

**Antifungal activity of Cowpea
(*Vigna unguiculata L. Walp*) proteins:
Efficacy, shelf life extension
and sensory effects in bread**

By

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A thesis submitted for the degree of
Doctor of Philosophy

Heriot-Watt University

School of Life Sciences

Edinburgh

June 2015

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ABSTRACT

Natural food preservatives extracted from plants are an emerging market in the food industry. Cowpea *Vigna unguiculata* L. Walp is a well-established crop around the world, and recent research has revealed that it contains multiple proteins, with potent antifungal and antiviral properties. Exploitation of the antifungal properties of cowpea protein is limited by the lack of characterisation of antifungal proteins, and the lack of knowledge about their efficacy when added to food. Furthermore, the mechanism of antifungal protein activity is not understood fully, and therefore requires further investigation. This study aimed to evaluate the antifungal potency of cowpea seed protein isolate (CPI) against bread yeasts and moulds using microbiological tests, and in a leavened bread application. The research investigated the effects of CPI on shelf life, and the sensory and textural acceptability of bread. Further aims included the characterisation of proteins in CPI with antifungal activity, and establishing an understanding of antifungal activity using computer modelling.

The first stage of the study (Chapter 2) involved the preparation of CPI, separation of high and low molecular weight protein fractions by ultrafiltration, and analysis of electrophoretic profiles using SDS PAGE. Analysis of CPI was performed using LC-MS/MS, and the proteomics results reported for the first time. Twenty- three proteins with lowest hit number (HN) and highest score were selected from the first ninety-nine hits of matching of LC/MS/MS results to an existing database. The two most abundant proteins (with highest score number and lowest HN) were identified as vicilin (mw 49654), which is a storage protein, and then lipoxygenase (mw 97284) which is a metabolism protein.

The second stage of the study (Chapter 3) involved testing CPI activity against fungal growth using a micro spectrophotometric assay (micro plate method) and the spread plate method. Activity was tested against known bread spoilage moulds: *Penicillium chrysogenum*, *Penicillium brevicompactum*, *Penicillium hirsutum*, *Aspergillus versicolor* and *Eurotium rubrum*. The results showed CPI exhibited high antifungal activity against *P. chrysogenum*, *P. brevicompactum*, *P. hirsutum* and *E. rubrum*; no statistically significant effect was seen against *A. versicolor*. CPI exhibited different ranges of inhibition towards the same species at different concentrations demonstrating that the antifungal effect was concentration dependent. The antifungal activity of CPI was unaffected by heat treatment or protease treatment, indicating the antifungal components are heat stable and protease resistant. The antifungal activity of

proteins in the CPI ultrafiltrate (10 kDa molecular weight cut off point) was increased by comparison with CPI and CPI retentate.

The third stage of the study (Chapter 4) involved testing the effect of CPI addition on shelf life, and the sensory and textural properties of leavened bread. A concentration of 2.3% of CPI showed the best resistance to fungal growth during the storage period. No growth was observed throughout the 8 day storage period at room temperature, whereas control samples began to show contamination on the fourth day of storage. The CPI filtrate (2.3%) showed better shelf life extension than CPI after 25 days, confirming the antifungal activity of low molecular weight proteins. Inclusion of 2.3% CPI did not significantly affect the sensory or textural acceptability of the bread. Although the hardness of bread containing CPI increased significantly after 3 days compared to the control, sensory acceptability was within acceptable limits.

The fourth stage of the study (Chapter 5) involved understanding the mechanism of action of defensins at the fungal cell wall. The hypothesis for defensin as a major antifungal protein in CPI was based on the results of the antifungal activity of the low molecular weight fraction of CPI, the heat stable and protease resistant antifungal properties of CPI, and confirmation of the presence of defensins in the proteomics results. The computer model detailing the simulation of defensin activity showed the adsorption of defensin molecules to the surface of a phospholipid bilayer membrane leading to a disordering of the membrane that would ultimately lead to disruption of cell metabolism.

ACKNOWLEDGEMENTS

Special thanks to my supervisor, Dr. Lydia Campbell, from the School of Life Sciences, Heriot-Watt University, who helped me complete this work through her continuous supervision. I also express my thanks to Dr. Stephen Euston, my second supervisor, for his assistance in the design of the computer model and his advice during the studies. I am also grateful to Dr. Peter C. Morris for always being there when I needed him.

Special thanks to my dear husband (Matab) for his support, encouragement and unlimited assistance, which has had a significant impact on my ability to complete this thesis.

My gratitude goes to my wonderful family, my sisters and my brother, for their never-ending support, and to my son (Bader) and my daughters (Adil and Bateel) for their support and unlimited encouragement, which enabled me to complete this work.

Words are powerless to express my gratitude to my mother. Your outstanding guidance, great support and encouragement throughout my life have helped me to achieve my goals and to become more than I dreamed. I cannot thank you enough.

I am very grateful to my friends for their support and advice during my study.

Thank you so much to all staff members and PhD students at the School of Life Sciences, Heriot-Watt University.

I would like to express my gratitude and appreciation to Mrs Vicky for her help and to Prof. Cait MacPhee at the University of Edinburgh for her assistance in the analysis of the CPI.

DECLARATION

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ABBREVIATIONS

AACC:	American Association of Cereal Chemists
AMPs:	Antimicrobial peptides
ARG:	Arginine
°C:	Degrees Celsius
CFU:	Colony forming units
CPI:	Cowpea protein isolate
DPPC:	Di-phosphatidylcholine
DSSP:	Hydrogen bond estimation algorithm
GROMACS:	GRONingen MACHine for Chemical Simulation
GSTs:	Glutathione S-Transferases
HN:	Hit number
HSPs:	Heat shock proteins
LC-MSMS:	Liquid-chromatography, coupled with tandem mass spectrometry
LOXs:	Lipoxygenases
LTPs:	Lipid transfer proteins
LYS:	Lysine
MIC:	Lethal or minimum inhibitory concentration
Mins:	Minutes
MOWSE:	Molecular weight search
MW:	Molecular weight
MWCO:	Molecular weight cut-off
NLS:	Nuclear localization signal
NMR:	Nuclear magnetic resonance
NVT:	Constant particle numbers N, volume V and temperature T
OEO:	Oregano essential oil
PDA:	Potato dextrose agar
PGK:	Phosphoglycerate kinase
PME:	Particle mesh Ewald
RG:	Radius of gyration

RMSD:	Root mean square displacement
SD:	Standard deviation
SDS:	Sodium dodecyl sulphate
SDS-PAGE:	SDS polyacrylamide gel electrophoresis
SPC:	Simple point charge
TCA:	Trichloroacetic acid
UF:	Ultrafiltration

Chapter 1

General introduction

Increasing consumer demands for safe foods free from chemical preservatives have generated interest in the use of natural antimicrobials. Many studies clearly demonstrate the importance of natural food preservatives as safe and acceptable additives (Cushnie and Lamb, 2005; Hauser et al., 2014). During the last decade, many researchers in different countries have been seeking to determine the efficacy of plant products that exhibit antimicrobial properties (see **Table 1.1**) (Burt, 2004; Burt and Reinders, 2003; Castello et al., 2002; Choi et al., 1997; Cruz et al., 2013; Cushnie and Lamb, 2005; Dorman and Deans, 2000; Giuseppe et al., 2009; Hauser et al., 2014; Ho et al., 2007; Magro et al., 2006; Mohameda et al., 2014; Negi, 2012; Ng, 2004; Terras et al., 1992; Wang and Ng, 2005). The antimicrobial compounds in plant extracts include polyphenols, flavonoids, tannins, alkaloids, terpenoids, isothiocyanates, lectins, polypeptides, and their oxygen-substituted derivatives (Choi et al., 1997; Devilliers et al., 2008; Nguefack et al., 2004; Olasupo et al., 2003; Ye et al., 1999; Ye and Ng, 2000).

To be accepted as a food additive, a natural food preservative must be easily extractable and cost-effective to produce. Cruz et al. (2013), Shao et al. (2011), and Singh et al. (2010) exemplify this by discussing the possible case of a useful compound present in a rare tropical plant. They propose that, if it was difficult to cultivate economically, it would be unlikely to be considered as a food preservative in the developed world, unless the gene controlling its production could be expressed in a microorganism, thereby allowing large-scale production by industrial fermentation. However, considerable advances in genetic and protein engineering provide scope for the use of such a technology, albeit that the strong consumer resistance to genetically modified organisms prevents this at present. Whether such consumer concern can be reversed in the future, as the world's food resources diminish, remains uncertain (Tiwari et al., 2009).

Table 1.1. Naturally occurring preservatives derived from plants.

Example of preservative	Typical target organism	References
Clove, (<i>Syzygium aromaticum</i>) derived from flower buds	Clove oil has antibacterial activity targeting gram-negative anaerobic bacteria. It is also active against <i>Aspergillus</i> , and <i>Penicillium</i> genera.	Shan et al. (2009)
Rosemary	Active against <i>Escherichia coli</i>	Soylu et al. (2006) Soylu et al. (2010)
Oregano	Active against <i>Listeria monocytogenes</i> <i>E. coli</i>	Soylu et al. (2006) Soylu et al. (2010) Zabka et al. (2009)
Malva (<i>Malva sylvestris</i> L.) aqueous extracts	<i>Aspergillus candidus</i> , <i>A. niger</i> , <i>Penicillium</i> sp., and <i>Fusarium culmorum</i>	Magro et al. (2006)
Sloe berries	<i>Salmonella</i> spp	Soylu et al. (2010)
Ginger (<i>Zingiber officinale</i>)	<i>E. coli</i> , <i>Bacillus</i> spp, <i>Salmonella</i> spp	Singh et al. (2007)

There has been much research into pathogenesis-related proteins, focusing on their ability to provide resistance to pathogenic fungi, bacteria, and viruses. Organisms such as fungi, bacteria, and plants produce a variety of antifungal proteins, which have played a critical role in food and medicinal applications (Wong et al., 2010).

In order to survive, plants have developed their own defence mechanisms to fight pathogenic fungi; this includes synthesising antifungal low-molecular-weight compounds, proteins and peptides (Aerts et al., 2008; Datta and Muthukrishnan, 1999; Selltrechnikoff, 2001).

Antifungal proteins are able to demonstrate a range of antimicrobial properties (Datta and Muthukrishnan, 1999; Rivillas and Soriano, 2007; Selltrechnikoff, 2001; Wang and Ng, 2002; Wang and Ng, 2005; Ye et al., 1999; Ye and Ng 2005), and some research publications have shown that cowpea seeds contain multiple proteins with potent antifungal and antiviral properties (Datta and Muthukrishnan, 1999; Gomes et al., 1998; Mine et al., 2010; Negi, 2012; Ng, 2004; Rose et al., 2006; Ye et al., 2000a; Ye et al., 2000b). Furthermore, an investigation into antifungal proteins extracted from esculent seeds found them to be promising, low-cost, and natural food preservatives, applicable for use in many food applications (Negi, 2012).

1.1 Background literature study

1.1.1 *Food spoilage*

Food spoilage can be defined as “any sensory change (tactile, visual, olfactory or flavour) which the consumer considers to be unacceptable”. Microbial food spoilage can occur in response to the growth of bacteria, yeasts and moulds.

The main mechanisms involved in food spoilage are:

- Microbiological spoilage, sometimes accompanied by the development of pathogens.
- Chemical and enzymatic activity, leading to lipid breakdown or changes in colour, odour, flavour or texture.
- Migration of moisture and/or other vapour, resulting in changes in texture, water activity and flavour.

Variables (both formulation and processing) that impact these mechanisms, and which can be implemented to control deterioration, include: (1) moisture and water activity; (2) pH; (3) heat treatments; (4) emulsifier systems; (5) preservatives and additives; and (6) packaging. The safety and quality of foods can be affected by chemical, physical and microbiological factors.

The corruption of food by microbial contamination is one of the main causes of financial losses in the food industry, and of food-borne diseases. Spoilage resulting from yeasts and moulds can often be clearly seen, appearing as discoloured areas on the surface of the food, enabling it to be removed before consumption. Spoilage is also evident from the production of gas, or alterations to the smell or taste of a product. This type of spoilage can go undetected until consumption. Cases of spoilage have a negative impact on both the consumer, and the manufacturer. The cost of spoilage is high for the manufacturers; moreover, unknowingly using raw materials contaminated with yeasts or moulds can lead to many batches of a final product being rejected. Moreover, disposal of spoiled products, and the subsequent investigations and cleaning procedures result in additional expenditure (Filtenborg et al., 1996).

1.1.2 *Yeasts and moulds*

Yeasts and moulds are able to survive in a wide range of environments; for example, in plants, animal products, soil, water, and insects. Their enormous capacity for coexistence can be explained by the fact that yeasts and moulds have the ability to consume a diversity

of substrates, such as pectins and other carbohydrates, organic acids, proteins and lipids. Furthermore, yeasts and moulds are comparatively tolerant to low pH, low water activity, low temperatures, and the presence of preservatives. In addition, yeasts can utilise food ingredients, such as organic acids like lactic, citric, and acetic acids, which are commonly considered to be effective against many kinds of microorganisms (Filtenborg et al., 1996).

1.1.3 Moulds (*Mycotoxins*)

It has been discovered that mould growth can cause numerous kinds of food spoilage: off-flavours, toxins, discolouration, and the formation of pathogenic or allergenic compounds and mycotoxins, which cause a loss of quality (Aziz et al., 1997; Chelkowski and Cierniewska, 1983; Jespersen et al., 1994; Nguefack et al., 2004; Nielsen and Rios, 2000; Perkowski et al., 1991; Prakash et al., 2015; Steyn and Stander, 1999).

Mycotoxins are secondary metabolites, with a low molecular weight, and are formed by filamentous fungi, generally in the late exponential or early stationary phases of growth. These can lead to many serious diseases, including some forms of cancer, immune suppression, and nerve defects (Steyn and Stander, 1999; Watanabe et al., 2006).

In addition to fungal growth, other factors play an important role in the production of toxins, including the nutritional level of food, environmental conditions, and the method of producing, handling and storing food products. Studies carried out by Arroyo (2003) found that many toxins are secreted by moulds, although the production of mycotoxins is common in food spoilage moulds. *Aspergillus*, *Penicillium* and *Fusarium* are considered the most important mycotoxigenic species present in food materials.

1.1.4 Factors involved in promoting food spoilage

The microbial activity of a wide range of possible microorganisms results in the spoilage of food and beverages. The microbial activity responsible for attacking a specific food or beverage depends on the features of the product, and the techniques used for processing and storing it. The groups of factors affecting the propagation of microorganisms in food described by Huis, (1996); Mossel and Westerdijk (1949) are: (i) intrinsic parameters; (ii) extrinsic parameters; (iii) methods of processing and preservation. It is essential to understand that any of the above-mentioned factors will impact the effect of others. Consequently, the effect of a combination of parameters is generally higher than the observed effect of a single parameter (Huis, 1996).

- Intrinsic parameters

Intrinsic parameters are considered as the physical, chemical and structural properties that are essential within the food itself. The most vital intrinsic factors are: water activity, pH, ionic strength, nutrients, and natural antimicrobial materials.

- Extrinsic parameters

Extrinsic parameters are those elements of the environment in which a food is stored, in particular, temperature, humidity, and ambient conditions.

- Mode of handling and preservation

Repeated physical or chemical treatments can result in modifications in the characteristics of food stuffs, as a result of the microorganisms associated with the products used in treatment.

According to Pitt and Hocking (1987), the principal intrinsic factors affecting microbial growth are:

- Water activity
- pH (Hydrogen ion concentration)
- Temperature of both processing and storage
- Gas tension, and
- Nutrient status

1.1.4.1 *Water activity*

Water availability in food stuffs is a critical factor associated with microbial spoilage. It affects the efficiency of chemical or physical reactions. Water availability is defined as the ratio of the vapour pressure of the water in the substrate (p) to that of pure water at the same temperature (p^0):

$$a_w = \frac{p}{p^0} \quad (1)$$

If water is tightly bound to food components, the tendency for it to escape from the food as a vapour will be reduced, limiting the partial pressure, and ensuring the water activity is low. a_w is associated with the physical properties of materials, such as: boiling and freezing points, equilibrium relative to humidity, and osmotic pressure.

Water activity ranges from zero (water is absent) to 1.0 (pure water). Higher a_w substances tend to support more microorganisms. Bacteria usually require at least 0.91, and fungi at least 0.7, so water activity can predict which microorganisms might be potential sources of spoilage and infection (Roos, 1993). Food designers use water activity to formulate shelf-stable food. If a product is kept below a certain level of water activity, then mould growth is inhibited, which results in a longer shelf life. Stability of shelf life means the product will not degrade easily (Arroyo, 2003; Powitz, 2007; Roos, 1993).

It is well known that the water activity plays an important role in several processes such as reducing non-enzymatic browning reactions, spontaneous autocatalytic lipid oxidation reactions, and affecting the activity of enzymes and vitamins. A large number of fungi species are xerotolerant (they have the ability to grow in environments with very low water activity as low as 0.65), and are able to grow in foods such as dried fruits, nuts, grains and spices (Arroyo, 2003; Sandulachi, 2012).

1.1.4.2 *pH (hydrogen ion concentration)*

Most fungi are not significantly affected by pH; some fungi are able to grow at pH levels as low as 2, while yeast can survive at a pH below 1.5. This allows them to live in highly acidic environments, for example, in fruit juices and pulps (Taniwaki et al., 2009).

1.1.4.3 *Temperature of both processing and storage*

The temperature of processing and storage is a key factor affecting microbial growth. Furthermore, there are some heat-resistant organisms, called thermo-tolerant fungi, which are able to grow in both moderate and high temperature conditions (Meyers et al., 1987). Some species can also exist across extreme temperature ranges, growing at temperatures from 0°C to 47°C. Numerous species can grow at low temperatures and in environments with a low pH, creating problems for the shelf life of fermented milk products.

1.1.4.4 *Gas tension specifically of oxygen and carbon dioxide*

According to Miller and Golding (1949) and Pitt and Hocking (1987), the concentration of oxygen dissolved in the substrate has a much greater influence on fungal growth than atmospheric oxygen. The impact of carbon dioxide is to increase both the lag phase and the generation time of spoilage microorganisms; however, the exact mechanism for the bacteriostatic impact is unknown so far. Generally, carbon dioxide effectiveness rises with concentration, but high levels increase the likelihood of conditions being created where

pathogenic organisms such as *Clostridium botulinum* can survive (Daniels et al., 1985; Taniwaki et al., 2009).

1.1.4.5 Nutrient status

In general, microorganisms require a variety of nutrients such as nitrogen, vitamins and minerals to grow; therefore, nutrient-rich food offers good conditions, allowing microorganisms to proliferate (Pitt and Hocking, 1987).

1.2 Food preservation

1.2.1 Methods used in food preservation

1.2.1.1 Physical methods

Over the years many mechanisms have been employed during food production to influence microbial growth, including heat treatment, refrigeration, irradiation, and dehydration.

Physical techniques have also been developed to obtain fresh and safe foods. Nowadays there are new and different techniques, such as high-pressure processing, aseptic packaging, and pulse electric fields, all of which have been used successfully to keep multiple types of food safe and fresh. It is also possible to combine two methods of preservation to heighten microbial inhibition. In some cases, heat treatments are used, such as pasteurisation and sterilisation, to inhibit or kill microbes, while heat-resistant microbes are managed by controlling other factors, such as pH and water activity.

1.2.1.2 Chemical methods

Chemical methods are chemical components that can inhibit the growth of microorganisms, known as antimicrobial agents. Antimicrobial agents are classified into two groups, either those inhibiting the growth of fungi, or those destroying it.

Clearly all the preservatives used to keep food safe from contamination must be completely safe for human consumption. For this reason, most countries are increasingly limiting the use of chemical preservatives in certain types of foods (Meyers et al. 1987). A study by Singh et al. (2010) reported that preservatives can be classified into two types depending on their source of origin: artificial preservatives, and natural preservatives.

1.2.1.3 Artificial preservatives

Artificial preservatives are synthetic chemical agents used to extend the shelf life of products, by protecting them from invading microbes (Arroyo, 2003; Rasooli, 2007). Examples of this category of preservatives permitted for use in food within the European Union are listed in **Table 1.2**.

Table 1.2. Preservatives permitted for use in food within the European Union.

E200 Sorbic acid	E224 Potassium metabisulfite	E261 Potassium acetate
E303 Potassium sorbate	E226 Calcium Sulfite	E262 Sodium acetate
E203 Calcium sorbate	E227 Calcium hydrogen sulphite	E263 Calcium acetate
E210 Enzonic acid	E228 Potassium hydrogen sulphite	E270 Lactic acid
E211 Sodium benzoate	E230 Biphenil	E280 Propionic acid
E212 Potassium benzoate	E231 Ortophenil phenol	E281 Sodium propionate
E213 Calcium benzoate	E232 Sodium ortophenil phenolate	E282 Calcium propionate
E214 Ethyl <i>p</i> -hydroxybenzoate	E233 Thiabendazol	E283 Potassium propionate
E215 Sodium ethyl <i>p</i> -hydroxybenzoate	E234 Nisin	E284 Boric acid
E216 Propyl <i>p</i> -hydroxybenzoate	E235 Natamycin	E285 Sodium tetraborate
E217 Soduim propyl <i>p</i> -hydroxybenzoate	E239 Hexamethylene	E290 Carbon dioxide
E218 Methyl <i>p</i> -hydroxybenzoate	E242 Dimethyl dicarbonate	E941 Nitrogen
E219 Soduim propyl <i>p</i> -hydroxybenzoate	E249 Potassium nitrite	E1105 Lysozyme
E220 Sulfur dioxide	E250 Acetic acid	
E221 Sodium sulphite	E251 Sodium nitrate	
E222 Sodium bisulfite	E252 Potassium nitrate	

Source: Luck and Jager (1997)

1.2.1.4 Natural preservatives

Naturally occurring preservatives include nisin and natamycin. Nisin is produced by the lactic acid bacterium, *Lactococcus lactis* sub sp. It is active against many Gram-positive bacteria, and especially against those that produce heat-resistant spores, but it has no or little activity against Gram-negative bacteria, yeasts and moulds. Nisin was first introduced as a food preservative in 1953 (Arroyo, 2003).

The polyene macrolide antifungal compound, natamycin (formerly pimaricin) can also be considered natural, because it is produced by fermentation of the bacterium *Streptomyces natalensis* and closely related species. The name, natamycin, is derived from the discovery

of this strain in 1955 in a soil sample from Natal Province in South Africa (Luck and Jager, 1997). Natamycin is now produced commercially, and is approved for use as food preservative worldwide, mainly for the surface treatment of cheese and dried sausages.

1.2.1.5 *Natural antimicrobials derived from plants*

Plants protect themselves against microorganisms and other predators by synthesising a wide range of compounds. These compounds include essential oils, phytoalexins, phenolics, and related compounds. Herbs and spices are often rich in such compounds.

A wide variety of spices and herbs possess antimicrobial activity (**Table 1.3**). There are a number of excellent reviews explaining the use of antimicrobials by plants (Aerts et al., 2008; Bertini et al., 2009; Cammue et al., 1995; Carvalho et al., 2001; Castello et al., 2002; Cruz et al., 2013; Dorman and Deans, 2000; Gonzalez et al., 2008; Kedia et al., 2014; Kedia et al., 2015; Liu and Yang, 2012; Lucera et al., 2012; Omidbeygi et al., 2007; Oussalah et al., 2006; Serra et al., 2008; Zampini et al., 2009). Typically, to achieve microbial inhibition, the addition of significant levels of a particular spice or herb will be required. As such, products preserved in this way usually exhibit strong flavours, with the result that their application has largely been confined to foods in which this specific taste, flavour or aroma is either appreciated or not noticed

Table 1.3. Plant compounds used as spices and herbs which have antimicrobial activity.

Plant compounds with antimicrobial activity			
Allspice	Marjoram	Caraway	Paprika
Coffee	Rosemary	Ginger	Turmeric
Lime	Basil	Onion	Clove
Peppermint	Fennel	Tea	Leek
Almond	Mustard	Cardamon	Parsley
Coriander	Sage	Hops	Vanillin
Mandarin	Bay	Orange	Cocoa
Pimento	Garlic	Thyme	Lemon
Anise	Nutmeg	Celery	Horseradish

Source: Beuchat (1994)

According to Arroyo (2003), more than 100,000 natural plant compounds have been investigated, revealing noteworthy structural diversity, and characterisation by low molecular weight. Some of the most commonly studied natural compounds are mentioned below. As can be seen below (**Figure 1.1**), some antimicrobial agents are found in nature.

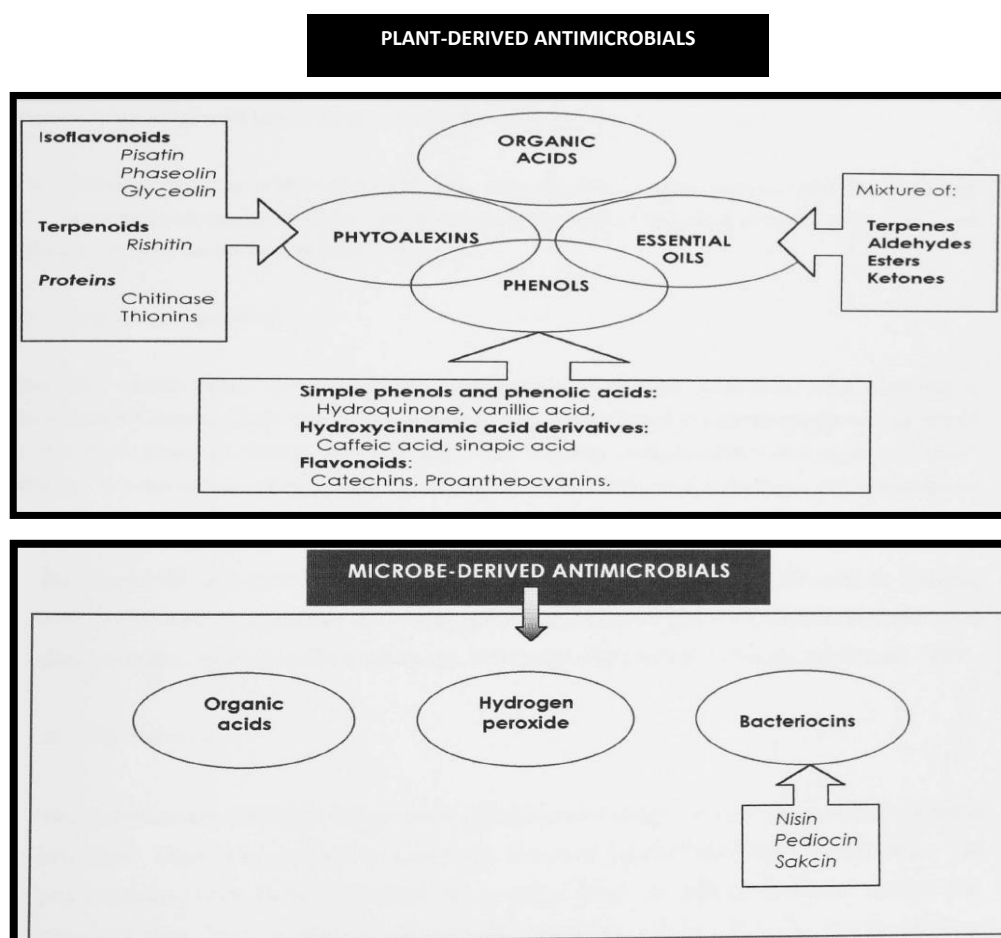


Figure 1.1. Antimicrobial agents found in nature (Source: Arroyo, 2003).

Terpenoids

Terpenoids are considered the main components of essential oils; these are characterised by structural diversity, and terpenes or terpenoids have been further classified into different classes: hemiterpenes (C₅), monoterpenes (C₁₀), sesquiterpenes (C₁₅), diterpenes (C₂₀), sesterterpenes (C₂₅), tetraterpenes/carotenoids (C₄₀). All terpenoid classes have recently been found to contain varying levels of antimicrobial activity. The accumulation of terpenoids in several plant types takes the form of antimicrobial glycosidics (Shao et al., 2011).

Aromatics

All plants produce natural compounds with a carbocyclic or heterocyclic aromatic ring, to which a hydroxyl substituent is attached. The majority of the aromatic compounds derived from plant products are phenols, which are considered a large class of compounds and can be divided into multiple subgroups, such as: simple phenols, phenylpropanoids, flavonoids, tannins and quinines. Examples of phenols with a positive effect in terms of antimicrobial activity are tannic acid, caffeic acid and vanillic acid (Shao et al., 2011).

Alkaloids

Alkaloids are nitrogen containing compounds produced by different plant species; there are two kinds of alkaloids (sanguinarine and chelerythine), which have been proven to be significant active antimicrobials working against bacteria and fungi. Berberine (alkaloid) from the 'smoke tree' which is considered to be representative of the alkaloids group, has shown a positive effect towards combatting *Staphylococcus aureus* (Shao et al., 2011).

Antimicrobial Peptides

Antimicrobial peptides (also called host defense peptides, AMPs) are part of the innate immune response, and are found among all classes of life, such as invertebrates, vertebrates, and plants. AMPs have broad spectrum opposition to a wide range of bacteria, fungi, and viruses (Gupta and Srivastava, 2014). According to Henderson and Lee (2013), AMPs cause the disruption of the cellular membrane of the target pathogen, destroying the cell membrane, and conferring resistance.

1.3 Mechanisms of action of the antimicrobial compounds

The permeability of the cytoplasmic membrane of microorganisms can be altered by different preservative compounds, such as essential oils and phenols, which leads to the

leakage of diverse substances such as ions, ATP, nucleic acids and amino acids (Aerts et al., 2008; Burt and Reinders, 2003; Cushnie and Lamb, 2005). Investigations into the antimicrobial action of phenolic compounds and their unique effects on cellular membranes showed that they cause structural and functional damage to plasma membranes (Lambert et al., 2001).

A study by Lambert et al. (2001) established that oregano essential oil (OEO), as well as thymol and carvacrol, interrupted the cell membrane.

1.4 Bread Spoilage Caused by Yeasts and Moulds

Bread is considered the most important staple food in the world, and is available in many different forms (Legan, 1993).

It is very difficult to evaluate bread losses caused by yeast and mould. Legan (1993) reported on US and German evaluations that 1% or more of total bread manufacture was lost to moulds. A later evaluation from one bakery in the US reported a 5% loss (Legan, 1993). Mould might be responsible for spoiling more than 23,000 tonnes of bread, worth approximately £20 million in the UK annually. In Western Europe, yearly losses may be around 225,000 tonnes of bread, worth nearly £242 million (Legan, 1993; Legan and Voysey, 1991).

The scale of these economic losses in the bread industry alone highlights the importance of conducting further research to identify new natural food preservatives to reduce food spoilage, whilst satisfying customer demands to provide safe, cheap, and healthy preservatives.

1.5 Objectives of the current study

This study will investigate the use of cowpea seed extracts as a natural food preservative in bread making, as summarised below.

Literature search

Cowpea proteins

- Preparation of cowpea seed extract using different methods to find the best extract providing good activity against the selected fungi;
- Preparation of CPI;

- Ultrafiltration of CPI; and
- Characterisation of proteins: SDS-PAGE and LC-MS/MS.

In vitro testing of anti fungal activity

- Testing of cowpea protein in an *in vitro* assay against selected fungi;
- Testing of filtrate and retentate; and
- Testing the effect of proteolysis and heat treatment on the antifungal properties of CPI.

Testing of CPI on shelf life and sensory properties of bread

- Characterisation of bread containing CPI protein in terms of texture, sensory analysis, and shelf life.

Computer simulation

- Computer simulation of the mechanism of the antifungal action of the CPI protein.

Chapter 2

Cowpea protein isolation and characterisation

2.1 Background literature study

Leguminous plants are valuable sources of protein, especially in developing countries, where they can be used as a food source for both animal and human consumption. They are high yield crops and can be produced at a low cost; they have the advantage of containing sufficient amounts of protein, carbohydrates, energy, vitamins and minerals. One such plant is cowpea (shown in **Figure 2.1** below).



Source: <http://r4dreview.org/2010/09/anyone-for-cowpea>.

Figure 2.1. Cowpea as an example of a leguminous plant.

The cowpea (*Vigna unguiculata*) is a leguminous plant, which originated in Ethiopia, and subsequently spread to other warm and dry regions around the world (Black et al., 2008). It is an essential pulse in many places around the world including Africa, India, South America, the USA and European nations. It is known by a variety of names, including niebe, wake, pink-eyes, black-eyed peas, field peas, ewa, and crowders.

2.1.1 Seed characteristics

Cowpea seeds vary in shape, size and colour. They can be globular, kidney, or square shaped, with lengths of between 2 and 12 mm. The weight of the seed can range from 5 to 30g/100 seeds (Black et al., 2008) and they can be red, white, green, brown, or black in colour, with either a smooth or a wrinkled texture as shown in **Figure 2.2** below.



Source: <http://phymap.ucdavis.edu/cowpea/>.

Figure 2.2. Different types of cowpea seeds

Cowpeas can be consumed as shelled green pulses or as dried seeds, and the plant leaves and pods can also be cooked and eaten as a green vegetable. Cowpeas are mainly used in the form of dried seeds, and are widely used in animal feed, playing a critical role in animal rearing (Black et al., 2008).

2.1.2 Nutritive value and composition

According to Singh (1997), a survey in 1991 showed the addition of cowpea to the diet of adults and children had a positive impact in reducing malnutrition. According to Phillips and Baker (1987), cowpea seeds can also be combined with cereals to improve the protein content of the diet. Some studies have reported that cowpea protein can be used in infant weaning foods, and in the preparation of food for diabetic patients, as well as in cold medication (Ellis, 2007; Mine et al., 2010).

However, cowpeas contain some anti-nutritional factors, such as phenolic compounds, which limit their use (Mokgope, 2007). These anti-nutritional factors result from the cowpea's capacity to form insoluble complexes with important food nutrients such as proteins and minerals (Mokgope, 2007).

In general, the composition of the cowpea is similar to that of other legume seeds, including protein, starch and sugars. Cowpea protein contains a high level of lysine, but a low level of sulphur amino acids (Rangel et al., 2003). The amino acid contents (mg/g N) are shown in **Table 2.1**.

Table 2.2 shows that the approximate composition of cowpea (*Vigna unguiculata*) is 18.3-35.0% protein, 0.7-3.5% fat, 2.7-7.0% fibre, 31.5-48.0% starch, and 2.5-4.9% ash and 12.0-15.0% moisture (Mokgope, 2007). This demonstrates that cowpea seeds contain high levels of proteins, as well as starch.

Table 2.1. The amino acid content (mg/g N of cowpea protein).

The amino acid content	(mg/g N)
Isoleucine	239
Leucine	440
Lysine	427
Methionine	73
Cysteine	68
Phenylalanine	323
Tyrosine	163
Threonine	225
Tryptophan	68
Valine	283
Arginine	400
Histidine	204
Alanine	275
Aspartic acid	689
Glutamic acid	1027
Glycine	234
Proline	244
Serine	268

Source: Fuccillo et al. (1997)

Table 2.2. Approximate composition of cowpeas

Constituents	Range (%)
Crude protein	18.3-35.0
Crude fat	0.7-3.5
Crude fibre	2.7-7.0
Starch	31.5-48.0
Ash	2.5-4.9
Moisture	12.0-15.0

Source: Mokgope (2007)

A study of cowpea proteins by Rangel et al. (2004) revealed that its *in vivo* protein digestibility is superior to that of other widely used legumes. Although they detected trypsin inhibitor, the researchers considered this non-detrimental to human health as long as the CPI is adequately processed using heat treatment before its addition to food products. None of the experimental diets used in this study showed evidence of toxicity. The study concluded, therefore, that CPI is a viable, cost-effective source of protein, which could be used to supplement the protein-poor diets of the malnourished, lower socio-economic classes in developing countries (Chan and Phillips, 1994; Rangel et al., 2004).

Lima et al. (2004) examined the effects of protease inhibitors, from mature and immature cowpea seeds, on the digestive system of mammals. Trypsin inhibitor activities were found to be low in both kinds of beans. The group were able to mitigate the risk posed by these anti-nutritional factors, which are widespread in the legumes and cereal seeds used in the human diet, by heating (cooking) them prior to consumption.

2.1.3 Types of proteins in cowpeas

Studies on the conditions required for the extraction of cowpea proteins were conducted by Cerdeira et al. (1985) and Khan et al. (1980) who also established that the majority of bean protein is present in the globulin fraction, which constitutes 72% of the extractable protein.

A study by Ragab et al. (2004) reported that the protein composition of cowpea is 71.4% albumin, 11.1% globulin, 2.20% prolamin, and 11.0% glutalin; meaning that approximately 95.7% of the total protein contained in the cowpea was extractable using liquid solutions (see **Figure 2.3**).

It has been discovered that the molecular sizes of cowpea proteins are similar to those of navy and kidney bean, which have major sizes ranging from 43 to 47kDa, and minor molecular sizes in the range of 26 to 28 kDa (Freitas et al., 2004; Horax et al., 2004). Some cowpeas produce defensive reactions against a large number of organisms, such as bacteria, fungi, and insects (Rose et al, 2006; Singh and Rachie, 1985). Horax et al. (2004), Pereira et al. (2009), and Rangel et al. (2003) showed that vicilin is the predominant storage protein fraction in legume protein isolates, with a molecular weight of 50 kDa. According to research by Paredes et al. (2006), the molecular size of chickpea proteins is similar to that of cowpea proteins. Previous research using SDS-PAGE also indicated similar molecular weight profiles for kidney and navy bean protein isolates to that of CPI (Horax et al., 2004; Kimura et al., 2008). The antifungal proteins in cowpea proteins are described in Chapter 5.

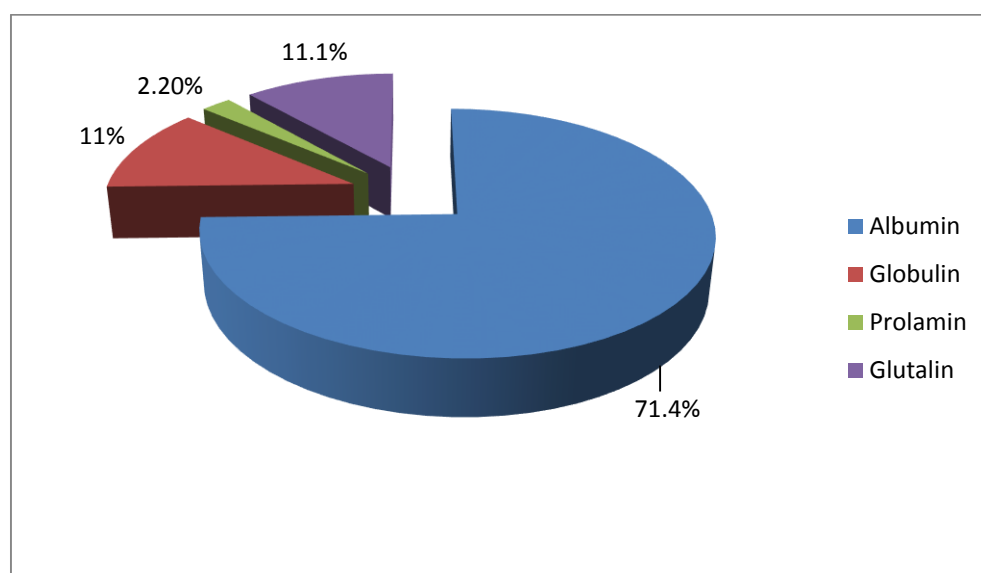


Figure 2.3. Protein compositions of cowpea beans (Source: Ragab et al., 2004).

2.2 Functional properties of cowpea protein

Previous studies by Rangel et al. (2004) have already demonstrated good functional properties for cowpea protein in terms of solubility, emulsification, and foaming. The solubility of a protein can have an important influence on properties such as emulsification, gelation, and foaming. Ragab et al. (2004) conducted a study to determine the extent to which pH and/or salt concentration affect the fractionation, solubility, and functional properties of cowpea proteins. They found the protein is highly soluble at alkaline and acidic pHs, and that salts increase the solubility only at the isoelectric pH; this is a significant factor in food processing. The main conclusions of this study were that the high

solubility of cowpea protein at alkaline pH indicates higher emulsifying and foaming properties than other proteins. When combined with its excellent water- and fat-holding capacities, these results suggest that CPI is eminently suitable for use in food formulation systems.

Upon examination of how pH or NaCl concentrations influenced its emulsifying properties CPI was found to have a minimum capacity of 40 ml oil/g protein, with an increase either side of pH 4 and 5, changing to 82 ml oil/g protein at pH 3, and 150 ml oil/g protein at pH 10. The addition of NaCl improved the solubility of the protein, whilst emulsification capacity decreased as the salt concentration increased. The foaming capacity of CPI was found to be pH dependent, with CPI unable to foam at pH 5.

According to the study of Ragab et al. (2004), heating CPI at 70°C for 15 mins affects its viscosity, causing it to rise from 3.90 cps (at 20°C) to 8.00 cps, in response to the conformational characteristics of the protein.

2.3 Analytical methods used in this chapter to analyse cowpea proteins

This section describes how cowpea seed proteins have been analysed using the following techniques:

2.3.1 *Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)*

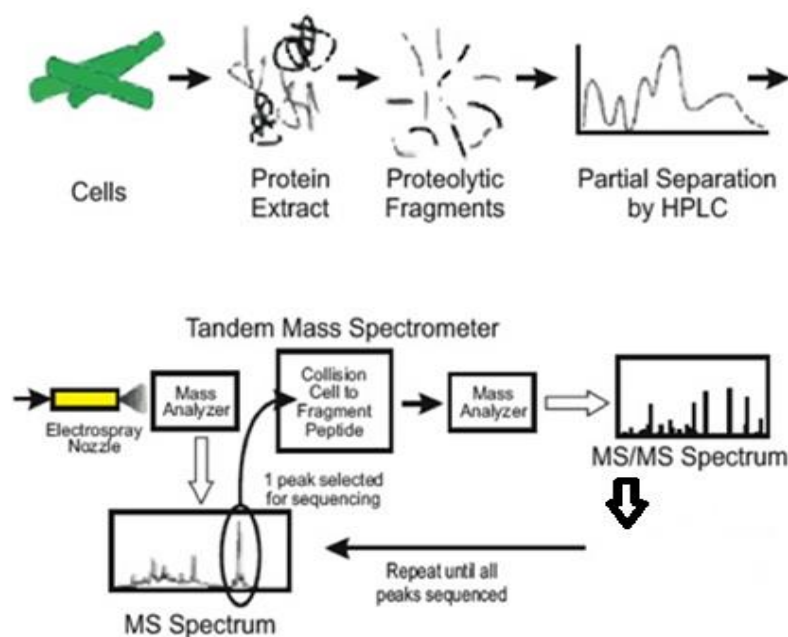
In the electrophoresis process the macromolecules are separated in an electric field. A method commonly used to separate proteins using electrophoresis consists of a polyacrylamide gel as a support medium, and sodium dodecyl sulfate (SDS) in order to denature the proteins. This method is known as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS (also known as lauryl sulfate) is an anionic detergent that denatures proteins. It exposes areas that are usually hidden, and coats the protein chain with negatively charged surfactant molecules. The complex structure of proteins is abolished by the negative charges on SDS, which will then be attracted to an anode (positively-charged electrode) in an electric field. Larger molecules are prevented from migrating as quickly as smaller molecules through polyacrylamide gels. As SDS-denatured polypeptides have a very similar charge-to-mass ratio, the final separation of proteins is determined by differences among the relative molecular mass of polypeptides. SDS-PAGE protein separation can enable the estimation of the relative molecular mass of proteins, and determine the relative abundance of major proteins in a sample. Proteins can be detected through the use of various staining compounds (Brunelle and Green, 2014).

2.3.2 *Liquid chromatography-mass spectrometry LC/MS/MS and proteomics analysis*

Several proteomic tools can be applied for the detection and identification of proteins, such as liquid chromatography mass spectrometry (LC/MS/MS) and Matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS). LC/MS/MS is beginning to play a critical role in many areas of clinical biochemistry, rivalling conventional liquid chromatography and other techniques such as immunoassay. Analysis of proteins using LC/MS/MS is a powerful tool. To the best of this author's knowledge, no analysis of cowpea seed protein composition using these tools is available in the literature.

LC/MS/MS is typically used for the proteomic analysis of complex samples, where peptide masses might be analysed with a high-resolution mass spectrometer. A modern LC/MS/MS can run samples of complex biological fluids (e.g. human serum or plant extracts) (Takac and Samaj, 2011). **Figure 2.4** presents the sequential method followed in a classic analysis using LC/MS/MS. The fraction eluted in the first column is introduced into the second column, which can then be directly linked to the mass spectrometer (Board, 2013). The analytical data are matched against an existing data base to enable characterisation of proteins. In order to determine which peptide sequence in a database of protein sequences gives the best match, the database is searched for each entry by using the masses of the intact peptides. In cases where the calculated mass of a peptide matches that of a known peptide in the data base the unknown peptide can be identified (Cattrell, 2011).

Protein Identification with Chromatographic Separation (LC/MS/MS)



(Source: Board, 2013).

Figure 2.4. Schematic diagram for the LC/MS/MS method. A classic analysis via LC/MS/MS is presented and the fraction eluted in the first column is introduced into the second column, which is directly linked to the mass spectrometer.

2.3.3 Ultrafiltration

Ultrafiltration (UF) is a technique in which separation through a semipermeable membrane occurs in response to forces, such as pressure or concentration gradients. The retentate contains suspended solids and solutes that have a high molecular weight. Both water and solutes with a low molecular weight filter through the membrane in the filtrate. Ultrafiltration membranes are defined according to the molecular weight cut-off (MWCO) of the membrane. Ultrafiltration has been used successfully for many years and offers a gentle method of purifying and concentrating protein samples. This technique can replace precipitation, evaporation, dialysis, lyophilisation, and gel filtration as a method to concentrate and desalt protein without losing a significant amount of protein.

However, ultrafiltration is not a fractionation technique, it is a separation technique. In general, it is not practical to use ultrafiltration for protein fractionation, unless there is a minimum ten-fold difference in the molecular weights of the two proteins being separated (Nath, 2008). **Figure 2.5** reveals the ultrafiltration system that was used for this study.

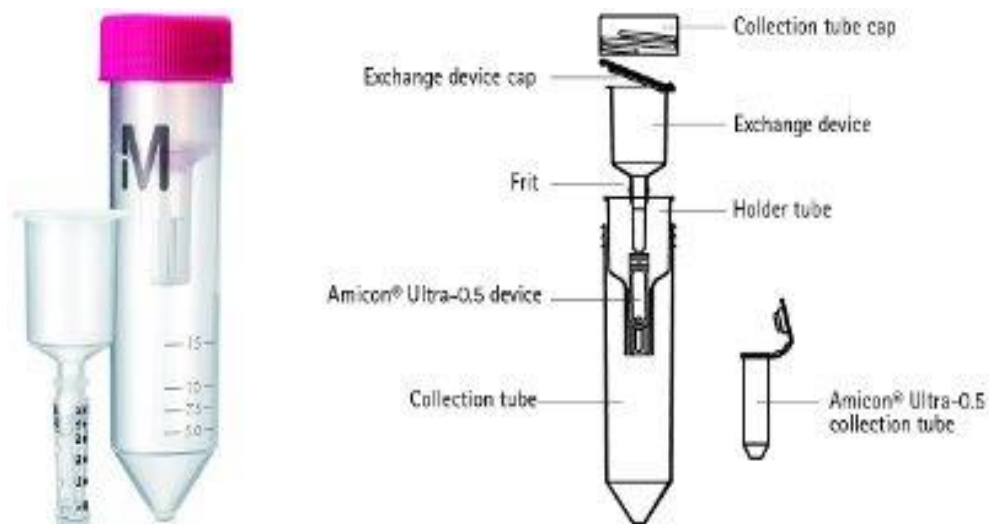


Figure 2.5. Amicon Ultrafiltration system manufactured by Millipore. Generally, small molecules are permitted through the membrane pores, but larger molecules might remain at the membrane surface

2.4 Materials and Methods

All materials, chemicals, reagents, such as glycine, bovine serum albumin, Bradford solution, 2-mercaptoethanol, Coomassie Brilliant Blue, acetic acid, Tris, SDS, 70% ethanol, media petri dishes (100×15 mm), micro-centrifuge tubes, ultrafiltration tubes, and buffer were obtained from Sigma-Aldrich, London (UK).

2.4.1 *Preparation of water soluble extract from cowpea seeds*

Water-soluble extracts from cowpea seeds were prepared as described by Giuseppe et al. (2009). Fifty grams of dry seeds were homogenised with 50 ml of distilled water in a blender (PBI International). Then 50 ml of distilled boiling water was added to the ground material and mixed well. The solution was then incubated at room temperature (20°C) for 20 mins, with stirring, and then centrifuged at 14,000 rpm for 20 mins at 4°C. Finally, the supernatants were collected for the antifungal assay.

2.4.2 *Preparation of crude extract and ammonium sulphate precipitation*

Adhering to the method carried out by Wang et al. (2004) the extract of cowpea seeds was prepared as follows. Exactly 100 g of cowpea seeds were soaked in distilled water for several hours. Then they were homogenised using 0.2M sodium acetate buffer, pH 5.4 before being centrifuged at 12,000 rpm for 20 mins at 4°C. The supernatant was then labelled as crude extract.

The crude extract was treated using ammonium sulfate at 20% saturation. After centrifugation at 12,000 rpm for 20 mins, the supernatant was disposed of and the precipitate collected and dissolved in 100 ml of 0.02M sodium acetate buffer (pH 5.4).

2.4.3 Preparation of cowpea protein isolate (CPI)

The method was carried out according to that developed by Ahmed (2014). As outlined in **Figure 2.6**, the cowpea seeds were first ground and then mixed with distilled water. The pH was adjusted to 10 using NaOH; the solution was stirred for 20 mins, and then centrifuged at 14,500 rpm at 4°C, for 30 mins. The supernatant obtained after centrifugation was then adjusted to pH 4 using HCl, and stirred for 20 mins before being centrifuged. The precipitate was resuspended in water and the pH adjusted to 7.0. The samples were then freeze dried (see **Figure 2.6**).

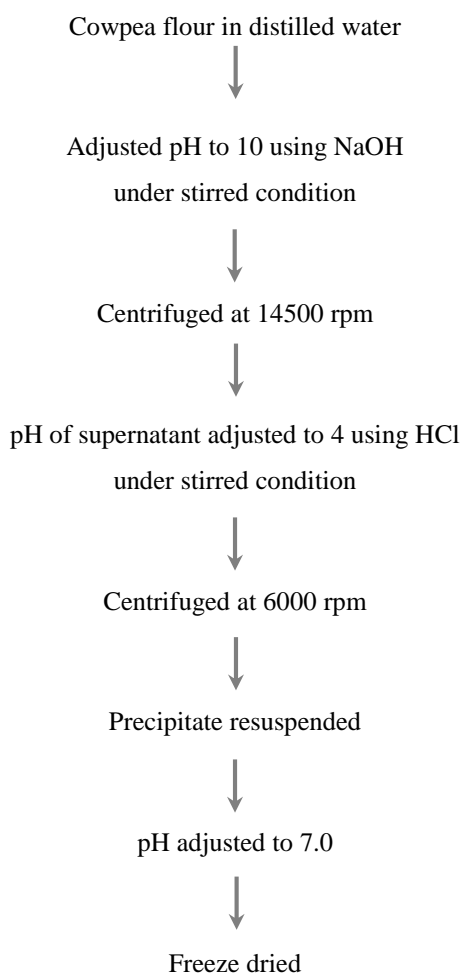


Figure 2.6. Preparation of cowpea protein isolate (CPI).

2.4.4 *Determination of cowpea protein concentration*

The total protein content of CPI (soluble and insoluble) was determined using the Kjeldahl method; while its soluble protein content was determined using the Bradford method.

2.4.5 *Kjeldahl method*

The Kjeldahl method (AOAC, 1995, method No. 988.05; Lynch et al., 1998) was used to determine crude protein content. Samples of 0.25-1.00 g were weighed into a digestion flask and two tablets of Antifoam S (each one containing 0.97 g sodium sulphate & 0.03 g silicone antifoam) and one tablet of KJELTAB S catalyst (containing 5 g potassium sulphate and 5 mg selenium (Se), added. 12 ml of sulphuric acid was carefully added to each digestion flask. Sulphuric acid digests the protein, releasing nitrogen from the sample, which is then converted to ammonium ions. The flasks were then transferred to a digestion unit (Tecator™ digester system 20, Foss-UK), and gradually heated up to 420°C for one hour until they became clear, after which the flasks were heated for a further 10 mins, then the digestion flasks were cooled to room temperature, and transferred to a distillation unit (Kjeltec™ 8100 distillation system, Foss-UK). 70 ml of water and 50 ml of sodium hydroxide (40% (w/v)) were added to each flask, and the samples were distilled for three minutes to release ammonia. The released ammonia was then reacted with 25 ml of boric acid, and kept in a clean and dried beaker to form ammonium borate. An excess of dilute hydrochloric acid (0.1 N) was added to perform back titration of the ammonium. The protein content was calculated as a percentage of the dry weight of the sample by multiplying the total nitrogen content by a conversion factor of 6.25. This value of 6.25 was used to convert nitrogen content to protein content (Hall and Schonfeldt, 2013).

2.4.6 *Bradford method*

Firstly, a range of 0.1-1 mg/ml of different concentrations of bovine serum albumin standard protein was prepared using a stock solution of mg/ml and all made up to a final volume of 1 ml with distilled water. Then 100 µl from each tube was transferred to spectrophotometer tubes. 5.0 ml of Bradford solution was added to each tube then gently vortexed. After setting the spectrophotometer to the required wavelength (595 nm) the tubes were read using blank tubes without the protein.

The absorbance was plotted against the protein concentration. Then the protein concentration range that resulted in a linear relationship was selected and used as standard curve to calculate the concentration of CPI.

Serial dilutions of CPI samples were prepared, and then 100 μ l from each tube was transferred to glass test tubes. Then 5.0 ml of Bradford solution was added to each tube of samples and standards and vortexed before being incubated at room temperature 20°C for 10 minutes. The absorbance of the samples (standard and CPI) was measured at a wavelength of 595 nm (Walker, 2002). The protein dilutions were selected that gave an absorbance that falls within the absorbance range of the BSA standard curve. The standard curve was used to calculate the concentration of the unknown CPI samples. The linear standard curve for the Bradford method is shown below (**Figure 2.7**).

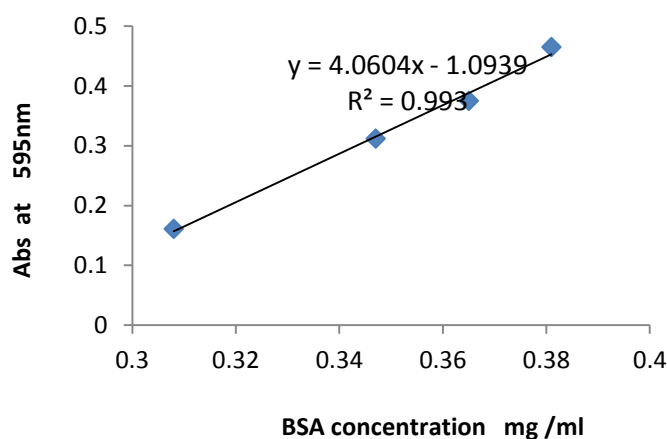


Figure 2.7. Linear standard curve of bovine serum albumin as determined by the Bradford assay.

2.4.7 Ultrafiltration method (UF)

This experiment was undertaken to create a purified extract, and obtain preliminary information regarding the molecular weight of the active compounds. The CPI extract was fractionated using ultrafiltration centrifugal tubes, different membrane sizes of filter units were used with 50, 30, 10 kDa cut off, obtained from Sigma-Aldrich (UK). According to the manufacturer's instructions, 500 μ l of CPI extract was applied to the centrifugal tubes, and then it was centrifuged at 10.000xg for 50 minutes. The samples were then freeze-dried, after which the effectiveness of each fraction was tested using micro plate assay.

2.4.8 Preparation of samples for SDS PAGE

To concentrate the CPI extract, the trichloroacetic acid (TCA) precipitation was applied. Firstly, 250µl of 100% TCA was mixed with 1.0ml CPI. This was then incubated for 30 minutes on ice and centrifuged for 30 mins. The pellet was carefully extracted and mixed with 0.5 ml cold acetone. It was vortexed briefly and then centrifuged at 4°C for 15 minutes. Finally, the pellet of protein was left to air dry, and then the 7 mg obtained from the extraction step was dissolved in distilled water (1 ml). This protein solution (100 µl) was added to an equal volume of non-reducing sample buffer (120M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, and 0.008% bromophenol blue solution). This was mixed thoroughly before 10–20 µl was loaded into the gel, which amounts to 35-70 µg protein per well.

2.4.9 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) method

SDS-PAGE was carried out according to the procedure outlined by Wu and Hojilla (2005), using Pre-cast SDS PAGE 10-20% Tris-glycine gradient gels in an electrophoresis unit (XCell Surelock™ Mini Cell, Invitrogen Life Technologies, Paisley, UK), at a constant voltage of 180V for approximately 45 minutes. The running buffer was 10x SDS-PAGE buffer (1% SDS, 0.25M Tris-HCl and 1.92M glycine). A protein molecular weight ladder was included in each gel as a marker to facilitate analysis of the gels, and to enable molecular weight determination of sample proteins. The molecular weight markers were Plus2 pre-stained (1x) 53 MW 4-250 KDa. Gels stained with Coomassie Brilliant Blue were scanned using a BIO-RAD Molecular imager® ChemiDoc™ XRS+ and results were analysed using gel analyser software GelAnalyzer 2010a.

2.4.10 CPI Identification by Liquid-Chromatography Coupled Tandem Mass Spectrometry (LC/MS/MS)

Prof. Cait MacPhee, of the School of Physics & Astronomy in the School of Chemistry at the University of Edinburgh kindly carried out LC/MS/MS analysis of the freeze-dried CPI sample.

2.5 Results

In this study, different methods were applied in order to find the best extraction method that gives good fungal inhibitory results using a microplate assay (Chapter 3).

- Water soluble extract of cowpea seeds
- Cowpea extract with ammonium sulphate precipitation
- Cowpea protein isolate CPI
- Ultrafiltration (UF) method

The results of antifungal activity are given in Chapter 3 section 3.3.1. No antifungal activity was exhibited by the water soluble extract and the ammonium sulphate precipitated fraction which had a protein content of 52.09% \pm 0.11 and 58.02% \pm 0.11 respectively as determined by Kjeldahl.

The best antifungal activity was obtained by CPI and the ultrafiltrate. For this reason the protein characterisation for the remainder of this chapter was focussed on CPI and its filtrate and retentate.

The content of protein in the freeze-dried CPI as determined by Kjeldahl was 71.2% \pm 0.40 of dry weight and is consistent with a study by Kinsella (1979) who reported protein content in CPI of 76.0%.

The soluble protein concentration for CPI and its filtrate and retentate as determined by Bradford is shown in **Table 2.3**.

Table 2.3. Soluble protein in ultrafiltration test as determined by the Bradford method. Data are presented as the mean of 3 triplicates and the standard error of the mean.

Sample	Protein concentration mg/ml
CPI	3 \pm 0.42
CPI filtrate after filtration (UF)	1.7 \pm 0.13
CPI retentate after filtration (UF)	5.3 \pm 0.22

The starting volume of the CPI solution that was ultrafiltered was 10 ml, the filtrate was 7 ml and the retentate was 3 ml. This shows that approximately 40 % of the protein was in the filtrate and 53% of the protein was concentrated in the retentate. However the

concentration of protein in the filtrate was low (1.7 mg/ml), it showed up as faint bands on gels (**Figure 2.8**, lanes 5 and 6), and so was concentrated by TCA treatment (**Figure 2.8**, lane 1). The proteins in crude CPI were also concentrated by TCA (lanes 2 and 3 compared to lanes 4 and 7 in CPI in **Figure 2.8**).

2.5.1 Electrophoresis (SDS-PAGE)

2.5.1.1 Electrophoretic Profile of CPI

The molecular mass of the proteins in CPI was determined by comparing their electrophoretic mobility with those of molecular mass marker proteins, details of the method are presented in section (2.4.9). The SDS-PAGE gels are shown in **Figures 2.8, 2.9, 2.10** and **2.11**.

Figure 2.8 (lanes 2, 3, 4 and 7) shows that CPI consists of polypeptides of molecular weight ranging from 10 to 115 kDa, with major polypeptides at 30, 40, and 50 kDa. These results confirm findings by Ahmed (2014), who reported a similar SDS PAGE profile for CPI.

The effects of ultrafiltration on the molecular sizes of proteins are shown in lanes 1, 5, 6, and 8 of **Figure 2.8**. To facilitate comparison with original CPI, these lanes were each cut out individually and compared to non UF treated CPI in **Figures 2.9, 2.10, and 2.11**. Copies of densitometric scans of each lane are displayed below each gel combination.

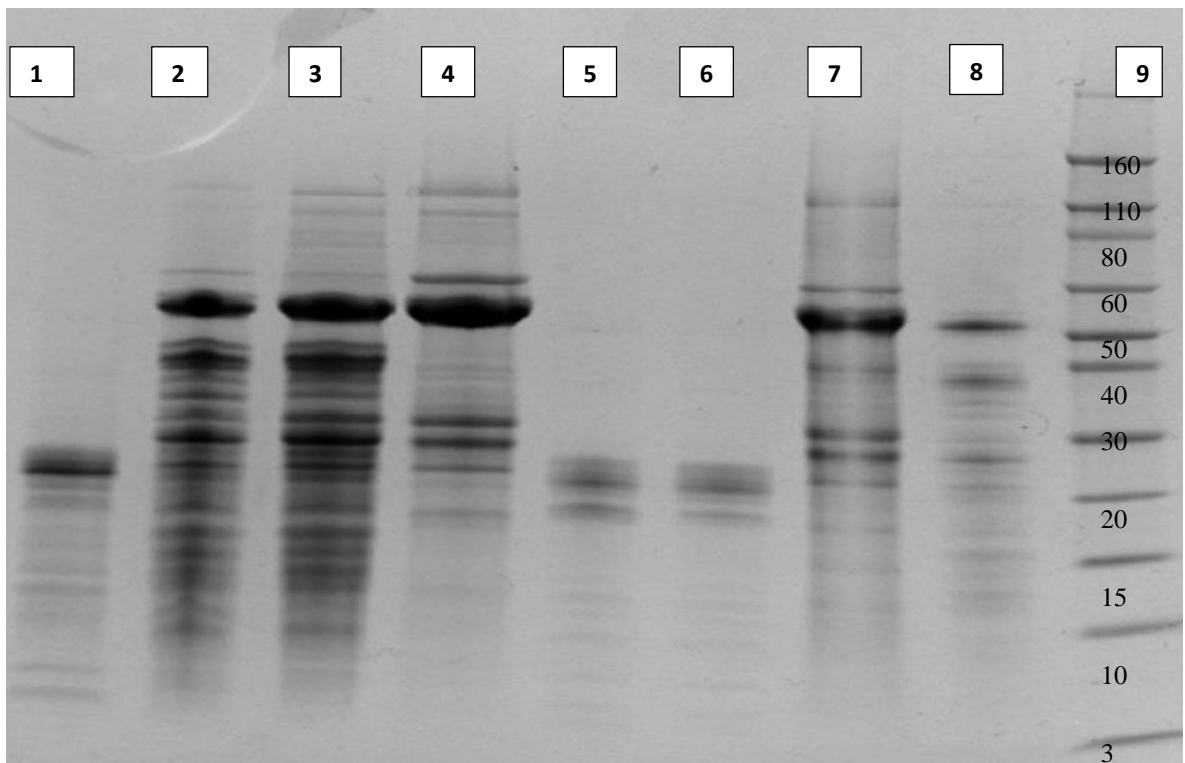


Figure 2.8 The effect of TCA treatment to concentrate proteins on electrophoretic profiles of CPI and its retentate and filtrate following ultrafiltration. Lane **1** CPI filtrate with TCA treatment. Lanes **5** and **6** CPI without TCA treatment. Lane **8** CPI retentate with TCA treatment. Lanes **2** and **3** crude CPI treated with TCA. Lanes **4** and **7** CPI without any treatment. Lane **9** molecular weight markers.

The results in **Figure 2.9** show that precipitation of the CPI sample with TCA before SDS-PAGE resulted in a concentration of low molecular weight bands smaller than 10 kDa. **Figure 2.10** shows that the polypeptides smaller than 30 kDa were removed from CPI by ultrafiltration. **Figure 2.11** shows proteins lower than 30 kDa were enriched in the filtrate, whereas all proteins larger than 30 kDa were removed by ultrafiltration. The molecular weight cut off point for the UF membrane was 10 kDa; however, the gel shows enrichment of a 25 kDa fraction. The reason could be due to limitations in the ultrafiltration procedure.

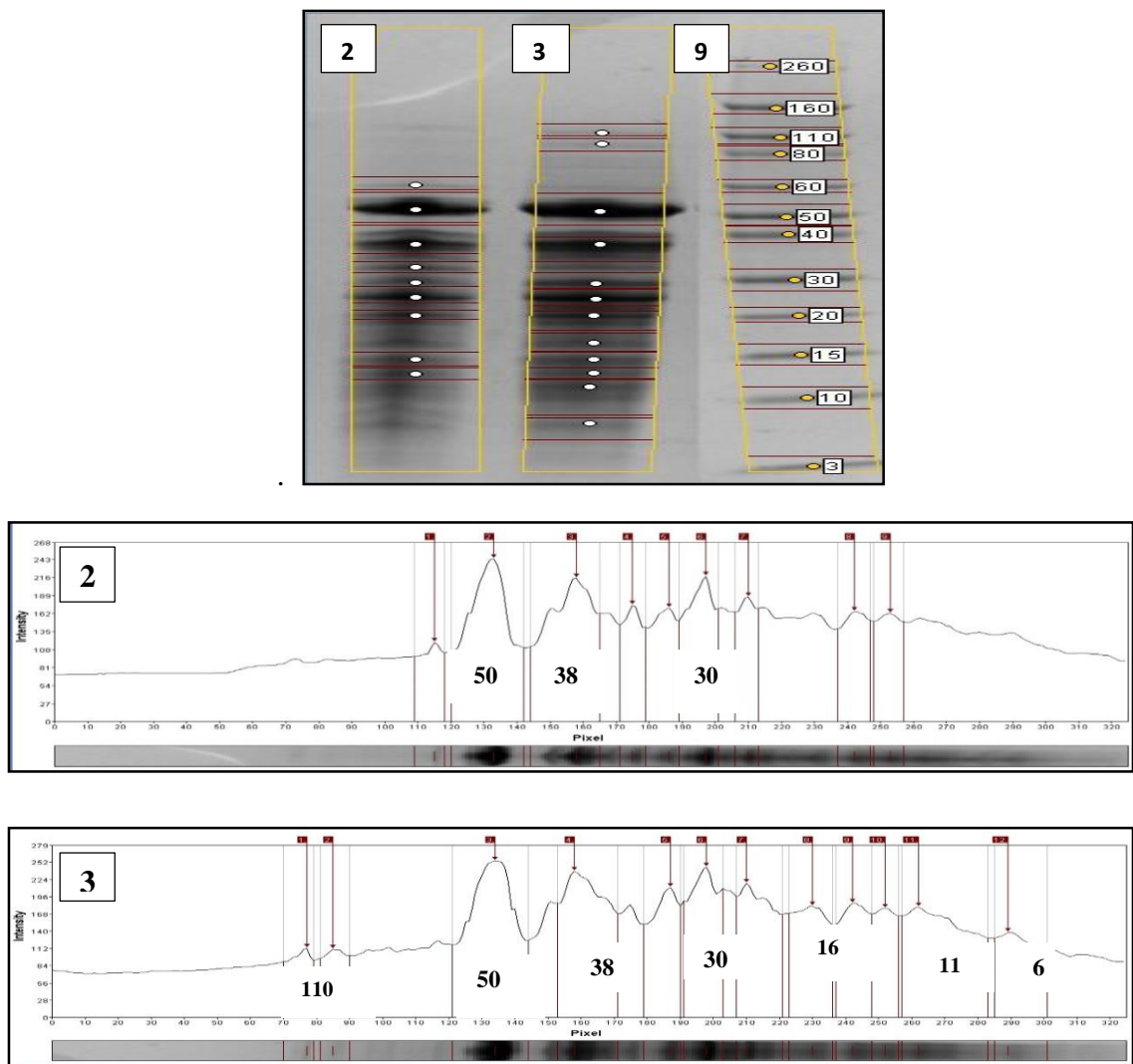


Figure 2.9 SDS-PAGE profiles of lanes 2 and 3 of **Figure 2.8**. Lane 2 crude protein CPI prepared according to section (2.4.3). Lane 3 TCA treated CPI sample (prepared according to section 2.4.8). Lane 9 molecular weight markers. Densitometric scans of lanes 2 and 3 represent the respective band intensities. Molecular weights (kDa) are indicated within the scans.

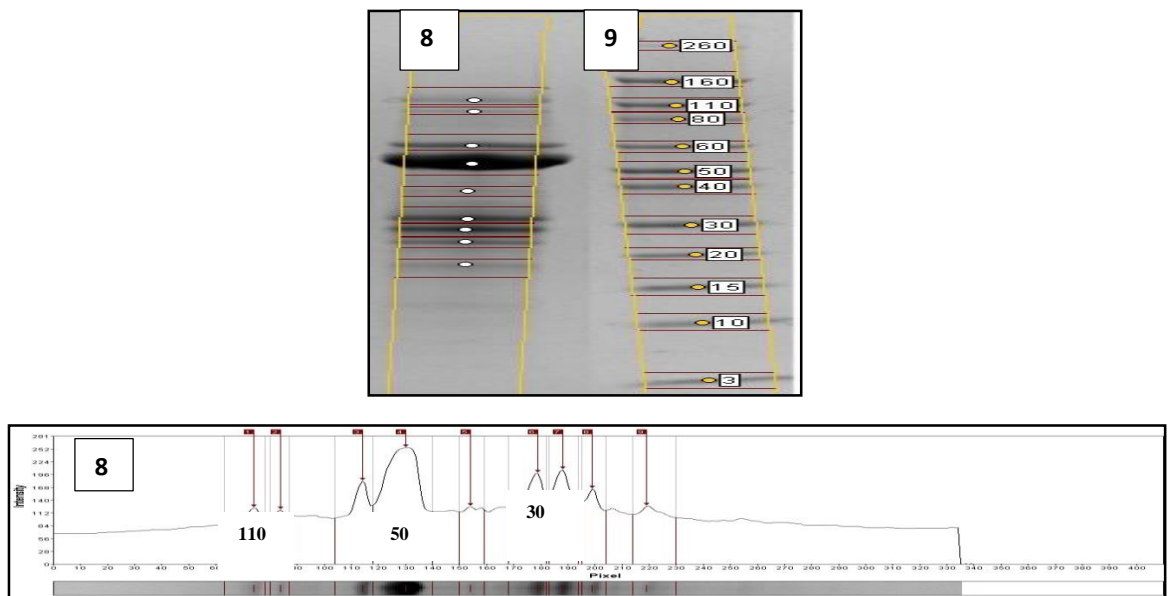


Figure 2.10 SDS-PAGE profile of lane 8 of **Figure 2.8**. Lane 8 CPI UF retentate treated with TCA according to section (2.4.7. and 2.4.8). Lane 9 molecular weight markers. Densitometric scan of lane 8 represents the respective band intensities. Molecular weights (kDa) are indicated within the scans.

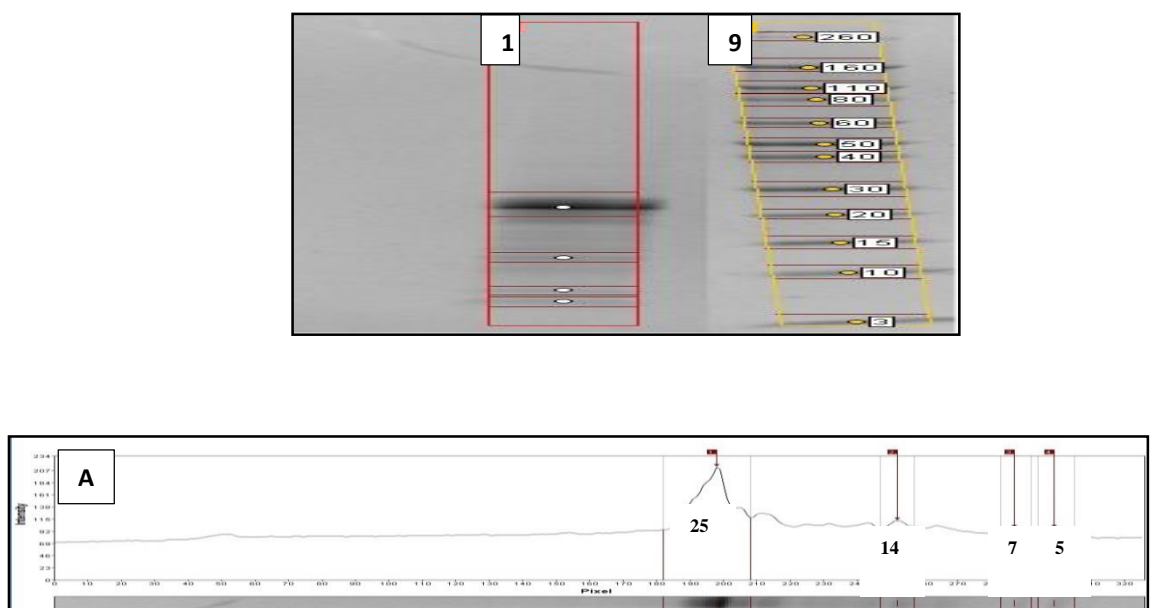


Figure 2.11 SDS-PAGE profile of lane 1 of **Figure 2.8**. Lane 1 CPI filtrate treated with TCA. Lane 9. Molecular weight markers. Densitometric scan of lane 1 represents the respective band intensities. Molecular weights (kDa) are indicated within the scans.

2.5.2 Identification of the Cowpea Seed Protein Isolates (CPI) by LC/MSMS

Mascot Version 2.4 (Matrix Science Ltd, UK) and the NCBI database were used to identify proteins according to the LC/MSMS results. There were approximately 600 hits resulting from a search using this database. The proteins were screened to rule out any hits resulting from probable contaminants, such as trypsin autolysis peptides, hair, skin keratins, matrix molecules, and clusters. Several candidate proteins were identified for each of the protein hits. The MOWSE (Molecular Weight Search) probability-based algorithm was used to identify the proteins, with at least two peptides being matched for every predicted peptide map for the protein sample, as shown in **Table 2.4**.

In order to get an accurate identification of the proteins two standards were used, the MOWSE score, and the condition that the identification relies on at least two peptides being matched to the known peptide map of a protein. MOWSE (Molecular Weight Search) is a process for recognising proteins depending on molecular weight of the peptides obtained from proteolytic digestion of the protein sample (Abdurhman, 2014). The method was developed first by Pappin et al. (1993) and in it a higher number is used to express an accurate identification. Results of proteomics analysis are shown in **Table 2.4** and they were categorised based on the hit number (HN), score number, molecular weight, and protein match indicator.

The hit number (HN) is a rough indicator of protein abundance in the sample, with a lower hit number indicating a more abundant protein. The score number reflects the combined scores of all observed mass spectra that can be matched to known amino acid sequences in a database. A higher score indicates a more confident match, and the number of peptide matches compared to the total number of peptides produced. In order to determine which peptide sequence in a database of protein sequences gives the best match, specific software is used. Several scoring algorithms have been devised to decide which peptide sequence best matches a given MS/MS spectrum (Cattrell, 2011). The first ninety-nine hits that have been identified are presented in **Table 2.4**. The twentythree proteins with lowest HN and highest score are shown in a separate table (**Table 2.5**).

Table 2.4 Results of Proteomics analysis of CPI identified with liquid-chromatography and with Coupled Mass Spectrometry (LC/MS/MS)

HN	Proteins name	Score	MW (Da)	Protein match
Functional category 1: Protein synthesis & translation factor				
6	Cyclophilin [<i>Phaseolus vulgaris</i>]	1896	49654	6/3
9	Ribosomal protein L2	214	28224	7/6
13	Alloalbumin venezia	165	71177	8/8
30	Hypothetical protein OsJ_11158 [<i>Oryza sativa japonica group</i>]	118	29287	4/3
31	Elongation factor	123	35556	5/3
33	Cysteine synthase	123	34362	6/3
44	Putative 40S ribosomal protein S5	114	16884	3/3
56	Elongation factor	98	18638	1/1
64	Methionine synthase [Glycine max]	93	84401	7/4
69	40S ribosomal protein S3 (zxrRPS3A)	91	27673	3/2
76	Inosine monophosphate dehydrogenase	86	53530	2/2
78	Protein phosphatase 2A	83	66079	3/3
80	Hypothetical protein LOC10038	82	32721	3/3
84	40S ribosomal protein S13	80	17187	3/2
85	Glycyl-tRNA synthetase, putative	80	77959	4/2
Functional category 2: Transporters				
24	Putative glutathione S-transferase	136	24782	2/2
25	Importin alpha 2	93	59059	2/2
26	Rack	133	35979	4/5
54	V-ATPase 66 kDa subunit	97	36297	3/4
74	SDH1-2; succinate dehydrogenase	87	70003	2/3
75	Transport protein	86	85862	2/2
Functional category 3: Energy/ ATP synthase/glycolysis/ electron transport/ stress				
5	RuBisCO subunit binding-protein alpha subunit, chloroplast precursor, putative, expressed [<i>Oryza sativa</i>]	176	50480	2/2
8	Glyceraldehyde 3-phosphate dehydrogenase [<i>Solanum tuberosum</i>]	223	36795	6/3
10	Dehydrogenase	133	41524	7/6
12	ATPase subunit [<i>Beta vulgaris subsp. vulgaris</i>]	177	55306	8/6
14	Enolase	170	47974	4/3
20	RuBisCO subunit binding-protein beta subunit	133	64491	6/8
32	Transitional endoplasmic reticulum ATPase - <i>Arabidopsis thaliana</i>	119	94357	7/5

HN	Proteins name	Score	MW (Da)	Protein match
37	ATP binding protein, putative [<i>Ricinus communis</i>]	120	89778	4/3
40	Chloroplast glyceraldehyde-3-phosphatedehydrogenase subunit B [<i>Ostreococcus tauri</i>]	118	47233	4/3
50	26S proteasomeregulatory subunitS5A [<i>Mesembryanthemum crystallinum</i>]	106	42960	3/3
63	PGK (phosphoglycerate kinase); phosphoglycerate kinase [<i>Arabidopsis thaliana</i>]	93	42162	5/3
70	Malate dehydrogenase	90	43498	2/2
73	Phosphoenolpyruvate carboxylase;	89	111147	5/3
79	HSP91; ATPbinding [<i>Arabidopsis thaliana</i>]	83	92491	4/4
96	Aconitase, putative	67	99108	5/3
108	Alcohol dehydrogenase-1CN	73	41624	4/2
Functional category 4: Protein destination and storage proteins				
1	Vicilin protein	1896	49654	8/6
Functional category 5: Transcription				
38	BiP isoform A [Glycine max]	116	73633	7/4
46	Beta-tubulin	109	50447	7/5
58	Prohibitin2	96	30702	1/1
90	Protein phosphatase type 2C	78	30955	1/1
Functional category 6: Metabolism/sugars and polysaccharides/amino acids/nucleotides/lipid				
2	Lipoxygenase [<i>Pisum sativum</i>]	282	97284	14/ 14
4	Alpha-1,4 glucan phosphorylase L isozyme	249	114079	11/11
7	Actin [<i>Caragana korshinskii</i>]	231	41880	9/7
16	Wheat adenosylhomocysteinase-like protein [<i>Oryza sativa Japonica Group</i>]	161	53860	6/6
19	Ketol-acid reductoisomerase [<i>Arabidopsis thaliana</i>]	148	64285	4/4
21	Tubulin+ A	143	50345	6/5
23	Malate dehydrogenase, cytoplasmic	138	35810	3/2
29	26S proteasome subunit S9, putative [<i>Ricinus communis</i>]	128	47437	4/2
36	ADP-glucose pyrophosphorylase alpha subunit IbAGPa1 [<i>Ipomoea batatas</i>]	111	57404	5/5
41	ATP citrate lyase a-subunit [<i>Lupinus albus</i>]	116	66481	5/3
45	Cotyledoneous yieldin-like protein [<i>Vigna unguiculata</i>]	113	29512	5/4
52	Serine hydroxymethyltransferase [<i>Gossypium hirsutum</i>]	102	52382	5/4
59	Mitochondrial F1-ATPase beta subunit [<i>Dimocarpus longan</i>]	82	59868	5/5

HN	Proteins name	Score	MW (Da)	Protein match
60	Glycine-rich RNA binding protein [<i>Medicago sativa</i>]	95	10835	1/1
68	UDP-glucose pyrophosphorylase [<i>Amorpha fruticosa</i>]	91	51666	11/6
71	Glucose phosphomutase	90	63369	5/4
7	Actin [<i>Phaseolus vulgaris</i>]	160	23678	5/3
Functional category 7: Stress responses/disease/defence/pathogenesis-related protein				
17	Heat shock protein	158	80380	7/7
18	Heat shock 70 kDa protein	152	71291	8/6
39	14-3-3-like protein A; AltName: SGF14A	119	29145	5/5
49	T2H3.8 [<i>Arabidopsis thaliana</i>]	107	92599	2/1
65	Heat shock protein 70 [<i>Arabidopsis thaliana</i>]	92	77230	4/3
102	Defensin-like protein	74	5695	2/2
Functional category 8 : Unclassified proteins				
11	Unnamed protein product	198	22972	4/3
13	Predicted: hypothetical protein	97	94701	2/4
22	Predicted protein	139	94982	6/6
27	Unknown	131	24915	3/3
28	Granule-bound starch synthase Ia precursor [<i>Vigna unguiculata</i>]	131	67287	5/4
34	Unknown	122	44992	3/2
35	OligopeptidaseA, putative [<i>Ricinus communis</i>]	121	88119	3/3
42	Os05g0516600 [<i>Oryza sativa Japonica Group</i>]	103	23324	3/4
43	Predicted: hypothetical protein	108	89292	2/3
47	Predicted protein [<i>Populus trichocarpa</i>]	108	193800	4 /4
48	Unknown	107	46929	4/4
51	Predicted protein	102	15824	3/3
55	Unknown	98	40368	3/2
57	Unknown	96	15345	4/4
62	Unknown	94	39228	2/3
66	Puromycin-sensitive	91	99308	2/2
72	Unknown	89	49788	2/2
82	Alpha-fibrinogen precursor [<i>Homo sapiens</i>] 70223 1/1	82	70223	1/1
83	Unknown	82	29508	2/2
86	26S proteasome subunit 8; Tat binding protein [<i>Fagus sylvatica</i>]	80	47621	2/1
87	87 predicted protein [<i>Populus trichocarpa</i>]	79	116361	2/2
88	Hypothetical protein [<i>Vitis vinifera</i>]	79	82971	1/1
89	Hypothetical protein [<i>vinifera</i>]	79	82971	1/1

HN	Proteins name	Score	MW (Da)	Protein match
91	Putative protein [<i>Arabidopsis thaliana</i>]	78	49039	1/1
92	Unknown	78	32625	1/1
93	Predicted protein	77	57568	1/3
94	Hypothetical protein	77	84062	1/1
95	Unnamed protein product	75	54538	3/3
97	20S proteasome alpha subunit	75	28577	1/1
98	Hypothetical protein [<i>Citrus x paradisi</i>]	75	32737	1/1
99	Unknown	75	54538	3/3
101	Predicted: hypothetical protein [<i>Vitis vinifera</i>]	74	30543	1/1
103	Unknown	74	19200	½
104	Putative alpha7 proteasome subunit [<i>Nicotiana tabacum</i>]	73	27466	2/1

The first ninety-nine proteins identified in this study were categorised into different function based groups using categories designated by Bevan et al. (1998). The percentages are shown in **Figure 2.12**. The two proteins with the highest score number and lowest HN were vicilin, which is a storage protein, and then lipoxygenase, which is a metabolism protein. As would be anticipated, the majority of these proteins appeared to be associated with energy and metabolic processes in the seed, followed by protein synthesis.

Various proteins identified have been described previously in several studies and their functions are known. It is impractical to discuss the function of all ninety- nine proteins, therefore only a selection was chosen for discussion as listed in **Table 2.5**. Those chosen for further discussion are the top twenty- three proteins from **Table 2.4**.

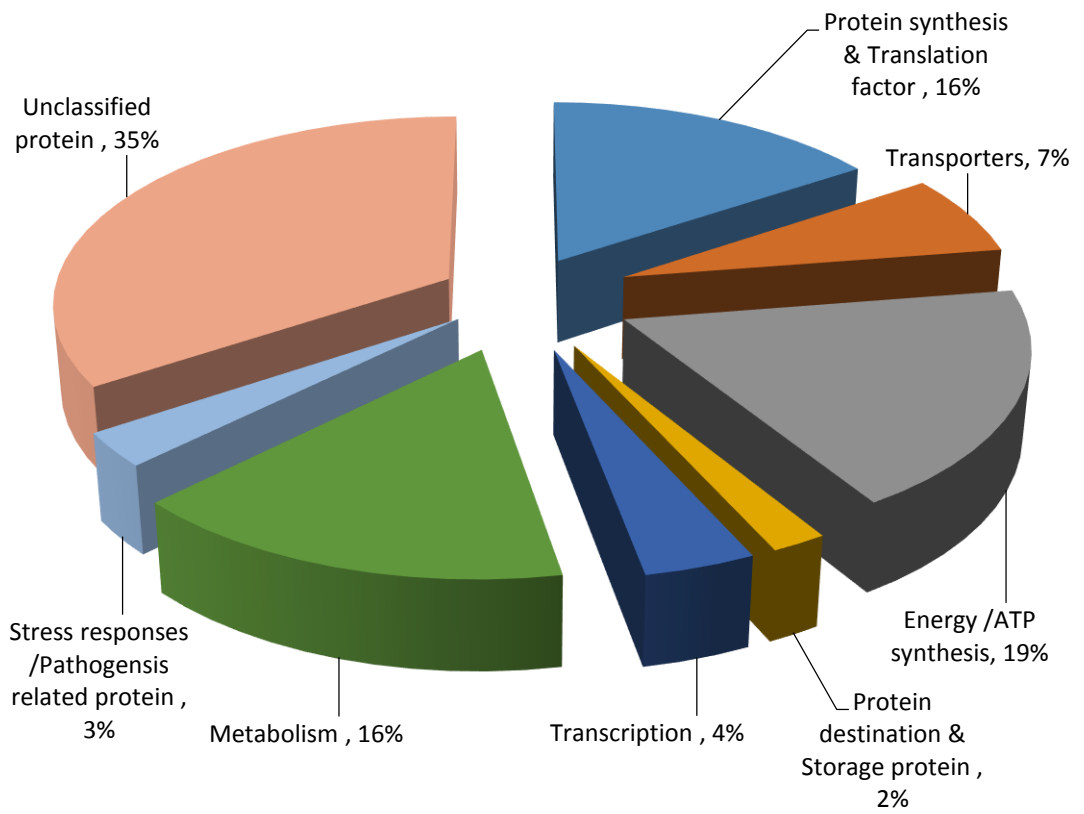


Figure 2.12 Classifications of the 99 proteins identified in CPI into eight different groups according to their functions using the categories based on the method described by Bevan et al. (1998).

Table 2.5 The twenty-three proteins of CPI with highest MOWSE score and lowest hit number.

HN	Proteins name	MOWSE score	MW (Da)	Protein match
1	Vicilin protein	1896	49654	81/76
2	Lipoxygenase	282	97284	14/14
4	Alpha-1,4 glucan phosphorylase L isozyme	249	114079	11/11
5	RuBisCO subunit binding-protein alpha subunit, [<i>Oryza sativa</i>]	176	50480	2/2
6	Cyclophilin	1896	49654	6/3
7	Actin	231	41880	9/7
8	Glyceraldehyde-3-phosphate dehydrogenase	233	36776	6/3
9	Ribosomal protein L2	214	28224	7/6
10	Dehydrogenase	133	41524	7/6
11	Unnamed protein product	198	22972	4/3
12	ATPase subunit [<i>Beta vulgaris subsp. vulgaris</i>]	177	55306	8/6
13	Predicted hypothetical protein	97	94701	2/4
14	Enolase	170	47974	4/3
15	Alloalbumin venezia	165	71177	8/8
16	Wheat adenosylhomocysteinase-like protein [<i>Oryza sativa Japonica Group</i>]	161	53860	6/6
17	Heat shock protein	158	80380	7/7
18	Heat shock 70 kDa protein	152	71291	8/6
19	Ketol-acid reductoisomerase	148	64285	4/4
20	Rubisco subunit binding-protein beta subunit	133	64491	6/8
21	Tubulin A	143	50345	6/5
22	Predicted protein	139	94982	6/6
23	Malate dehydrogenase, cytoplasmic	138	35810	3/2
24	Putative glutathione S-transferase	136	24782	2/2

2.5.3 Description of important proteins in cowpea, classified according to their function

Functional category 1: Protein synthesis/Translation factors

6 Cyclophilin

Cyclophilins form a subcategory of a large protein family, named immunophilins (Hacker and Fischer, 1993). They are remarkably preserved across all genera, playing a pivotal role

in important cellular processes. In plants with several cyclophilin members (e.g. *Arabidopsis* and rice), the cyclophilin members have been found to be linked with many functions and controlling pathways through foldase, scaffolding, chaperoning or other unidentified activities. However, despite suggestions concerning the several functions of plant cyclophilins, the physiological relevance and molecular basis of the stress-responsive expression of plant cyclophilins is still generally unknown. The extensive spread and ubiquitous nature of these cyclophilins does specify their essential importance in plant survival (Kumari et al., 2013).

9 Ribosomal protein L2

Ribosomal protein L2 (RPL2) is one of thirty-four proteins that form part of the larger 50S subunit of the prokaryotic ribosome, or one of forty-nine proteins in the large 60S subunit of the eukaryotic ribosome (Stelzl et al., 2001).

Functional category 2: Transporters/Transport ATPases

24 Putative glutathione S-transferase

Glutathione S-transferases (GSTs) are a family of eukaryotic and prokaryotic phase II metabolic isozymes, best known for being able to catalyse the conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates, in order to perform detoxification. GSTs catalyse the conjugation of GSH through a sulfhydryl group across a wide variety of substrates to increase the water solubility of the compounds. This detoxifies endogenous compounds (e.g. peroxidised lipids) and allows the breakdown of xenobiotics. GSTs can also bind to toxins and operate as transport proteins, resulting in GSTs originally being called ligandin (Leaver and George, 1998; Litwack et al., 1971).

Functional category 3:Energy/ ATP synthase/ Glycolysis/ Electron transport/ Gluconeogenesis/ Photosynthesis/ Pentose phosphate

20 RuBisCo subunit binding-protein beta subunit and 5 RuBisCo subunit binding-protein alpha subunit, chloroplast precursor, putative, expressed [Oryza sativa]

The rates of photosynthesis and photorespiration in the cell are determined by ribulose biphosphate carboxylase-hydrogenase (RuBisCo), by catalysing two reactions: the carboxylation of D-ribulose 1,5-bisphosphate, which is the main event in carbon dioxide fixation, and the oxidative fragmentation of pentose substrate in the photorespiration process (Ellis et al., 1988).

8 Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

An enzyme of ~37kDa catalyses the sixth step of glycolysis, thereby breaking down glucose into energy and carbon molecules (Tao Lu et al., 2009).

10 Dehydrogenase

A dehydrogenase is an enzyme from the group of Oxidoreductases, which oxidises a substrate by a reduction reaction, transferring one or more hydrides (H⁻) to an electron acceptor (Strommer, 2011).

12 ATPase subunit [Beta vulgaris subsp. vulgaris]

ATPases (or ATP synthases) are membrane-bound enzyme complexes/ion transporters that combine ATP synthesis and/or hydrolysis with the transport of protons across a membrane. ATPase can harness energy from a proton gradient, using the flux of ions across the membrane via the ATPase proton channel to drive the synthesis of ATP (Toei et al., 2010).

14 Enolase

Enolase is present in all tissues and organisms capable of glycolysis or fermentation. It is also known as phosphopyruvate hydrolase and catalyses the penultimate step of the glycolysis pathway; the conversion of 2-phosphoglycerate to phosphoenolpyruvate (Zhang et al., 1997).

Functional category 4: Protein destination and storage/storage protein

1 Vicilins/Stress protein

Vicilins are oligomeric seed storage proteins, (7S) globulins, which have a molecular mass of 50 kDa and are expressed during germination (Gomes et al., 1997). Vicilins 7S globulins are one of the major classes of seed storage proteins (Rangel et al., 2003). The vicilins in CPI are polypeptides migrating in SDS PAGE gel of 50 and 52 kDa. According to Rangel et al. (2003), purified cowpea vicilin consists of a doublet of polypeptides ranging from 30 to 52 kDa. A study carried out by Gomes et al. (1997) demonstrated that the vicilins from cowpea (*Vigna unguiculata*) and other legume seeds can be strongly linked with chitin, chitosan, and fully acetylated chitin. It is well established that chitin present in fungal cell walls attaches to many different proteins, called chitin-binding proteins. These are the cause of inhibition of microbial growth (Gomes et al., 1997).

Functional category 6: Metabolism/sugars and polysaccharides/amino acids/nucleotides/lipid

2 Lipoxygenase

Lipoxygenase is an iron-containing enzyme with the ability to catalyse the formation of hydroperoxides in fatty acids that contain a pentadiene segment (Andreou and Feussner, 2009). Lipoxygenases comprise a large family, spread widely across both the plant and animal kingdoms and play a role in plant growth, resistance to pests, senescence, and responses to damage (Braidot et al., 2004; Croft et al., 1994; Ha et al., 2014).

21 Tubulin

Limited studies have examined microtubules from plants that host pathogenic fungi. Microtubules are a preserved cytoplasmic structure found in all eukaryotes. Their function is varied in essential roles, ranging from cellular morphogenesis to cell division, cell movement and even signal transduction. They consist of a protein known as tubulin. Tubulin is a highly preserved heterodimeric protein, comprised primarily of monomeric globular polypeptides designated A-and B-tubulins. Microtubules exhibit antifungal activity against plant pathogenic fungi (Bokros et al., 1993; Hall and Minton, 2005; Koo et al., 2009; Landvik et al., 2001).

7 Actin

Action does not possess enzymatic activity and its contribution to various cellular processes resides in its ability “to offer a dynamic filamentous scaffold upon which several regulatory and motor proteins react” (Carrier, 1991; Sheahan et al., 2004).

Functional category 7: Stress responses/Disease/defence/pathogenesis-related protein

17 Heat shock protein

Heat shock proteins (HSPs) have been detected in all type of cells and tissue and are induced in cells exposed to sub-lethal heat shock. The literature study highlights that exposure to environment and pathological stresses contribute to the production of HSPs, particularly the inducible form of HSP-70, HSP-72 (Kiang and Tsokos, 1998). The degree of induction in response to heat shock, heavy metals, metabolic inhibitors, amino acid analogs, bacterial infection, viral infection, and Oxidant injury depends on the level and length of exposure to stress. The increase is temporary, but how long it remains high varies for different cell types. The major HSPs are expressed at 38°C in the absence of heat shock, and there are different reasons leading to the production of HSP proteins.

A study carried out by Wehmeyer and Vierling (2000) reported that the HSPs in maize, pea and fava beans have distinct regulatory controls and probably distinct functions throughout seed maturation as contrasting to during heat stress. This indicates that developing seeds can support a full heat shock response with the appearance of all of the heat-inducible class I and class II HSPs (Wehmeyer et al., 1996). In the heat stress tolerance the prominence of the HSPs is still not totally understood but by any means they are contributory to tolerance to a number of environmental stresses. These reasons may have led to the identification of two classes of heat shock proteins in the CPI sample.

18 Heat shock 70 kDa Proteins

Heat shock proteins HSP-70 and -90 are detected in all organisms, whereas HSP-110 is present generally in mammalian cells. Their extremely conserved structure suggests they play a role in essential cellular processes (Kiang and Tsokos, 1998). The main conclusions of a study carried out by Kiang and Tsokos (1998) are that, the mechanisms by which HSPs protect cells remain unclear. HSPs with molecular masses of 60, 70, 90, and 110 kDa are considered the most understood. HSPs have the ability to function as molecular chaperones in processes such as protein development and degradation. Their gene expression is triggered by physiological stimuli, pathological intruders, and environmental stressors (Kiang and Tsokos, 1998).

2.6 Discussion

The aim of this chapter was to characterise the proteins that exhibited antifungal activity described in the next chapter (Chapter 3). In the present chapter, different methods of protein extraction were applied to cowpea seeds and also subjected to ultrafiltration. These protein fractions had been tested for antifungal activity in Chapter 3 and the results indicated that the water soluble extract and ammonium sulphate precipitation fraction exhibited no antifungal activity, whereas cowpea protein isolate (CPI) as prepared by salt solution extraction and acid precipitation showed the best antifungal activity. CPI solution subjected to 10 kDa molecular weight cut off ultrafiltration resulted in the highest antifungal activity in the filtrate compared to the retentate. The aims of present chapter were to examine the electrophoretic profile of CPI, retentate and filtrate and to characterise the proteins in CPI by LC/MS/MS and to match the results to an existing data base to enable characterisation of proteins.

SDS PAGE analysis revealed that CPI consists of polypeptides of molecular weight ranging from 10 to 115 kDa, with major polypeptides at 30, 40, and 50 kDa (**Figure 2.8**). These

results confirm findings by Ahmed (2014), who reported a similar SDS PAGE profile for CPI. The electrophoretic profiles also showed that the polypeptides smaller than 30 kDa were removed from the retentate of CPI by ultrafiltration and enriched in the filtrate as shown by SDS electrophoretic profiles and corresponding densitometric scans (**Figures 2.10 and 2.11**). Chapter 3 will describe how the ultrafiltrate has increased antifungal activity compared to CPI and the retentate, which is attributed to proteins with molecular weight lower than 30 kDa.

The twenty-three proteins with lowest HN and highest score were selected from the first ninety-nine hits of matching of LC/MS/MS results to an existing database. The two most abundant proteins (with highest score number and lowest HN) were identified as vicilin (mw 49654), which is a storage protein, and then lipoxygenase (mw 97284) which is a metabolism protein. The vicilin identification confirms the results of SDS PAGE which shows a major protein with a molecular weight at 50 kDa (**Figure 2.9**) and the corresponding densitometric scans. This molecular weight was previously reported by Horax et al (2004) to be vicilin that is present in many pulses.

The identification of lipoxygenase being an abundant protein in CPI is surprising because the SDS PAGE profile of CPI shows a faint band at 98 kDa compared to vicilin (50 kDa) which is confirmed by the densitometric scan (**Figure 2.9**). The molecular weight of 98-100 kDa for plant lipoxygenase has been reported (Braidot et al., 2004). A possible explanation could be that the lipoxygenase was degraded by contaminating proteases during purification as no protease inhibitors were added to the purification buffers (section 2.4.3). Evidence reported of proteolytic stability of plant vicilin (Astwood et al., 1996) would explain the more extensive degradation of lipoxygenase compared to vicilin. As the complete preparation of CPI was analysed by LC/MS/MS, proteolytic fragments of lipoxygenase could also be matched to lipoxygenase in the database. Another explanation could be inadequate staining of lipoxygenase in the gel. Kwanyuen and Wilson (2007) reported differences in uptake of Coomassie Blue stain by different molecular weight proteins of soy bean protein isolate due to differences in density of the gradient gel.

Figure 2.11 shows that ultrafiltration of CPI enriched a peptide fraction with molecular weight 20-25 kDa and results in Chapter 3 show that this fraction exhibited high antifungal activity. Proteomics analysis of CPI resulted in an “unnamed protein” with molecular weight of 23 kDa and HN of 11 in (**Table 2.5**) Ye et al. (2002a) reported antifungal activity of a 28 kDa peptide purified from cow peas by CM Sepharose. The N-terminal amino acid

sequence of the 28kDa protein resembled that of a chitinase, but the protein remains unidentified.

Many of the proteins identified in the cowpea protein isolate have been reported previously in reference to other legume proteins and their functions have been determined (Boye et al., 2010; Mune et al., 2008; Pereira et al., 2009; Rangel et al., 2003; Rangel et al., 2004). A study carried out by Moura et al. (2014) describes the proteomics of proteins extracted from the leaves of the cowpea plant. However, in the present study the results of proteomics analysis of protein extracted from cowpea seeds (CPI) are reported for the first time.

Further studies would include proteomics analysis of the protein in the filtrate of CPI with the aim to characterise the low molecular weight proteins that demonstrated increased anti-fungal activity as described in Chapter 3.

Chapter 3

Antifungal Activity of Cowpea Seed Proteins

3.1 Introduction

3.1.1 *Brief introduction to fungi*

Fungi are a group of approximately 80,000 known species of organisms, which include yeasts, rusts, smuts, mildews, moulds, and mushrooms.

In addition, there are numerous fungus-like organisms, such as slime moulds and oomycetes, which are not part of the kingdom of fungi, being chromista, but which are often referred to as fungi. Fungi are very important, both for the environment and in medical applications, and are widely distributed worldwide. Many fungi live freely, in either the soil or water, while others form relationships, which may be parasitic or symbiotic, with plants or animals (Webster and Weber, 2007).

Fungi are eukaryotic organisms, meaning their cells contain membrane-bound organelles and clearly defined nuclei. They were once included in the plant kingdom, however as many lack chlorophyll and possess unique structural and physiological features they are now considered distinct. Moreover, fungi's main method of vegetative growth and nutrient intake distinguished them from all other living organisms, including animals.

Fungi use extracellular digestion to take in nutrients, assisted by enzymes, with the subsequent absorption of the solubilised breakdown products. This blend of extracellular digestion and absorption is the ultimate indicator of the fungal lifestyle. Fungi grow as a system of branching tubes, known as the hyphae (**Figures 3.1 A, B**), which form the mycelium.

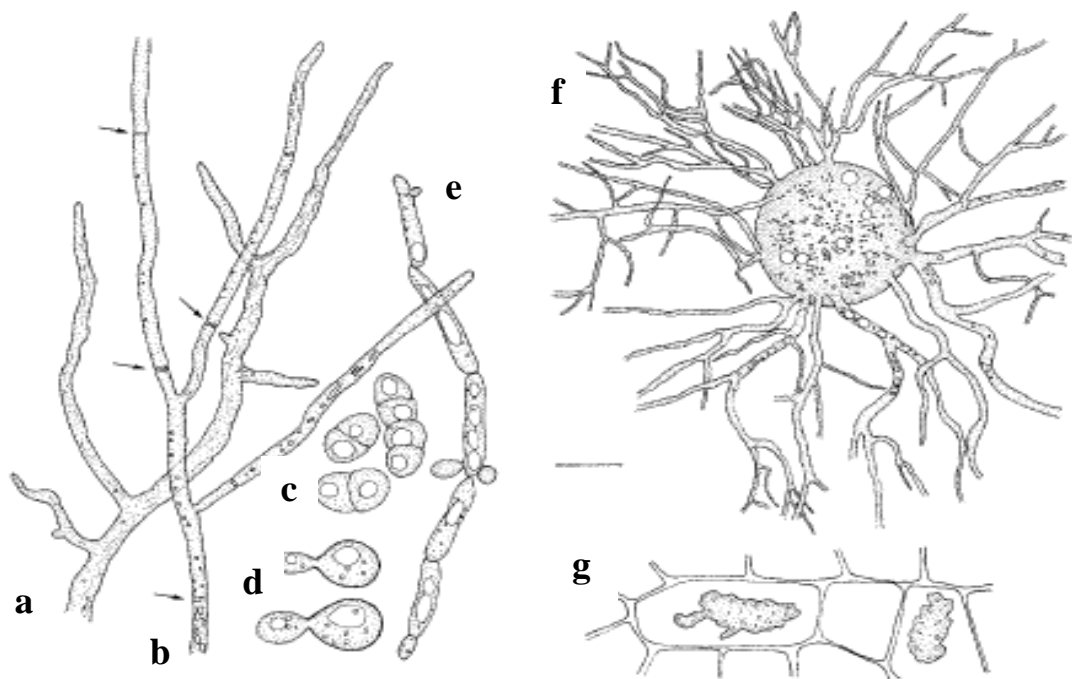


Figure 3.1 Various growth forms of fungi. **(a)** Aseptate hypha of *Mucormucedo* (Zygomycota). The hypha branches form a mycelium. **(b)** Septate branched hypha of *Trichoderma viride* (Ascomycota). Septa are indicated by arrows. **(c)** Yeast cells of *Schizosaccharomyces pombe* (Ascomycota) dividing by binary fission. **(d)** Yeast cells of *Dioszegia takashimae* (Basidiomycota) dividing by budding. **(e)** Pseudohypha of *Candida parapsilosis* (Ascomycota), which is regarded as an intermediate stage between yeast cells and true hyphae. **(f)** Thallus of *Rhizophlyctis rosea* (Chytridiomycota) from which a system of branching rhizoids extends into the substrate. **(g)** Plasmodia of *Plasmodiophora brassicae* (Plasmodiophoromycota) inside cabbage root cells (Webster and Weber, 2007).

Not all fungi have hyphae; some are discrete yeast cells, divided by either fission (**Figure 3.1 c**) or, more commonly, budding (**Figure 3.1 d**). Yeasts are common, particularly where there is no need for efficient penetration of the substratum, including on plant surfaces or in animals' digestive tracts (Carlile et al., 2001). Some species are dimorphic, meaning they can switch between hyphal and yeast-like growth forms. There are also stages between yeast cells and true hyphae, known as pseudohyphae (**Figure 3.1 e**). Some lower fungi grow as a thallus, a walled structure in which the protoplasm is concentrated in one or more centres, from which root-like branches (rhizoids) ramify (**Figure 3.1 f**). Some plant-pathogenic fungi and fungus-like organisms grow as naked plasmodium (**Figure 3.1 g**).

Higher fungi's hyphae extend through tip growth, and this is followed by cross-wall formation or septation, while the lower fungi stay aseptate (aside from the segregation of spores or damaged colony areas) (Carlile et al., 2001; Webster and Weber, 2007).

3.1.2 *Naming and Classification*

The fungi kingdom is incredibly large, with a combination of both identified species and species yet to be categorised exceeding 300,000. The majority of these species are microscopic fungi (including yeasts, and moulds). A smaller number of species have “mushroom” reproductive systems, which can easily be seen.

There are three kingdoms of fungi: eukaryota (exclusively contains fungi), and protozoa and chromista (both of which have organisms not studied by mycologists and grouped together under the term protocista). Protozoa are very difficult to resolve using phylogenetic means, and they form a diverse and ancient intermediate group between the eukaryotes and the prokaryotes (Webster and Weber, 2007).

3.1.3 *Physiology and taxonomy*

The eukaryotic nature of fungal cells is determined by the cell nucleus, which is surrounded by a double membrane, enclosing the chromosomes in a nucleoplasm. The majority of yeasts and fungi have haploid life cycles, although some can alternate between haploid and diploid life cycle. Chromosomes contain DNA-protein structures, which duplicate and segregate into newly divided cells or hyphal compartments at mitosis, thereby ensuring the genetic material transfers to daughter cells or septate compartments during cell division. Normally yeasts have a single nucleus per cell, although in filamentous fungi, the hyphal compartments may have more than one nucleus. Monokaryotic basidiomycetes have one nucleus type per compartment, while dikaryons or heterokaryons have two or more genetically distinct haploid nuclei. Multiple nuclei in individual hyphal compartments enable fungi to benefit from both haploid and diploid lifestyles (Carlile et al., 2001). **Table 3.1** summarises the physiological function of various fungal cell organelles.

Table 3.1 Functional components of an idealised fungal cell.

Organelle or cellular structure	Function
Cell envelope	Comprising: the plasma membrane, which acts as a selectively permeable barrier for the transport of hydrophilic molecules in and out of the fungal cells; the periplasm, containing proteins and enzymes unable to permeate the cell wall; the cell wall, which provides protection and shape and is involved in cell–cell interactions, signal reception, and specialised enzyme activities; fimbriae involved in sexual conjugation; capsules to protect cells from dehydration and immune cell attack
Nucleus	Relatively small, containing chromosomes (DNA–protein complexes), which pass genetic information to daughter cells on cell division, and the nucleolus, which is the site of ribosomal RNA transcription and processing
Mitochondria	Site of respiratory metabolism under aerobic conditions and, under anaerobic conditions, for fatty acid, sterol and amino-acid metabolism
Endoplasmic reticulum	Ribosomes on the rough ER are the sites of protein biosynthesis
Proteasome	Multi-subunit protease complexes involved in regulating protein turnover
Golgi apparatus and vesicles	Secretory system for import (endocytosis) and export (exocytosis) of proteins
Vacuole	Intracellular reservoir (amino acids, polyphosphate, metal ions); proteolysis; protein trafficking; control of cellular pH. In filamentous fungi, tubular vacuoles transport materials bi-directionally along hyphae
Peroxisome	Oxidative utilization of specific carbon and nitrogen sources (contain catalase, oxidases). Glyoxysomes contain enzymes of the glyoxylate cycle

Source: Kavanagh (2011)

3.1.4 Nutritional requirements

In the case of nutrition, fungi have simple needs, and the majority of species can survive well in aerobic conditions, provided that they have a supply of glucose, ammonium salts, inorganic ions and several other growth factors. Macronutrients, in millimolar concentrations contain sources of carbon, nitrogen, oxygen, sulphur, phosphorus, potassium, and magnesium; and micronutrients, in micromolar concentrations containing trace elements including calcium, copper, iron, manganese, and zinc, all of which would be needed for fungal cell growth. Sugars are commonly used in fungal growth, ranging from simple hexoses (e.g. glucose) to polysaccharides (e.g. starch, cellulose, aromatic hydrocarbons). Fungi are non-diazotrophic (cannot fix nitrogen) and therefore must be supplied with compounds that contain nitrogen, either in inorganic form (e.g. ammonium

salts), or organic form (e.g. amino acids). A common source of nitrogen in fungal growth media is ammonium sulphate, as it is also a source of utilisable sulphur. Nitrate can also facilitate the growth of many fungi (except the yeast *S. cerevisiae*), and they may also utilise nitrite. Nitrate reductase, followed by nitrite reductase, are the enzymes that convert nitrate to ammonia. The majority of fungi can assimilate amino acids, amines, and amides as sources of nitrogen. Urea is also commonly used by fungi, and certain basidiomycetous yeasts are categorised as urease positive (able to utilise urea), although the majority of ascomycetous yeasts are urease negative. As regards the need for oxygen, the majority of fungi are aerobes, although yeasts like *S. cerevisiae* are sometimes termed facultative anaerobes, as they can only grow in strictly anaerobic conditions if they have a supply of fatty acids and sterols (which they are unable to synthesise without molecular oxygen). Oxygen is necessary as the terminal electron acceptor in aerobically respiring yeasts and fungi (Garraway and Evans, 1984).

3.1.5 *Reproduction*

Fungi reproduction occurs in complex ways, mirroring the variations in lifestyle and genetic makeup within the wide and varied kingdom of fungi. Reproduction can be asexual, sexual, and via dispersing spores or spore-containing propagules (Webster and Weber, 2007).

3.1.5.1 *Asexual reproduction*

Asexual reproduction occurs either through vegetative spores (conidia) or mycelial fragmentation. The fungal mycelium divides into components, with each growing into an individual mycelium. The asexual structures of oomycetes for dispersal can be either flagellate motile zoospores, or sporangia, where the zoospores develop. The zoospores can be released by the dispersing zoosporangia, or the latter can germinate and develop coenocytic hyphae once they have found a suitable surface. Certain species use both processes, and are dependent on factors such as the environment and temperature. In certain species, sporangia can also disperse, with the structures sharing similarities with conidiophores and conidia. Conidiophores are simple or branched hyphae that bear spores (conidia) such as in *Penicillium* fungi (Kavanagh, 2005).

3.1.5.2 *Sexual reproduction*

Apart from glomeromycota, all fungal phyla can reproduce sexually. There are considerable differences in the sexual reproduction among the primary fungal groups, and within these

groups. Species may be identified according to biological species, based on concepts used in mating experiments. The structures developed during sexual reproduction result in the names of the principal groups of fungi. Oomycetes produce gametangia. The oogonium comprises haploid oocytes produced by meiosis. Along with the oogonium, antheridia develop and produce haploid nuclei through meiosis. These nuclei migrate to oogonia through a fertilization tube produced by the oogonium, in order to fertilise an oocyte. This fusion results in diploid oospores that germinate and produce coenocytic non-septate hyphae with multiple nuclei. This is the diploid life cycle. There are life cycle variations within the group: some oocytes develop in the oogonia, and the oospores produce both coenocytic hyphae and sporangium-producing zoospores. The oogonia and the antheridia are able to produce hormones that stimulate and regulate development reciprocally (Kavanagh, 2005).

3.1.6 *Spore dispersal*

In both asexual and sexual dispersion, spores from a large number of fungal species are dispersed following forcible ejection from their reproductive structures. The ejection process ensures that the spores leave the reproductive structures and travel through the air over a long distance. Consequently, several fungi have specific mechanical and physiological mechanisms, in addition to the spore-surface structures (e.g. hydrophobins), in order to expel spores. There are different mechanisms to release spores for example by external mechanical forces, for example puffballs (Carlile et al., 2001).

3.1.7 *The fungal cell wall*

3.1.7.1 *Fungal cell wall structures*

The cell envelope is the peripheral structure around the cytoplasm in fungi, comprising the cell membrane, cell wall and other extracellular structures. The cell wall (a dynamically forming exoskeleton) protects the fungal protoplast from the external environment and defines growth, cellular strength, shape, and interactive properties. In filamentous fungi, apical growth is linked closely to the creation and organisation of the cell wall. The plasma membrane in the fungal cell envelope is a phospholipid bilayer, interspersed with globular proteins, which control the entrance of nutrients and the exit of metabolites, thereby forming a selective barrier for their translocation. The main sterol in fungi membranes is ergosterol; this differs from the cholesterol found in animal membranes, and phytosterols in plants.

The cell wall structure in yeasts contains polysaccharides (primarily β -glucans for rigidity) and proteins, and some lipid and inorganic phosphate material. Typically, hyphal cell walls have fewer mannans than yeast cells, thus changes in their composition can be observed in the transition from unicellular to mycelial growth of dimorphic fungi. **Figure 3.2** shows the structure of the fungal cell wall (Hardison and Brown, 2012; Kavanagh, 2005).

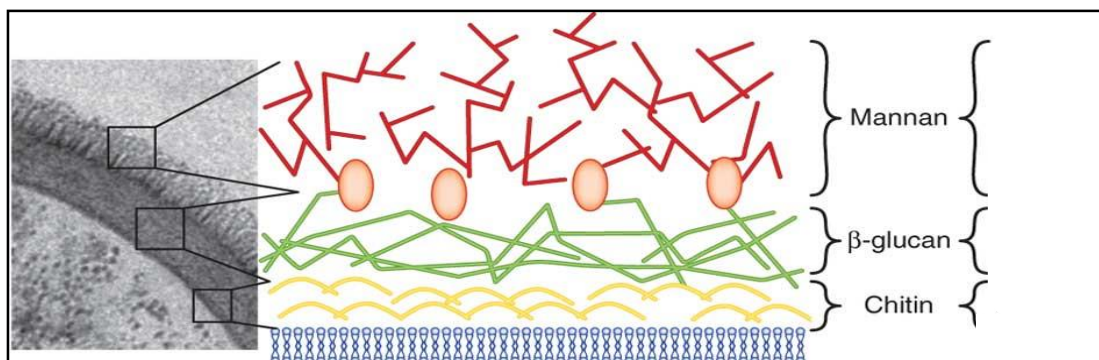


Figure 3.2 Electron micrograph of the fungal cell wall (*Candida albicans*). The carbohydrate-rich layers of the fungal cell wall are highlighted: mannan (mannosylated proteins), β -glucan and chitin (Hardison and Brown, 2012).

The fibrillar polysaccharides, chitin, chitosan, chitin-glucan complexes, and cellulose comprise the inner layer of the wall. These are embedded in more gel-like matrix polymers, such as glucans and glycoproteins, which extend outwards to comprise the outer layer of the wall (Hardison and Brown, 2012).

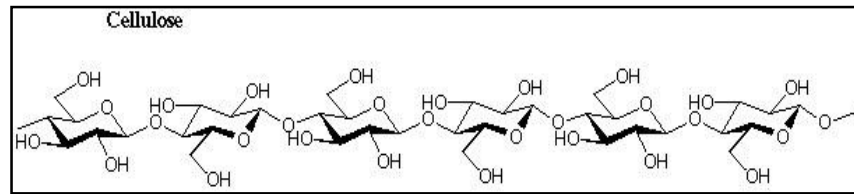
Filamentous fungi cell walls consist of various polysaccharides according to taxonomic groups (chitin, glucans, mannoproteins, chitosan, polyglucuronic acid or cellulose) along with smaller amounts of proteins and glycoproteins (**Table 3.2**).

Table 3.2 The major polymers found in different taxonomical groups of fungi.

Taxonomic Grouping	Fibrillar Polymers	Matrix polymers
ColumOomycetes	$\beta(1,3)$, $\beta(1,6)$ – Glucan; Cellulose	Glucan
Chytridomycetes	Chitin; glucan	Glucan
Zygomycetes	Chitin; chitosan	Polyglucuronic acid Glucuronomannoproteins
Basidiomycetes;	Chitin; $\beta(1,3)$ - $\beta(1,6)$ Glucans	$\alpha(1,3)$ -glucan; xylomannoproteins

Source: Hanson (2008)

The chemical structures of the major polymers found in fungi cell walls are displayed in **Figures 3.3, 3.4 and 3.5.**



(Source: <http://www.clarku.edu/faculty/dhibbett/tftol/content/1introprogress.html>).

Figure 3.3 Structure of cellulose

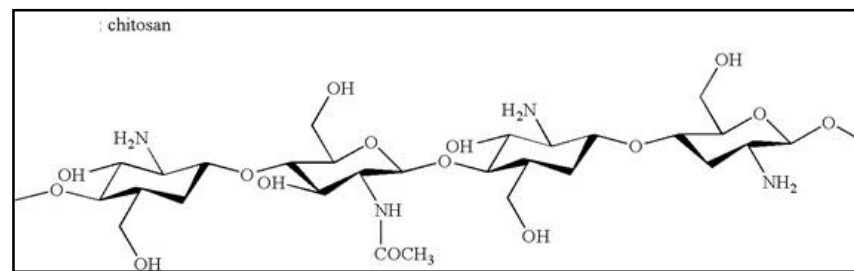


Figure 3.4 Structure of chitosan (Hanson, 2008).

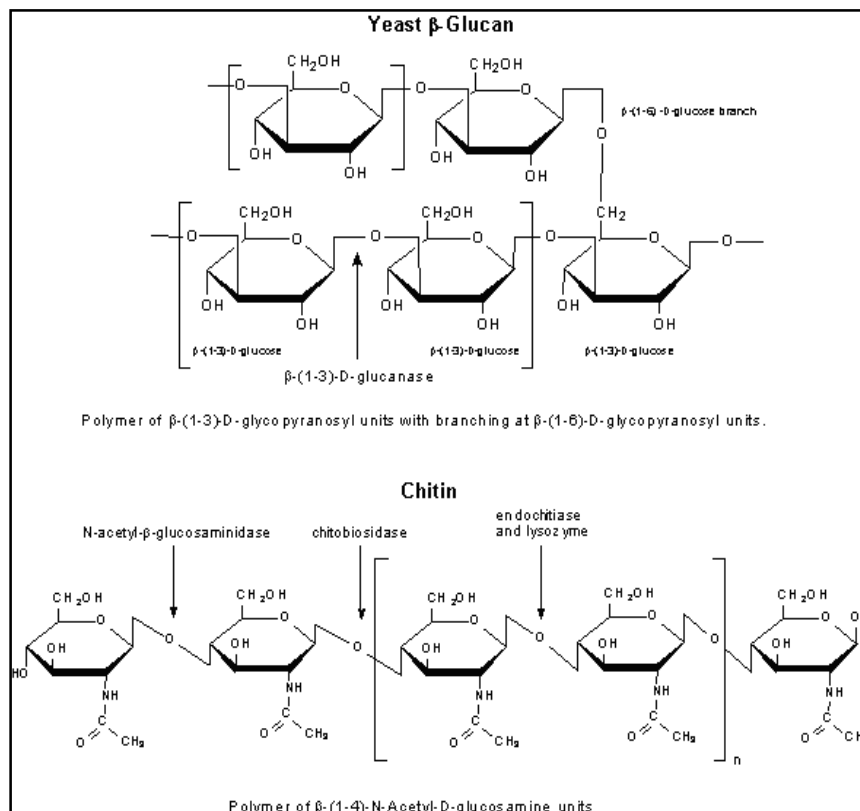


Figure 3.5 Structure of glucan & chitin (Hanson 2008).

3.1.8 Mould spoilage

Spoilage and contamination of foods by fungi is a major problem, especially in developing countries (Saranraj and Geetha, 2011). Mould spoilage is considered a serious and costly problem for bakeries, and consequently, using preservatives is an attractive way to limit spoilage and ensure food safety. Nevertheless, consumers are increasingly demanding a reduction in the use of additives as preservatives. To date, mould growth is the major factor limiting the shelf life of bakery foodstuffs. Generally, most moulds prefer high a_w values (>0.8), whereas a few xerophilic moulds grow at a_w values as low as 0.65. Mould growth on bakery foodstuffs results in serious commercial losses.

The average losses in the bakery industry attributable to mould spoilage amount to around 200 million pounds of product each year in the UK (Seiler 1993). The most common moulds found in bakery products are *Rhizopus* sp., *Aspergillus* sp., *Penicillium* sp., *Monilia* sp., *Mucor* sp and *Eurotium* sp. (Jago and Jago, 1911; Seiler, 1983; Legan, 1993; Seiler, 1993).

3.1.9 Microbiological tests used in this study

In this chapter, CPI was tested to investigate its effectiveness against *Penicillium chrysogenum*, *P. brevicompactum*, *P. hirsutum*, *Aspergillus versicolor* and *Eurotium rubrum*, which are the moulds most responsible for bread spoilage (Legan, 1993).

Different methods were adopted in this study to determine the activity of cowpea protein extract against bread mould. These were the disk-diffusion method, the microspectrophotometric assay (microplate method), and the agar dilution method.

A. Disk diffusion method

In the first stage, the antifungal activity of CPI was estimated according to the disk-diffusion agar method, which is applicable as a simple and low cost test. The specific advantage of this method is that it makes it possible to test several extracts per plate against particular microorganisms, using small volumes of test samples. In addition, it is recommended as the general standard method for testing natural products (Burt, 2004; Scorzoni et al., 2007; Pillay et al., 2011).

B. The microspectrophotometric assay (microplate method)

The purpose of this method was to determine the strength of antifungal properties and the minimal inhibitory concentration (MIC). Growth is measured according to optical density at 590nm.

A microplate containing a series of wells was used. For each of the different proteins, the extract was mixed with an appropriate medium, potato dextrose broth (PDB), and then inoculated with a fungal strain. The growth was then measured according to optical density at 590nm using an ELISA plate scanner. When applying this method, it is possible to determine the MIC, which is defined as the lowest concentration capable of inhibiting visible fungal growth. This method resulted in the most reproducible results for the MIC (Scorzoni et al, 2007). In addition, it was recommended as the standard method for testing natural products (Benaducci et al., 2007; Scorzoni et al., 2007).

C. Agar dilution method

The purpose of this method was to determine the strength of antifungal properties according to visible growth and also to determine the minimal inhibitory concentration (MIC). The agar dilution method is the first technique to have been standardised for MIC and is currently the recommended method for *Campylobacter* sensitivity testing. In this test observable growth of microorganisms in a series of dilutions is tested on agar plates (Luber et al, 2003).

3.2 Materials and Methods

3.2.1 Materials

Strains of fungi were supplied by Leibniz-Institut DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen Gmb centres (Germany), and the cowpea (*Vigna unguiculata*) seeds were obtained from a local market (ASDA). A 10 mM Tris-HCl buffer, Petri dishes (100 x 15 mm), a sterile blank paper disks (0.64 cm in diameter), a cell-counting chamber, potato dextrose agar, potato dextrose broth, and microplates were obtained from Sigma-Aldrich, London (UK).

The following cowpea protein extracts were tested:

- Cowpea water soluble extract, prepared as described in Chapter 2, section 2.4.1
- Cow pea ammonium sulphate precipitation fraction (Chapter 2, section 2.4.2)
- CPI as described in Chapter 2, section 2.4.3
- CPI filtrate prepared as described in Chapter 2, section 2.4.7
- CPI retentate prepared as described in Chapter 2, section 2.4.7

3.2.2 Methods

3.2.2.1 Preparation of spores

The fungi tested in this study were *Penicillium chrysogenum*, *Penicillium brevicompactum*, *Penicillium hirsutum*, *Aspergillus versicolor* and *Eurotium rubrum*. Potato dextrose agar was used to grow the fungus at 27°C for 7 days. The petri dish containing fungal growth was examined under a dissecting microscope. A lot of germinating conidia appeared on the sites of inoculation and by using the low power of the microscope, single conidia could be observed, which were removed using a sterile Pasteur pipette and immersed in 3 ml of sterile water. The spore concentration was determined by using a cell-counting chamber and adjusted to 2×10^6 spores /ml (Dellavalle et al., 2011).

Antifungal activity was performed in this study using three methods; 1) disk diffusion assay, 2) agar dilution method and 3) microspectrophotometric assay (microplate method). This method was also used to measure the minimal inhibitory concentration (MIC) of CPI.

3.2.2.2 Disk diffusion assay

The purpose of this method was to test the effect of different concentrations of CPI on the growth of various fungal species on an agar plate. The disk diffusion method was carried out in accordance with that described by Ye et al. (2000a). Petri dishes (100 x 15 mm) containing 10 ml of potato dextrose agar were used. These were then inoculated with different species of fungi, including: *Penicillium chrysogenum*, *Penicillium brevicompactum*, *Penicillium hirsutum*, *Aspergillus versicolor* and *Eurotium rubrum*. A sterile blank paper disk (0.64 cm in diameter) was placed in the middle, and 100 µl of the sterile CPI test sample (0.05g/ml; 0.8g/ml 0.1g/ml and 0.8 g/ml) that had been subjected to microfiltration in 10 mM Tris-HCl buffer (pH 7.2), was added to the disc. The negative control was a disk with water. The Petri dish was then incubated at 25°C for 72 hrs.

3.2.2.3 Agar dilution method

The purpose of this method was to test the effect of different concentrations of CPI on growth of *Penicillium chrysogenum* on an agar plate. This method was conducted according to that reported by Luber et al. (2003). Firstly, 5 ml of each of the test samples (0.02g/ml; 0.05g/ml and 0.08 g/ml) of CPI were added to 10 ml of potato dextrose agar and poured into petri dishes (100 x 15 mm) and allowed to solidify. Then 10 µl of spore suspension (2×10^6 spores/ml) of test organism was inoculated on the surface of the

solidified medium, which was incubated at 25°C and then the antifungal activity was tested by determining the visible growth of fungi during 4 weeks.

3.2.2.4 *Microspectrophotometric assay (microplate method)*

The purpose of this method was to measure the inhibition activity of CPI (0.05 g/ml) against different species of fungi, including; *Penicillium chrysogenum*, *Penicillium brevicompactum*, *Penicillium hirsutum*, *Aspergillus versicolor* and *Eurotium rubrum*. The methodology of Dellavalle et al. (2011) was followed. All the media and equipment were autoclaved at 121°C for 15 mins for the antifungal assay. First, a 96-well microlitre plate was used to measure the growth inhibition of 10 µl of spore suspension (2×10^6 spores/ml) in 70 µl of potato dextrose broth (PDB) by 20 µl of CPI protein at a concentration of 0.05 g/ml. The negative control was 10 µl of spore suspension in 70 µl of PDB and 20 µl of distilled water. The ELISA plate reader was used to measure the absorbance at 595 nm, after 2, 24, 48, and 72 hours. All the experiments were undertaken in triplicate under sterile conditions.

3.2.2.5 *Minimal inhibitory concentration (MIC) using the microspectrophotometric assay (microplate method)*

To determine the minimum inhibitory concentration (MIC) of CPI the microspectrophotometric assay (microplate method) was also conducted according to Dellavalle et al. (2011) as described in section 3.2.2.4. In this method 20 µl CPI of different concentrations of (0.01 g/ml; 0.02 g/ml; 0.05 g/ml; 0.08 g/ml; 0.1 g/ml) was inoculated with a 10 µl suspension (2×10^6 spores/ml) of *Penicillium chrysogenum*, *Penicillium brevicompactum*, *Penicillium hirsutum* and *Eurotium rubrum* respectively in 70 µl of potato dextrose broth (PDB). The negative control was 10 µl of spore suspension in 70 µl of PDB and 20 µl of distilled water. Samples were incubated at 25°C for 72 hours. The MIC was defined as the lowest concentration of CPI that inhibited fungal growth at 25 °C for 72 hours.

3.2.2.6 *Proteolysis of CPI*

CPI (2%) was treated with proteinase K and trypsin based on the work of Rizzello et al. (2011). Trypsin and proteinase K were dissolved in Tris-HCl 0.25 M, pH 8 (0.1% wt/vol, final concentration). One hundred microlitres of CPI extract was mixed with 100 µl of the enzyme solution and incubated for 6 hours at 25°C. The mixture was boiled for 3 mins to stop the

reaction; thereafter the residual activity of the CPI extract was determined using the microplate method as described in section 3.2.2.4.

3.2.2.7 *Determination of thermal stability of CPI compounds related to antifungal activity*

This method was carried out according to that described by Yu et al. (2007). The CPI extract was treated at different temperatures: 70°C and 120°C for a period of 15 mins, then the remaining antifungal activity was tested using the microplate method as described in section 3.2.2.4.

3.2.2.8 *Statistical analysis*

The determinations were performed in triplicate (n=3) and mean \pm standard deviation (SD) values calculated. The data obtained were analysed using a one-way ANOVA. A comparison between the means (3 replications) was performed at the 95% significance level ($p \leq 0.05$), applying the least significant difference test (LSD). The analyses were carried out using SPSS version 10 for Windows (SPSS Inc., NY, and USA).

3.3 Results

3.3.1 *Antifungal activity of different extracts*

Table 3.3 Antifungal activity of different extracts of cowpea with different test methods.

Type of extract	Test method	Antifungal activity
Soluble extract	Microplate	Negative
	Disk diffusion	Negative
	Agar dilution	Negative
Ammonium sulphate precipitate	Microplate	Negative
	Disk diffusion	Negative
	Agar dilution	Negative
CPI	Microplate	Positive
	Disk diffusion	Negative
	Agar dilution	Positive
CPI retentate	Microplate	Weak activity
	Diskdiffusion	Negative
CPI filtrate	Microplate	Positive
	Disk diffusion	Negative

The result presented in **Table 3.3** above depicts that CPI and CPI filtrate showed positive inhibitory activity using the microplate assay. The cowpea soluble extract and ammonium sulphate precipitate showed no inhibitory activity. This might be because the active antifungal fraction is not extractable with water (soluble extract), or because the high salt concentration of the ammonium sulphate precipitate could have masked the activity of the antifungal proteins.

3.3.1.1 Efficiency of different methods

In this section different antimicrobial methods were used to test the antifungal activity of CPI. The disk diffusion method concerned addition of different concentrations of CPI to agar plates inoculated with different fungal species and a paper disk infused with CPI was placed on each plate to estimate the degree of inhibition during incubation over 72 hrs. The agar dilution method concerned the addition of different concentrations of CPI to potato dextrose agar which was poured into petri dishes. After solidification the agar was inoculated with *Penicillium chrysogenum*, and visible growth of fungi was monitored visually during 4 weeks. The microspectrophotometric assay (microplate method) was used to monitor spectrophotometrically the multiplication of fungal spores in potato dextrose broth (PDB) containing 0.05g/ml CPI. This method was also used to determine the minimum inhibitory concentration (MIC) of CPI.

The disk diffusion method was not reproducible for cowpea protein extracts, because of dissimilarities in the physical properties, for instance solubility and diffusion in agar. Some compounds have low antimicrobial activity but a high diffusion coefficient or solubility, and migrate through the agar swiftly, so they have similar effect to those for active compounds with low diffusion. This problem arises when zones of inhibition are associated with different classes of compounds. In addition, many factors could affect the size of the inhibition zones, including evaporation of antimicrobial substances, disk size, the volume of compound added to the disk, adsorption by the disk, the kind of agar, the agar's strength, the pH, the volume of the agar, and the microbial strains tested (Luber et al., 2003; Burt, 2004; Scorzoni et al., 2007). The microspectrophotometric assay gave the most reliable and reproducible results and the agar dilution method gave a good visual presentation of the effect of CPI on fungal growth on an agar plate.

3.3.2 Antifungal Activity of CPI

3.3.2.1 Microspectrophotometric Assay

The microspectrophotometric assay method was performed to measure the effect of 0.05 g/ml CPI on fungal growth spectrophotometrically. Multiplication of fungal spores with CPI was measured as increase in absorbance at 595 nm. The negative control was fungal spores without CPI in the growth medium.

The antifungal activities of CPI are presented in **Figure 3.6**. The growth of the species *Penicillium chrysogenum*, *Penicillium brevicompactum*, *Penicillium hirsutum* and *Eurotium rubrum* demonstrated some degree of sensitivity to the CPI extract tested, although the growth of *Aspergillus versicolor* was not inhibited by CPI extract. From this, the CPI with the widest spectrum of activity was found to be against *E. rubrum*, followed by *P. brevicompactum*, *P. chrysogenum* and *P. hirsutum* with a statistically significant variation compared to the negative control.

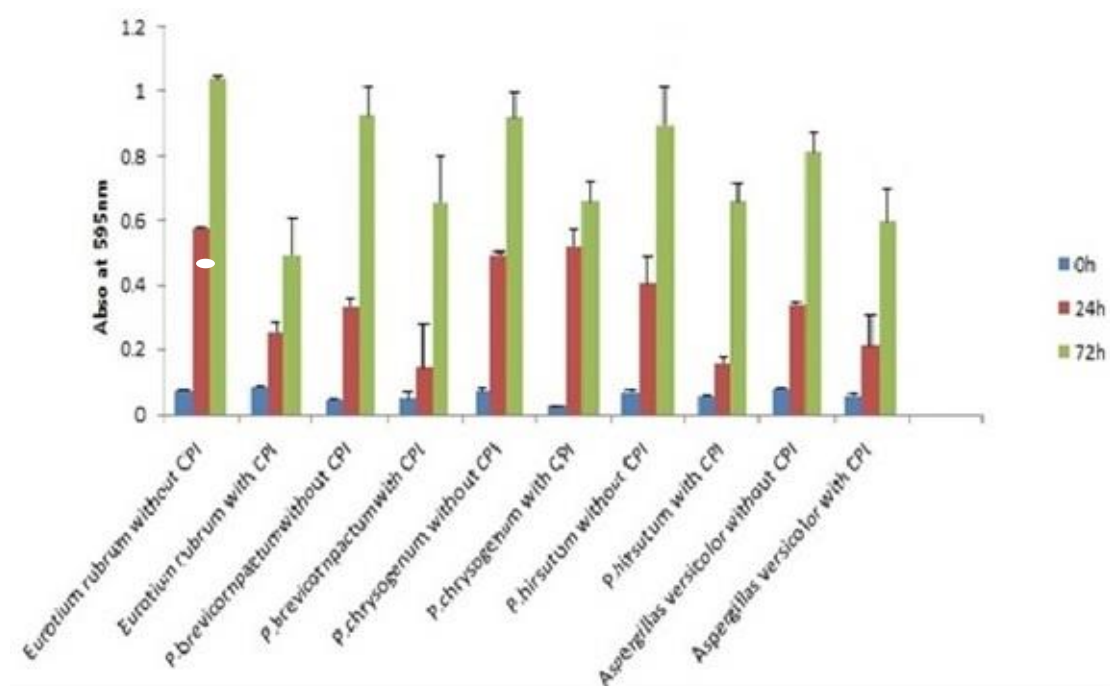


Figure 3.6 Antifungal efficacy of 0.5g/ml of CPI extract as a function of time after incubation with *P. hirsutum*, *P. chrysogenum*, *P. brevicompactum*, *E. rubrum* and *A. versicolor* at pH7.2 at 25°C as determined by the microplate method. The blue bar indicates 24 hours, the red bar indicates 48 hours and the green bar indicates 72 hours. The growth was tested in the absence of CPI as a negative control.

The experiment was undertaken in triplicate and standard errors indicated. A statistical difference at $p < 0.05$ was considered significant after 72 hours. **Figure 3.6** shows that *E. rubrum*, *P. brevicompactum*, *P. hirsutum* and *P. chrysogenum* treated with CPI showed statistically significant different lower values compared to the control that was not treated with CPI (p values: $p < 0.001$, $p < 0.037$, $p < 0.04$, $p = 0.043$ respectively), confirming inhibition of fungal growth by CPI. *A. versicolor* treated with CPI showed no significant difference in growth compared to the control ($p = 0.137$, respectively), indicating that CPI did not inhibit growth of *A. versicolor*.

3.3.2.2 Minimal inhibitory concentration (MIC) of CPI using microplate method

In order to determine the MIC of CPI, the method described in section 3.2.2.5 was performed. The CPI concentrations used were (0.01 g/ml, 0.02g/ml, 0.05 g/ml, 0.08 g/ml and 0.1 g/ml) against strains of *P. hirsutum*, *P. chrysogenum*, *P. brevicompactum* and *E. rubrum*. This experiment confirmed that the inhibitory activity of CPI depends on both the species of fungi and the CPI concentration

As can be seen in **Figures 3.7, 3.8, 3.9 and 3.10**, CPI at a low concentration (0.05 g/ml) exhibited a high level of inhibitive activity against *P. chrysogenum*, at concentrations of 0.1 and 0.05 g/ml it presented a high level of inhibitive activity against *P. brevicompactum*, at concentrations of 0.05 and 0.02 g/ml it inhibited *E. rubrum* and at 0.05, 0.01 and 0.1 g/ml it showed a strong ability to inhibit *P. hirsutum*.

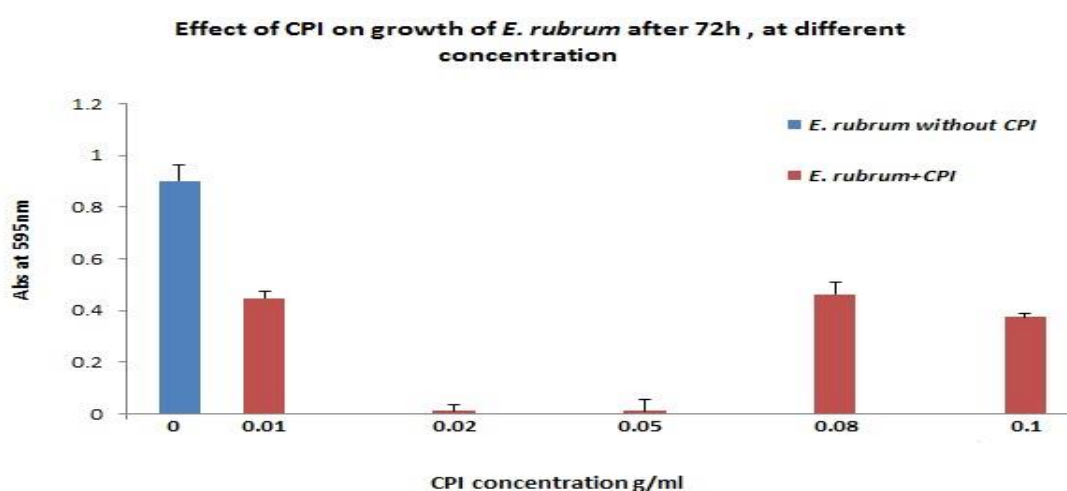


Figure 3.7 Antifungal efficacy of different concentrations of CPI extract after incubation with *E. rubrum* for 72 hours.

The inhibitory effects of CPI depicted in **Figure 3.7** were statistically significant compared to the control: $p=0.026$, $p<0.001$, $p<0.001$, $p=0.027$, $p=0.024$) respectively.

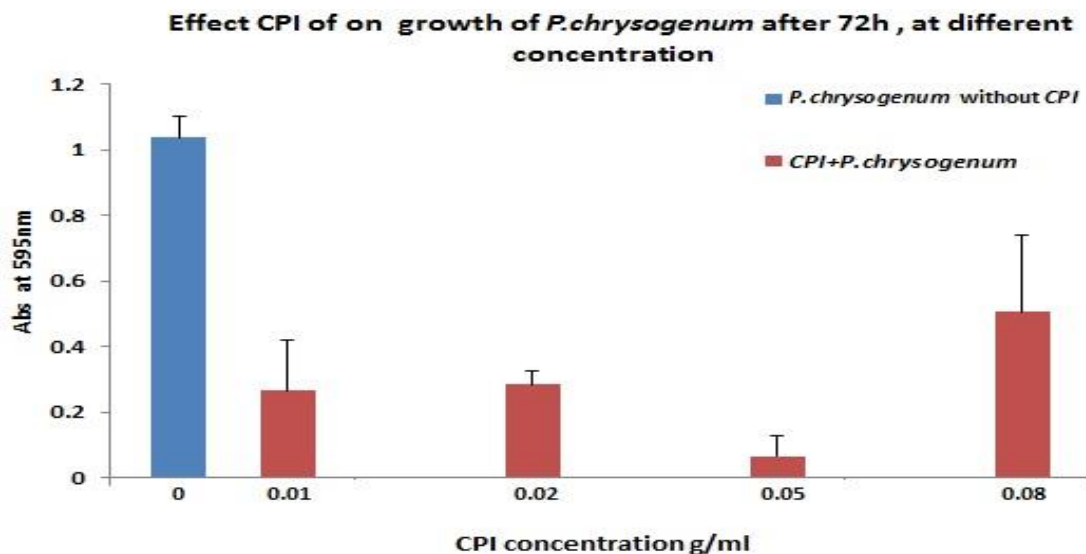


Figure 3.8 Antifungal efficacy of different concentrations of CPI extract after incubation with *P. chrysogenum* for 72 hours.

The different concentrations of CPI in **Figure 3.8** all showed statistically significant inhibition against *P. chrysogenum* when compared to the control: $p=0.004$, $p=0.006$, $p<0.001$, $p=0.023$, respectively.

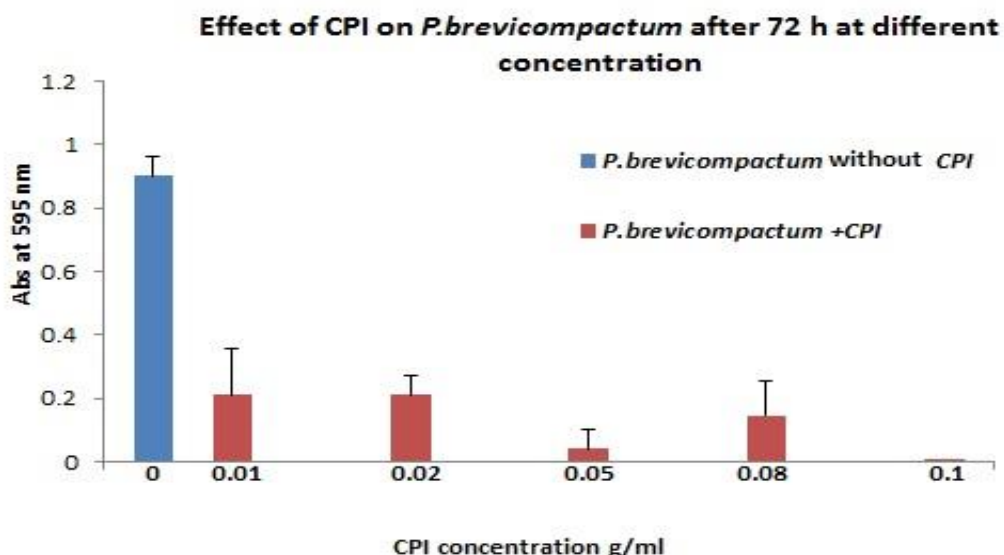


Figure 3.9 Antifungal efficacy of different concentrations of CPI extract after incubation with *P. brevicompactum* after 72 hours.

All the different concentrations of CPI in **Figure 3.9** showed statistically significant inhibition against *P. brevicompactum*, when compared to the control: $p=0.004$, $p=0.003$, $p<0.001$, $p<0.001$, $p<0.001$, respectively.

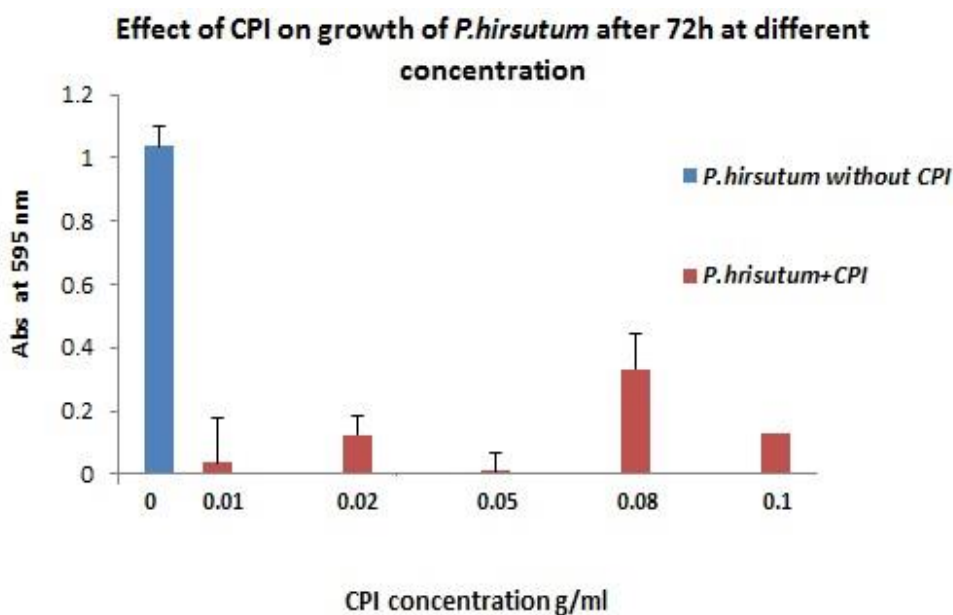


Figure 3.10 Antifungal efficacy of different concentrations of CPI extract after incubation with *P. hirsutum* for 72 hours.

All the different concentrations of CPI in **Figure 3.10** showed statistically significant inhibition against *P. hirsutum* compared to the control: $p<0.001$, $p<0.001$, $p<0.001$, $p=0.025$, $p<0.001$ respectively.

This experiment was interpreted to indicate that CPI extract had minimal inhibitory concentration (MIC) of 0.05g/ml against *E. rubrum*, *P. brevicompactum*, *P. hirsutum* and *P. chrysogenum*. The degree of activity was interpreted according to the criteria proposed by Morales et al., (2008) as moderate activity (MIC: 0.01-0.05 g/ml). An inactive extract would have an MIC >0.1 g/ml and high antifungal activity would be exhibited at MIC ≤ 0.01 g/ml (Morales et al., 2008).

3.3.2.3 Agar dilution method

The agar dilution method was used in this study to determine the the effect of different concentrations of CPI on growth of *P. chrysogenum*. Whereas the microplate method was useful to measure multiplication of spores over a period of 72 hours, the agar dilution

method would give an indication of the effect of CPI on the growth of fungi over a longer period.

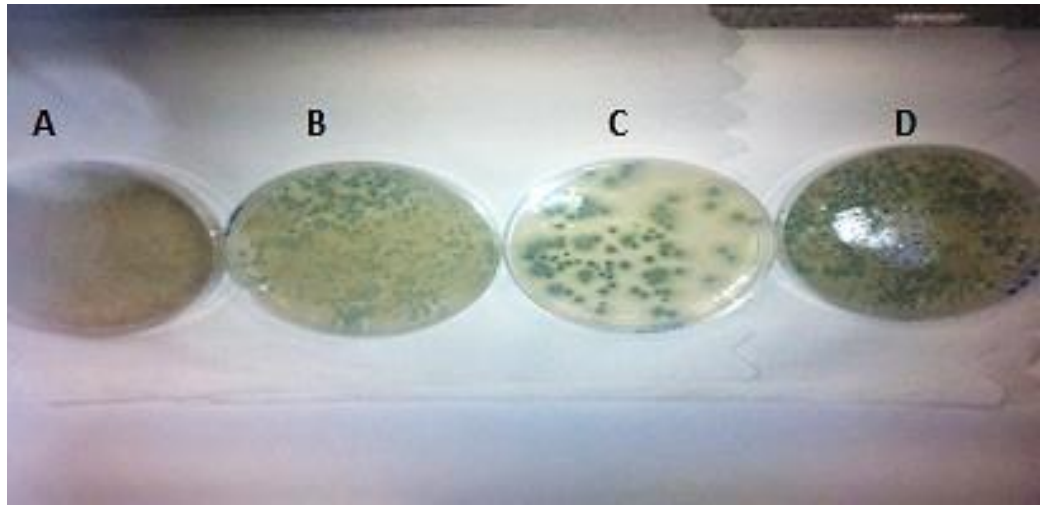


Figure 3.11 The effect of different concentrations of CPI on the growth of *P. chrysogenum* after 8 days. **A-** The control, *P. chrysogenum* without CPI. **B-** *P. chrysogenum* inoculated with 0.02 g/ml CPI; **C-** inoculated with 0.05 g/ml CPI and **D-** inoculated with 0.08 g/ml CPI.

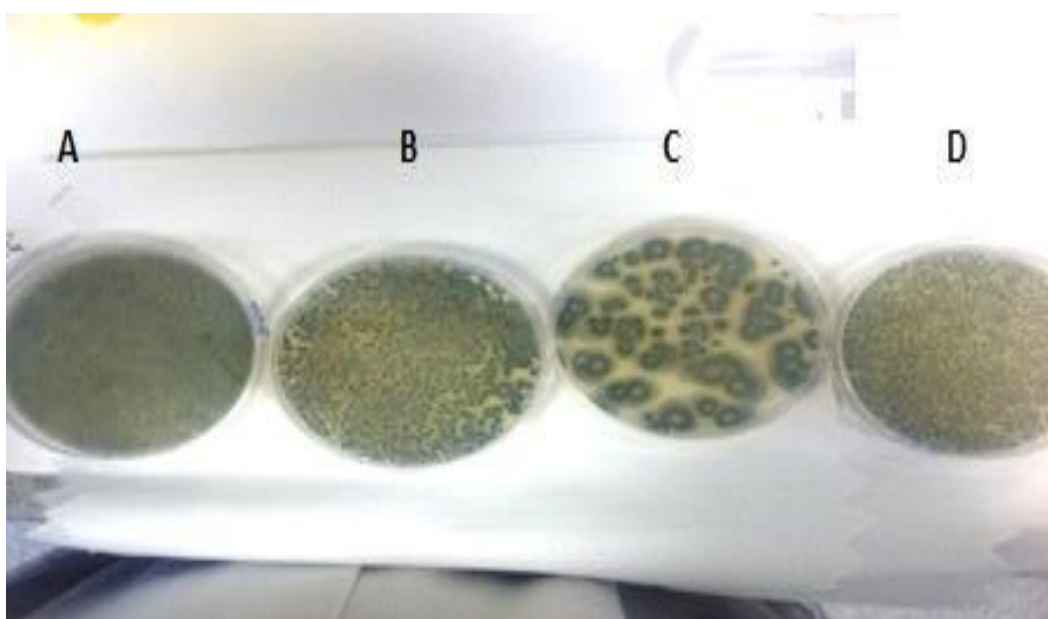


Figure 3.12 The effect of different concentrations of CPI on the growth of *P. chrysogenum* after 2 weeks. **A -** The control, *P. chrysogenum* without CPI. **B-** *P. chrysogenum* inoculated with 0.02 g/ml CPI; **C-** inoculated with 0.05 g/ml CPI and **D-** inoculated with 0.08 g/ml CPI.

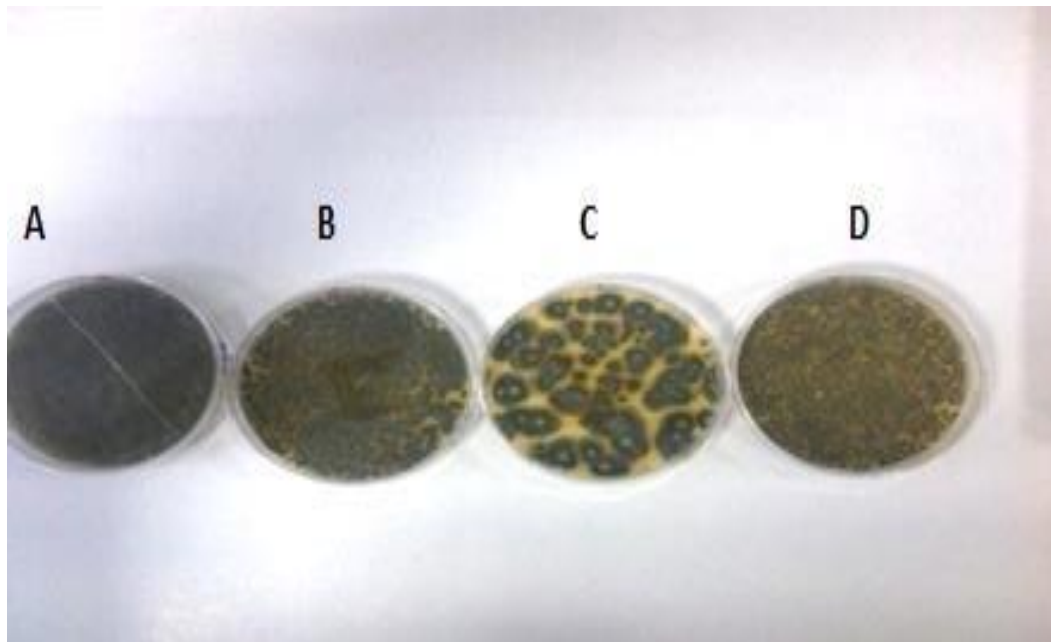


Figure 3.13 The effect of different concentrations of CPI on the growth of *P. chrysogenum* after 4 weeks. **A-** The control, *P. chrysogenum* without CPI. **B:** *P. chrysogenum* inoculated with 0.02 g/ml CPI; **C-** inoculated with 0.05 g/ml CPI and **D-** inoculated with 0.08 g/ml CPI.

Three different concentrations of CPI: 0.02, 0.05 and 0.08 g/ml were tested against *P. chrysogenum*. The dishes were then monitored after periods of: 8 days (**Figure 3.11**), 2 weeks (**Figure 3.12**), and 4 weeks (**Figure 3.13**).

Figures 3.11 to 3.13 show that the highest inhibition of *P. chrysogenum* was obtained by using 0.05 g/ml CPI. This result remained consistent up to 4 weeks of incubation. The higher concentration of 0.08 g/ml showed reduced inhibition, indicating that the most effective inhibitory concentration of CPI is 0.05 g/ml. This is consistent with what was found in determination of the MIC of 0.05g/ml of CPI against *P. chrysogenum* as shown in **Figure 3.8**.

3.3.2.4 Photographs of microscopic slides of *Penicillium chrysogenum*

Photographs of the microscopic slides of *Penicillium chrysogenum* are shown in **Figures 3.14 A and B**. The results indicate a clear difference between the control in the absence of the CPI extract and in the presence of the extract; at a concentration of 0.02 g/ml. (**A**) Mycelium was clear in the absence of CPI, whereas (**B**) showed a smaller number of conidia, in the presence of the CPI. The production of toxins by fungi generally depends

on the formation of the mycelium (Mossini et al., 2009). These photographs demonstrate that CPI inhibits the formation of mycelia, and thereby could inhibit the production of toxins.

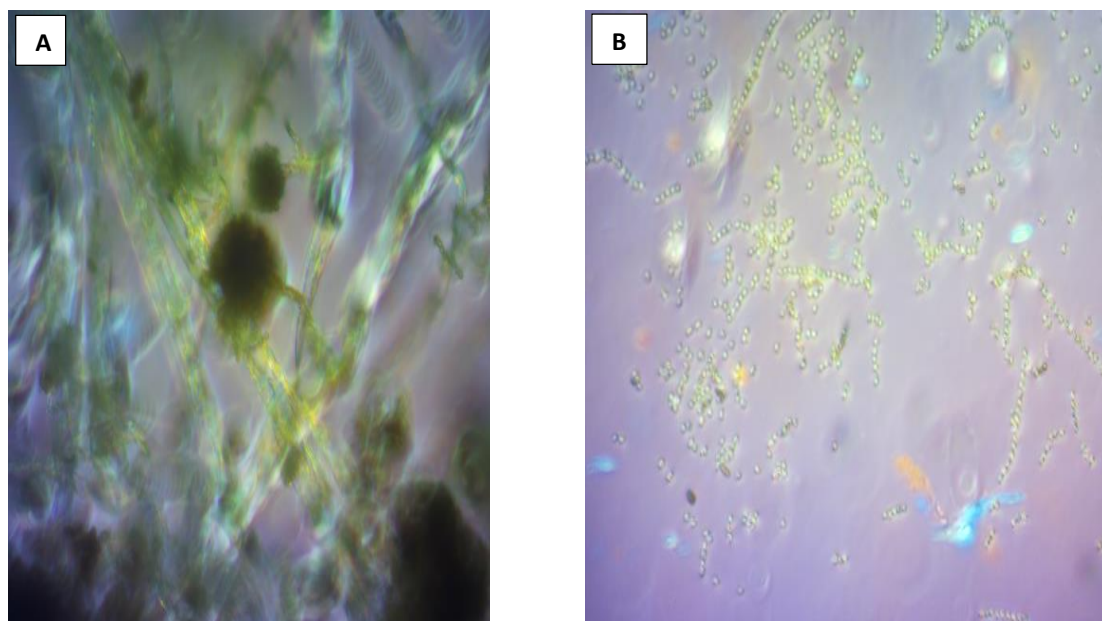


Figure 3.14. Photographs of microscopic slides of *Penicillium chrysogenum*.

A - *P. chrysogenum* without CPI. **B** - *P. chrysogenum* with (0.02 g/ml) of CPI. The fungal concentration was 2×10^6 spores/ ml.

3.3.3 *Antifungal activity of CPI filtrate and retentate (UF) using the microplate method*

In this experiment the antifungal efficiency of the retentate and filtrate of CPI obtained after ultrafiltration was measured. As this was a feasibility experiment, only one concentration of each fraction was tested (0.08g/ml) and only one fungal species was chosen (*E. rubrum*).

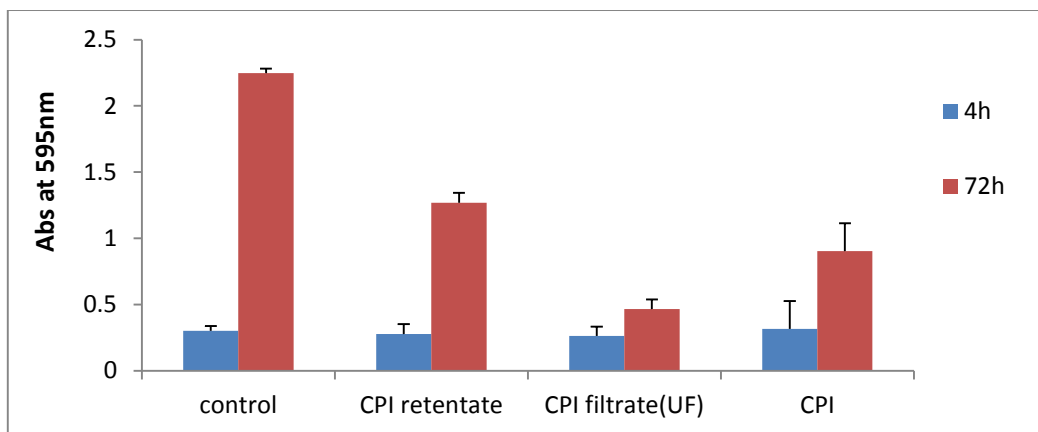


Figure 3.15. The effect of CPI retentate, CPI filtrate and CPI without UF treatment at concentrations of 0.08 g/ml on the growth of *E. rubrum* using the microplate method after 4 hours (blue bars) and 72 hours (red bars).

The retentate and filtrate of CPI subjected to ultrafiltration showed different levels of antifungal activity compared to CPI. As shown in **Figure 3.15** CPI (0.08g/ml) filtrate exhibited statistically significantly higher antifungal activity against *E. rubrum* than the control (no CPI), the retentate and CPI, in decreasing order. This indicates that the proteins with molecular weight smaller than 30kD that are present in the filtrate (**Figure 2.8**) might contain higher antifungal activity than higher molecular weight proteins in the retentate (**Figure 2.8**).

3.3.4 Stability of antifungal activity of CPI

In order to confirm that the antifungal activity of CPI is indeed a protein and not non protein antifungal agents such as phenolic compounds, two feasibility tests were carried out. The first was proteolytic digestion of CPI, which would potentially disrupt antifungal protein structures. CPI extract was treated with proteinase K and trypsin based on the work of Rizzello et al. (2011); thereafter the remaining activity of the CPI extract was determined using the microplate method as described in section 3.2.2.4.

The second approach was heat treatment of CPI, which would potentially denature antifungal proteins and render them inactive. On the other hand, thermal stability of CPI extract as regards to its antifungal ability would be a significant factor for food applications undergoing heat treatment such as baking. Therefore, a feasibility study was undertaken to test the activity for heat-treated CPI after heat treatment at temperatures of 70°C and 100°C for 15 mins respectively by using the microplate method as described in section (3.2.2.4).

For both feasibility tests only the one concentration of heat treated CPI (or digested CPI) was tested (0.02g/ml) against one fungal species (*P. hirsutum*). The control was CPI without any heat or proteolytic treatment.

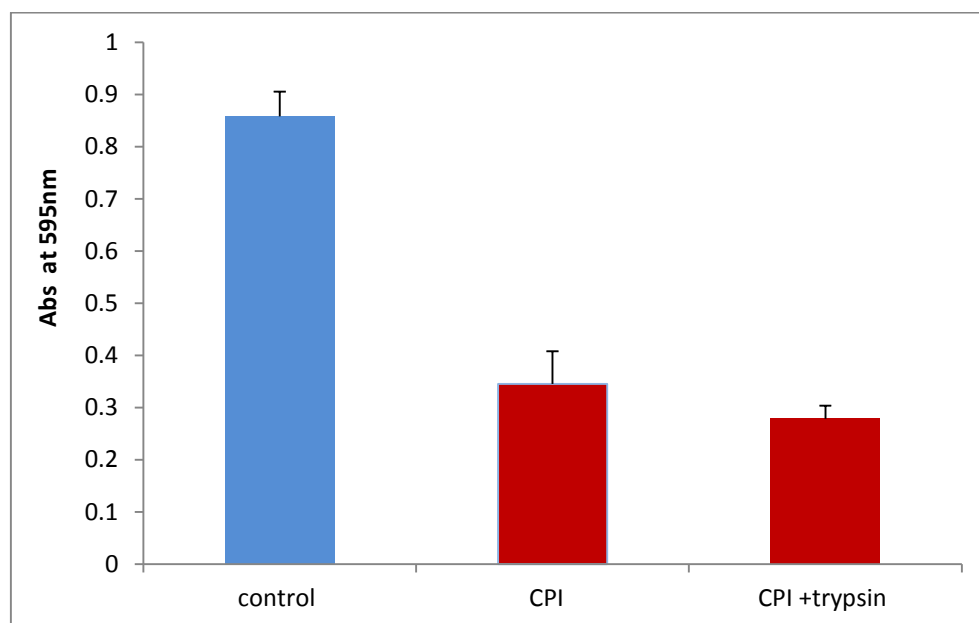


Figure 3.16. Effect of trypsin treatment of CPI on antifungal activity against *P. hirsutum* using the microplate method. A concentration of 0.02g/ml of CPI and 0.02 g/ml enzyme treated CPI was tested. Water was used as negative control and CPI without any treatment was used as positive control.

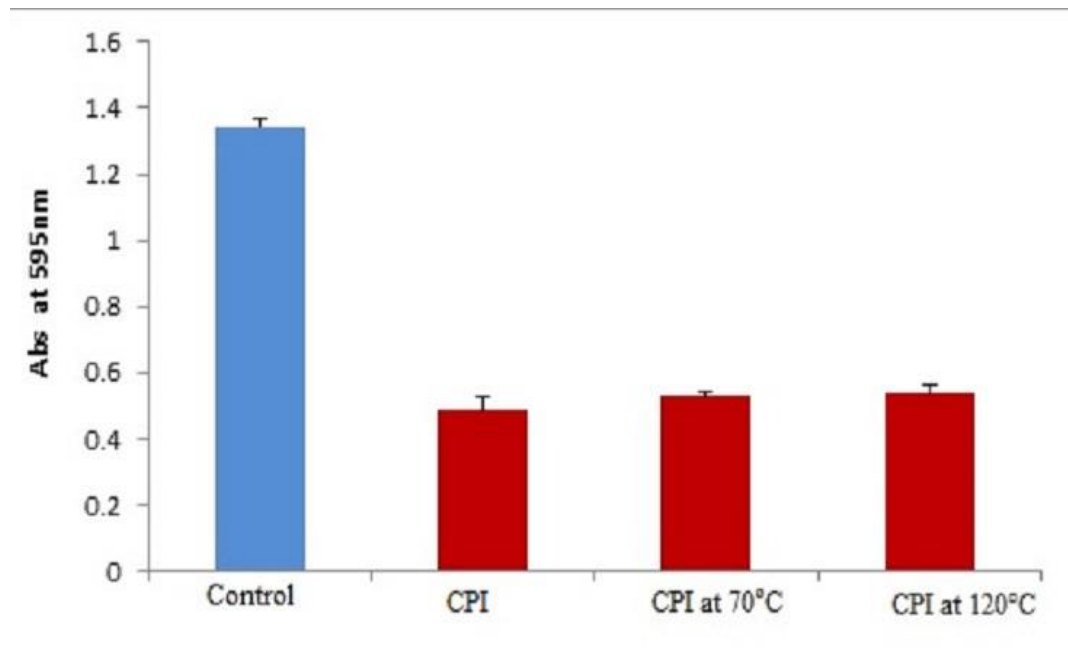


Figure 3.17. Effect of heat treatment of CPI (0.02g/ml) on antifungal activity against *P. hirsutum* using the microplate method, with water and CPI without any treatment as a positive control.

Figure 3.16 shows that proteolytic treatment did not affect the antifungal activity of CPI compared to the non digested CPI sample. **Figure 3.17** shows that heat treatment at either 70°C or 120°C for 15 minutes did not affect the antifungal activity of CPI compared to the non heated CPI sample. These findings could be correlated with results from Wang et al., (2009) and Segura et al., (1998) who showed that defensin peptides with antifungal activity were heat resistant and stable against protease treatment.

3.4 Discussion

This chapter detailed the effectiveness of different cowpea extracts by conducting microbiological tests against fungal species considered to be the most important contaminants of bread (Rizzello et al., 2011). The soluble extract and ammonium sulfate precipitate of cowpea revealed no antifungal activity, whereas CPI showed significant antifungal activity when tested against various species.

CPI exhibited high inhibition, particularly towards *E. rubrum* and *P. brevicompactum*, with good inhibition towards *P. chrysogenum* and *P. hirsutum*. However, there was no statistically significant inhibition of *A. versicolor*.

The difference in effectiveness of the CPI against different fungal species may be due to the differences in their physiology, which will be affected differently by the antifungal mechanism of CPI. Different fungal defense mechanisms such as membrane permeability and resistance to structural damage due to difference in composition of the fungal cell walls might be affected differently by CPI (Burger et al., 2014; Ng, 2004; Wong et al., 2010; Vriens et al., 2014).

As can be seen in **Figures 3.7, 3.8, 3.9** and **3.10** the MIC of CPI extract was around 0.05 g/ml, this value presented a strong ability to inhibit all species. This could be rated as moderate antifungal activity according to criteria established by Morales et al., (2008).

The photographs presented in **Figure 3.14**, of microscopic slides of *P. chrysogenum*, indicated a clear difference between the control in the absence of CPI and in the presence of CPI at a concentration of 0.02 g/ml. Mycelia were visible in the absence of the cowpea extract, and a small number of conidia only grew in the presence of the cowpea proteins. The production of toxins by fungi generally depends on the formation of mycelia (Mossini et al., 2009); these photographs demonstrate that CPI inhibits the formation of mycelia and shows visible evidence for inhibition of growth which confirms the findings of Ye et al. (2000a.)

The major component of CPI consists of vicilin (50 kDa) peptide (**Figure 2.8**), which has been reported to exhibit antifungal activity by binding to yeast and fungal cell walls (Gomes et al. (1998). CPI filtrate (UF) showed a statistically significant inhibition against *Eurotium rubrum* compared to the CPI retentate (UF), and CPI without UF treatment. Ye et al. (2000a) reported antifungal activity of two low molecular weight peptides (28 kDa and 12 kDa) purified from cowpeas and separated by CM Sepharose, against *Fusarium oxysporum*, *Rhizoctonia solani*, *Coprinus comatus*, *Physalospora piricola*, *Mycosphaerella arachidicola* and *Pleurotus ostreatus*. The N-terminal amino acid sequence of the 28kDa protein resembled that of a chitinase but that of the 12 kDa peptide remains unidentified.

Antifungal activity of CPI was not reduced after proteolysis or heat treatment. Although this finding does not rule out the possible role of contaminating non protein antifungal compounds in the CPI extract, the scientific literature does report the existence of antifungal peptides that are protease and heat resistant. Wang et al. (2009) reported the presence of a trypsin inhibitor of 8kDa in CPI, which could explain the results of antifungal activity of CPI

after treatment with trypsin (**Figure 3.16**). Furthermore, Gomes et al., (1997) proposed that cowpea vicilin should be classified as family of plant lectins, based on structural similarities. Lectins are well known to be resistant to digestive proteases and are slowly hydrolysed even after extensive heat treatment.

CPI also retained its antifungal activity after heat treatment at 70°C and 120°C. These findings are consistent with results from many other studies (Yu et al., 2007; Skouri and Gargouri, 2008; Wang et al., 2009) that showed peptides with antifungal activity were heat resistant and stable against protease treatment. Wang et al., (2009) isolated a thermostable chitinase from cranberries with antifungal properties. The finding of thermal stability of antifungal properties of CPI would be of advantage in food application such as bread that is subjected to high temperatures during baking.

To date, much of the work undertaken on cowpea seed extracts has been within the field of functional properties, demonstrating their potential health benefits. However, the present study is novel as far as this author is aware, in being the first study to report the effectiveness of CPI against bread spoilage by fungi.

Further investigation would include:

- Testing of antifungal activity at different concentrations and determination of the MIC of CPI ultrafiltrate;
- Testing the antifungal activity of CPI ultrafiltrate against different fungal species; and
- Testing the heat and proteolytic stability of antifungal activity of CPI ultrafiltrate.

Chapter 4

Study of the effect of CPI addition to bread on shelf life, textural and sensory properties

4.1 Introduction

In bread making, the use of plant extracts as preservatives has been the focus of much attention. Many of the compounds identified are still limited in their usefulness, which appears to be due to several reasons: adverse sensory properties, high cost, low effectiveness, questionably safety, or adverse effects on the bread's texture. Numerous plant extracts exhibit antimicrobial activity against a range of bacteria, yeasts, and moulds, however, the difference in the quality and quantity of their bioactive components is an important detriment when considering them for use in food. Plants are comprised of numerous components and are valuable sources of new and biologically active fragments, possessing antimicrobial properties. As regards international guidelines for evaluating the safety of food additives, because of the problems with the standardisation of plant components, typical toxicological standards remain unspecified. Improved cost effective isolation procedures to yield standardised extracts, as well as safety and toxicological evaluations of antimicrobials require further research (Butt and Batool, 2010; Negi, 2012; Prakash et al., 2015).

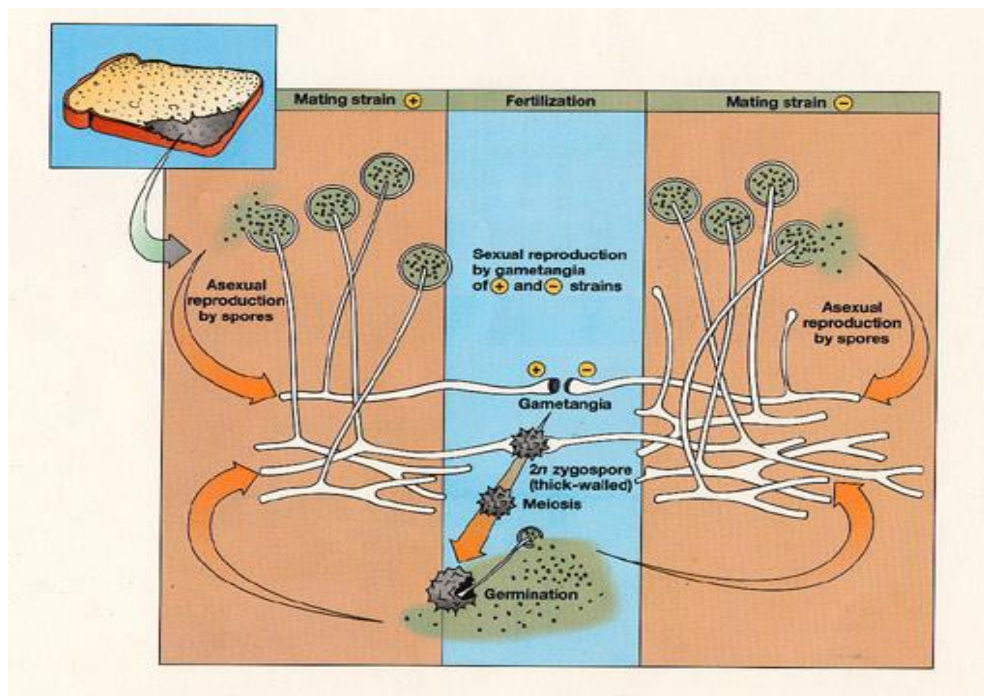
Moreover, some compounds are not available in sufficient quantities, indicating a need to expand the list of natural preservatives that can be successfully added to bread (Negi, 2012). Furthermore, the lack of reproducibility of their activity is a major obstacle, despite the great diversity of compounds that the plants contain.

4.1.1 *How mould spoils bread*

Fungal growth is considered the most important factor resulting in the corruption of bakery products; this often occurs because of the presence of specific species, including *Aspergillus*, *Fusarium*, and *Penicillium*. In addition to the unlimited economic losses resulting from the presence of mould, the production of mycotoxins resulting in public health problems is a concern (Legan, 1993).

The contamination of bread occurs after baking, during the cooling, slicing, or wrapping processes. The high temperature during cooking is sufficient to eliminate contaminants from earlier processes. Thus, the occurrence of any contamination must result from bakery

equipment, the environmental conditions, or flour containing fungal spores, which can spread easily throughout the bakery (see **Figures 4.1** and **4.2**). It has been estimated that 1 g of flour contains as many as 8,000 mould spores (Legan and Voysey, 1991; Legan, 1993; Cauvain and Young, 2007; Gerez et al., 2009).



Source: <http://leavingbio.net/fungus/fungi2.htm>

Figure 4.1. A schematic presentation showing the cycle of fungi reproduction on bread



Source: <http://website.nbm-mnb.ca/mycologywebpages/Moulds/Characteristics.htm>

Figure 4.2. A photograph showing the appearance of mould on bread

4.1.2 Microbiological spoilage of bread

Mould growth is one of the most common sources of microbial spoilage in bread. Bacterial spoilage is comparatively less common, despite still causing problems in warm conditions. Bacterial spoilage usually takes the form of so-called ‘rope’, which is caused by growth of the *Bacillus* species. Yeast is the least common cause of microbial spoilage in bread (Jago and Jago, 1911).

4.1.2.1 Mould Spoilage

Penicillium spp. are the commonest bread spoilage moulds; although a wide range of spoilage moulds, including *Penicillium*, *Aspergillus*, *Cladosporium*, *Mucorales* and *Neurospora*, have been observed in wheat-based breads (Legan, 1993).

The conjoint black bread mould is *Rhizopus (nigricans) stolonifer*. It appears as a fluffy white cottony mycelium and black sporangia. *Neurospora sitophila* is a different type of mould, and is reddish in colour. It becomes established in bread stored in high humidity conditions or wrapped while still warm. Storage temperature plays an important role in the type of moulds that grow in bread. For example, *Aspergillus* spp was the dominant mould causing spoilage of bread in India, whereas 90% of the moulds isolated from a variety of breads in Northern Ireland were *Penicillium* spp (Jago and Jago, 1911; Legan, 1993).

As well as spoilage problems, some moulds present a hazard to public health, owing to their ability to produce mycotoxins. Exposure to mycotoxins can happen either as a direct result of eating bread spoiled by mycotoxigenic moulds, or indirectly by consuming the products of animals fed contaminated bread (Jago and Jago, 1911).

Some types of mould that can attack bread are listed below in **Table 4.1**.

Table 4.1. Characteristics of bread moulds.

Mould	Colony colour	Colony appearance	Comments
<i>Penicillium</i> spp.	Blue/green	Flat, spreads rather slowly	The most common type of bread mould frequently present
<i>Aspergillus niger</i>	Black	Fluffy, spreading with spore heads often clearly visible	
<i>Aspergillus flavus</i>	Olive green		
<i>Aspergillus candidus</i>	Cream	Flat, spreads slowly	
<i>Aspergillus glaucus</i>	Pale green	Very fluffy and fast spreading	Often present on damp bakery walls, commonly encountered
<i>Cladosporium</i> spp.	Dark olive green	Very fluffy and fast spreading	Will grow very rapidly on moist bread
<i>Neurospora Sitophila</i>	Salmon pink		Will grow very rapidly on moist bread
<i>Rhizopus nigricans</i>	Grey/black		
<i>Mucor</i> spp.	Grey		

Source: Cauvain and Young (2007).

4.1.2.2 Bacterial Spoilage

Bacillus subtilis or *Bacillus licheniformis* cause rope, which is a spoilage problem affecting bread and other bakery products. The causative organism, *B. subtilis*, occurs naturally in the soil and therefore rope bacteria may be present on the external parts of grains and vegetables. They may also exist in the air, and so can be present in dust in the bakery environment. The main source of contamination is from raw ingredients. The incidence of rope is rare in several countries because adding calcium propionate, good hygiene, and good bakery practice limit it, so that it remains under control. Conversely, in some countries where there is a demand for reduced salt levels (e.g. the UK) there is a higher risk of rope growth, particularly if microbial growth inhibitors are not added to the dough recipe (Jago and Jago, 1911).

4.1.2.3 Yeast spoilage

Contamination with wild yeasts occurs rarely in bread made following a short process; however, it can sometimes happen with a long fermentation process, or when sponge or dough processes are applied (Seiler, 1993). Most sources of contamination involve physical contact with dirty equipment or infected high-sugar foods, which are considered ideal substrates for osmophilic yeasts. Generally, there are two types of yeasts involved in the spoilage of bread (Legan and Voysey, 1991).

1. Fermentative yeasts. These yeasts ferment sugars present in the bread. Several types of yeast have the ability to grow on bread and cause such defects, but *Saccharomyces cerevisiae*, which is bakers' yeast, is the mostly commonly encountered.
2. Filamentous yeasts; also known as 'chalk moulds', because they have ability to form a white, spreading growth on the surfaces of bread, and because it is difficult to distinguish between them and mould growth. They are considered as yeasts and not moulds because they produce single cells and reproduce by budding. There are varying numbers of chalk moulds, but *Pichia burtonii* is considered the most common and troublesome, having the ability to grow rapidly on bread and being resistant to preservatives and disinfectants compared to the other moulds (Jago and Jago, 1911).

4.1.3 Control of Microbiological Spoilage of Bread

4.1.3.1 Preservatives

The most commonly used preservatives in bread, to avoid or reduce microbial growth, are propionates, as shown in **Table 4.2**.

Table 4.2. Anti-mould agents most commonly used in bread and approved in the EU
<https://www.food.gov.uk/science/additives/enumberlist>

Code	Anti-mould agents	Recommended level of use (%) ^a
E280	Propionic acid	0.1
E282	Calcium propionate	0.2
E281	Sodium propionate	0.2
E5000	Sodium dipropionate (70% solution)	0.2

^a % of flour weight

(Source: Seiler, 1983).

Organic acids, for instance propionic acid, act by changing the pH balance of microorganisms (Cauvain and Young, 2007). The modulation of internal cellular pH, by ionisation of the acid molecules leads to the inhibition of microbial growth (Cauvain and Young, 2007). Because their effectiveness on yeasts is minimal, propionates can be used in bread without disrupting the fermentation activity of the dough (Seiler., 1993; Cauvain and Young, 2007).

Ethyl alcohol has been widely used as an effective preservative substance. A bread study by Legan (1993) found adding about 0.5% and 3.5% ethanol, based on loaf weight caused

a significant extension to the shelf life of the bread. Another study by Seiler (1983) established that when the alcohol is added to loaves their shelf life increases correspondingly with the ethanol concentration, reporting a 50% extension of life with a 0.5% addition of ethanol, based on loaf weight (Brul and Coote, 1999).

4.1.3.2 *Modified Atmosphere Packaging*

According to Cauvain and Young (2007), a modified atmosphere refers to the storage of foodstuff in atmospheres where there are increased concentrations of carbon dioxide. The application of modified atmosphere packaging (MAP) in bakery products became widespread in the late 1970s, mainly in Europe, as new labelling regulations required preservatives to be declared (Cauvain and Young, 2007).

4.1.3.3 *Irradiation*

Irradiation can be used to destroy mould spores, which might be present on the surfaces of bread. The types of irradiation used in bread preservation are ultraviolet, microwave, and infrared (Cauvain and Young, 2007).

4.1.3.4 *Applications of plant extract in bread making*

The inclusion of plant extracts in food making has many advantages in terms of safety, being inexpensive, available, and promising to make a positive difference to the nutritional value of the product potentially. However, natural does not always mean safe. Consequently, any new preservative derived from plants must be tested using a full toxicological evaluation before it is declared suitable and can receive the necessary legislative approval for use. Toxicological assessments are very expensive, and would be too expensive for most additive and ingredient suppliers. An option, which avoids high cost toxicity testing, is to use additives already present in the human diet, with a long history of use, which are recognised as safe and edible at the concentrations recommended for food preservation (Negi, 2012).

The same study indicates that an ideal and applicable natural food preservative would:

- be active at low concentrations;
- be heat stable;
- be unaffected by either low or high pH;
- impart no flavour or odour property to the food;

- possess no toxicity;
- be easily assayable;
- have no pharmaceutical or veterinary application;
- not allow microorganisms to develop resistance; and
- not require labelling by E or INS numbers.

The present study aims to test the potential of CPI in bread as a natural preservative that would fulfil these criteria.

4.1.4 *Effect of concentration of natural preservatives in bread*

Preservative agents are essential in manufactured food, as they render products safe and prevent spoilage. They also satisfy consumers' requirements for high quality, safe food with an extended shelf life. The level of natural preservatives essential for adequate effectiveness is significantly higher in foodstuffs in comparison with laboratory media, and this can impact negatively on the organoleptic properties of food (Nascimento et al., 2000; Olasupo et al., 2003; Ng, 2004; Nguetack et al., 2004; Sorensen et al., 2010; Negi, 2012; Rai et al., 2012).

In this study we tested low CPI concentrations (0.02-0.08%) in bread, similar to the range tested in Chapter 3 using microbiological tests, and these showed no antifungal activity. Therefore, higher concentrations were tested in bread. Ahmed, (2014) tested concentrations of 2, 4 and 6% of CPI in bread and found that concentrations of 2 and 4% did not adversely affect texture or sensory properties of bread. Therefore, to test for antifungal activity on bread in the present study, concentrations of 1.5-12% were chosen to be tested (section 4.2.1.1).

4.2 Materials and methods

Potato dextrose agar PDA and Petri plates were obtained from Sigma-Aldrich London (UK). Flour, sugar, salt; yeast, sesame seed and vegetable oil were bought from ASDA supermarket, Edinburgh (UK).

Methods

4.2.1 *Preparation of the bread*

Bread samples were prepared using the straight dough method 10-10 B of AACC (2000). A range of 1.5-12% CPI was tested in bread and the flour was reduced accordingly. Based on the outcome of the results described in section 4.3, a concentration of 2.3% CPI was

selected to prepare bread for the purpose of sensory evaluation and texture analysis, using the recipe given in **Table 4.3**. The control was bread containing no CPI.

Table 4.3. Ingredients used for making the bread.

	CPI	CPI filtrate	Control
Ingredient	%	%	%
Flour	58.4	58.4	60.7
CPI	2.3	2.3	0
Water	33.1	33.1	33.1
Sugar	1.8	1.8	1.8
Salt	0.6	0.6	0.6
Yeast	0.6	0.6	0.6
Fat	3.2	3.2	3.2
Total	100	100	100

When preparing the breads, 200 g quantities were mixed. The dough was prepared from wheat flour or wheat-cowpea composite flours. The ingredients were mixed in a food mixer (Breville, SHM2) and kneaded at setting 2 for 5 minutes, followed by incubation at 30°C for 50 mins to allow for the yeast to begin the fermentation process. Each dough preparation was rolled into a ball, put into a bread pan, covered and allowed to rise at 35°C for 30 mins. The risen dough preparations were then baked at 200°C for 30 mins in a Russell Hobbs 14552 Mini Oven and allowed to cool for 1 hour. Then the bread evaluation methods mentioned below were carried out.

4.2.1.1 Testing of different CPI flour ratios and CPI filtrate

The bread was baked according to the recipe indicated in **Table 4.3**. Doughs were prepared from wheat flour with the addition of different quantities of cowpea flour (CPI). Concentrations of 1.5 to 12% w/w were tested by mixing CPI with the wheat flour and the amount of flour was reduced accordingly. To test the CP ultrafiltrate fraction, 2.3% freeze-dried powder was added to the recipe.

4.2.2 Bread evaluation

4.2.2.1 Specific volume measurement

The volume of bread was measured after 24 hours' storage and the loaf specific volume (ml/g) measured during the storage time (24h to 192h). To measure the specific volume of

the bread samples, the method described in AACC (2000) was used, using sesame seed displacement. The sesame seeds were decanted into a volume container to measure the volumes and then a sample was completely covered with seeds. The sesame seeds were then measured again and the following equation was used to calculate the sample volume.

$$\text{Loaf volume (ml)} = V1 - V2$$

V1= volume of sesame seeds in the empty container (ml)

V2 = volume of sesame seeds in the container with sample

The specific volume (cm³/g) was calculated as follows:

$$\text{Specific volume } \text{cm}^3/\text{g} = \frac{\text{Loaf volume of bread}}{\text{Weight of bread}}$$

All measurements were undertaken in triplicate.

4.2.2.2 Moisture content of bread samples

The moisture content of each loaf of bread was measured during the storage period; all bread types were tested in triplicate. The moisture content was determined by drying a 3 g sample in an air oven to a temperature maintained at 105 ± 3 °C for 5 hours as per procedure given in AACC (2000) method.

4.2.2.3 Textural profile analysis of bread samples

The texture of the bread samples was analysed after 24 hours of storage, using a Zwick/Roell type Z010 texture analyser as described in method 74-09 of AACC (2000); all the bread types were tested in triplicate during the storage period.

4.2.2.4 Sensory evaluation

A sensory analysis of the bread samples was performed 24 hours after baking to investigate the overall of acceptability of the bread samples. The bread samples were served with water. Twenty panellists were randomly selected from among the staff and students at the School of Life Sciences, Heriot Watt University. The evaluation of the bread was based on a 9 point hedonic scale, as described by Larmond (1977), with 1 = disliked extremely, 2 = disliked very much, 3 = disliked, 4 = disliked slightly, 5 = neither liked nor disliked, 6 = liked slightly, 7 = liked, 8 = liked very much and 9= liked extremely; bread without CPI was used as a control.

4.2.3 Study of the shelf life of bread containing CPI and CPI ultra filtrate

4.2.3.1 Visual inspection

The bread samples containing the cowpea protein and control (duplicated) were stored at room temperature 20°C. Spoilage was observed by the appearance of the samples during the storage period, which lasted for more than 35 days.

4.2.3.2 Microbiological analysis

Bread samples preparation

10 g of bread was homogenised with 90 ml of sterilised water for 2 mins using a lab scale homogeniser (ultra turrax); after which one ml of the bread sample was added to 9 ml of sterilised water to obtain a 10⁻¹ dilution. Then serial dilutions: 10⁻², 10⁻³, 10⁻⁴, and 10⁻⁵ were prepared, and subsequently 0.1 ml of each serial dilution was spread on the surface of the plate to determine growth, using the spread-plate method.

Spread-plate method

Fungi were counted using the spread-plate method: 0.1 ml of bread samples dilution as described above was spread on the surface of 10 ml of potato dextrose agar (PDA). The Petri dishes were then incubated at 25°C for 72 hours, and then the colony forming units (CFU) were counted. All experiments were undertaken in triplicate under sterile conditions.

4.2.4 Statistical analysis

The determinations were performed in triplicate (n=3) and mean ± standard deviation (SD) values calculated. Data obtained were analysed with a one-way ANOVA. The comparison between means (3 replications) was performed at the 95% significance level ($p \leq 0.05$) by the least significant difference test (LSD). The analyses were performed using SPSS version 10 for Windows (SPSS Inc., NY, and USA).

4.3 Results

4.3.1 Effect of different CPI concentrations on fungal growth

Testing of low CPI concentrations (0.02-0.08%) in bread, similar to the range tested in Chapter 3 using microbiological tests showed no antifungal activity. Therefore, higher concentrations were tested in bread. The higher concentrations tested in bread ranged from (1.5 to 12%). The ranges from 7 to 12% showed no inhibitory activity, possibly because the higher concentrations of CPI raised the concentration of the compounds that could be

utilised by microorganisms, and therefore stimulated microbial growth. Therefore, the remainder of the bread making experiment only focused on concentrations that had shown growth inhibition during the storage period, which were 2.3, 4.5 and 6.7%.

As can be seen from **Figures 4.3 and 4.4**, the best inhibition was in bread with 2.3% CPI, which showed good resistance to fungal growth over the storage period, compared with the other samples.



Figure 4.3. Photographs of bread that had been stored for 12 days, showing reduced bread spoilage caused by different concentrations of CPI. **1** - Bread containing 6.7% CPI. **2** - Bread containing 4.5% CPI. **3** - Bread containing 2.3% CPI.

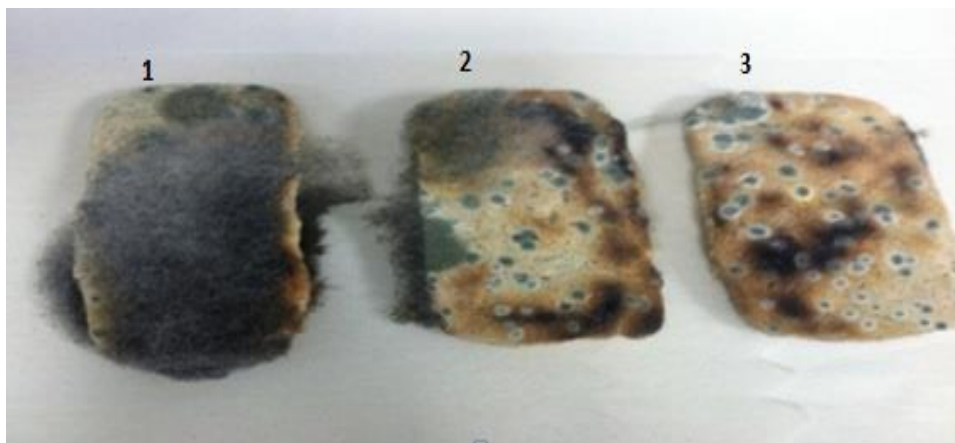
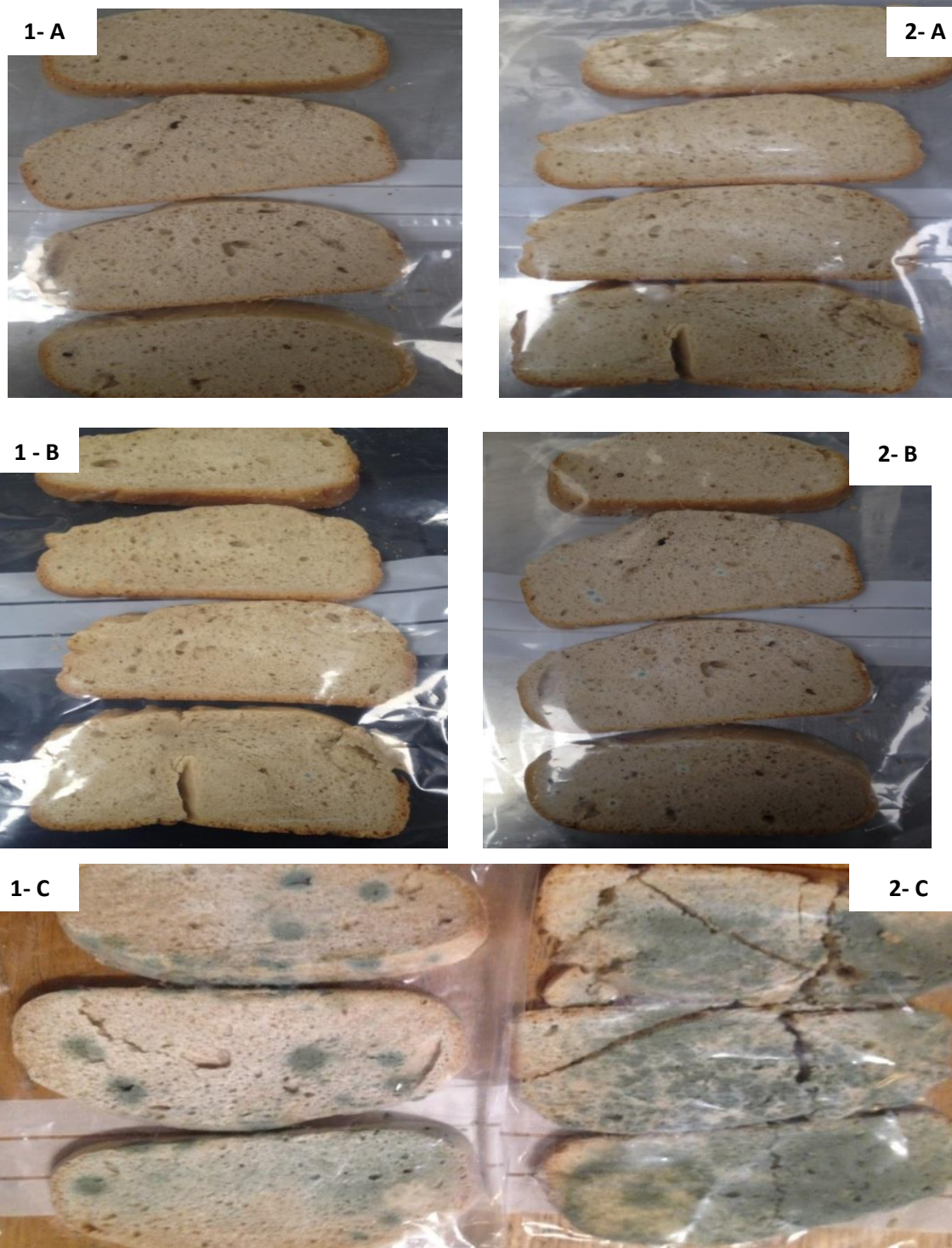


Figure 4.4. Photographs of bread that had been stored for 21 days, showing reduced bread spoilage caused by different concentrations of CPI. **1** - Bread containing 6.7% CPI. **2** - Bread containing 4.5% CPI. **3** - Bread containing 2.3% CPI.

Figure 4.3 shows that 2.3% CPI caused inhibition of fungal growth after 12 days compared to bread containing 6.7% CPI, whereas 4.5% CPI caused intermediate inhibition of growth.

Figure 4.4 shows the inhibition of growth from 2.3% CPI after 21 days compared to the other concentrations. The results show that a concentration of 2.3% of CPI resulted in the best inhibition of growth.

4.3.2 *Effect of CPI (2.3%) w/w on fungal growth at different storage times*



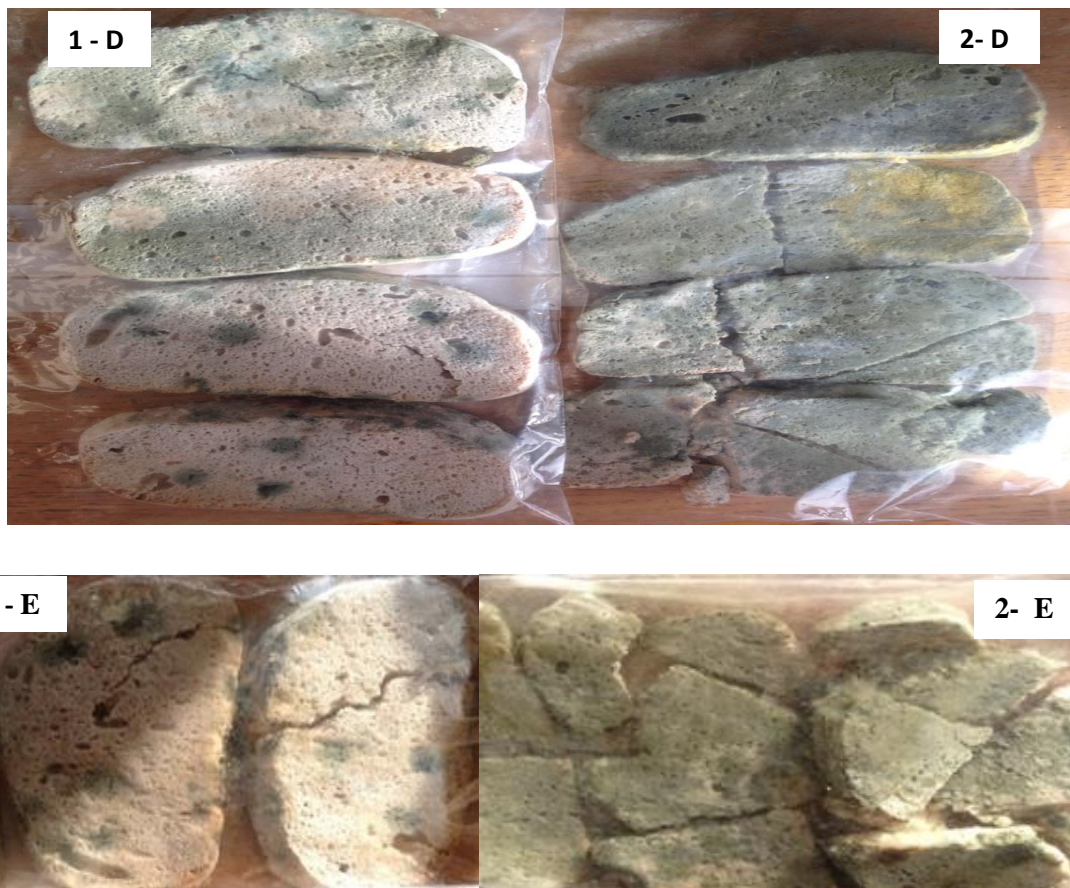


Figure 4.5 (A-E) Effect of CPI (2.3%) in baked bread on shelf life during storage time of 60 days. (1) is CPI containing bread and (2) is the control without CPI. **A-** after 2 days of storage, **B-** after 8 days of storage, **C-** after 25 days of storage, **D-** after 38 days of storage, **E-** after 60 days of storage.

Figures 4.5 (A-E) shows photographs of the effect of 2.3% CPI on the shelf life of bread over a period of 60 days' storage at room temperature in sealed closed containers. No fungal growth of CPI containing bread (sample) was detectable after 8 days' storage, whereas the control bread without CPI showed significant fungal growth. Fungal growth was visible on the sample after 25 days and progressively increased after 38 and 60 days. The control showed significantly more fungal growth than the sample after 25 days and was covered in fungi after 38 and 60 days respectively. The antifungal effect of CPI is clearly visible in the figures in comparison to the control. The results show that 2.3% CPI strongly inhibited the growth of fungi, extending the shelf life of the bread.

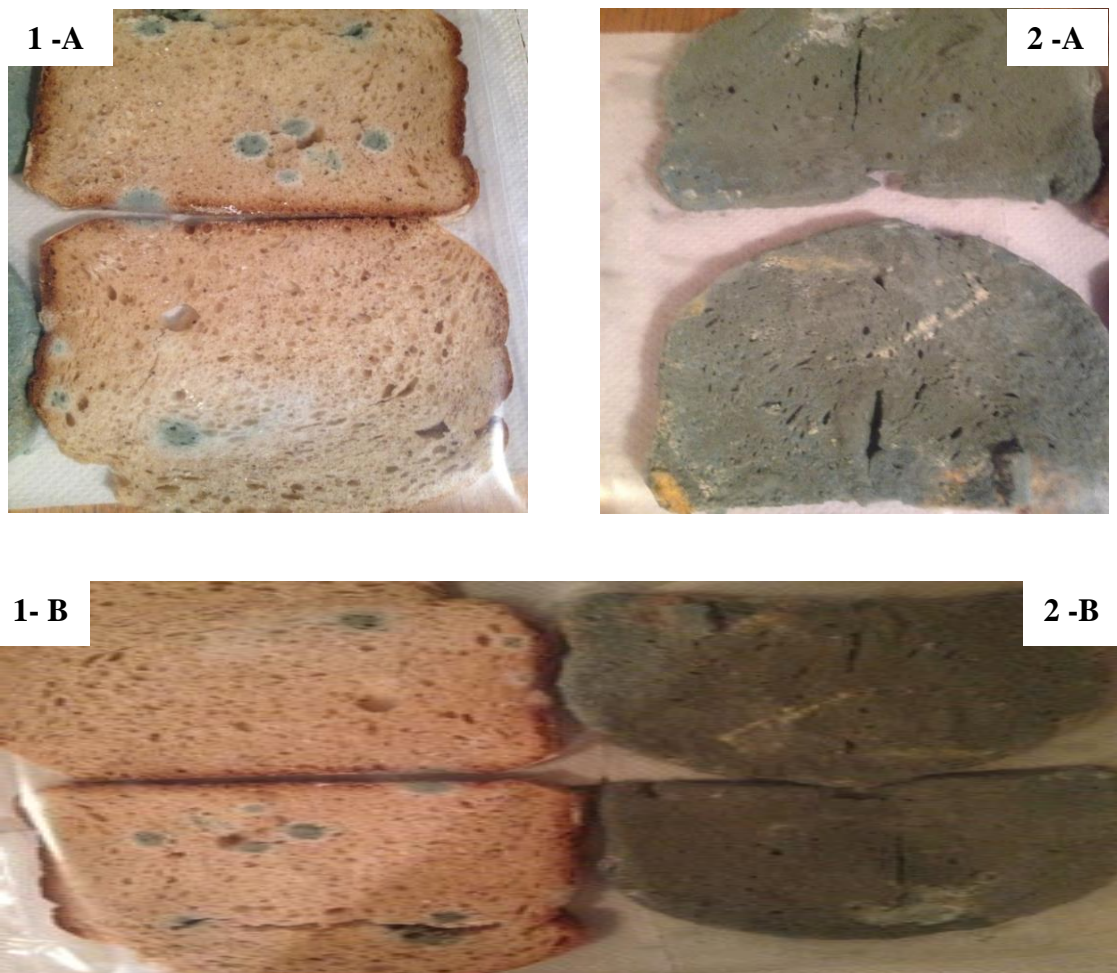


Figure 4.6 Bread samples with protein from CPI filtrate (2.3%) after 60 days of storage. CPI containing bread (1) and the control (2). **A-** Bread samples after 28 days of storage, **B-** after 60 days' storage time.

The effect of 2.3% of the freeze dried filtrate of CPI solution that was subjected to ultrafiltration (section 2.4.7), was evaluated also in the bread samples (**Figure 4.6.A and B**). The results show weak fungal contamination was visible in the bread with CPI UF filtrate after 28, 60 days of storage time compared to control breads that were completely covered with microbial growth. These results are consistent with the microbial growth inhibition of 2.3% CPI in **Figure 4.5 A-E**, but the inhibitory effect of the filtrate appears to be stronger than that of CPI after 60 days.

4.3.3 Effect of CPI on the microbial count of bread samples

A further microbiological analysis was performed to investigate the activity of CPI on the microbial count of bread samples by using the spread plate method which was prepared as mentioned in section 4.2.3.2. As can be seen in **Figure 4.7** during days 1-9 of storage, CPI

strongly inhibited the growth of fungi, and that there was a significant difference ($p < 0.05$) in the yeast and mould count of the bread. This result appears to agree with the visually apparent storage stability of bread after 8 days' storage compared to the control without CPI (**Figure 4.5 B**).

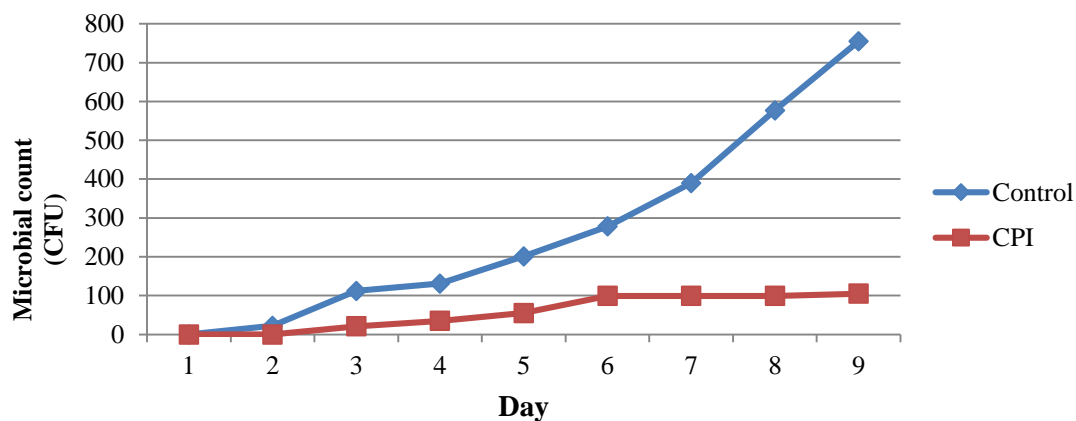


Figure 4.7. The effect of CPI (2.3%) on microbial count of yeast and mould on bread during the storage period. The samples were measured in duplicate, and there was a statistical difference ($p < 0.05$).

The results show that 2.3% CPI resulted in an approximately 86% inhibition of microbial growth in bread after 9 days of storage at room temperature. The results could have a significant potential impact on extending the shelf life of bread containing CPI as natural preservative, as the limiting factor would not be determined by microbial growth, but rather by retrogradation and staling affecting the sensory shelf life of bread (depending on the packaging).

4.3.4 Sensory testing evaluation of bread

4.3.4.1 Effect of 2.3% of CPI on sensory acceptability of bread texture

As shown in **Figure 4.8**, CPI at a concentration of 2.3% CPI did not affect sensory acceptability. Bread samples with 2.3% CPI did not differ statistically compared to the control ($p = 0.100$), and the CPI bread samples showed a result somewhere between disliked and liked very much. Of the 20 panellists, 8 included a comment about the texture, including “good fluffy”, “soft”, and” not crumbly”, and “good and nice loaf and quite soft”. Thus, it can be observed that the texture of the CPI bread received positive responses compared to the control bread.

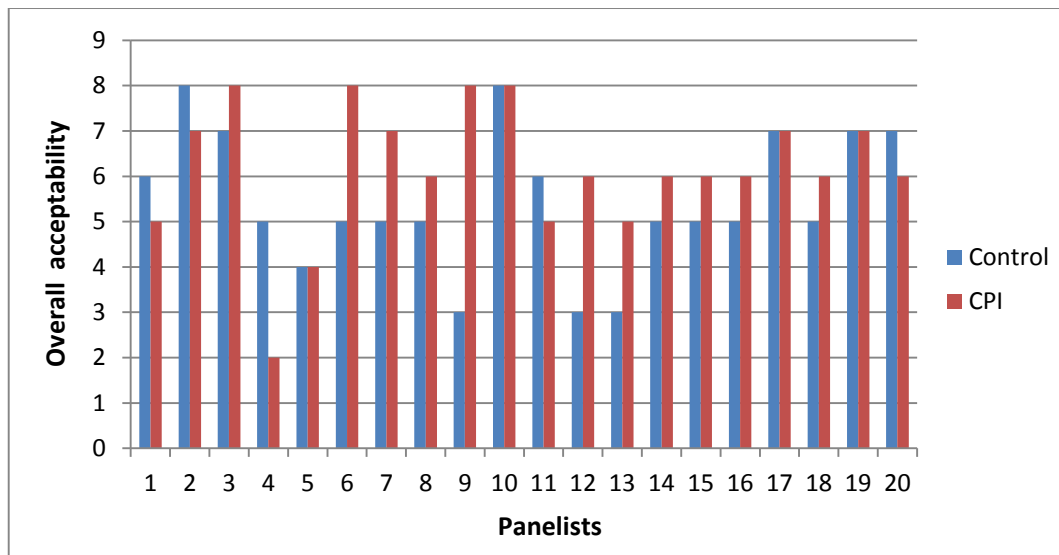


Figure 4.8. Sensory acceptability of the texture of breads baked with CPI (2.3%) compared to the control. There was not a statistically significant difference between the control and test samples ($p = 0.100$).

4.3.4.2 Effect of 2.3% of CPI on the taste and overall acceptability of bread

Bread samples with 2.3% of CPI did not differ statistically ($p = 0.378$) in taste compared to the control bread, and the bread samples showed results between liked slightly and liked very much. Three panellists indicated that there was a bean flavour to the CPI bread; however, they also stated the bread was acceptable. Another 2 panellists observed there seemed to be a nutty taste; 3 panellists noticed a slight sweetness, but also stated the CPI bread tasted good and acceptable. The taste results are presented in **Figure 4.9**.

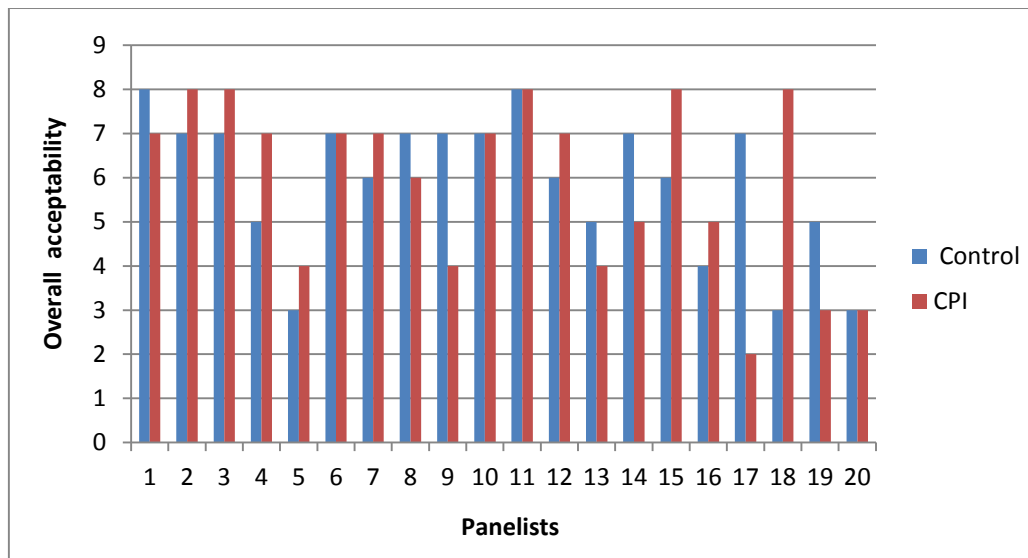


Figure 4.9. The taste evaluation of bread baked with CPI (2.3%) and bread without CPI. There was not a statistically significant difference between the input groups ($p = 0.378$).

4.3.4.3 Effect of 2.3% CPI on sensory evaluation of bread colour

The analysis of the effect of adding 2.3% of CPI on the colour of the bread found bread with CPI achieved results between disliked slightly and liked extremely, whereas the control bread received a score ranging from disliked slightly to liked very much. Therefore, no significant difference was observed for CPI bread compared to control bread. The results are presented in **Figure 4.10**.

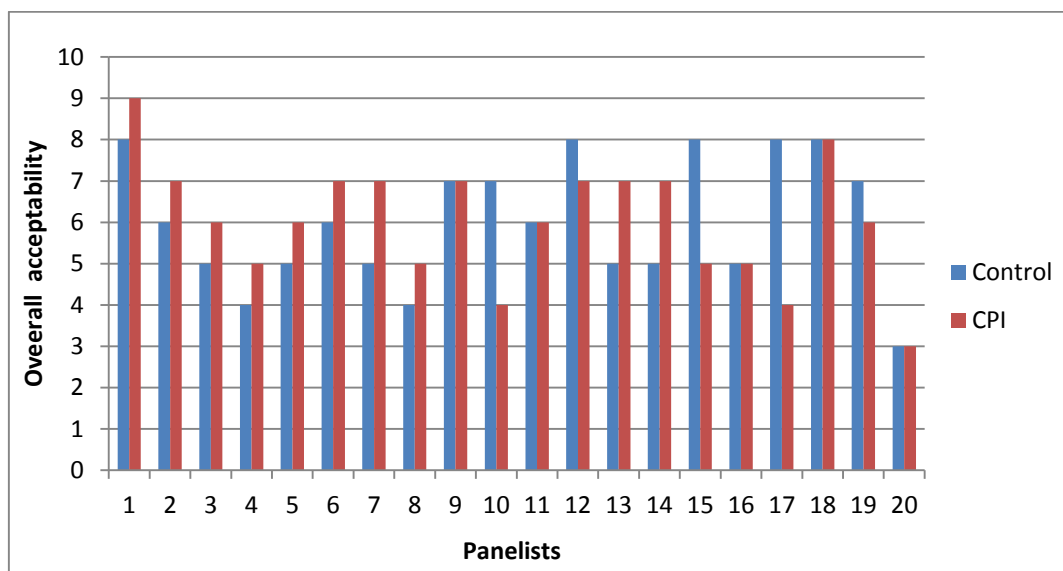


Figure 4.10. The colour evaluation of breads baked with CPI (2.3%) and without CPI. There is not a statistically significant difference ($p = 0.976$).

4.3.4.4 Effect of CPI (2.3%) on the hardness of the bread

Hardness is considered an important index for determining the quality of bread; this changes during the storage period as a result of the loss of elasticity (Ulziijargal et al., 2013). The texture was measured with a texturometer (4.2.2.3) at different storage times following baking. The effect on texture at different storage times by addition of 2.3% CPI to bread compared to the control is shown in **Figure 4.11**. There was an obvious and statistically significant ($p = 0.050$) difference in hardness between the CPI bread and the control samples after 24, 48, 72 and 192 hours' storage. ($p = 0.117$), ($p = 0.100$), ($p = 0.076$) and ($p=0.100$) respectively.

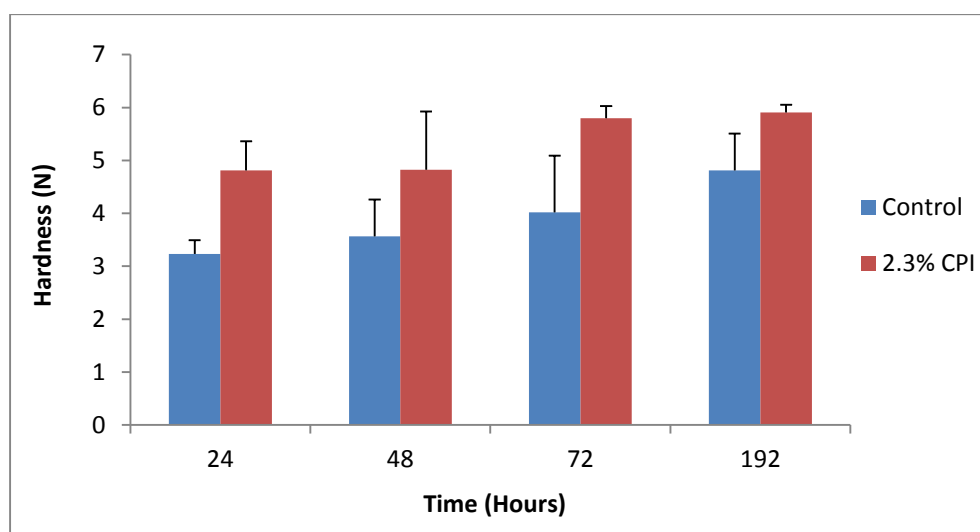


Figure 4.11. Hardness of breads baked with CPI (2.3%). (Samples were measured in duplicate).

Although the inclusion of CPI increased the hardness of bread the difference compared to the control diminished after 72 and 192 hours. The increased hardness also did not appear to affect the sensory acceptability of the bread (**Figure 4.9**). The results support the findings of Ahmed (2014) who reported a similar effect on texture of bread supplemented with 2 and 4% CPI.

4.3.4.5 Effect of adding CPI (2.3%) on bread moisture

The moisture content of the CPI bread was about 37% after 24 and 72 hours' storage respectively, and that of the control bread was about 42% after both storage times (see **Figure 4.12**). It seems that the control bread had slightly higher moisture content. Whilst the addition of 2.3% CPI to the bread caused a slight decrease in the moisture content,

however the differences were not statistically significant. Ahmed (2014) reported no significant moisture loss compared to the control for bread supplemented with either 2 or 4% CPI.

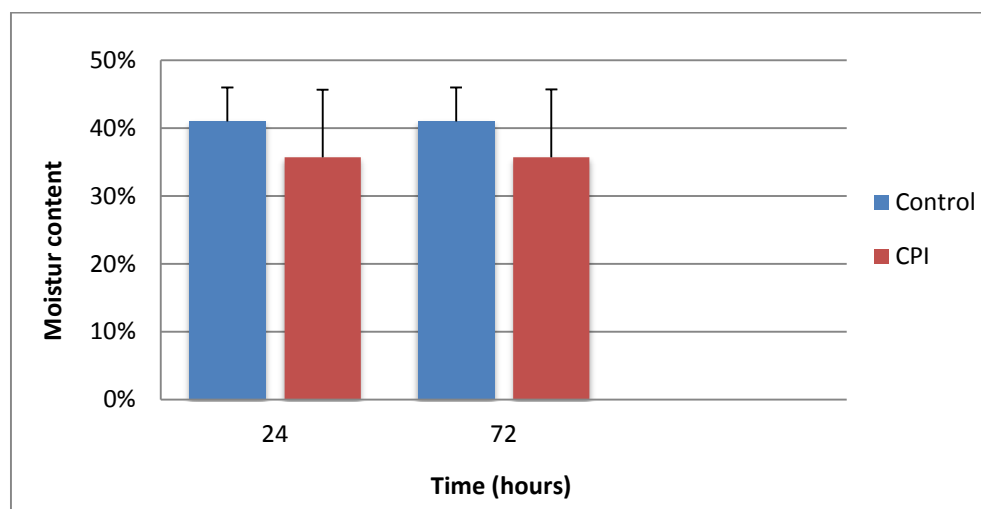


Figure 4.12. Moisture content of bread baked with CPI (2.3%) during storage time.

The retention or loss of water during storage of bread will have an effect on the sensory perception of bread (Mondal and Datta, 2008). The results presented here indicate that the addition of CPI to bread does not significantly affect the moisture content compared to the control content after 24 and 72 hours' storage control. As moisture content directly affects staling of bread (Mondal and Datta, 2008), it can be concluded that addition of CPI to bread would not accelerate the staling process compared to the control.

4.3.4.6 Specific volume measurement

The specific volume of cowpea protein bread was analysed during the storage time; all the bread types were tested in triplicate, and it was discovered that cowpea protein bread and control bread were not statistically different, as can be seen in **Figure 4.13**.

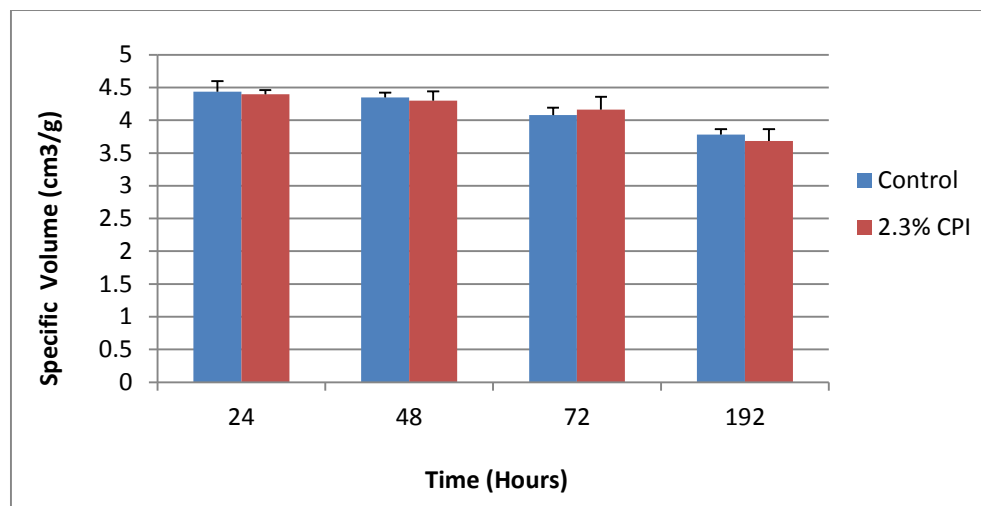


Figure 4.13. Influence of CPI (2.3%) addition on the specific volume of bread samples during the storage time.

All data in **Figure 4.13** display a general trend in reduction of specific volume with storage time. The reason could be shrinkage of the pore size of bread due to gradual moisture loss and retrogradation (Mondal and Datta, 2008). The results presented here confirm that the addition of 2.3% CPI to bread does not affect the specific volume compared to the control.

4.4 Discussion

In this study, different concentrations (1.5, 2.3, 4.5, 6.5 and 12%) of CPI were applied to bread to find out the best concentration to extend the shelf life. A pattern of inhibition of fungal contamination on bread versus concentration showed the shape of an inverted bell curve where concentrations of 1.5 and 12% showed no inhibition of growth (results not shown), 4.5% and 6.5% showed intermediate inhibition whereas maximum inhibition was found with 2.3% CPI. A similar inverted bell curve shaped effect of growth inhibition against several fungal species as a function of CPI concentration was found with the microplate method (section 3.3.2.2) and the agar dilution method (section 3.3.2.3). These microbial tests showed that the minimum inhibitory concentration (MIC) for CPI ranged between 0.02 and 0.05 g/ml for several known fungal species. The tests in bread showed that a CPI concentration of 2.3% appears to be the MIC for many non identified microbial organisms which could include moulds, yeasts and fungi. It can thus be concluded that results of the tests to determine maximum effective concentration for CPI application in bread are supported by the results of the microbial tests in Chapter 3.

Although the hardness of the bread increased significantly after 3 days compared to the control, sensory acceptability was not significantly affected. These results confirm those of Ahmed (2014), who reported that the addition of 2 and 4% CPI to bread did not significantly affect sensory or textural properties. The same author optimised the extraction process for CPI, and the resultant process gave results that were similar to those of using soy protein isolate (SPI) (a commercially available product). The functional properties of CPI were found to be comparable, and in some cases better than SPI. The data showed that the hardness of the bread samples increased significantly after 3 days' storage. The reason for the increased hardness during the storage time could be a loss of moisture (Tong et al., 2010).

Although there was no significant difference detected when conducting a sensory analysis of the bread's texture (see **Figure 4.8**), the result of the textural analysis showed a significant difference between the CPI bread and that of the control sample on the first day of storage; whereas, no significant difference was observed between day 2 and day 8 (**Figure 4.11**).

As shown in **Figure 4.12**, the control bread apparently had slightly higher moisture content during days 1 to 3 of storage; the addition of 2.3% CPI to the bread caused a slight decrease in moisture during days 1 to 3, which was not statistically significant.

The specific volume of the bread is considered an important indicator of the quality of bread. It is better to have average sized bread, because being too small or too big will negatively affect the quality of the bread. According to Kirby (2007), the specific volume of bread directly relates to the ability of the dough to rise. The specific volume of standard bread is in the range $3.5\text{-}6\text{ cm}^3/\text{g}$ (Ulzijiargal et al., 2013). The specific volume of cowpea protein bread was analysed during the storage time, and no statistically different variation was found, as can be seen in **Figure 4.13**.

According to the results of this study, the addition of 2.3% of CPI to the bread has no impact on bread volume or colour. However, the hardness of all the bread samples increased significantly after 3 days of storage which did not seem to impact the sensory evaluation of the hardness. Therefore, it is concluded that the addition of 2.3% CPI to bread does not cause any significant differences in sensory acceptability. Consequently, the addition of CPI to bread could be a successful application in the food industry.

In comparison, several studies have showed the addition of other natural extracts may impact the quality of food products. For example, a study carried out by Ho et al. (2013) showed that addition of banana pseudo-stem flour to bread adversely affects bread quality. According to a study by Ulziijargal et al. (2013), breads supplemented with 5% mushroom mycelia were less acceptable. Skric et al. (2009) also found the sensory acceptability of bread supplemented with 5% barley flour was reduced.

The results of this study show that CPI could satisfy the majority of the aforementioned criteria. These include, being active at low concentrations, being heat stable and imparting no adverse sensory properties. As cowpea is a traditional food, the use of CPI as natural preservative requires no E-number labelling which is appealing to the consumer. This implies great potential for its use as a natural preservative in the food industry.

From another perspective, the addition of CPI to bread could raise its nutritional value by increasing the protein and fibre content. Ahmed (2014) reported that addition of 4% CPI to leavened bread, leads to an increase in total fibre from 0.08% to 0.46% and in protein content from 7.5% to 9.5%. According to Singh et al. (1997), a survey in 1991 showed the addition of cowpea to the diet of adults and children had a positive impact in reducing malnutrition. According to Phillips and Baker (1987), cowpea seeds can also be combined with cereals to improve the protein content of the diet.

To the best of the author's knowledge, the application of cowpea protein isolate as a natural preservative in bread remains unreported, making this study the first to do so.

Further studies would involve comparison of the relative efficiency of CPI as preservative in bread compared to preservatives that require labelling such as propionate. Further studies could also include optimising the effective antifungal concentrations of the low molecular weight CPI fraction in bread.

Chapter 5

Mechanism of antifungal action and computer modelling

5.1 Introduction

Many authors have indicated that the antimicrobial effects of peptides, proteins, and lytic enzymes are dependent on their ability to attack the cell walls and membranes of their target. Regardless, the precise mechanisms informing the action of many antimicrobial compounds are not understood fully, or remain unidentified. Therefore, additional investigation is needed to clarify their active mechanisms.

5.1.1 *Mechanisms of fungal growth inhibition*

5.1.1.1 *General introduction*

The mechanisms of antimicrobial agents' actions can be divided into two fundamental groups: Inhibition of the growth of spoilage microorganisms (fungistatic or bacteriostatic effect), or destruction of the microorganisms (fungicidal or bactericidal effect) (Dorman and Deans, 2000; Shao et al., 2011; Negi, 2012; Henderson and Lee, 2013). Inhibition of microbial growth works by affecting the microbes' environment; e.g. sugars or salts that reduce the water activity. The other groups exert a direct effect on the cell, as summarised below:

- Effect on the DNA
- Impact on protein synthesis
- Control of the enzyme activity
- Control of the cell membrane and cell wall stability
- Control of the transfer mechanisms for nutrients

Many categories of antifungal proteins inhibit the synthesis of the fungal cell wall, or alter the cell wall structure and/or function, whilst others disturb the structure of the fungal membrane, causing lysis of the fungal cells (Selitrennikoff, 2001).

As many studies show, the antifungal proteins a plant produces when it is attacked by pathogens provide immunity. Plants have the potency to fight these fungal pathogens; this plays a crucial role in the medicinal properties of the plants, which can then be used in the

treatment of health problems (Datta and Muthukrishnan, 1999; Dorman and Deans, 2000; Ng, 2004; Wang et al., 2004; Negi, 2012; Hauser et al., 2014).

Antifungal proteins are classified into different groups, according to their structural and functional characteristics. These groups include chitinases and chitinase-like proteins, chitin-binding proteins, cyclophilin-like proteins, defensins and defensin-like proteins, deoxyribonucleases, embryo-abundant protein-like proteins, protein glucanases, lectins, lipid transfer proteins, peroxidases, protease inhibitors, ribonucleases, ribosome-inactivating proteins, storage 2S albumins, and thaumathin-like proteins (Wang et al., 2004). See **Table 5.1**.

Table 5.1. Some antifungal proteins of plants and their possible functions.

Name	Origin	Size/ structure	Action against fungi	References
Chitinases and chitinase-like protein	Tobacco, cowpea	29-kDa, 30-kDa and 15-kDa.	Hydrolyse chitin, an N-acetylglucosamine polymer (the most important constituent of the fungal cell wall)	Wong et al., (2010) Carlile et al., (2001) Rose et al., (2006)
Lectins	Chinese herb (<i>Pouteria torta</i>) Seeds	66-kDa 14-kDa	Plant lectins are capable of changing the structure of the fungal cell wall by binding to carbohydrates on the fungal membrane	Wong et al., (2010)
Protease inhibitors	Chilli seeds Acacia plumose seeds	20-kDa	Protease inhibitors suppress the fungal protease	Wong et al., (2010)
Thaumatin-like proteins (ILPs)	Chickpea Cowpea French bean Kiwi fruits Banana fruits	20-kDa	ILPs can hydrolyze β -1,3glucans	Wong et al., (2010) Rose et al., (2006)
Vicilins 7S globulin storage proteins	Cowpea seeds		Bind to chitin	Rose et al., (2006)
Deoxyribonuclease proteins		30-kDa	Act by hydrolysing the DNA of invading foreign organisms	Wong et al., (2010)
Ribonucleases			Antifungal effects when hydrolysing RNA from intruding foreign organisms	Wong et al., (2010)

5.2 Classification groups of antifungal proteins

5.2.1 *Chitinases and chitin binding proteins*

Chitin is a primary element of the fungal cell wall. Hydrolysis of this element by chitinases creates antifungal properties. A study carried out by Wong et al. (2010) revealed a large proportion of the antifungal activity of chitinases is a consequence of chitin binding and not the chitinase activity itself.

Different chitinase proteins, with antifungal activity, have been isolated from a variety of plants, including a 29-kDa protein from Indian squill bulbs, and a 42-kDa protein from fern leaves. Many leguminous plants, including chickpeas, rice beans, pinto beans, and cowpeas contain chitinase-like proteins of approximately 30-kDa (Wong et al., 2010). Chitinases and β -1,3-glucanases can hydrolyse the β -1,3 linked glucan and β -1,4 linked N-acetyl-D-glucosamine polymer respectively. These are the main elements of the fungal cell wall, and the enzymes can therefore inhibit fungal growth (Carlile et al., 2001; Rose et al., 2006).

5.2.2 *Cyclophilin-like proteins*

Cyclophilin-like proteins have been found in many organisms such as plants, yeasts, and rats. These proteins have been isolated from many kinds of plants, including cowpeas (Ye and Ng, 2001; Wong et al., 2010). Cyclophilin proteins from chickpeas, mung beans, and field beans exhibit translation-inhibitory activity (Ye and Ng, 2000; Ye and Ng, 2002).

5.2.3 *Defensins and defensin-like peptides*

Plant defensins are small (45-54 amino acids) cysteine-rich proteins thought to be involved in a host plant's defence mechanism against fungal pathogens (Wong et al., 2010). Some seeds contain 7 kDa defensin-like peptides with antifungal activity.

The inhibitory quality of these peptides is also evident in cowpeas (*V-anguliculata*), which can potently inhibit the α -amylases of weevils, and slightly inhibit mammalian α -amylases. However, they lack the ability to inhibit the action of α -amylases from *Aspergillus* and *Callosobruchus maculatus* (Wong et al., 2010).

Defensins of 6.8 kDa have been isolated from lima bean legumes, and have been shown to possess antifungal activity (Wong et al., 2010) has been isolated from pea seeds. Psd1 is an antifungal defensin that has been isolated from pea seeds; RsAFP1 and RsAFP2 are antifungal defensins found in radish seeds (Vriens et al., 2014).

The possible mechanisms of the action of plant defensins include internalisation by binding to receptors, membrane translocation and membrane permeabilisation (Vriens et al., 2014). The conclusion has been that plant defences use different mechanisms of antifungal action, but that their mode of action is not entirely clear, so demands further research.

5.2.4 *Glucanases*

Glucans are the second most important element of the fungal cell wall. The indirect antifungal activity of glucanases leads to partial digestion of glucans and chitin. Glucanases fight fungi through the hydrolysis of glucan in the fungal cell wall (Wong et al., 2010).

5.2.5 *Lipid transfer proteins (LTP)*

Lipid transfer proteins delay the growth of fungal pathogens. The work of Wong et al. (2010) postulated that LTPs permeabilise the membrane by inserting themselves into the membrane. The hydrophobic pocket of the protein forms a pore that causes the leakage of intracellular material, although the mechanism of action has not been demonstrated (Wong et al., 2010).

5.2.6 *Peroxidases*

French bean legumes contain a 37-kDa peroxidase, which inhibits mycelial growth in fungi (Ye and Ng, 2002; Wong et al., 2010). These are heme-proteins, metalloprotein containing a heme prosthetic group, using H₂O₂ to oxidise a large variety of hydrogen donors, such as phenolic substances, amines, ascorbic acid, indole, and certain inorganic ions.

5.2.7 *Storage 2S albumins*

2S albumins from different origins are known to have antifungal activity (Wong et al., 2010). 2S albumins have a low molecular weight (4.6, 6.6, and 12 kDa) (Moreno et al., 2004), and are water-soluble storage proteins, rich in glutamine. The secondary protein structure comprises four α -helices and there are four disulfide bonds, as found in the α -amylase/trypsin inhibitors and nonspecific lipid transfer proteins. 2S albumins from radish seeds were shown to cause permeabilisation of the phytopathogens plasma membranes (Moreno et al., 2004).

5.2.8 *Non-classified antifungal proteins*

There are many antifungal proteins, which cannot easily be classified into the aforementioned categories. These proteins offer different functions in different areas, with various results. Two such proteins, isolated from red lentil seeds, are a 32kDa antifungal protein, with inhibitory activity against *Botrytis. Cinera* and *Fusarium oxysporum* and an 11kDa antifungal protein with inhibitory activity against *Cercospora arachidicola* and *F. oxysporum* (Wang and Ng, 2005; Wong et al., 2010).

New antifungal peptides and proteins are being discovered almost daily, and tests are currently being carried out on antifungal proteins and peptides to discover if they can be used to treat human and animal diseases.

5.3 **Cowpea antifungal properties**

Under normal growth conditions, cowpea seeds suffer attack from cowpea weevil and other pathogens. One of the main fungal pathogens is *Fusarium oxysporum*, which causes *fusarium* wilt (Rose et al., 2006). Some cowpeas produce defensive responses to a large number of organisms, such as bacteria, fungi and insects (Rose et al., 2006).

Ye et al. (2000a) and Mine et al. (2010) demonstrated that multiple proteins with antifungal and antiviral potency are present in cowpeas seeds; these include trypsin and papain inhibitors, variant vicilins, 7S globulin storage proteins glucan hydrolases represented by chitinases, β -1,3-glucanase, and a lipid transfer protein (LTP) (Rose et al., 2006).

Ye et al. (2000a) reported that the antifungal protein in cowpea could potently inhibit HIV-1 reverse transcriptase and α -glucosidase, which could be beneficial in both agricultural and medicinal contexts. The molecular weight of α and β antifungal proteins were reported as 28 kDa and 12 kDa respectively. Mine et al. (2010) characterised the α -antifungal protein as a chitinase-like protein, based on N-terminal sequencing.

The LC-MS/MS analysis of CPI detected several proteins with possible antifungal activity (**Chapter 2 Table 2.4**). Based on the results of this study and the literature search, the proteins with potential antifungal activity that can be included in bread are listed in **Table 5.2**.

Table 5.2. Proposed proteins in CPI with antifungal activity.

CPI proteomics	Molecular weight MW (Da)	Antifungal activity	Possible mechanism
Vicilins (7S globulin storage proteins)	49654	+	Bind to chitin cell wall
Defensins	5695	+	Described in section 5.2.3
Lipoxygenase	97284	+	Not fully understood
Cyclophilin	49654	+	Still generally unknown
Unnamed Protein	22972	+	Chitinase

(+) means have positive antifungal activity

5.4 Hypothesis of defensins as antifungal proteins in CPI

The hypothesis to be tested in this study is that the main active compound in CPI is a defensin. This is supported by the following results from the current study:

- CPI filtrate showed statistically significant inhibition of fungal growth compared to the CPI retentate (section 3.3.3) indicating a protein with low molecular weight;
- The antifungal activity of CPI containing low molecular weight proteins was stable against proteolysis and heat treatment (**Figures 3.16 and 3.17**); and
- Proteomics analysis identified defensins as components of CPI.

These findings are consistent with results from Wang et al., (2009) and Segura et al., (1998) who showed defensin peptides with antifungal activity were heat resistant and stable against protease treatment.

A study carried out by Stotz et al. (2009) reported that the majority of plant defensins are active towards a wide range of fungi; e.g. *Fusarium culmorum* and *Botrytis cinerea*, baker's yeast, and human pathogenic fungi. The same study indicated that to date most plant defensin isolated are seed-derived. This correlates with the findings in relation to CPI, which is a seed extract with good activity towards fungi in bread.

The experimental work and results of the previous chapters indicate that the antifungal protein in CPI has low molecular weight. It also showed that the antifungal activity of low molecular weight proteins is resistant to protease and thermal treatment which are typical characteristics of defensins (Wang et al., 2009; Segura et al., 1998). For this reason, we postulate that the antifungal components in CPI are defensins and we proceeded to construct a model to simulate the possible mechanism of their antifungal activity.

This chapter provides a computer model for the mechanism and action of plant defensins.

5.5 Computer modelling

Plant defensins are a group of small globular peptides typically comprising of 45-54 amino acids. Their secondary structure is comprised of a single α -helix and three antiparallel β -sheets, which are highly conserved across species (Liu et al., 2006). The structure is held together by four disulphide bridges, which are again strictly conserved between species (Liu et al., 2006). The available structure of mung bean defensin was downloaded and used as a sample of leguminous defensin because there is a great similarity in their structure. In addition, building up a new structure of cowpea defensin would be time consuming. In this modelling section, the adsorption of mung bean defensin VrD1 into the phosphatidylcholine bilayer interface is simulated using a molecular dynamics simulation, to try to understand the mechanism by which plant defensins attack fungal cells better.

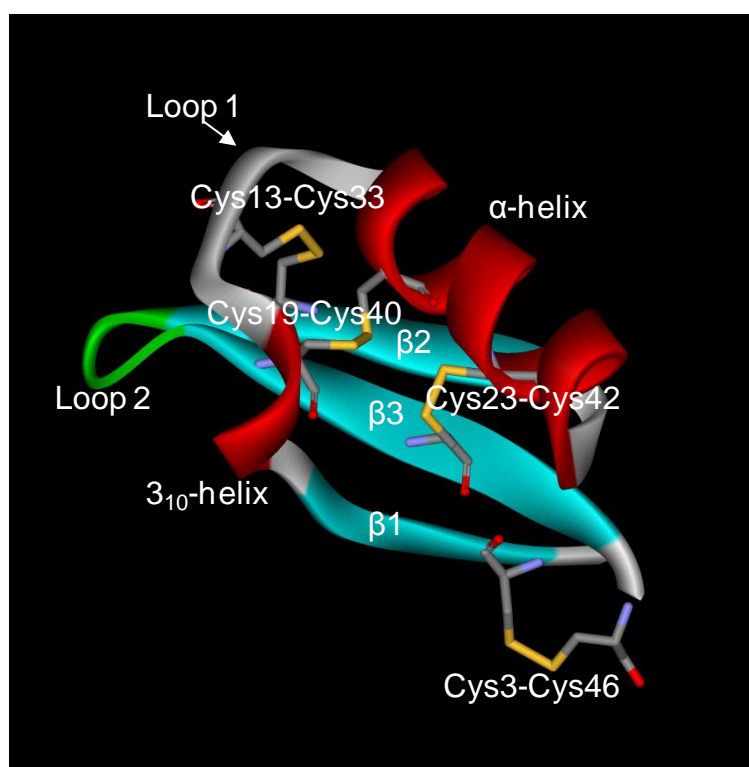


Figure 5.1. Structure of mung bean defensin VrD1 showing the secondary structure and disulphide bonds.

Mung bean defensin (**Figure 5.1**) has a secondary structure comprised of an α -helix, a short 3_{10} helix, and an anti-parallel β -sheet with three strands ($\beta 1$, $\beta 2$, and $\beta 3$). The α -helix and the $\beta 3$ strand of the β -sheet are joined by two disulphide bonds between Cys19-Cys40 and

Cys23-Cys42, with a further disulphide bond (Cys13-Cys33) connecting the β 2 strand and loop 1 (which joins the α - and 3_{10} helices). The fourth disulfide bond is between Cys3-Cys46, and brings the N and C terminal ends close together. The four disulfide bonds maintain a close-packed tertiary structure in the molecule. A further loop section (loop 2) between the β 2 and β 3 strands completes the structure. A relatively high proportion of amino acids in defensins (around 17%) are charged, with positively-charged arginine and lysine accounting for more than 70% of all charged amino acids (Wang and Wang, 2004).

The mechanism by which defensins kill fungal cells is not well understood. The most favoured hypothesis is that cationic parts of the molecule bind electrostatically to negatively-charged regions of the cell membrane (e.g. the phospholipid head groups), thereby disrupting the membrane (Yeaman and Yount, 2003). Once bound, the peptides act by forming membrane pores, resulting in the loss of cellular ions and metabolites, thus interrupting metabolism and causing cell death. The formation of these pores may differ between defensins (Yeaman and Yount, 2003). Three models have been put forward regarding plant defensins. These are the barrel-stave, toroid pore (or wormhole), and carpet mechanisms (Pelegriani et al., 2011). In the barrel-stave mechanism, when the protein interacts with the membrane, a hydrophobic region is inserted into the membrane, and this interacts with the acyl chains of the phospholipid. As more peptides bind, they eventually reach critical a concentration, at which point they associate with, and form a barrel ring structure, that opens a pore in the membrane, where the hydrophobic parts of the peptide are on the outside of the barrel (interacting with the phospholipid acyl chains), and the hydrophilic parts of the molecules line the inside of the pore. This is illustrated in **Figure 5.2 A**. An alternative mechanism is the toroidal pore. In this case, a barrel-shaped pore is also formed, but is formed by the overlap of proteins and membrane phospholipid head groups, which leads to a curvature of the membrane, resulting in rupture. The carpet mechanism involves binding of the defensins to the membrane via electrostatic interactions, until a monolayer (carpet layer) forms at the cell membrane surface (**Figure 5.2 B**). This protein layer causes the displacement of membrane phospholipids, altering the membrane's fluidity, leading to membrane disruption and cell death. In this mechanism, no discrete pores form in the membrane.

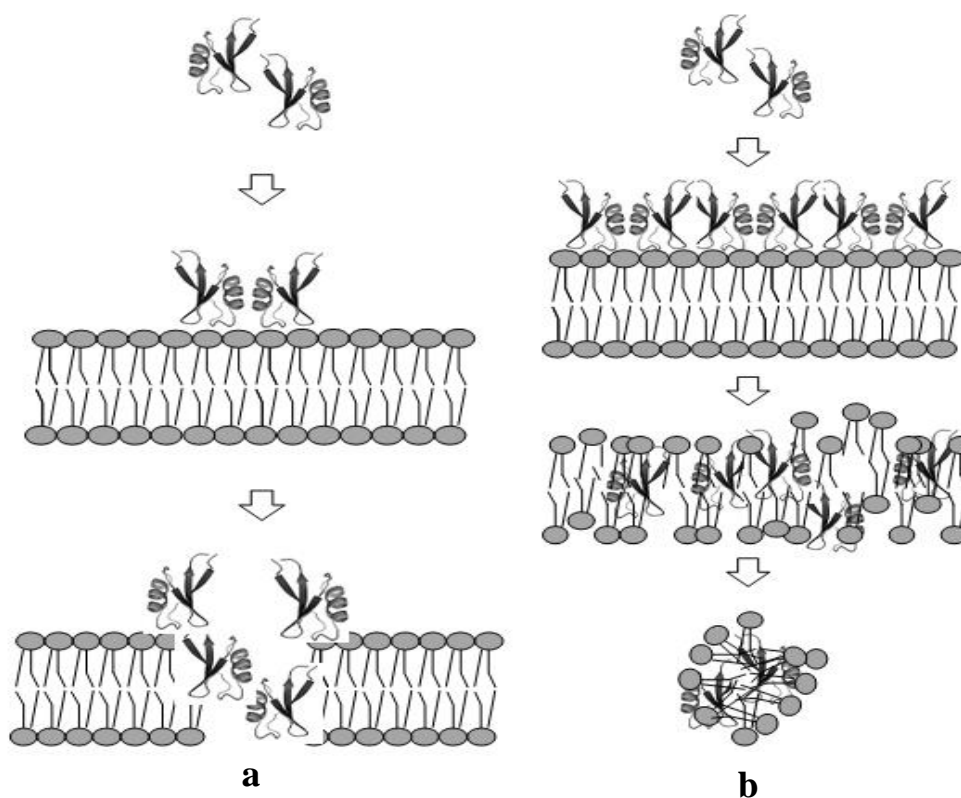


Figure 5.2. Two mechanisms for the mechanism of action of defensins. **a** The barrel-stave model; **b** the carpet model. (Pelegrini et al., 2011)

In this section a molecular dynamics simulation was used to study the interaction of a typical defensin molecule (mung bean defensin VrD1) with a diphosphatidylcholine bilayer membrane. This allowed probing of the mode of binding to the bilayer, to understand the mechanism by which the defensin disrupts the membrane more fully.

5.5.1 *Methods of computer modelling*

Simulation Methodology

The structure of the VrD1 defensin isolated from mung beans was downloaded from the Brookhaven Protein database (www.rcsb.org). This structure was determined by Liu et al. (2006) using a soluble form. The molecular dynamics package GROMACS (version 4.05) was used to construct a phospholipid bilayer-water interface, and to simulate the adsorption of the defensin at this interface. To construct the di-phosphatidyl-choline (DPPC) bilayer interface, a method developed previously was used (Euston, 2014).

A pre-equilibrated DPPC bilayer was obtained from the GROMACS website (www.gromacs.org). This system, containing 100 DPPC molecules, 50 in each of the two leaflets of the bilayer, was originally developed by Chiu et al. (2009). The bilayer

simulation box was expanded in the z-dimension to form a vacuum space, into which a single defensin molecule was inserted on one side of the bilayer. Explicit water (simple point charge, SPC, water) (Berweger et al., 1995) was added into the vacuum space until a density of 1000 g/L was reached. Energy minimisation of the bilayer-defensin-water system was conducted using a conjugate gradients method (Hestenes and Stiefel, 1952). Production simulation runs were carried out in the canonical (NVT) (constant particle numbers N, volume V and temperature T) ensemble for a time period of up to 360ns. The following simulation parameters were used:

- Modified 43a2 force field parameterised for lipids (43a2-S3) (Chiu et al., 2009);
- Particle mesh Ewald (PME) method (Darden et al. 1993; Essmann, et al., 1995) to sum coulomb interactions (cut-off of 1.0 nm), and Van der Waals interactions (cut-off of 1.6 nm);
- Nose-Hoover thermostat (Nose, 1984; Hoover, 1985) set to 300K; and
- Parrinello-Rhman barostat (Parrinello and Rahman, 1981, Nose and Klein, 1984) to control the pressure at 1 bar with semi-isotropic pressure coupling, allowing independent control of the pressure in the x-y plane, in the z-direction normal to the interface.

The simulation trajectories were analysed for the following properties, using the in-built analysis functions in GROMACS:

- The root mean square displacement (RMSD) of backbone atom positions relative to the starting native conformation was calculated to probe changes in the tertiary structure;
- Radius of gyration (Rg) of the backbone atoms was also calculated to probe secondary structure changes;
- DSSP (Kabsch and Sander, 1983) a secondary structure prediction programme was used to calculate changes in ordered secondary structure (helices, sheets, and turns); DSSP defines the proportion of secondary structure in the protein based on geometric bond torsion angle, H-bonding propensity considerations. DSSP output is in the form of a colour coded graph (see **Figure 5.7**) that assigns secondary structure as a function of amino acid position in the primary sequence of the protein, and as a function of the time in the simulation;
- Density profiles for the proteins, DPPC and water were calculated in the normal interface (z) direction to probe adsorption of the protein at the interface;

- Mean square displacement of the DPPC molecules in the plane of the DPPC bilayer, to allow calculation of the lateral diffusion coefficient; and
- Order parameter of the acyl chains of the DPPC molecules in the bilayer

5.6 Results

Two simulations were carried out, with two different orientations of the protein allowed to adsorb at the interface. These were labelled orientation 1 and orientation 2. The defensin molecule adsorbed to the DPPC bilayer after 5 ns for both orientations 1 and 2.

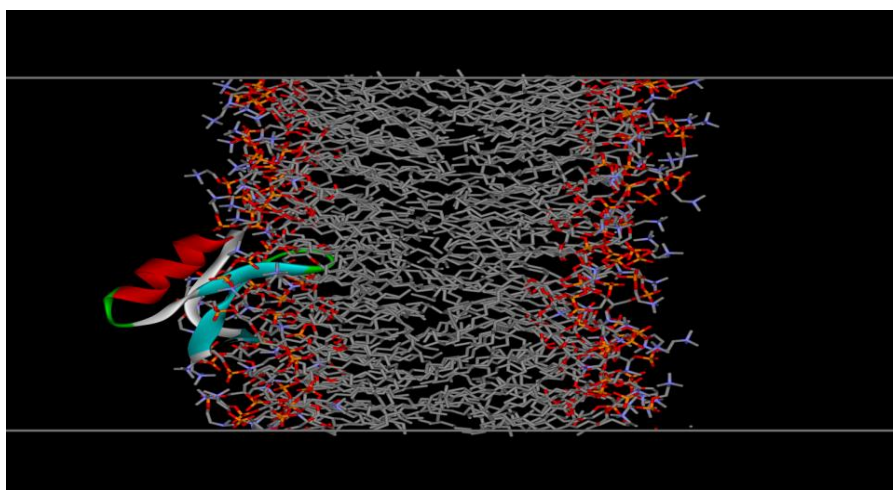


Figure 5.3. Snapshot conformation of mung bean defensin in orientation 1 adsorbed to the DPPC bilayer-water interface. The total simulation time was 320ns. Water molecules were removed from the simulation box for clarity.

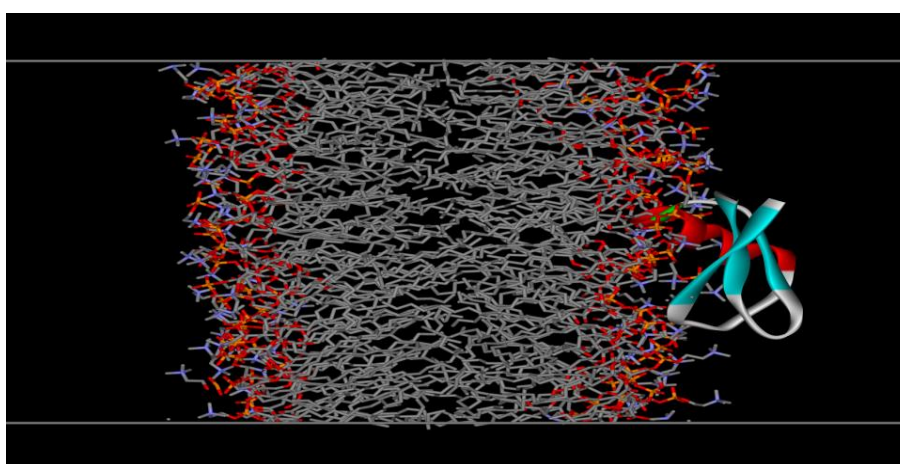


Figure 5.4. Snapshot conformation of mung bean defensin in orientation 2 adsorbed to the DPPC bilayer-water interface. The total simulation time was 360ns. Water molecules were removed from the simulation box for clarity.

Figures 5.3 and **5.4** show snapshot conformations of mung bean defensin, adsorbed to the DPPC bilayer-water interface. The final conformations for the adsorbed structures of both orientations of defensin show the protein penetrates into the DPPC bilayer. The fluctuations in protein conformation may occur as the protein diffuses across the phosphatidylcholine head groups “looking” to insert itself into a gap in the bilayer.

This adsorption of the defensin molecule to the phospholipid bilayer molecule is accompanied by a small but significant decrease in the root mean square displacement (RMSD, **Figure 5.5**) of the backbone atoms, because the molecule changes conformation as it touches the surface.

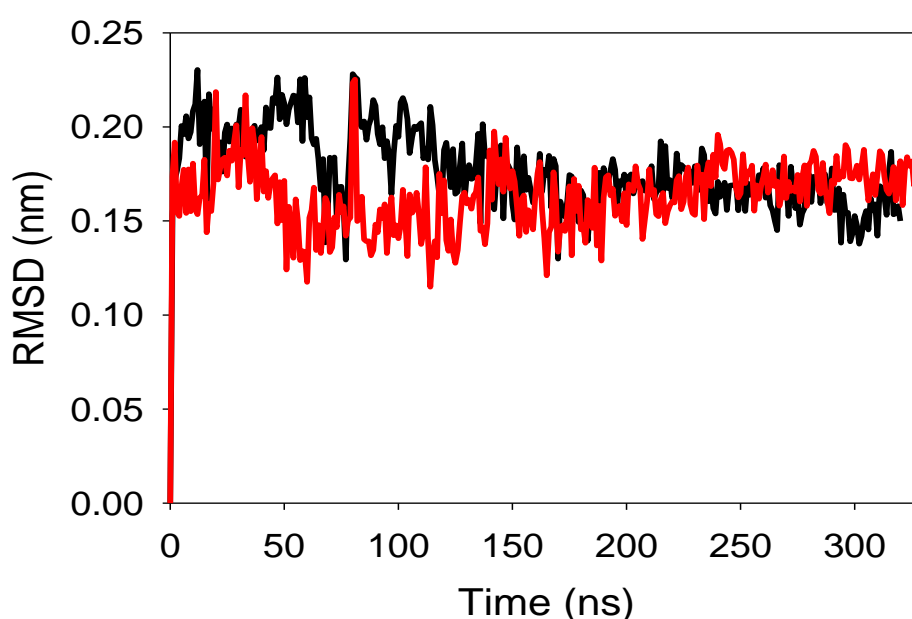


Figure 5.5. Root mean square displacement (RMSD) of the backbone atoms of the defensin chains. The red and black lines are results for two separate simulations of the same molecule starting from different initial conformations. These two conformations represent different orientations of the defensin molecule with respect to the DPPC surface, and are designed to explore the effect of the orientation of the protein on the adsorption and unfolding at the DPPC-water interface.

This process does not lead to a large change in the volume of the protein, as the total radius of gyration (R_g total in **Figure 5.6**) is approximately constant throughout the simulation. The molecule does undergo some conformational rearrangement however, since the

components of the radius of gyration in the x, y and z axes change significantly during the course of the simulation. The x, y and z components R_g of defensin adsorbed in both orientations show a high degree of variability, more so than the overall variability of R_g . This suggests that the protein is changing its shape at the interface, but that there is little overall change in molecular volume.

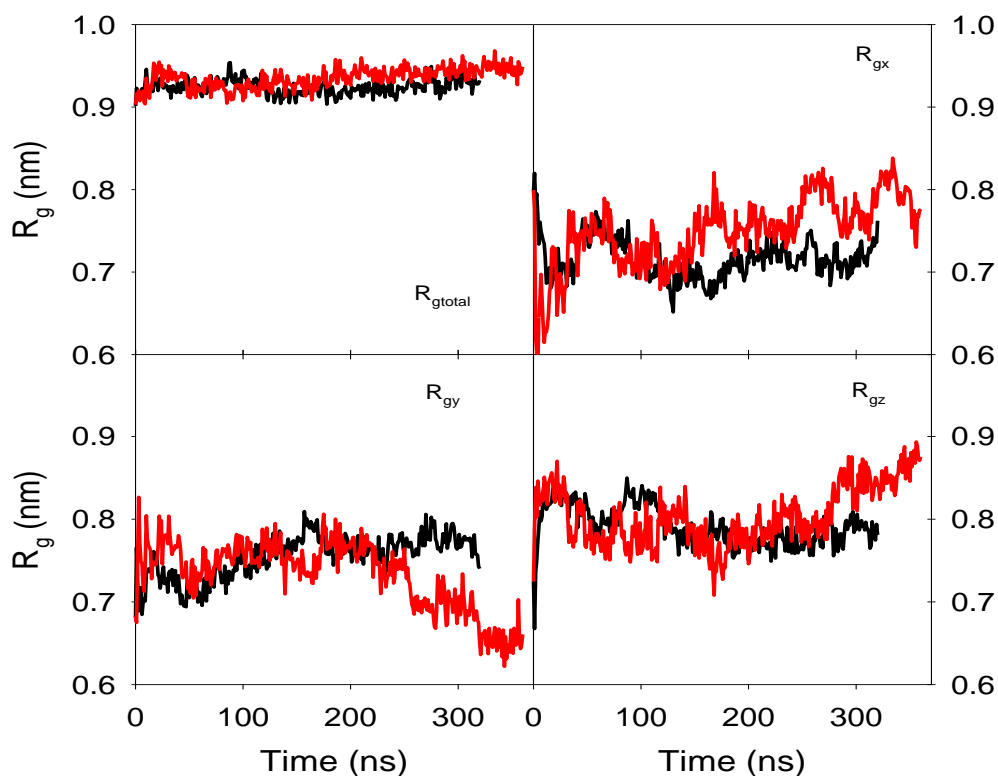
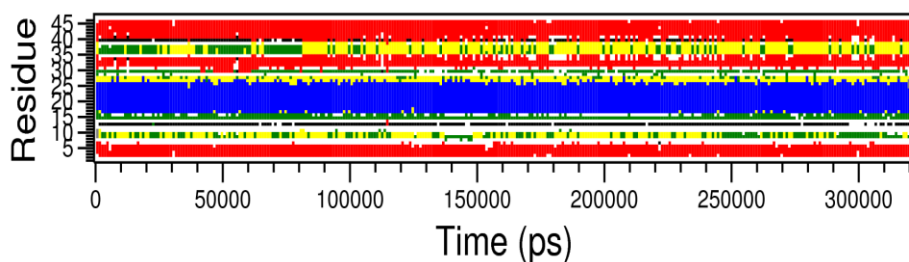


Figure 5.6. Radius of gyration of the defensin molecules over the course of the simulations. The overall radius of gyration (R_{gtotal}), and the x, y and z components (R_{gx} , R_{gy} and R_{gz}). The red and black lines are for the two starting orientations of the defensin molecule with respect to the DPPC-water interface.

The defensin molecules adsorb to the DPPC bilayer, with little change in secondary structure, as indicated by the secondary structure plots in **Figure 5.7**. This shows how the number of residues in the helices, sheets, random coils etc. change throughout the course of the simulation. Clearly, the α -helix and β -sheet structure remain stable throughout the course of the simulation.

Orientation 1

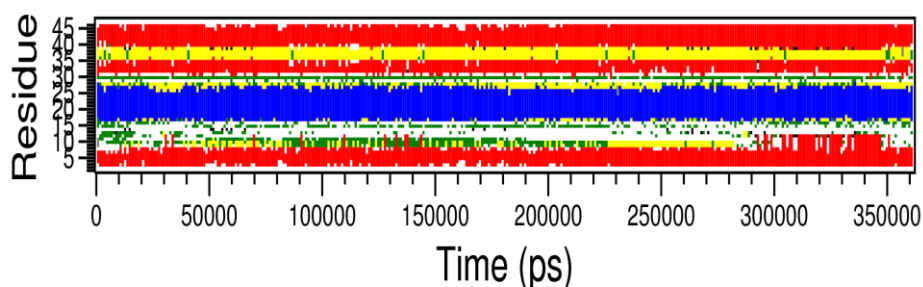
Secondary structure



□ Coil ■ B-Sheet ■ B-Bridge ■ Bend ■ Turn ■ A-Helix ■ 3-Helix

Orientation 2

Secondary structure



□ Coil ■ B-Sheet ■ B-Bridge ■ Bend ■ Turn ■ A-Helix ■ 3-Helix

Figure 5.7. Secondary structure assignment for defensin adsorbed in the second orientation. Orientations 1 and 2 are depicted in the figure. This shows the effect on the orientation of the protein structure. In these plots each amino acid is assigned to a secondary structure type or random coil according to geometric considerations. The amino acid number is plotted on the y-axis and the simulation time on the x-axis. This allows visualization of the position of different secondary structure elements in the protein chain, and to see how these vary with time as the protein adsorbs to the DPPC-water-interface.

The amino acids adsorbed to the interface for both orientations of the defensin molecule are listed in **Table 5.3**.

Table 5.3. Amino acid residues adsorbed to the DPPC-water interface for both orientations of the adsorbed defensin molecule. Amino acids with cationic side chains are highlighted in green

Adsorbed Amino-acids in orientation 1	Adsorbed Amino-acids in orientation 2
MET4	ILE5
ILE5	LYS6
LYS6	LYS7
LYS7	GLU8
LSY12	GLY9
CYS13	TRP10
LEU14	HIS21
ILE15	SER22
ASP16	CYS23
ASN13	LYS24
CYS33	ASN25
LYS34	ARG26
GLY35	GLY27
MET36	TYR28
THR37	VAL44
ARG38	
THR39	
CYS40	
LEU43	
CYS46	

This Table reveals several of the amino acids contain positively charged side chains; this would be anticipated from defensin theories, which suggests that the modelling is able to

reproduce this aspect of defensin interaction with the DPPC membranes (Yeaman & Yount, 2003).

The effect of the defensin molecules on the structure of the DPPC bilayer can be assessed by looking at various parameters. The density profile for the DPPC of protein and water across the simulation box in a direction normal to the interface is shown in **Figure 5.8**. From this, it is clear that the presence of the protein leads to structural changes in the interface, as the shape of the DPPC profile on the side where the protein adsorbs differs from the DPPC profile on the side where there is no protein.

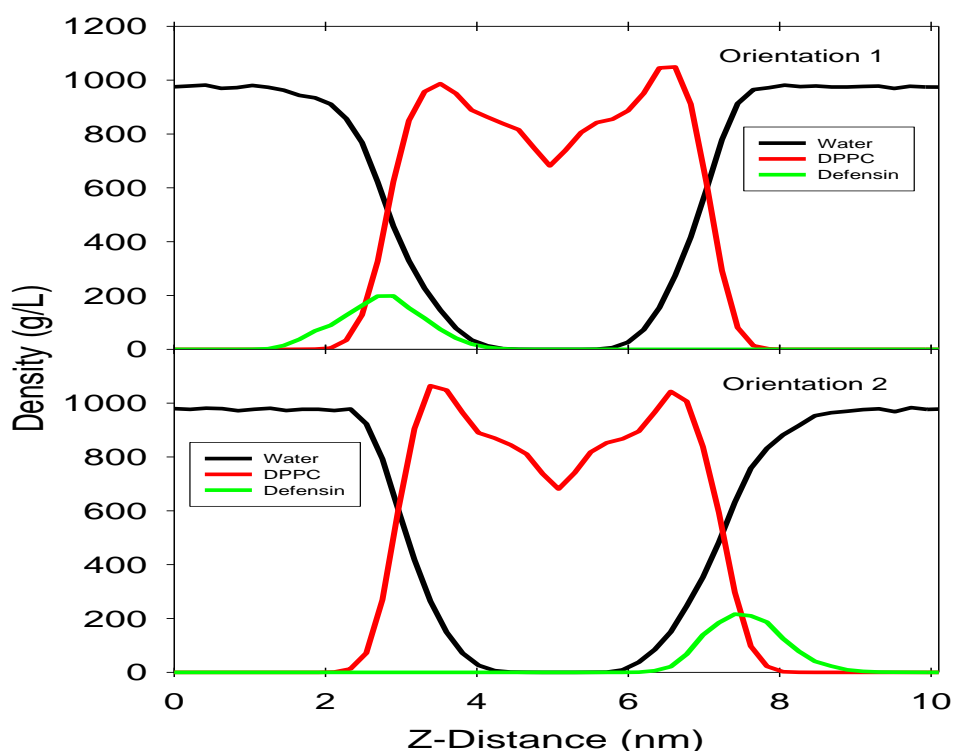


Figure 5.8. Density profiles for water, DPPC, and defensin protein in a direction normal to the bilayer interface. The upper figure represents orientation 1 and the lower figure represents orientation 2.

The effect of defensin molecules on the structure of the DPPC bilayer can be assessed by observing various parameters. For example, the structural changes in the DPPC bilayer can be quantified by looking at two additional parameters: the ordering of the carbon chains in the DPPC bilayer (the order parameter), and the diffusion coefficient for the DPPC molecules in the bilayer. **Figure 5.9** shows the order parameter for acyl chains in the DPPC bilayer when defensin is adsorbed in orientation 1 or orientation 2, and for a DPPC bilayer with no adsorbed defensin.

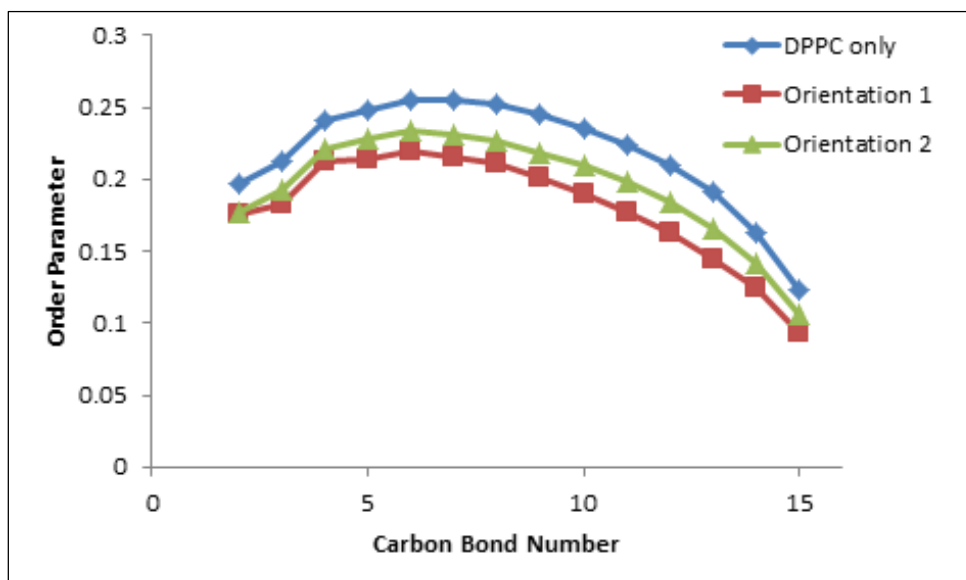


Figure 5.9. Order parameter for the acyl chains in the DPPC bilayer.

The (second-rank) order parameter is defined as:

$$P_2 = \frac{1}{2}(3\cos^2\langle\theta\rangle - 1) \quad (3)$$

Where θ is the angle between the bond and the normal bilayer. $P_2 = 1$ means there is perfect alignment and a normal bilayer, $P_2 = -0.5$ anti-alignment, and $P_2 = 0$ random.

From **Figure 5.9** the order parameter for all DPPC acyl chains is found to decrease the further the bond is from the glycerol backbone. This is because the bilayer becomes slightly more disordered when away from the phosphatidyl group layer. This indicates that the defensin molecules disrupt and disorder the DPPC bilayer, in line with the hypothesised mechanisms for the action of defensins. In addition, the diffusion coefficient for the DPPC molecules in the bilayer also suggests the membrane has been disrupted. It is noticeable that the order parameter for the DPPC molecules in the bilayer in which a defensin is adsorbed is lower than those where there is no protein. The lateral diffusion coefficient (DL), i.e. in the plane of the bilayer, can be calculated from the mean square displacement of the DPPC molecules over time. The DL for DPPC molecules in a bilayer with no adsorbed defensin is larger ($4.95 \text{ cm}^2/\text{s}^{-1}$) than for DPPC molecules in the presence of defensin ($4.02 \text{ cm}^2/\text{s}^{-1}$), which indicates a disordering of the bilayer. In the DPPC bilayer without protein, the DPPC molecules align along the normal bilayer as normal, and are able to diffuse past each other through the ordered gaps between the molecules. When defensin

is present at the bilayer surface, the DPPC molecules are more disordered, and it becomes more difficult for the DPPC molecules to diffuse through the gaps and pass each other.

5.7 Conclusions

The adsorption of defensin molecules to the surface of a DPPC bilayer membrane leads to a disordering of the membrane, which would ultimately disrupt the cell's metabolism. The simulations show positively charged amino acid residues (lysine (LYS) and arginine (ARG)) are prominent in the group of amino acids that anchors the defensins to the DPPC surface, and presumably this means that they play some role in binding the protein to the surface, in agreement with the current theories for defensin action (Yeaman & Yount, 2003). In conclusion, the simulation results are consistent with current theories regarding the ability of defensin to bind to and disrupt the bilayer membrane.

Further work should include isolation, characterisation and sequencing of defensins from CPI, comparison of physical and antifungal properties of CPI defensin to that of mung bean defensin, and repeating the computer modelling experiment using CPI defensins.

Chapter 6

Conclusions

6.1 Novel results of this study

- It is the first time that the antifungal properties of CPI have been demonstrated in bread.
- It is the first time that it has been demonstrated that CPI can be used as an antifungal protein without affecting the sensory or textural properties of bread. This could be of future commercial benefit, as it could be used as a natural preservative in bread.
- It is the first time that the antifungal activity in bread has been associated with a low molecular weight protein compound in CPI.
- It is the first time that a proteomics analysis of cowpea protein isolate CPI has been performed.
- Heat stability, proteolytic stability and the proteomics studies indicate that the active compound in CPI could be a defensin.
- It is the first time that a computer model has been used to design a simulation of the antifungal properties of defensin.

6.2 Overview of research findings

Various protein extracts of cowpea protein were tested for activity against fungal growth using a micro spectrophotometric assay (micro plate method) and the spread plate method. Activity was identified in CPI, and a low molecular weight fraction of CPI. CPI exhibited high antifungal activity against *Penicillium chrysogenum*, *Penicillium brevicompactum*, *Penicillium hirsutum*, and *Eurotium rubrum*, whereas no statistically significant effect was seen against *Aspergillus versicolor*. CPI exhibited different ranges of inhibition toward the same species at different concentrations. The antifungal activity of CPI was unaffected by heat treatment, or protease treatment, indicating that antifungal components are heat stable and protease resistant. The antifungal activity of proteins in the CPI ultrafiltrate (10 kDa molecular weight cut off point) was increased compared to that of CPI and CPI retentate.

CPI was subjected to analysis by LC-MS/MS, and proteomics results reported for the first time. The two most abundant proteins were identified as vicilin, which is a storage protein, and lipoxygenase, which is a protein involved in cell metabolism.

The addition of CPI at 2.3% to leavened bread resulted in significant inhibition of fungal growth during the storage period. No growth was observed throughout 8 day storage period at room temperature, whereas the control samples began to show contamination on the fourth day of storage. The CPI filtrate (2.3%) showed a similar shelf life extension to CPI after 25 days, confirming the antifungal activity of low molecular weight proteins. Inclusion of 2.3% CPI did not significantly affect the sensory or textural acceptability of the bread produced. Although the hardness of bread containing CPI increased significantly after 3 days compared to the control, the effect on sensory acceptability was not significant.

A hypothesis was established that defensin is the major antifungal protein in CPI, based on the results of antifungal activity of the low molecular weight fraction of CPI, the heat stable and protease resistant antifungal properties of CPI and confirmation of the presence of defensins from proteomic results.

A computer model was designed to simulate the antifungal activity of defensin. This demonstrated that the adsorption of defensin molecules to the surface of a phospholipid bilayer membrane leads to a disordering of the membrane, which ultimately would lead to the disruption of the cell's metabolism.

6.3 Impact of the results

This study confirms that CPI could be utilised as a natural preservative in bread. The inclusion of a concentration of 2.3% significantly increases the shelf life of bread, without affecting its sensory acceptability. This could be of considerable commercial value, as cowpea is a widely consumed product in the Middle East and Africa. Introduction of the protein extract to bread would not only increase its nutritional value, but would also increase its shelf life. These findings could raise awareness of the future potential of this ingredient in Europe and other developed countries.

The results of this study form the basis for the commercialisation of a new plant derived preservative. The extraction process for CPI was previously optimised in a PhD study conducted in the School of Life Sciences at Heriot Watt University (Ahmed 2014). The process was similar to that used for soy protein isolate (SPI). Because cowpeas have a long history of use as a food source, the use of CPI would be recognised as safe at the concentration recommended for bread preservation (2.3%), and the need for full toxicological evaluation and legislative approval could therefore be avoided.

Identification of a low molecular weight protein fraction with high antifungal activity could provide the foundation for the purification or enrichment of the compound and support the commercialisation of it as a natural preservative for bread applications. The concentrated low molecular compound would have less sensory impact and could be tested in other bakery applications, and alleviate the losses attributed to mould spoilage.

The suggestion that defensin could be a key antifungal compound in CPI based on proteolytic and thermal stability of the low molecular protein, and the construction of a computer model of its antifungal activity are significant contributors to the fast growing scientific field of novel antifungal protein analysis.

The results of this thesis should be sufficient to trigger wider studies on the effect of CPI towards a larger number of microbes that cause food spoilage. Certainly, further study is needed to test different concentrations of CPI, and investigate its sensory impact on products.

It is predicted that the data presented in this thesis could be used to produce a new natural food preservative and the utilisation of cowpea protein isolate on a large-scale basis.

6.4 Recommendations for future study

Further work needs to be undertaken to identify, purify, and characterise the antifungal compounds from cowpea and describe their activity towards the fungi that cause food spoilage. Further work also needs to be done to investigate the commercial possibilities of CPI, and to expand on the applied aspects of spoilage prevention, perhaps including cowpea in other food products and expanding the list of acceptable food products.

Future research should also aim to increase knowledge about natural antifungal compounds, and to determine their properties and possible applications in an industrial setting.

Further investigation would include:

- Testing of the antifungal activity of different concentrations and determination of the MIC of CPI ultrafiltrate;
- Testing of the antifungal activity of the CPI ultrafiltrate against different fungal species; and
- Testing the heat and proteolytic stability of the antifungal activity of CPI ultrafiltrate.

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