. | F pr. |
|---------------------|------------|--------|-------|------|-------|
| Prot_dose           | 3          | 5240.  | 1747. | 0.71 | 0.553 |
| Residual            | 27(1)      | 66122. | 2449. |      |       |
| Total               | 30(1)      | 71248. |       |      |       |

Tables of means

Variate: ALT

Grand mean 152.5

| Prot_dose | 0     | 1000mg pcy | 100mg pcy | 10mg pcy |
|-----------|-------|------------|-----------|----------|
|           | 158.6 | 130.6      | 163.0     | 157.7    |

Least significant differences of means (1% level)

|        |           |
|--------|-----------|
| Table  | Prot_dose |
| rep.   | 8         |
| d.f.   | 27        |
| l.s.d. | 81.31     |

Analysis of variance

Variate: AST

| Source of variation | d.f. | s.s.    | m.s.   | v.r. | F pr. |
|---------------------|------|---------|--------|------|-------|
| Prot_dose           | 3    | 3659.   | 1220.  | 0.12 | 0.946 |
| Residual            | 28   | 280347. | 10012. |      |       |
| Total               | 31   | 284006. |        |      |       |

Tables of means

Variate: AST

Grand mean 366.

| Prot_dose | 0mg  | 1000mg pcy | 100mg pcy | 10mg pcy |
|-----------|------|------------|-----------|----------|
|           | 361. | 385.       | 359.      | 361.     |

Least significant differences of means (1% level)

|        |           |
|--------|-----------|
| Table  | Prot_dose |
| rep.   | 8         |
| d.f.   | 28        |
| l.s.d. | 148.6     |

Analysis of variance

Variate: CL

| Source of variation | d.f. | s.s.   | m.s.  | v.r. | F pr. |
|---------------------|------|--------|-------|------|-------|
| Prot_dose           | 3    | 21.250 | 7.083 | 2.58 | 0.073 |
| Residual            | 28   | 76.750 | 2.741 |      |       |
| Total               | 31   | 98.000 |       |      |       |

Tables of means

Variate: CL

Grand mean 100.75

|           |        |            |           |          |
|-----------|--------|------------|-----------|----------|
| Prot_dose | 0mg    | 1000mg pcy | 100mg pcy | 10mg pcy |
|           | 100.50 | 99.62      | 101.00    | 101.88   |

Least significant differences of means (1% level)

|        |           |
|--------|-----------|
| Table  | Prot_dose |
| rep.   | 8         |
| d.f.   | 28        |
| l.s.d. | 39.8      |

Analysis of variance

Variate: CREA

|                     |            |         |        |      |       |
|---------------------|------------|---------|--------|------|-------|
| Source of variation | d.f.(m.v.) | s.s.    | m.s.   | v.r. | F pr. |
| Prot_dose           | 3          | 49.736  | 16.579 | 3.89 | 0.020 |
| Residual            | 27(1)      | 114.937 | 4.257  |      |       |
| Total               | 30(1)      | 162.855 |        |      |       |

Tables of means

Variate: CREA

Grand mean 13.89

|           |       |            |           |          |
|-----------|-------|------------|-----------|----------|
| Prot_dose | 0mg   | 1000mg pcy | 100mg pcy | 10mg pcy |
|           | 15.22 | 12.53      | 12.77     | 15.05    |

Least significant differences of means (1% level)

|        |           |
|--------|-----------|
| Table  | Prot_dose |
| rep.   | 8         |
| d.f.   | 27        |
| l.s.d. | 3.117     |

Analysis of variance

Variate: GLU

|                     |      |        |        |      |       |
|---------------------|------|--------|--------|------|-------|
| Source of variation | d.f. | s.s.   | m.s.   | v.r. | F pr. |
| Prot_dose           | 3    | 33.929 | 11.310 | 6.92 | 0.001 |
| Residual            | 28   | 45.747 | 1.634  |      |       |
| Total               | 31   | 79.676 |        |      |       |

Tables of means

Variate: GLU

Grand mean 7.43

|           |      |            |           |          |
|-----------|------|------------|-----------|----------|
| Prot_dose | 0    | 1000mg pcy | 100mg pcy | 10mg pcy |
|           | 5.88 | 8.40       | 8.32      | 7.11     |

Least significant differences of means (1% level)

|        |           |
|--------|-----------|
| Table  | Prot_dose |
| rep.   | 8         |
| d.f.   | 28        |
| l.s.d. | 1.309     |

Analysis of variance

Variate: NA

|                     |      |         |        |      |       |
|---------------------|------|---------|--------|------|-------|
| Source of variation | d.f. | s.s.    | m.s.   | v.r. | F pr. |
| Prot_dose           | 3    | 587.38  | 195.79 | 5.14 | 0.006 |
| Residual            | 28   | 1067.50 | 38.12  |      |       |
| Total               | 31   | 1654.88 |        |      |       |

Tables of means

Variate: NA

Grand mean 134.8

|           |       |            |           |          |
|-----------|-------|------------|-----------|----------|
| Prot_dose | 0     | 1000mg pcy | 100mg pcy | 10mg pcy |
|           | 128.1 | 137.1      | 139.6     | 134.4    |

Least significant differences of means (1% level)

|        |           |
|--------|-----------|
| Table  | Prot_dose |
| rep.   | 8         |
| d.f.   | 28        |
| l.s.d. | 6.32      |

Analysis of variance

Variate: PHOS

|                     |      |         |        |      |       |
|---------------------|------|---------|--------|------|-------|
| Source of variation | d.f. | s.s.    | m.s.   | v.r. | F pr. |
| Prot_dose           | 3    | 8.2261  | 2.7420 | 2.94 | 0.050 |
| Residual            | 28   | 26.1047 | 0.9323 |      |       |
| Total               | 31   | 34.3308 |        |      |       |

Analysis of variance

Variate: PHOS

|                     |      |         |        |      |       |
|---------------------|------|---------|--------|------|-------|
| Source of variation | d.f. | s.s.    | m.s.   | v.r. | F pr. |
| Prot_dose           | 3    | 8.2261  | 2.7420 | 2.94 | 0.050 |
| Residual            | 28   | 26.1047 | 0.9323 |      |       |
| Total               | 31   | 34.3308 |        |      |       |

Tables of means

Variate: PHOS

Grand mean 4.62

|           |      |            |           |          |
|-----------|------|------------|-----------|----------|
| Prot_dose | 0    | 1000mg pcy | 100mg pcy | 10mg pcy |
|           | 5.38 | 4.75       | 4.28      | 4.05     |

Least significant differences of means (1% level)

|        |           |
|--------|-----------|
| Table  | Prot_dose |
| rep.   | 8         |
| d.f.   | 28        |
| l.s.d. | 0.989     |

Analysis of variance

Variate: Plt

|                     |      |         |       |      |       |
|---------------------|------|---------|-------|------|-------|
| Source of variation | d.f. | s.s.    | m.s.  | v.r. | F pr. |
| Prot_dose           | 3    | 17236.  | 5745. | 0.76 | 0.524 |
| Residual            | 28   | 210570. | 7520. |      |       |
| Total               | 31   | 227806. |       |      |       |

Tables of means

Variate: Plt

Grand mean 810.

|           |      |            |           |          |
|-----------|------|------------|-----------|----------|
| Prot_dose | 0mg  | 1000mg pcy | 100mg pcy | 10mg pcy |
|           | 815. | 843.       | 779.      | 801.     |

Least significant differences of means (1% level)

|        |           |
|--------|-----------|
| Table  | Prot_dose |
| rep.   | 8         |
| d.f.   | 28        |
| l.s.d. | 88.8      |

Analysis of variance

Variate: Plt

|                     |      |      |      |      |       |
|---------------------|------|------|------|------|-------|
| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---------------------|------|------|------|------|-------|

|           |    |         |       |      |       |
|-----------|----|---------|-------|------|-------|
| Prot_dose | 3  | 17236.  | 5745. | 0.76 | 0.524 |
| Residual  | 28 | 210570. | 7520. |      |       |
| Total     | 31 | 227806. |       |      |       |

#### Tables of means

Variate: Plt

Grand mean 810.

|           |      |            |           |          |
|-----------|------|------------|-----------|----------|
| Prot_dose | 0mg  | 1000mg pcy | 100mg pcy | 10mg pcy |
|           | 815. | 843.       | 779.      | 801.     |

Least significant differences of means (1% level)

|        |           |
|--------|-----------|
| Table  | Prot_dose |
| rep.   | 8         |
| d.f.   | 28        |
| l.s.d. | 88.8      |

#### Analysis of variance

Variate: Plt

|                     |      |         |       |      |       |
|---------------------|------|---------|-------|------|-------|
| Source of variation | d.f. | s.s.    | m.s.  | v.r. | F pr. |
| Prot_dose           | 3    | 17236.  | 5745. | 0.76 | 0.524 |
| Residual            | 28   | 210570. | 7520. |      |       |
| Total               | 31   | 227806. |       |      |       |

#### Tables of means

Variate: Plt

Grand mean 810.

|           |      |            |           |          |
|-----------|------|------------|-----------|----------|
| Prot_dose | 0mg  | 1000mg pcy | 100mg pcy | 10mg pcy |
|           | 815. | 843.       | 779.      | 801.     |

Least significant differences of means (1% level)

|        |           |
|--------|-----------|
| Table  | Prot_dose |
| rep.   | 8         |
| d.f.   | 28        |
| l.s.d. | 88.8      |

#### Analysis of variance

Variate: TBIL

|                     |      |         |        |      |       |
|---------------------|------|---------|--------|------|-------|
| Source of variation | d.f. | s.s.    | m.s.   | v.r. | F pr. |
| Prot_dose           | 3    | 94.180  | 31.393 | 4.16 | 0.015 |
| Residual            | 28   | 211.136 | 7.541  |      |       |
| Total               | 31   | 305.316 |        |      |       |

#### Tables of means

Variate: TBIL

Grand mean 8.57

|           |      |            |           |          |
|-----------|------|------------|-----------|----------|
| Prot_dose | 0mg  | 1000mg pcy | 100mg pcy | 10mg pcy |
|           | 5.88 | 8.46       | 9.46      | 10.49    |

Least significant differences of means (1% level)

|        |           |
|--------|-----------|
| Table  | Prot_dose |
| rep.   | 8         |
| d.f.   | 28        |
| l.s.d. | 2.812     |

#### Analysis of variance

Variate: Caecum\_full

| Source of variation | d.f. | s.s.    | m.s.   | v.r. | F pr. |
|---------------------|------|---------|--------|------|-------|
| Prot_dose           | 3    | 1.9978  | 0.6659 | 1.24 | 0.315 |
| Residual            | 28   | 15.0598 | 0.5378 |      |       |
| Total               | 31   | 17.0575 |        |      |       |

#### Tables of means

Variate: Caecum\_full

Grand mean 2.00

| Prot_dose | 0mg  | 1000mg mcy | 100mg mcy | 10mg mcy |
|-----------|------|------------|-----------|----------|
|           | 1.86 | 2.34       | 1.68      | 2.11     |

#### Analysis of variance

Variate: Empty\_Caecum

| Source of variation | d.f. | s.s.    | m.s.    | v.r. | F pr. |
|---------------------|------|---------|---------|------|-------|
| Prot_dose           | 3    | 0.01338 | 0.00446 | 0.29 | 0.832 |
| Residual            | 28   | 0.43004 | 0.01536 |      |       |
| Total               | 31   | 0.44342 |         |      |       |

#### Tables of means

Variate: Empty\_Caecum

Grand mean 0.532

| Prot_dose | 0     | 1000mg mcy | 100mg mcy | 10mg mcy |
|-----------|-------|------------|-----------|----------|
|           | 0.510 | 0.555      | 0.512     | 0.549    |

#### Least significant differences of means (1% level)

| Table  | Prot_dose |
|--------|-----------|
| rep.   | 8         |
| d.f.   | 28        |
| l.s.d. | 0.1269    |

#### Analysis of variance

Variate: Heart

| Source of variation | d.f.(m.v.) | s.s.     | m.s.     | v.r. | F pr. |
|---------------------|------------|----------|----------|------|-------|
| Prot_dose           | 3          | 0.001518 | 0.000506 | 0.10 | 0.957 |
| Residual            | 26(2)      | 0.127169 | 0.004891 |      |       |
| Total               | 29(2)      | 0.128429 |          |      |       |

#### Tables of means

Variate: Heart

Grand mean 0.444

| Prot_dose | 0mg   | 1000mg mcy | 100mg mcy | 10mg mcy |
|-----------|-------|------------|-----------|----------|
|           | 0.450 | 0.433      | 0.444     | 0.450    |

#### Least significant differences of means (1% level)

| Table  | Prot_dose |
|--------|-----------|
| rep.   | 8         |
| d.f.   | 26        |
| l.s.d. | 0.0719    |

#### Analysis of variance

Variate: Kidney

| Source of variation | d.f. | s.s.    | m.s.    | v.r. | F pr. |
|---------------------|------|---------|---------|------|-------|
| Prot_dose           | 3    | 0.05930 | 0.01977 | 1.09 | 0.371 |
| Residual            | 28   | 0.50977 | 0.01821 |      |       |
| Total               | 31   | 0.56907 |         |      |       |

Tables of means

Variate: Kidney

Grand mean 0.980

| Prot_dose | 0mg   | 1000mg mcy | 100mg mcy | 10mg mcy |
|-----------|-------|------------|-----------|----------|
|           | 1.041 | 0.924      | 0.994     | 0.963    |

Least significant differences of means (1% level)

| Table  | Prot_dose |
|--------|-----------|
| rep.   | 8         |
| d.f.   | 28        |
| l.s.d. | 0.1382    |

Analysis of variance

Variate: Liver

| Source of variation | d.f. | s.s.    | m.s.   | v.r. | F pr. |
|---------------------|------|---------|--------|------|-------|
| Prot_dose           | 3    | 2.0967  | 0.6989 | 1.70 | 0.190 |
| Residual            | 28   | 11.5349 | 0.4120 |      |       |
| Total               | 31   | 13.6316 |        |      |       |

Tables of means

Variate: Liver

Grand mean 4.49

| Prot_dose | 0    | 1000mg mcy | 100mg mcy | 10mg mcy |
|-----------|------|------------|-----------|----------|
|           | 4.40 | 4.89       | 4.47      | 4.19     |

Least significant differences of means (1% level)

| Table  | Prot_dose |
|--------|-----------|
| rep.   | 8         |
| d.f.   | 28        |
| l.s.d. | 0.657     |

Analysis of variance

Variate: Lymphnodes

| Source of variation | d.f. | s.s.     | m.s.     | v.r. | F pr. |
|---------------------|------|----------|----------|------|-------|
| Prot_dose           | 3    | 0.015153 | 0.005051 | 2.26 | 0.103 |
| Residual            | 28   | 0.062599 | 0.002236 |      |       |
| Total               | 31   | 0.077752 |          |      |       |

Tables of means

Variate: Lymphnodes

Grand mean 0.1085

| Prot_dose | 0mg    | 1000mg mcy | 100mg mcy | 10mg mcy |
|-----------|--------|------------|-----------|----------|
|           | 0.1011 | 0.1440     | 0.0849    | 0.1039   |

Least significant differences of means (1% level)

| Table  | Prot_dose |
|--------|-----------|
| rep.   | 8         |
| d.f.   | 28        |
| l.s.d. | 0.04843   |

Analysis of variance

Variate: Pancreas

| Source of variation | d.f. | s.s.     | m.s.     | v.r. | F pr. |
|---------------------|------|----------|----------|------|-------|
| Prot_dose           | 3    | 0.069362 | 0.023121 | 2.76 | 0.061 |

|          |    |          |          |
|----------|----|----------|----------|
| Residual | 28 | 0.234725 | 0.008383 |
| Total    | 31 | 0.304088 |          |

Tables of means  
 Variate: Pancreas  
 Grand mean 0.303

|           |       |            |           |          |
|-----------|-------|------------|-----------|----------|
| Prot_dose | 0mg   | 1000mg mcy | 100mg mcy | 10mg mcy |
|           | 0.285 | 0.369      | 0.318     | 0.241    |

Least significant differences of means (1% level)

|        |           |
|--------|-----------|
| Table  | Prot_dose |
| rep.   | 8         |
| d.f.   | 28        |
| l.s.d. | 0.0938    |

Analysis of variance

Variate: Spleen

|                     |      |         |         |      |       |
|---------------------|------|---------|---------|------|-------|
| Source of variation | d.f. | s.s.    | m.s.    | v.r. | F pr. |
| Prot_dose           | 3    | 0.01806 | 0.00602 | 0.31 | 0.818 |
| Residual            | 28   | 0.54272 | 0.01938 |      |       |
| Total               | 31   | 0.56079 |         |      |       |

Tables of residuals

Variate: Spleen

Tables of means

Variate: Spleen

Grand mean 0.356

|           |       |            |           |          |
|-----------|-------|------------|-----------|----------|
| Prot_dose | 0     | 1000mg mcy | 100mg mcy | 10mg mcy |
|           | 0.338 | 0.395      | 0.354     | 0.336    |

Least significant differences of means (1% level)

|        |           |
|--------|-----------|
| Table  | Prot_dose |
| rep.   | 8         |
| d.f.   | 28        |
| l.s.d. | 0.1426    |

Analysis of variance

Variate: Testes

|                     |            |         |         |      |       |
|---------------------|------------|---------|---------|------|-------|
| Source of variation | d.f.(m.v.) | s.s.    | m.s.    | v.r. | F pr. |
| Prot_dose           | 3          | 0.18202 | 0.06067 | 2.07 | 0.154 |
| Residual            | 13(15)     | 0.38180 | 0.02937 |      |       |
| Total               | 16(15)     | 0.47731 |         |      |       |

Tables of means

Variate: Testes

Grand mean 1.308

|           |      |            |           |          |
|-----------|------|------------|-----------|----------|
| Prot_dose | 0mg  | 1000mg mcy | 100mg mcy | 10mg mcy |
|           | 1.26 | 1.205      | 1.385     | 1.376    |

Least significant differences of means (1% level)

|        |           |
|--------|-----------|
| Table  | Prot_dose |
| rep.   | 8         |
| d.f.   | 13        |
| l.s.d. | 0.1851    |

Analysis of variance

Variate: Thymus

| Source of variation | d.f. | s.s.     | m.s.     | v.r. | F pr. |
|---------------------|------|----------|----------|------|-------|
| Prot_dose           | 3    | 0.011575 | 0.003858 | 0.75 | 0.531 |
| Residual            | 28   | 0.143825 | 0.005137 |      |       |
| Total               | 31   | 0.155400 |          |      |       |

#### Tables of means

Variate: Thymus

Grand mean 0.315

| Prot_dose | 0mg   | 1000mg mcy | 100mg mcy | 10mg mcy |
|-----------|-------|------------|-----------|----------|
|           | 0.340 | 0.293      | 0.326     | 0.301    |

Least significant differences of means (1% level)

| Table  | Prot_dose |
|--------|-----------|
| rep.   | 8         |
| d.f.   | 28        |
| l.s.d. | 0.0734    |

#### Analysis of variance

Variate: Caecum\_full

| Source of variation | d.f. | s.s.    | m.s.   | v.r. | F pr. |
|---------------------|------|---------|--------|------|-------|
| Prot_dose           | 3    | 1.0546  | 0.3515 | 0.93 | 0.437 |
| Residual            | 28   | 10.5333 | 0.3762 |      |       |
| Total               | 31   | 11.5879 |        |      |       |

#### Tables of means

Variate: Caecum\_full

Grand mean 2.14

| Prot_dose | 0    | 1000mg pcy | 100mg pcy | 10mg pcy |
|-----------|------|------------|-----------|----------|
|           | 1.86 | 2.31       | 2.10      | 2.29     |

Least significant differences of means (1% level)

| Table  | Prot_dose |
|--------|-----------|
| rep.   | 8         |
| d.f.   | 28        |
| l.s.d. | 0.628     |

#### Analysis of variance

Variate: Empty\_Caecum

| Source of variation | d.f. | s.s.    | m.s.    | v.r. | F pr. |
|---------------------|------|---------|---------|------|-------|
| Prot_dose           | 3    | 0.03356 | 0.01119 | 0.78 | 0.513 |
| Residual            | 28   | 0.39929 | 0.01426 |      |       |
| Total               | 31   | 0.43285 |         |      |       |

#### Tables of means

Variate: Empty\_Caecum

Grand mean 0.507

| Prot_dose | 0mg   | 1000mg pcy | 100mg pcy | 10mg pcy |
|-----------|-------|------------|-----------|----------|
|           | 0.510 | 0.527      | 0.454     | 0.537    |

Least significant differences of means (1% level)

| Table  | Prot_dose |
|--------|-----------|
| rep.   | 8         |
| d.f.   | 28        |
| l.s.d. | 0.1223    |

# Analysis of variance

Variate: Heart

| Source of variation | d.f. | s.s.     | m.s.     | v.r. | F pr. |
|---------------------|------|----------|----------|------|-------|
| Prot_dose           | 3    | 0.025375 | 0.008458 | 3.52 | 0.028 |
| Residual            | 28   | 0.067225 | 0.002401 |      |       |
| Total               | 31   | 0.092600 |          |      |       |

## Tables of means

Variate: Heart

Grand mean 0.4175

| Prot_dose | 0      | 1000mg pcy | 100mg pcy | 10mg pcy |
|-----------|--------|------------|-----------|----------|
|           | 0.4500 | 0.3725     | 0.4263    | 0.4213   |

Least significant differences of means (1% level)

| Table  | Prot_dose |
|--------|-----------|
| rep.   | 8         |
| d.f.   | 28        |
| l.s.d. | 0.05018   |

# Analysis of variance

Variate: Kidney

| Source of variation | d.f. | s.s.     | m.s.     | v.r. | F pr. |
|---------------------|------|----------|----------|------|-------|
| Prot_dose           | 3    | 0.187020 | 0.062340 | 8.44 | <.001 |
| Residual            | 28   | 0.206851 | 0.007388 |      |       |
| Total               | 31   | 0.393871 |          |      |       |

## Tables of means

Variate: Kidney

Grand mean 0.911

| Prot_dose | 0     | 1000mg pcy | 100mg pcy | 10mg pcy |
|-----------|-------|------------|-----------|----------|
|           | 1.041 | 0.886      | 0.868     | 0.849    |

Least significant differences of means (1% level)

| Table  | Prot_dose |
|--------|-----------|
| rep.   | 8         |
| d.f.   | 28        |
| l.s.d. | 0.0880    |

# Analysis of variance

Variate: Liver

| Source of variation | d.f. | s.s.    | m.s.   | v.r. | F pr. |
|---------------------|------|---------|--------|------|-------|
| Prot_dose           | 3    | 3.3260  | 1.1087 | 2.61 | 0.071 |
| Residual            | 28   | 11.9039 | 0.4251 |      |       |
| Total               | 31   | 15.2299 |        |      |       |

## Tables of means

Variate: Liver

Grand mean 4.86

| Prot_dose | 0mg  | 1000mg pcy | 100mg pcy | 10mg pcy |
|-----------|------|------------|-----------|----------|
|           | 4.40 | 5.30       | 4.94      | 4.80     |

Least significant differences of means (1% level)

| Table  | Prot_dose |
|--------|-----------|
| rep.   | 8         |
| d.f.   | 28        |
| l.s.d. | 0.668     |

# Analysis of variance

Variate: Liver

| Source of variation | d.f. | s.s.    | m.s.   | v.r. | F     | pr. |
|---------------------|------|---------|--------|------|-------|-----|
| Prot_dose           | 3    | 3.3260  | 1.1087 | 2.61 | 0.071 |     |
| Residual            | 28   | 11.9039 | 0.4251 |      |       |     |
| Total               | 31   | 15.2299 |        |      |       |     |

## Tables of means

Variate: Liver

Grand mean 4.86

| Prot_dose | 0mg  | 1000mg pcy | 100mg pcy | 10mg pcy |
|-----------|------|------------|-----------|----------|
|           | 4.40 | 5.30       | 4.94      | 4.80     |

Least significant differences of means (1% level)

| Table  | Prot_dose |
|--------|-----------|
| rep.   | 8         |
| d.f.   | 28        |
| l.s.d. | 0.668     |

# Analysis of variance

Variate: Lymphnodes

| Source of variation | d.f. | s.s.     | m.s.     | v.r. | F     | pr. |
|---------------------|------|----------|----------|------|-------|-----|
| Prot_dose           | 3    | 0.013219 | 0.004406 | 3.14 | 0.041 |     |
| Residual            | 28   | 0.039236 | 0.001401 |      |       |     |
| Total               | 31   | 0.052455 |          |      |       |     |

## Tables of means

Variate: Lymphnodes

Grand mean 0.1293

| Prot_dose | 0mg    | 1000mg pcy | 100mg pcy | 10mg pcy |
|-----------|--------|------------|-----------|----------|
|           | 0.1011 | 0.1200     | 0.1425    | 0.1537   |

Least significant differences of means (1% level)

| Table  | Prot_dose |
|--------|-----------|
| rep.   | 8         |
| d.f.   | 28        |
| l.s.d. | 0.03834   |

# Analysis of variance

Variate: Pancreas

| Source of variation | d.f. | s.s.     | m.s.     | v.r. | F     | pr. |
|---------------------|------|----------|----------|------|-------|-----|
| Prot_dose           | 3    | 0.047184 | 0.015728 | 4.29 | 0.013 |     |
| Residual            | 28   | 0.102687 | 0.003667 |      |       |     |
| Total               | 31   | 0.149872 |          |      |       |     |

## Tables of means

Variate: Pancreas

Grand mean 0.319

| Prot_dose | 0mg   | 1000mg pcy | 100mg pcy | 10mg pcy |
|-----------|-------|------------|-----------|----------|
|           | 0.285 | 0.316      | 0.292     | 0.383    |

Least significant differences of means (1% level)

| Table  | Prot_dose |
|--------|-----------|
| rep.   | 8         |
| d.f.   | 28        |
| l.s.d. | 0.0620    |

# Analysis of variance

Variate: Spleen

| Source of variation | d.f. | s.s.    | m.s.    | v.r. | F pr. |
|---------------------|------|---------|---------|------|-------|
| Prot_dose           | 3    | 0.03798 | 0.01266 | 1.03 | 0.396 |
| Residual            | 28   | 0.34539 | 0.01234 |      |       |
| Total               | 31   | 0.38337 |         |      |       |

Tables of means

Variate: Spleen

Grand mean 0.344

| Prot_dose | 0mg   | 1000mg pcy | 100mg pcy | 10mg pcy |
|-----------|-------|------------|-----------|----------|
|           | 0.338 | 0.391      | 0.295     | 0.353    |

Least significant differences of means (1% level)

| Table  | Prot_dose |
|--------|-----------|
| rep.   | 8         |
| d.f.   | 28        |
| l.s.d. | 0.1138    |

Analysis of variance

Variate: Testes

| Source of variation | d.f.(m.v.) | s.s.    | m.s.    | v.r. | F pr. |
|---------------------|------------|---------|---------|------|-------|
| Prot_dose           | 3          | 0.04154 | 0.01385 | 0.56 | 0.653 |
| Residual            | 13(15)     | 0.32375 | 0.02490 |      |       |
| Total               | 16(15)     | 0.34465 |         |      |       |

Tables of means

Variate: Testes

Grand mean 1.288

| Prot_dose | 0mg   | 1000mg pcy | 100mg pcy | 10mg pcy |
|-----------|-------|------------|-----------|----------|
|           | 1.268 | 1.253      | 1.286     | 1.347    |

Least significant differences of means (1% level)

| Table  | Prot_dose |
|--------|-----------|
| rep.   | 8         |
| d.f.   | 13        |
| l.s.d. | 0.1705    |

Analysis of variance

Variate: Thymus

| Source of variation | d.f. | s.s.     | m.s.     | v.r. | F pr. |
|---------------------|------|----------|----------|------|-------|
| Prot_dose           | 3    | 0.013112 | 0.004371 | 0.58 | 0.636 |
| Residual            | 28   | 0.212575 | 0.007592 |      |       |
| Total               | 31   | 0.225688 |          |      |       |

Tables of means

Variate: Thymus

Grand mean 0.308

| Prot_dose | 0mg   | 1000mg pcy | 100mg pcy | 10mg pcy |
|-----------|-------|------------|-----------|----------|
|           | 0.340 | 0.291      | 0.311     | 0.290    |

Least significant differences of means (1% level)

| Table  | Prot_dose |
|--------|-----------|
| rep.   | 8         |
| d.f.   | 28        |
| l.s.d. | 0.0892    |