SCREENING, ISOLATION AND CHARACTERIZATION OF LECTINS EXTRACTED FROM MUSHROOMS INDEGENOUS TO SOUTHERN AFRICA

by

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DECLARATION

I declare that the dissertation hereby submitted to the University of Limpopo, for the degree of Master of Science in Microbiology has not previously been submitted by me for a degree at this or any other university; that this is my work in design and in execution, and that all material contained herein has been duly acknowledged.

R. MARIBENG _____ _____

Initials and Surname Date

[redacted]

DEDICATION

I dedicate this work to my wonderful family:

My forever supportive, incomparable and loving parents; Dikeledi and William, and my amazingly caring siblings; Khutso, Lebea, Temo, Modjadji, Matheti, Tumisang and Mundzedzi. You are always the best!

Kgodisho, Phetogo, Kano and Temosho, you always bring a smile to my face.

My adorable baby boy, Phodisho. I love you!

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ABSTRACT

Lectins are among a large number of proteins produced by mushrooms. Mushroom lectins with important biological functions have been isolated and studied. However, none of the studies were reported on lectins isolated from mushrooms indigenous to southern Africa. A galactose-specific lectin from one of the common mushroom species in southern Africa, *Schizophyllum commune,* was isolated and characterized. Initially, twenty mushroom samples were collected and their crude extracts screened for lectin activities. Assays involved in the screening procedures included heamagglutination, carbohydrate inhibition, enzyme linked glycoprotein (ELGA) and various stability assays. Four different mushroom samples exhibited positive lectin activities with varying stabilities towards thermal treatment and susceptibility to proteolytic degradation. Further screening assays resulted in ZHR1 being selected for identification and purification of the lectin. This was due to its ability to agglutinate rabbit erythrocytes. In addition to its activity being destroyed after 3 hours of treatment with trypsin-NIPAAM conjugate, the activity of this lectin was also completely destroyed after an hour incubation in boiling water. In contrast to other mushroom extracts assayed, heamagglutination activity of the crude extract of ZHR1 was not inhibited by glycoproteins only but also by the sugars such as galactose, lactose and mannose. ZHR1 was identified as *S. commune. S. commune* lectin (ScL) was purified using affinity chromatography on a fetuin-agarose column and further purified using gel-filtration chromatography on Biogel P-100 column. ScL was characterized as a glycosylated, galactose-specific dimeric lectin with a molecular weight of approximately 32 and 33 kDa. ScL is a thermolabile lectin which loses its activity as early as 5 minutes after being incubated at 60°C. Anti-ScL antibodies, to be employed in screening for the presence of ScL in the protein extracts, were developed in the rabbits and their interaction with ScL was detected using the double immunodiffusion assays whereas their specificity towards the lectin was detected using Western blot. ScL is one of the first mushroom lectins to be isolated and studied in southern African region. If the lectin is found to be exhibiting important biological functions, ScL can be of commercial importance in the region.

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LIST OF ABBREVIATIONS

APS	ammonium persulfate
BCA	bicinchoninic acid
BSA	bovine serum albumin
Con A	Concanavalin A
DAB	3,3’ diaminobenzidine tetrahydrochloride
DMSO	dimethyl sulfoxide
DTT	$_{DL}$-dithiothreitol
EDTA	ethylenediamine tetraacetic acid
ELGA	enzyme linked glycoprotein assay
HCL	hydrochloric acid
HRP	horse-raddish peroxidase
KCL	potassium chloride
NAS	*N*-acrylosuccinimide
NIPAAM	*N*-isopropylacrylamide
OPD	o-phenylenediamine
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
RIP	Ribosome inactivating protein
SDS	sodium dodecyl sulphate
TBS	tris-buffered saline
TEMED	N,N,N’,N’-tetramethyl-ethylendiamine
UV	ultra violet

CHAPTER 1

INTRODUCTION

1.1 Background information

Mushrooms may be defined as the fleshy, spore-bearing fruiting bodies of fungi typically produced above the ground on soil or on any of their sources of food (Ramsbottom, 1954). A large number of proteins can be produced by mushrooms. These include the ribosome inactivating proteins (RIP's), proteases, antifungal proteins, ribonucleases, ubiquitin-like peptides, laccases (Ng, 2004), and lectins (Wang *et al.*, 2003; Ng, 2004). Out of all these mushroom proteins, lectins are the most examined due to their many usable biological activities (Wang *et al*., 2000).

Lectins can be defined as a group of proteins or glycoproteins of non-immunological origin that can recognise specific carbohydrate structures and are able to reversibly bind with free sugars or with sugar residues of polysaccharides, glycoproteins or glycolipids (Goldstein and Poretz, 1986). Because lectins contain two or more carbohydrate binding sites, they can cause cross-linking of red blood cells and successive precipitation reactions (Su *et al*., 2009).

A large number of proteins and peptides produced by the mushrooms have shown some interesting biological activities. Wang *et al*. (1996) have illustrated some various interesting biological functions revealed by mushroom lectins and they include mitogenic, anti-proliferative, antitumor, hypotensive as well as immunomodulatory activities.

Novel properties have been reported mostly on lectins isolated from mushrooms collected in Asia (Han *et al*., 2005; Su *et al*., 2009; Wang and Ng, 2003). It is believed that mushrooms indigenous to southern African might as well be exhibiting a number of biologically important lectins. However, little is known about these mushrooms and their biological activities because there have been no studies reported on mushrooms collected from southern Africa.

The current study is focussed on isolation and characterisation of lectins from mushrooms collected from southern Africa. Isolation is done among others, from an edible mushroom, *Schizophyllum commune*. *S. commune* is a small, ashy-grey bracket fungus that is characterized by the hairy fruiting structures on the top and gills on the lower, hymenial surface. The fungus is widely spread and one of the most common bracket fungus in southern Africa (http://home.intekom.com/ecotravel/south-african/wildlife-guides/fungi-mushrooms-list.html- visited on 10-12-09).

The study therefore, contributes in adding knowledge about lectins from mushrooms that are indigenous to southern Africa. Lectins found to be exhibiting novel properties, will be of potential commercial value to the region.

1.2 Motivation for the study

There are a large number of mushrooms growing in the southern Africa, and those mushrooms may be producing a number of proteins (including the lectins) whose activities remain uninvestigated. It has been indicated previously by many studies conducted on the Asian mushroom lectins, that the mushroom lectins have important biological activities such as antiproliferative, immunomodulatory, antitumor activities etc. It is therefore, important to conduct more research on mushroom lectins that are indigenous to southern Africa and their activities in order to contribute to knowledge on lectins and their applications.

1.3 Aims and objectives

The main aim of the study is to screen, isolate and characterise lectins from mushrooms indigenous to southern Africa.

In order to reach the main aim of this project the following objectives will be met:

- Collection of mushroom samples from different regions in southern Africa.
- Extraction of mushroom samples using saline azide.
- Screening of aqueous fractions for lectin activity by hemagglutination and glycoprotein binding assays.
- Determination of the specificities of lectins in crude fractions by screening for the most inhibiting sugar or glycoprotein.
- Chromatographic purification of lectins.
- Characterization of the purified lectins.

CHAPTER 2

LITERATURE REVIEW

2.1 Lectins

Lectins may be defined in many different ways; according to Rudiger and Gabius (2001) for a protein to qualify as a lectin, it must meet the following requirements: a lectin must firstly, be a protein that binds carbohydrates and this part of definition ensures exclusion of the tannins, certain lipids, cationic substances and associated carbohydrates in carbohydrate-carbohydrate interactions. Secondly, lectins must be separated from immunoglobulins, which explains that lectins do not exclusively exist as a result of an immune response but they can also be present as a result of stress or a change in the environment as well. A third requirement is that a lectin does not biochemically modify the carbohydrates which it binds.

Damjanov (1987) and Vijayan and Chandra (1999) however, defined a lectin in simple terms as a carbohydrate-binding protein or glycoprotein of non-immune origin which agglutinates cells or precipitates glycoconjugates or both. Damjanov (1987) also classified lectins into five groups based on their affinity for sugars. The groups are: (i) glucose/mannose; (ii) galactose and N-acetyl-D-galactosamine; (iii) N-acetylglucosamine; (iv) L-fucose, and (v) sialic acids. The binding of lectins with simple or complex carbohydrate conjugates is reversible and noncovalent, whether free in solution or on cell surfaces (Brown and Hunt, 1972; Ghazarian *et al*., 2010).

2.2 Detection of lectins

According to Sharon (2007), the simplest way to detect the presence of a lectin in a biological material is to prepare an extract from the material and examine its ability to agglutinate red blood cells. It is believed that a screening procedure becomes even more convincing if the procedure is based on the ability of the lectins to precipitate polysaccharides or glycoproteins (Goldstein *et al*., 1976). If positive results are obtained from hemagglutination assays, it is then necessary to indicate that agglutination or precipitation is specifically inhibited by mono- or oligosaccharides, that is, it is sugar specific. Hemagglutination is commonly performed by the serial dilution technique using, in most cases, human or rabbits erythrocytes. Lectins that have been treated with trypsin or sialidase are mostly used because treated red blood cells were found to be more sensitive to agglutination than untreated cells (Lis and Sharon, 1993).

Mostly, agglutination is said to have occurred if the lectin binds the cells and form cross-bridges between them. However, there is no simple relation between the amount of lectin bound and agglutination activity. According to Lis and Sharon (1993), cases have been reported where significant amounts of lectin are bound to cells without causing agglutination. This is believed to be as a result of the fact that agglutination is affected by many factors which include; accessibility of receptor sites, membrane fluidity as well as metabolic state of the cells. External conditions of the assay such as temperature, concentration of the cell and mixing, also affect agglutination. When agglutination occurs and it is inhibited by monosaccharide or oligosaccharides, it indicates that carbohydrate structures, for which the lectin is specific to, are present on the surface of the cell.

A number of other methods for detection of lectins are currently available. These include microarrays of different carbohydrates that are coupled to wells of a microtiter plate (Bryan *et al*., 2002). Such glycochips greatly facilitate the screening for lectins in biological materials as well as defining their z

Another method for detecting lectins is based on sequence similarities of the newly discovered proteins to known lectins. This is done by performing homology searches in databases at the protein or cDNA level. This method has had a big impact in the field of

animal lectins where it is said to have led to the identification of many new proteins of this class. Drickamer and Dodd (1999) reported that the method also resulted in the discovery of large numbers of various lectin-like proteins, some of which however possess carbohydrate-binding activity.

2.3 Binding of lectins to carbohydrates

In order to obtain a better understanding of how lectins combine with carbohydrates, an extensive site-directed mutagenesis of lectin ECorL was carried out. After making a thorough examination of the lectin's specificity as well as considering the three-dimensional structure of the ECorL-ligand complex, it was then concluded that a combination of the key amino acid residues, an aspartic acid, an asparagine and an aromatic one is essential for galactose binding (Adar and Sharon, 1996; Moreno *et al*., 1997). It is assumed that the first two residues form hydrogen bonds with hydroxyls of the ligand and the third residue interact with the ligand hydrophobically. In addition to that, it was also proven that an identical combination of amino acid residues is also involved in the binding of mannose by other legume lectins such as Concanavalin A. It was then discovered that homologous lectins with different specificities could bind different monosaccharides primarily by the same set of invariant residues that are similarly positioned in their tertiary structures (Sharon and Lis, 2001). It was also proven that this combination of amino acid residues that is present in the combining sites of legume lectins is also found in certain animal lectins such as the mannose-specific ERGIC-53, a lectin that serves as a carrier of a specific subset of nascent glycoproteins between ER and Golgi compartments (Velloso *et al*., 2002).

When comparing the combining sites of lectins from most sources, including those of animals, it was concluded that lectins that differ structurally but with similar specificities might bind the same saccharide by different sets of combining site residues (Sharon and Lis, 2001). The literature also showed that lectins bind their ligands mostly by hydrogen bonds and hydrophobic interactions and that in rare cases electrostatic interactions, which are, ion pairing and coordination with metal ions also play a critical role (Sharon, 2007).

2.4 Lectins in cytochemistry

Lectins bind specifically to carbohydrates, for this reason they have been found to be powerful tools for investigating the cell surface architecture (Komath *et al*., 2001). Many studies dealing with the interaction of different lectins with plant, animal and microorganism cells were increasingly reported after it was demonstrated that Concanavalin A could be used as a cytochemical reagent when studying the cell surface coat of mammalian cells (Seftalioglu and Dalcik, 1994). The studies were aimed on not only allowing the mapping of the cell surface carbohydrates but also on contributing to a better understanding of various surface related biological phenomenon, such as immune response, morphogenesis drug resistance, cell attachment as well as cell-cell interactions.

Lectins are not electron-opaque and therefore they cannot be directly visualised at the electron microscope level, they then have to be conjugated to an electron-dense marker. It has been proved that several possibilities exist for the type of marker that can be used, for example, among the enzymatic markers; peroxidase (HRP) has been the most widely applied. Once bound to lectins, HRP is easily detected through an osmiophilic reaction using 3, 3'-diaminobenzidine as the substrate (Horisberger, 1984). Lectin binding sites can also be visualised by different particulate markers such as ferritin (Nicolson and Singer, 1971), hemacyanin (Smith and Revel, 1972), iron-dextran (Martin and Spicer, 1974) and iron-mannan (Roth, 1975). Colloidal gold has been introduced recently in the field of cytochemistry as an alternative to the particulate markers (Hughes, 2005). Gold particles are said to be electron dense, uniform in size and can be reproducibly and easily prepared, they are therefore believed to represent most markers for both the scanning and transmission electron microscopy (De Mey, 1983).

2.5 Physiological functions of lectins

The physiological role of lectins in plants is not well understood. However, there is evidence that lectins are involved in the recognition between cells or cells and various carbohydrate-containing molecules. This suggests that lectins may therefore, be involved in regulating physiological functions of plants. They seem to play an important role in the defense mechanism of plants against the attack of microorganisms, pests, and insects. Fungal infection or wounding of the plant seems to increase lectins. Lectins are hence believed to be nature's own insecticides and because of this, they have attracted the attention of scientists who are genetically engineering them to produce food plants, containing specific lectins, which will not only have an insecticide effect, but on ingestion will also be hostile to harmful bacteria in the human and animal gut (D'Adamo, 2006).

Some plant lectins are believed to be involved in the binding of symbiotic rhizobia to form the root nodules (van Rhijn *et al*., 1998; Lodeiro *et al*., 1990). Lectins are common in food products such as tomatoes, where they bind to the mucosal cells of these food products and resists denaturation by acid and by proteolytic enzymes and this may be a defensive mechanism for the plant (Lis and Sharon, 1986).

Due to their role in adhesion, lectins have been considered to be important in symbiotic and pathogenic interactions between some microorganisms and hosts. Microbial lectins play an important role in mediating microbial adhesion to surfaces colonized by these microorganisms (Slifkin and Doyle, 1990).

In animals, lectins serve many different biological functions that include the regulation of cell adhesion, glycoprotein synthesis as well as the control of protein levels in the blood. Some lectins, which specifically recognize galactose residues, are found on the surface of mammalian liver cells. It is believed that these cell-surface receptors are responsible for the removal of certain glycoproteins from the circulatory system. These proteins are also known to play important roles in the immune system by recognizing carbohydrates that are found exclusively on pathogens or those that are inaccessible on host cells (http://www.wikipedia.org/Biological functions of lectins, visited on 23-02-09).

2.6 Applications of lectins

Some lectins are very effective toxins and have been applied as therapeutic agents (Minko, 2004; Thies *et al*., 2005). For example, lectins; ricin and abrin have been combined to specific monoclonal antibodies and applied as immunotoxins in cancer therapy (Lis and Sharon, 1986; Nicolson, 1994). Lectins can as well be used as carriers for the delivery of chemotherapeutic agents and are also the significant reagents for investigating cell surface receptors in bacteria, protozoa, and higher organisms (Etzler, 1986; Nicolson, 1994) The interactions of plant lectins with microorganisms have been applied for the typing of bacteria, fungi, and protozoa (Lis and Sharon, 1986). The usefulness of lectins is also found in the characterization of bacterial cell components and in the detection of bacteriophage receptors (Lis and Sharon, 1986).

Lectins may offer different important biological applications, for example, in medicine and medical research, purified lectins are useful in a clinical setting since they may be utilised in blood typing, for example, a lectin isolated from *Dolichos biflorus*, is used for identification of cells that belong to the A1 blood group (Etzler and Kabat, 1970). The *Ulex europaeus* lectin is the used to identify the H blood group antigen while a lectin isolated from *Vicia graminea* is used to identify the N blood group antigen (Sharon and Lis, 2004).

It has been shown by investigators that lectins are useful reagents for the study of fungal cell surfaces and may as well be of value as important aids in the classification of fungi (Barkai-Golan and Sharon, 1978). Chitin, a polymer of β-(1→4)-N-acetyl-D-glucosamine, is characterised as one of the major components of fungal cell walls (Badreddine *et al.*, 2008).

A fluorescein-conjugated wheat germ agglutinin has been shown to be an effective probe in detecting chitin on hyphal surfaces (O'Connell and Ride, 1990). Wheat-germ agglutinin has furthermore been reported to inhibit growth and spore germination of the fungus *Trichoderma viride* (Mirelman *et al*., 1975).

One of the distinct advantages of applying lectins is that cellular or surface receptor sites can be partially characterised by hapten inhibition studies (Damjanov, 1987). Unlike the production of antisera which requires pretreatment of microorganisms for antigen

preparation, injection of the microorganism and glycoconjugate into animals, such as rabbits, as well as absorption of antisera to eliminate non-specific antibody reactions (Slifkin and Cumbie, 1987), lectins are considered simpler to use. Conjugation of lectins to a histochemical label such as flourescein, peroxidase or colloidal gold, helps in making lectins to be used as histochemical probes for identification and localization of specific carbohydrate residues in microorganisms by light or electron microscopy as well as by blotting methods (Malcolm and Doyle, 1990).

2.7 Mushrooms

Mushrooms are umbrella-shaped fungi that have two main parts: the mycelium and the fruiting body**.** The mycelium grows below the surface of the soil or any food source, while the umbrella-like top, or fruiting body, grows on a stalk from the mycelium. Mushrooms belong neither to the animal nor plant kingdom, but they form a phylum on their own. Unlike green plants, mushrooms have no chlorophyll to make food; they survive by absorbing food from decaying or living plants in their surroundings. It is believed that there are over 3,000 species of mushrooms throughout the world (Http: // www. Answerbag.com/ articles/ visited on 07-01-2009).

Mushrooms differ greatly in both shape and size. Some can be very small and others can be over 30.5 cm tall. Some mushrooms are edible while others are extremely poisonous. Mushrooms were described by Ng (2004) as one class of fungi that is very rich in proteins.
According to Mattila *et al*. (2001), the exact number of mushroom species that exist is not known and yet they are believed to be distributed worldwide. Toth, (1995) proved that of all the existing mushroom species, less than 10% of them are edible while a roughly equal proportion is considered to be poisonous. A huge amount of different cultivated mushrooms is believed to be produced and sold throughout the year. *Agaricus bisporus* and Shiitake (Lentinus *edodes*) are considered as the two most popular cultivated mushrooms (Miles and Chang, 1997).

Mushrooms have long been consumed by humans not only as part of the normal diet but also as a delicacy because of their highly desirable taste and aroma. In addition, the tonic, nutritional as well as medicinal properties of mushrooms have long been recognised; for

example, mushrooms were considered by Romans as the foods of the Gods while the Chinese declared them to be the Elixir of life (hypothetical substances believed to maintain life indefinitely) (Johl *et al*., 1995).

Mushrooms are high in proteins (19-35%, including all the essential amino acids) and low in fat. They also contain a relatively large amount of carbohydrates and fiber ranging from 51 to 88% and from 4 to 20%, respectively. Breene (1990) indicated that mushrooms contain significant amounts minerals and vitamins, such as thiamine, riboflavin, ascorbic acid and vitamin D_2. In addition to their nutritional value, some mushrooms in the far East also have a medicinal value; antitumor, antiviral and hypolipidemic effects have been reported (Toth, 1995; Johl *et al*., 1995; Miles and Chang, 1997).

2.7.1 Development of mushrooms

The reproduction unit of all fungi is referred to as the spore. When the spore lands on a suitable base or substrate with ideal growth conditions, it will germinate by sending out a germ tube which then becomes attached to the base or the substrate. The tube then develops into the hyphae which then expand and develop into a network of hyphal threads, known as mycelium. Mycelium, which is hardly seen, is the vegetative body of the fungus and it is responsible for its nutrition and formation (home.intekom.com/ecotravel/fungi-mushrooms-list.htm- visited on 20-09-09).

The mycelium continues to grow and branch throughout the substrate. This will happen for as long as it is still able to obtain nutrients from the substrate, with the temperature and moisture conditions remaining favourable. The mycelium may even continue to grow for a long time without forming any sex organs, but, once two sexually differentiated mycelia meet and plasmas of the conjugating cells unite and if external conditions are ideal, a fruit-body will appear. Then a mushroom or toadstool pops out of the ground (home.intekom.com/ecotravel/.../fungi-mushrooms-list.htm- visited on 20-09-10).

2.7.2 Cultivation of mushrooms

There is a variety of methods in which mushrooms can be cultivated. Some of these methods are extremely simple and demand little or no technical skills whereas some are much more technically demanding. Technically demanding mushroom cultivations are those that require aspects of sterile handling technology (Chang and Miles, 1989). Cultivation of mushrooms involves different operations each of which must be performed for cultivation to be successful. Operations involved include selection of mushroom spores or strains, maintenance of mycelia cultures, development of inoculums, preparation of growing medium, spawn inoculation and colonisation of substrate as well as management of crop for mushroom production (Mushroom cultivation technology, 2009).

In any mushroom cultivation process, the first stage is to obtain a pure mycelia culture of the specific mushroom strain. Such cultures can be obtained from mushroom enterprises, mushroom institutes and mushroom specialists (Stamets, 1993). Such types of cultures have originally been derived from a single of multispore cultures or by tissue culture from a mushroom of a high yielding and dynamic strain.

The desired mushroom must be able to colonise the substrate before other fungi or bacteria do so. To achieve this, pre-grown mycelium (free of any contaminants) of the mushroom is inoculated on a sterile substrate. This material is referred to as spawn. Using spawn will give the cultivated mushroom an advantage in growth over other fungi. The mycelium will colonize the substrate and use the available nutrients. After having colonised the substrate, the mycelium is capable of producing fruiting bodies (Oei, 2005).

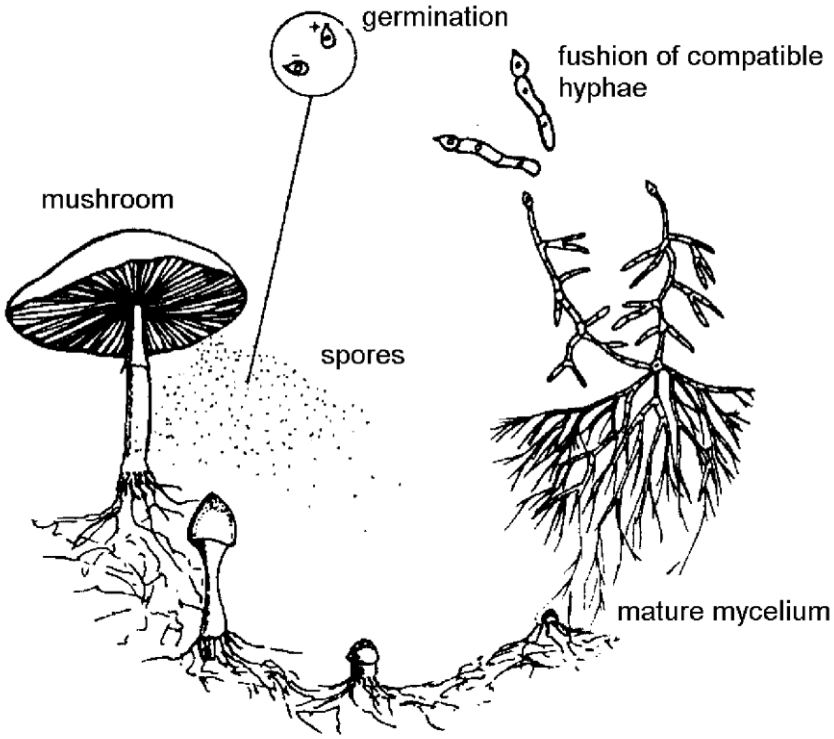

Figure 1: Life cycle of a mushroom grown in nature (Oei, 2005).

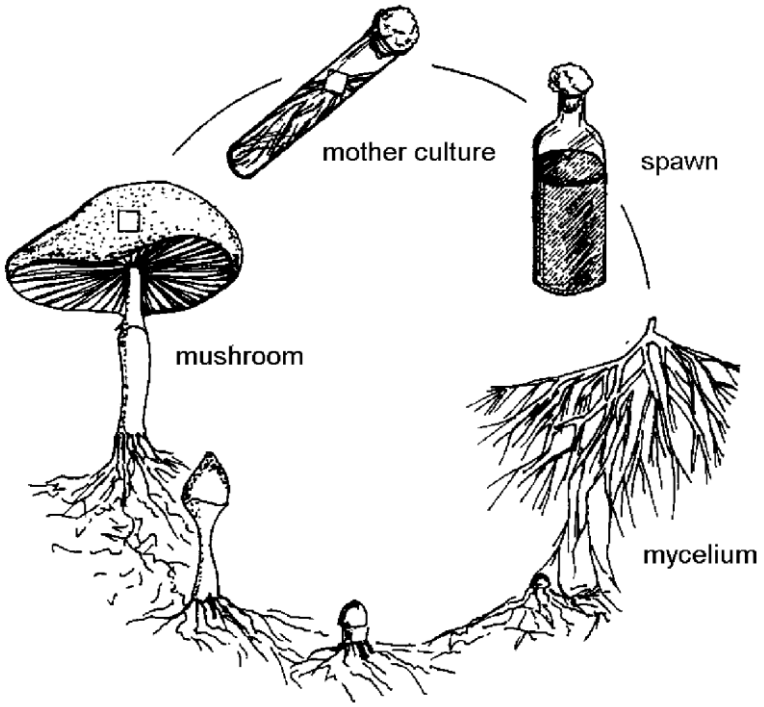

Figure 2: Life cycle showing the development of mushrooms to a spawn. Tissue cultures are isolated from a mushroom and propagated on a suitable substrate. The full-grown substrate is then used in mushroom growing (Oei, 2005).

2.7.3 Medicinal uses of mushrooms

Medicinal mushrooms are now being studied mostly by ethnobotanists and medical researchers. The ability of some of mushrooms to inhibit tumor growth and enhance aspects of the immune system has been a subject of research for about 50 years (Borchers *et al*., 2008). Today, mushroom research continues worldwide, with a focus on mushrooms that may have hypoglycaemic activity, anticancer activity, antipathogenic activity as well as immune system enhancing activity. So far, it has been discovered that the oyster mushroom contains the significant amounts of lovastatin, a member of the drug class of statins, used for lowering cholesterol (hypolipidemic agent) in those with hypercholesterolemia and hence preventing cardiovascular diseases (Gunde-Cimerman and Cimerman, 1995) and that some fungi may be a future source of taxol, an anti-cancer chemotherapy drug (Ji *et al*., 2006). Penicillin, lovastatin, ciclosporin, griseofulvin, cephalosporin and ergometrine are the most famous pharmaceuticals which have been isolated from the fungi kingdom.

Psilocybin mushrooms, commonly known as magic mushrooms is a group of mushrooms that is believed to be possessing psychedelic properties. These mushrooms have psychoactive properties and hence, have played a role in native medicine where they were used in an attempt to effect mental and physical healing as well as to facilitate visionary states. Psilocybin, a naturally occurring chemical in certain psychedelic mushrooms like *Psilocybe cubensis*, is being studied for its ability to help people suffering from psychological disorders, such as obsessive-compulsive disorder. Minute amounts have been reported to stop cluster and migraine headaches (Sewell *et al*., 2006).

A study, done by the John Hopkins Hospital (Sewell *et al*., 2006), showed that psychedelic mushrooms could provide people an experience with substantial personal meaning and spiritual significance. In the study, one third of the subjects reported that ingestion of psychedelic mushrooms was the one most spiritually significant event of their lives. Over two-thirds reported it among their five most meaningful and spiritually significant events and on the other hand, one-third of the subjects reported extreme anxiety (Griffiths *et al*., 2006).

2.7.4 Mushroom species common in southern Africa

Clitopilus prunulus, also known as the miller or the sweetbread mushroom is one of the most commom mushroom found in Southern Africa (http://home.intekom.com /ecotravel /south-African /wildlife-guides/ fungi-mushrooms list.htm). It is an edible pink-spored basidiomycete mushroom that is also common in grasslands in Europe. The mushroom is mostly differentiated by its grey to white cap and decurrent gills (*Clitopilus prunulus:* The mushroomExpert.com. visited on 10-12-09).

The cap is initially convex when young, but in maturity flattens out, usually with a shallow central depression. It is white or light gray or yellow, sticky when moist, and 3 to 10 cm (1.2 to 3.9 inches) in diameter with a characteristic feel to the touch of chamois skin.

The gills are decurrent in attachment to the stipe, spaced together rather closely, and whitish, although they often develop a pinkish hue in age. This mushroom has a mealy odor, somewhat like cucumber (Wood *et al*., 1994).

Schizophyllum commune is also one of the common mushrooms in Southern Africa. *S.commune* is an edible mushroom belonging to the phylum basidiomycetes, order agaricales and family Schizophyllaceae. The fungus grows mostly during rainy season and appears frequently on dead wood (Zoberi, 1978). The mushroom is believed to be a very good source of proteins, vitamins, lipids and mineral elements that might be needed by those who value the mushroom. As with most of edible fungi, *S. commune* is also seasonal; not available all year round.

Adejoye *et al. (*2007) conducted a physicochemical study on the mushroom. The aim was to evaluate the suitable conditions needed for growing this fungus so that it can be available to the consumers all the time. They then discovered that in order to obtain maximum mycelium growth of *S. commune,* the fungus should be cultivated on pH 5.5 and a temperature of 25°C. Riboflavin and pyridoxine promoted good vegetative growth while, mannitol and sorbitol were found to be the best utilizable carbon sources that are most suitable for cultivation of *S. commune*. *S. commune* is also considered as one of the most widely distributed and common mushrooms on earth and as a result, it is considered as one of the most studied mushrooms on earth.

It is easy to recognise this mushroom because its tiny fruiting bodies do not have stems and they attach themselves like tiny bracket fungi on dead wood. *S. commune*, unlike a bracket fungus, has what appear to be gills on its underside, rather than pores or a simple, flat surface (Kuo, 2003).

Other mushroom species common in southern Africa include *Coprinus micaceus, Podaxis pistillaris, Coriolopsis polyzona, Leucoagaricus bisporus, Phlebopus sudanicus, Amanita pantherina, Amanita phalloides, Amanita rubescens, Coprinus micaceus, Coprinus plicatilis, Pycnoporus sanguineus, Laccaria placate, Lactarius deliciosus, Lactarius hepaticus, Panaeolus papilionaceus, Scleroderma citrinum, Laccaria amethystine, Amanita pantherina, Amanita phalloides, Amanita rubescens* as well as *Boletus edulis* (http://home.intekom.com /ecotravel /south-African /wildlife-guides/ fungi-mushrooms list.htm visited on 15/10/11).

2.8 Lectins obtained from mushrooms

According to Guillot and Konska (1997), in mushrooms, lectins are found on the caps, stapes as well as mycelia and variation in lectin content occurs depending on the time and place of harvest and on the corpophone age. Lectins, in mushrooms, play an important function in dormancy (Guillot *et al.,* 1991), growth and morphogenesis, morphological changes due to parasitic infections and molecular recognition during the early stages of mycorrhization (Giollant *et al*., 1993).

Lectins isolated from mushrooms were found to have specificities towards sugars such as, N-acetylglucosamine (Chumkhunthod *et. al*., 2006), D (+)-galactosamine (Ngai and Ng, 2004), D (+) galactose (Ngai and Ng, 2004), N-acetylglucosamine (Wang and Ng, 2003), N-acetylneuraminic acid (Ueda *et al*., 2003), arabinose (Wang and Ng, 2005) and lactose (Han *et al*., 2005).

Several studies have been conducted on mushroom lectins and their applications (Presant and Kornfeld, 1972; Sueyoshi *et al*. 1985; Yu *et al.*, 1993). The study conducted by Sueyoshi *et al*. (1985) revealed that *Agaricus bisporus* mushroom produces more than one lectin then, Presant and Kornfeld (1972) showed that the molecular masses of those tetrameric lectins which are produced by *A. bisporus* were all 64 kDa. *A. bisporus* lectins display some biological applications including lectins antiproliferative action towards various tumor cell lines (Yu *et al.*, 1993).
On the other hand, two lectins were isolated from the mushroom *Agaricus edulis* (Eifler and Ziska, 1980). The two lectins differed vastly in molecular mass and carbohydrate content. One of them is dimeric with a molecular weight of 32 kDa while the other one is a tetramer with a molecular weight of 60 kDa. It was then indicated that unlike a 60 kDa lectin, the hemagglutinating activity of the 32 kDa lectins is not inhibited by the common simple sugars (Eifler and Ziska, 1980).

LZ-8 is an immunomodulatory lectin that was isolated from the mushroom *Garnoderma lucidum* (Ooi *et al.,* 2002**).** The protein exhibits a molecular weight which is similar to the *F. velutipes* lectin and its amino acid sequence is highly homologous to the *Volvariella volvacea* immunomodulatory protein (Tanaka *et al*., 1989). Haak *et al*. (1993) and Van-Der-Hem *et al*.

(1995) also conducted studies on LZ-8 and discovered that the lectin manifested some mitogenic activities on mouse splenocytes as well as on the human peripheral lymphocytes.

She *et al*. (1998) have isolated a novel lectin from both the fruiting bodies and mycelia of the straw mushroom *V. volvacea*. The lectin is a homodimeric non-glycoprotein with a molecular mass of 32 kDa. The hemagglutinating activity of this lectin was found to be inhibited by thyroglobulin and not by simple sugars. It was also shown that the lectin exhibits the potent stimulatory activities towards the spleen cells and increases the expression of interleukin-2 (IL-2) and interferon.

A mushroom lectin was isolated from ascomycete *Cordyceps militaris* by Jung *et al*. (2007). *C. militaris* is one of the most popular mushrooms in eastern Asia and it is used as a nutraceutical and in traditional Chinese medicine. This lectin, known as CML, exhibited hemagglutination activity in mouse and rat red blood cells but not in human ABO red blood cells. Hemagglutination activity of CML was inhibited by sialoglycoproteins and not by monosaccharides, disaccharides and asialoglycoproteins. The activity of the protein was found to be maximal at pH 6.0-9.1 and at temperatures below 50˚C. SDS-PAGE of CML showed a single band with a molecular mass of 31.0 kDa under the reducing and non-reducing conditions. CML is also reported to be exhibiting mitogenic activity against the mouse splenocytes (Jung *et al*., 2007).

A novel lectin with a molecular mass of 17 kDa has been isolated from the dried fruiting bodies of the toxic mushroom *Inocybe umbrinella.* The lectin was purified by ion-exchange chromatography on DEAE-cellulose, and CM-cellulose, and gel filtration on Superdex 75. Hemagglutination activity of the lectin was inhibited by raffinose, D-Mellibiose, α-lactose and D (+)-galactose. The activity was inhibited by Ca^{2+}, Mn^{2+} and Mg^{2+} but was unaffected by Fe^{3+}, Zn^{2+} and Al^{3+} ions. The lectin inhibited proliferation of tumor cells, including hepatoma HepG2 cells and breast cancer MCF7 cells with an IC_{50} of 3.5±0.2 μM and 7.4± 0.3 μM, respectively (Zhao *et al*., 2009).

A dimeric lectin with a molecular mass of 29.4 kDa was isolated by Feng *et al.* (2006) from the dried fruiting bodies of the mushroom *Armillar luteo-virens.* Its purification procedure involved ammonium sulphate precipitation $(NH4)_2SO_4$ precipitation, ion-exchange chromatography on DEAE-cellulose, CM-cellulose, and Q-Sepharose, and gel filtration by

fast protein liquid chromatography on Superdex 75. The polysaccharide, inulin, inhibited the hemagglutination activity of the lectin and the activity could not be inhibited by simple sugars. The lectin stimulated mitogenic response of mouse splenocytes with the maximal response achieved by 1 µM lectin. The lectin also inhibited proliferation of tumor cells including MBL2 cells, HeLa cells, and L1210 cells with an IC_{50} of 2.5, 5, and 10 µM, respectively. However, the lectin did not affect proliferation of HepG2 cells (Feng *et al*., 2006).

Immunomodulatory and antitumor activities of the two lectins TML-1 and TML-2 isolated from *Tricholoma mongolicum* were investigated. When tested for immunomodulatory activities in mice, the two lectins stimulated nitrite ion (NO_2-) production and activated macrophages to produce tumor necrosis factor (TNF) and macrophage activating factor and were therefore regarded as immunomodulatory substances. TML-1 and TML-2 reduced the growth of sarcoma 180 cells implanted in mice by 68.84% and 92.93%.

A two 16 kDa subunits lectin isolated from the wild mushroom *Xerocomus spadiceus* (Liu *et al*., 2004). The lectin exhibits the mitogenic activity towards mouse splenocytes and among most of the carbohydrates tested; only inulin was able to inhibit the hemagglutinating activity of this lectin.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Mushroom samples

Mushrooms used in this experiment were collected from different parts of southern Africa, including Debengeni falls, Tzaneen in the Limpopo Province of South Africa, University of Limpopo (Turfloop campus), South Africa and No. 20 Hillside road, Harare in Zimbabwe. Different samples collected from Tzaneen, Harare and University of Limpopo were labelled TZ 1 to 8, ZHR 1 to 7 and TRFP 1 to 5, respectfully.

Identification of selected species was done by the centre for Applied Mycological Studies, University of Pretoria, South Africa.

3.1.2 Reagents

Sodium azide, Sodium chloride, Ammonium sulphate, Sodium phosphate (monobasic and dibasic), Bovine Serum Albumin (BSA), Bicinchonicic acid reagent (BCA) protein assay reagent, Copper (II) sulphate solution, Phosphate buffered saline tablets, Citric acid, Sodium citrate, Ovalbumin, Thyroglobulin, Fetuin, Asiolofetuin, Mucin type I, Mucin type II, Mucin type III, Horse-Raddish peroxidase, Mellibiose, Galactose, Lactose, Glucose, Maltose, Fructose, L-Fucose, D(+) glucosamine, N-acetyl-D-galactose, N-acetyl-D-glucosamine, Methyl-β-D-glucopyranoside, Methyl-α-D-mannopyranoside, α-Lactose monohydrate, Magnesium chloride Hexahydrate, Manganese Chloride, Calcium chloride Dihydrate, Azocasein, Cytochrome c, Blue Dextran, Carbonic anhydrase, β-amylase, Alcohol dehydrogenase, Freund's reagent (complete and incomplete), Potassium phosphate, Agarose, Ammonium persulphate (APS), Sodium dodecyl Sulphate (SDS), N, N, N', N'-tetramethyl-ethylediamine (TEMED), Trizma base, Dimethyl sulfoxide (DMSO), $_{DL}$-Dithiothreitol (DTT), Tween 20, 3,3' diaminobenzidine tetrahydrochloride (DAB), *N*-isopropylacrylamide (NIPAAM), HRP anti-rabbit IgG, and *N-acrylosuccinimide* (NAS), Dialysis Tubing

Cellulose membrane were obtained from Sigma-Aldrich (Pty) Ltd, Gauteng, South Africa. Potassium Chloride (KCL) was obtained from Chemies Suiwer, Coomasie Brilliant blue R-250, Coomasie Brilliant blue G-250 and Acrylamide were obtained from Fluka Biochemika. Bis N, N'-Methylene-bis-acrylamide, Affi-Gel 15 Gel and Biogel P-100 were obtained from Bio-Rad Laboratories (Bio-rad laboratories (Pty) Ltd, South Africa). Glycine and Glycerol were obtained from Saarchem UniLAB (PTY) LTD. (Saarchem (Pty) Ltd, Midrand, South Africa). Xylene, Acetic acid, Ethanol, Methanol, Hydrochloric acid, Ammonium nitrate, Hexane, Isopropanol, Acetone, Diethyl ether were obtained from Rochelle chemicals (Rochelle chemical and laboratory equipments cc, Johannesburg, RSA). Protein molecular Weight marker was obtained from Fermentas (Ferments- Inqaba biotec, Pretoria, South Africa). Bromophenol blue was obtained from Amersham Life Science, (Pty) Ltd, SA. Trypsin was obtained from Invitrogen and Hydrogen peroxidase was obtained from Pakmed, Johannesburg, South Africa.

3.1.3 Equipment

BIO-RAD BioLogic LP chromatography system apparatus, Speedy Autoclave (vertical type), Beckman Coulter Allegro X 22R centrifuge, Bio-rad mini-protean multicasting chamber, Beckman Coulter DTX 800 Multimode Detector microplate reader.

3.2 Methods

3.2.1 Extraction of lectins from mushroom samples

Mushroom samples were collected and stored at 4˚C until use. To extract lectins, samples were firstly ground to fine powder using a commercial blender. The powdered mushrooms were extracted overnight at 4˚C using saline-azide solution (0.9% sodium chloride and 0.02% sodium azide) at a volume that is five times the weight of each mushroom sample. Aqueous extracts were obtained after centrifugation at 3398 xg for 10 minutes.

3.2.2 Haemagglutination assays

Mushroom crude extracts were screened for lectin activities using hemagglutination assays. The assays were performed using 4% rabbit red blood cells. The blood was collected into an equal volume of Alsever solution (0.055% citric acid, 0.8% sodium citrate, 2.05% D-glucose, 0.42% sodium chloride) (Alsever and Ainslie, 1941) by bleeding a rabbit through a small opening made in the lower vein of the ear. Washed erythrocytes were prepared from rabbit blood by repeated suspension in saline-azide and centrifugation at 1204 xg until the washings were clear. Saline-azide solution was then used to prepare 4% (v/v) red blood cells suspension.

Hemagglutination assays were performed by adding 25 µL of saline-azide solution containing 2 mM metal ions (Ca^{2+}, Mg^{2+}, Mn^{2+}) into the wells of 96 well U-shaped microtiter plates. This was followed by addition of 25 µL of lectin samples in the wells of the second column of each row. Serial dilutions were then made from where the lectin samples were added, mixed and transferred to the next well, up to the last wells. The extra 25 µL in the wells of the last columns was then discarded. Saline-azide was used as a negative control in the wells of the first columns, while, 1 mg/mL of Concanavalin A, prepared in saline-azide solution, was used as a positive control. As a final step in the assay, 50 µL of 4% rabbit erythrocytes (prepared in saline-azide solution) was then added. The plates were incubated at room temperature for 60 minutes. Thereafter, agglutination of erythrocytes was estimated visually and expressed as agglutination titer, which is defined as the reciprocal of the maximum dilution that gives a visible agglutination.

3.2.3 Determination of lectin specificity for sugar/glycoprotein

3.2.3.1 Carbohydrate/ glycoprotein inhibition assays

Inhibition assays were performed in order to determine the specificities of the lectin samples towards sugars and glycoproteins. The sugars used in the assays include; lactose, galactose, maltose, fructose, fucose, mannose, glucose, mellibiose, D (+) glucosamine, methyl-β-D-glucopyranoside, methyl-α-D-mannopyranoside, N-acetyl-galactosamine and N-acetyl-glucosamine. The glycoproteins used include; asialofetuin, fetuin, mucin type I, mucin type II, mucin type III and ovomucoid. Concentrations of 0.2 M and 5 mg/mL in saline-azide solution were prepared for sugars and glycoproteins, respectively.

In the assays, 25 µL of lectin samples were added in duplicates into wells of the U-shaped microtitre plates. Then, 25 µL of the different sugars and glycoproteins was added to the wells followed by incubation at room temperature for 30 minutes. Following incubation, 50 µL of 4% erythrocytes was added. The plates were further incubated for 30 minutes. The results were visually analysed and the sugar/glycoprotein specificity to the lectin was taken as the one to which inhibition of agglutination occurred.

In addition to carbohydrates inhibition assays, the lowest concentrations of the sugars inhibiting hemagglutination of lectin samples were determined. This was performed by firstly, adding saline-azide solution in each well of the U-shaped microtiter plate except for the wells in the first column. A volume of 50 µL of the sugars that were previously able to inhibit the lectins was then added to the empty wells of the first column.

Serial dilutions of the sugars/glycoproteins were made by transferring 25 µL of sugars/glycoproteins from the wells in the first column to the second column, mixed and transferred to the next column up until the last column. The extra 25 µL in the last column was then discarded. An equal volume of 25 µL of the lectin samples was then added to all the wells. The plates were thereafter incubated at room temperature for 30 minutes after which 50 µL of 4% rabbit red blood cells in saline azide solution was added. The plates were further incubated for 30 minutes. The results were analysed visually and the lowest concentration of a sugar/ glycoprotein inhibiting hemagglutination was recorded from the last wells in which inhibition occurred.

3.2.3.2 Enzyme linked glycoprotein assays (ELGA)

This is a sensitive assay that is done to detect the possible low levels of lectins in the mushroom samples. The biotin-glycoprotein conjugates were firstly prepared for the assay. Glycoprotein solutions (3 mg/mL) were prepared in 0.2 M sodium borate buffer pH 8.8. Solutions were dialyzed overnight against 0.2 M sodium borate buffer pH 8.8. In order to prepare the biotin conjugates, N-hydroxysuccinimidobiotin was dissolved in Dimethyl sulfoxide (DMSO) at 200 mg/mL. While vortexing, a volume of 5 µL biotin ester was added in 2.5 µL aliquots to the dialyzed glycoproteins solutions. Biotin-glycoprotein conjugates were then stored at 4˚C until use.

Enzyme linked glycoprotein assays were performed by firstly coating overnight, flat-bottomed, 96-well plates with 100 µL of different mushroom (lectin) samples. To block the plates, 1% bovine serum albumin (BSA) prepared in 0.5 M Tris-buffered saline (TBS) pH 7.6 (TBS-BSA) with ions was added. The plates were washed twice with TBST (0.1% Tween 20 in TBS). Biotin glycoproteins, in the ratio of 1: 200 in TBS with 2 mM metal ions, were added to the plates, incubated for 90 minutes and then washed three times with TBS+ions. Streptavidin peroxidase, prepared in the ratio 1:1000 in TBS without metal ions was then added. Thereafter, the plates were washed three times with TBS. O-Phenylenediamine (OPD) (1 tablet equivalent to 200 µmoles (Sigma) in 12 mL of 0.1 M citrate-phosphate buffer pH 5.0) was added. Absorbance reading was taken at 405 nm after 20 minutes of incubation at room temperature.

3.2.4 Stability assays on crude lectin extracts

3.2.4.1 Thermal stability assays

Thermal stabilities were performed to determine the heat stabilities of the lectin crude samples treated over specific period of time. Heat stabilities of lectin extracts was initially assayed at high temperature whereby extracts were placed in boiling water (95˚C) water bath and then assayed for lectin activities at 5, 10, 15, 30, 60, 120 and 180 minutes. Lectin extracts were again placed in water baths of different temperatures (25˚C, 60 ˚C, 80˚C and 95˚C) over 5, 10, 15, 30, 60, 120 and 180 minutes. After every incubation period, samples

were assayed. Lectin activities of treated extracts were assayed using hemagglutination assays using 4% rabbit erythrocytes.

3.2.4.2 Proteolysis of lectins

Proteolysis of lectins was done to assess the degradation of lectins by trypsin-N-isopropylacrylamide (NIPAAM) conjugate. *N*-Acriylosuccinimide (NAS) was used to activate trypsin via the succinimide. The activated trypsin was then covalently attached to poly-NIPAAM, a thermoresponsive polymer, by copolymerisation with NIPAAM. Initially, Trypsin-NAS was prepared by adding 1.75 mL of 2.5% trypsin solution into 0.25 mL of NAS (2 mg/mL prepared in dimethyl sulfoxide (DMSO)). The solution was then incubated at 30 ˚C for an hour. In order to separate trypsin from NAS, the solution was passed through a Sephadex G-25 (15 cm X 1 cm) column. The protein was eluted with 0.01 M phosphate buffered saline (PBS) pH 7.2 at a flow rate of 0.5 mL/min. Fractions containing trypsin were pooled and copolymerized with NIPAAM by dissolving 1.25% of NIPAAM in 10 mL of trypsin solution.

In the assay, 500 µL of the trypsin-NIPAAM conjugate was used to treat an equal volume of the lectin samples. For untreated samples, 500 µL of PBS was added to an equal volume of lectin samples. Both treated and untreated solutions were incubated in water bath at 30˚C for different time intervals. The conjugates were precipitated by incubation at 45˚C for 1 minute followed by centrifugation at 5878 xg for 1 minute. A volume of 25 µL of the supernatant was taken immediately after centrifugation and assayed for lectin activity using hemagglutination assay.

3.2.5 Purification of the lectin using chromatographic methods

3.2.5.1 Affinity chromatography

Affinity Chromatography was done in order to isolate a pure *S.commune* lectin from the crude sample. Mucin and fetuin columns were prepared for purification of the lectin because they were both found as the strongest inhibitors when performing the glycoprotein/sugar inhibition assays. To prepare each column, mucin type II and fetuin were dissolved in 20 mL of 10 mM acetate buffer pH 6.0 to a concentration of 2 mg/mL. The glycoprotein solutions

were dialysed overnight against the same buffer. To prepare the gel, the stock gel (Affi Gel-15 active ester Agarose) was washed with deionised water twice and the washings were discarded. The glycoprotein solutions (20 mL each) and 20 mL of gel were transferred into 50 mL centrifuge tubes and mixed. After mixing, the suspensions were split into two tubes, each tube with 20 mL, and left overnight on a shaker. The tubes were centrifuged at 771 xg for 15 minutes to collect the gel. From each preparation, the two 10 mL gels were mixed into one tube and filled with 10 mM acetate buffer pH 6.0 to 50 mL, and washed three times. The gel was then washed with distilled water and kept in saline-azide solution at 4°C until use.

A volume of 2 mL of the crude sample that had been concentrated ten times using Microcorn centrifugal filter devices (3 kDa cut-off membranes) was loaded onto affinity columns equilibrated with saline-azide solution. Unbound proteins were eluted using saline-azide solution at a flow rate of 0.5 mL/min. The strongest monosaccharide inhibitor, 0.3 M galactose (in saline-azide solution) was used to elute the bound proteins at a flow rate of 0.5 mL/min. Fractions in which bound lectin was eluted were collected and dialyzed overnight using a cellulose-based dialysis tubing membrane (12 kDa cut-off membrane) in saline-azide overnight at 4˚C. To analyse purity of the lectin fractions, 15% sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). Protein bands were visualised by staining the gels with 0.1% Coomasie Brilliant Blue R250 for an hour and destained by heating in tap water using a microwave oven.

3.2.5.2 Gel Filtration chromatography

Lectin from affinity chromatography was further purified by gel filtration chromatography using a Biogel P-100 column (2 cm x 64 cm). The column was firstly equilibrated with saline-azide solution. The protein that was eluted with 0.3 M galactose solution from affinity chromatography was concentrated ten times. A volume of 2 mL of the lectin sample to be purified was loaded on the column using 5 mL sterile syringes. Bound lectin was eluted with saline-azide solution at a flow rate of 0.5 mL/min. The lectin sample was eluted into two fractions. Pooled fractions were concentrated using Microcorn centrifugal filter device before they were tested for lectin activities by hemagglutination assay. Purity of the lectin was analysed on 15% SDS-PAGE gels.

3.2.6 SDS-PAGE analysis of purified protein

SDS-PAGE was done to check purity of the lectins obtained from chromatography. SDS-PAGE was done also to estimate molecular weight of the lectins. Fractions separated by chromatography were visualised on 15% SDS-PAGE gels. Lectin samples were concentrated 10 times using Amicon Ultra 15 Millipore centrifugal filter devices (10 kDa cutout membranes). Proteins to be electrophoresed were added together with equal volumes of sample buffer (0.06 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.025% Bromophenol blue). Samples were then boiled for 5 minutes before they were loaded on the gels. Molecular weight markers included were β-galactosidase (116 kDa), bovine serum albumin (BSA) (66.2 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa), restriction endonuclease (25 kDa, β-lactoglobulin (18.4 kDa) as well as lysozyme (14.4 kDa). After electrophoresis was completed, 0.1% Coomasie Brilliant Blue R250, in 50% methanol and 10% acetic acid, was used to stain the gels. The gels were suspended in tap water and destained by boiling several times using a microwave oven.

3.2.7 Glycosylation detection

Glycosylation assay was done on the pure lectin sample eluted from gel filtration chromatography. BSA in a concentration of 1 mg/mL, 1 mg/mL Ovalbumin as well as 1 mg/mL Fetuin were also included in the test. Separation of the proteins was done on 12% SDS-PAGE gels, where the gels were run in duplicates. Proteins were transferred from one of the gels onto a nitrocellulose membrane by electroblotting. Transferring was done according to Invitrogen Life Technologies Instruction Manual (2003), where the surface of the gel was firstly made wet with transfer buffer (0.3% Trizma base, 1.44% glycine, 20% methanol) and then a pre-soaked transfer membrane was placed on the gel. A pre-soaked transfer membrane was then placed on top of the transfer membrane. Two pre-soaked blotting pads were placed into the cathode core of the blot module. Two more pre-soaked blotting pads were added to rise over the rim of the cathode core and the anode core was then placed on top of the pads. When transferring, the blot module was filled with transfer buffer until the gel/membrane combination was covered in transfer buffer. The outer buffer chamber was filled with 650 mL distilled water by pouring in the gap between the front of the blot module and front of the lower buffer chamber. The lid was placed on top of the unit

and transferring method using an Invitrogen Zoom Dual Power supply. The proteins were transferred for 60 minutes with an electric current of 25 volts and 100 milliamperes.

After transferring, 4% Bovine serum albumin (BSA) in TBST (TBS with 0.02% Tween 20) pH 7.6 was added to block the membrane for 45 minutes. The membrane was then incubated in 1 mg/mL biotin labelled Con A (prepared in the ratio of 1:2500 in 4% BSA TBS-T) for 45 minutes with gentle shaking. The membrane was then washed 3 times with TBS-T for 15 minutes. The membrane was then incubated for 45 minutes in Streptavidin peroxidase (prepared in the ratio 1:1000 in TBS-T). After the membrane was again washed 3 times, the colour was developed by adding the substrate 3, 3 diaminobenzidine tetrahydrochloride (DAB). A concentration of 0.6 mg/mL DAB was prepared in 50 mM Tris buffer pH 7.6 into which 10 µL of hydrogen peroxidase was added just before use.

3.2.8 Bicinchoninic acid (BCA) assay for protein quantitation

The protein concentrations were determined by BCA protein assay according to Smith *et al*. (1985). The assay was performed in a flat-bottomed microtiter plates using BCA assay kit. BSA standards were prepared in concentrations ranging from 10-1000 mg/mL from a stock solution of 1000 mg/mL. An amount of 10 µL of each protein sample (*S.commune* lectin together with the standards) was firstly added into the wells of the microtiter plate. To each sample, 200 µL of BCA reagent (50 parts reagent A: BCA mixed with 1 part reagent B: Copper sulphate) was added. The plate was then incubated for 60 minutes at room temperature. After the incubation, absorbance was read at 550 nm on a Bio-rad model 550 micro-plate reader. The concentration of the lectin was then estimated by interpolating from the BSA standard curve.

3.2.9 Raising anti-ScL antibodies in rabbits

3.2.9.1 Immunization of rabbits.

Pure *S. commune* lectin solution from Biogel P-100 column was used for development of rabbit antibodies. For initial immunization, 750 µL of 0.6 mg/mL lectin was mixed with an equal volume of Freund's complete adjuvant solution to a total volume of 1.5 mL. Rabbits were immunised after every two weeks over eight weeks. Except for the first immunization, all immunizations were done by adding the lectin with an equal volume of Freund's incomplete adjuvant solution. After eight weeks of immunization, the blood was collected and allowed to clot by incubating at room temperature for an hour. The blood was then centrifuged at 771 xg for 10 minutes and the supernatant was collected and stored at -20 ˚C.

3.2.9.2 Detection of developed antibodies against *S. commune* lectin

The Ouchterlony double immunodiffusion method was used to detect the reactivity of the antisera against the lectin sample. One percent agarose gel (w/v) was prepared in PBS. The gel was poured on the microscopic slide and allowed to set before the three wells were cut on the gel according to Walker (1994). Volumes of 10 µL each of antisera, crude sample as well as the pure lectin sample from Biogel P-100 column were added to the wells. The gel was incubated overnight at room temperature in a petri dish. To prevent the gel from drying out, a wet filter paper was placed together with the slide in the petri dish. The gel was then placed in excess saline-azide solution for 48 hours at room temperature before it was stained (0.1% Coomasie Brilliant Blue R250 (w/v) prepared in 40% methanol (v/v) and 10% acetic acid (v/v)). To distain the gel, a destaining solution containing 10% acetic acid and 40% methanol was used. Activity of the antisera against the two lectin samples was checked by viewing a precipitin formed between the lectins and antisera.

3.2.10 Purification of antibodies by chromatographic methods

To prepare an *S. commune* lectin column, the lectin was firstly dialysed against 10 mM acetate buffer pH 6.0 overnight. The stock gel (Affi Gel-15 active ester Agarose) was washed with deionised water twice and the washings were discarded. The lectin solution in saline azide solution (2 mL) and 2 mL of gel were transferred into a 15 mL centrifuge tube and mixed. After mixing, the suspension was split into two tubes; 2 mL into each tube and left overnight on a shaker. The tubes were centrifuged at 771 xg for 15 minutes to collect the gel. The gels (1 mL each) were mixed into one tube and filled with 10 mM acetate buffer pH 6.0 to 15 mL. The gel was then washed three times. The gel was then washed with distilled water and kept in saline-azide solution at 4˚C until used.

When running the column, a 2 mL column bed was firstly equilibrated by washing with four column volumes of binding buffer, pH 7.2 (0.01 M sodium phosphate, dibasic and 0.5 M sodium chloride). A clear, filtered antibody solution was diluted in the binding buffer in the ratio 1:1 and then applied to the column. To optimise the loading, the column sample loaded was collected and reapplied to the column two more times. The column was then washed with five bed volumes of the binding buffer. The antibody was eluted with 0.5 M acetic acid solution pH 2.4. Eluted fractions were collected in separate tubes, and then a protein assay was performed on each fraction to determine the fractions that contained the protein. The antibody fractions were pooled, concentrated 100 times using Amicon Ultra 15 Millipore centrifugal filter devices (3 kDa and 10 kDa cut-off membranes) and then stored at -60˚C until use. The column was regenerated for reuse by washing with ten column volumes of elution buffer followed by five column volumes of the binding buffer. The binding buffer was then replaced with storage buffer (100 mL binding buffer + 0.01% v/v thimerosal) and the column was stored at 4˚C.

3.2.11 Dot blot assay to determine fractions that contained the antibody

To determine eluted fractions that contained the antibody, 10 µL of the fractions were blotted on the 0.45 µm pore size nitrocellulose membrane. The membrane was blocked by adding 5% skim milk in TBS-T (20 mM Tris-HCL + 150 mM with 0.05% Tween 20) pH 7.5, for 60 minutes at room temperature. After the incubation period, secondary antibody, horseradish peroxidase (HRP) anti-rabbit IgG at the ratio 1: 1000 in TBS-T, was added to the membrane

and incubated for 30 minutes at room temperature. The membrane was washed 3 times with TBS-T and then once with TBS for 20 minutes. The substrate was then added and the antibody-containing fractions were determined by the colour reaction after the addition of the DAB substrate.

3.2.12 Western blot analysis of anti-ScL

Proteins in extracts and chromatography fractions were firstly separated on 15% SDS-PAGE gels. Immunoblot for the detection of antibodies was done by transferring the proteins from one of the gels to the nitrocellulose membrane. To block the membrane, 5% skimmed milk in TBS-T was added to the nitrocellulose membrane followed by incubation for 60 minutes at room temperature. The membrane was then incubated for 30 minutes with the primary antibody, i.e., the anti- ScL (prepared in the ration 1:2000 in TBS-T containing 5% skim milk) at room temperature. The membrane was then washed 3 times with TBS-T, each wash being 5 minutes long. The membrane was incubated with secondary antibody, HRP anti-rabbit IgG (prepared in the ratio 1: 1000 in TBS-T) for 30 minutes at room temperature. The membrane was then washed 3 times with TBS-T and then once with TBS for 20 minutes. The substrate, DAB was then added to the membrane for colour development.

CHAPTER 4

RESULTS

4.1 Screening for lectin activities in crude mushroom extracts

Crude extracts of mushroom samples collected from Tzaneen (TZ), Zimbabwe (ZHR) and University of Limpopo (TRFP) were tested for lectin activities using haemagglutination assays. Assays were performed using 4% rabbit red blood cells. Results were recorded when erythrocytes in the negative control had fully sedimented and appeared as dots at the bottom of the wells. Haemagglutination titer, defined as the reciprocal of the lowest dilution exhibiting haemagglutination, was recorded. Most of the crude extracts tested were able to agglutinate rabbit red blood cells with titers ranging from 2 to 64 (Figure 3). Heat stabilities of the protein extracts were then tested by incubating the extracts for 60 minutes at 95˚C. All the extracts lost 100% of their agglutination activity after an hour incubation at 95˚C (Table 1).

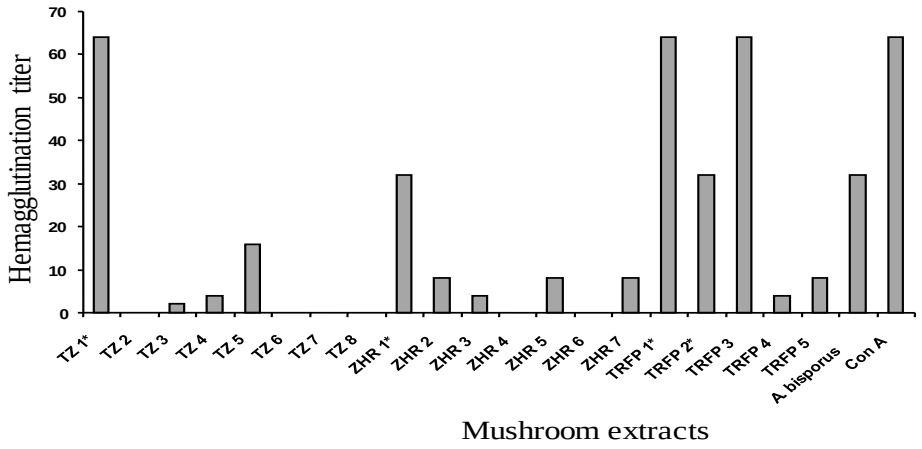

Figure 3: Haemagglutination activities of crude mushroom extracts.

Table 1: Thermal stabilities of crude mushroom extracts after an hour incubation at 95˚C.

Extracts	**Titer for Unboiled extracts**	**Titer for boiled extracts**
TZ 1	64	0
TZ 3	2	0
TZ 4	4	0
TZ 5	16	0
ZHR 1	32	0
ZHR 2	8	0
ZHR 3	4	0
ZHR 5	8	0
ZHR 7	8	0
TRFP 1	64	0
TRFP 2	32	0
TRFP 3	64	0
TRFP 4	4	0
TRFP 5	8	0
Agaricus bisporus	32	0
Con. A	64	0

TZ = Mushrooms collected from Tzaneen, South Africa; ZHR= Mushrooms collected from Harare, Zimbabwe; TRFP= Mushrooms collected from University of Limpopo (Turfloop campus); Con A= Concanavalin A.

4.2 Sugar/ glycoprotein specificities of lectins in selected mushroom extracts.

Heamagglutination inhibition assays were performed on lectin extracts which exhibited positive heamagglutination activities. Activity of most of the lectin extracts was inhibited by glycoproteins and not by carbohydrates (Table 2). ZHR1 was the only extract whose activity was inhibited by carbohydrates, that is, lactose, fructose and galactose.

Table 2: Sugar/ glycoprotein specificities of lectins in selected mushroom extracts.

Extracts assayed	Inhibiting sugars/glycoproteins
TZ4	- Mucin I, Mucin II, Mucin III
TZ 5	- Asiolofetuin, Fetuin, Mucin I, Mucin II, Mucin III
TZ 1	- Mucin I, Asialofetuin, Mucin II, Mucin III, Fetuin
ZHR 1	- Fetuin, Mucin I, Lactose, Fructose,Galactose
ZHR 2	-Thyroglobulin, Fetuin, Mucin I
ZHR 3	- Thyroglobulin
ZHR 5	- Fetuin, Asialofetuin
ZHR 7	- Mucin I, Mucin II
TRFP 1	- Mucin III, Fetuin, Thyroglobulin
TRFP 2	- Thyroglobulin, Fetuin, Asialofetuin, Mucin I
TRFP 3	- Mucin II, Mucin III, Fetuin
TRFP 4	- Thyroglobulin, Asialofetuin, Fetuin, Mucin III
TRFP 5	- Thyroglobulin, Fetuin, Mucin III

Glycoprotein binding specificities of lectin extracts were further investigated using the Enzyme Linked Glycoprotein Assay (ELGA). The assay detects possible low levels of lectins in the extracts. The assay, however, requires biotin-labelled glycoproteins, to which the enzyme conjugate, Streptavidin-peroxidase can bind. To test if the glycoproteins were labelled, a nitrocellulose membrane, from which glycoproteins were blotted, was used. The colour development observed after addition of the substrate confirmed that all the glycoproteins were labelled with biotin (Figure 4). Following the reading of absorbance at 405 nm, it was found that the crude extracts had high affinity towards glycoproteins, mostly, asialofetuin and fetuin (Figure 5).

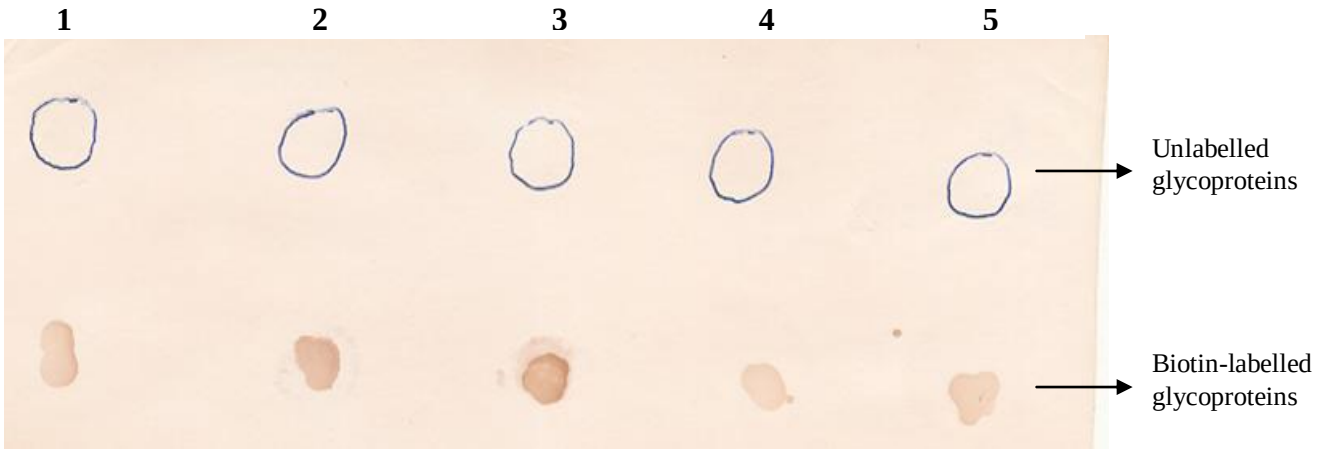

Figure 4: Detection of biotin labelled glycoproteins. 1: Asialofetuin; 2: Fetuin; 3: Mucin I; 4: Mucin II; 5: Mucin III.

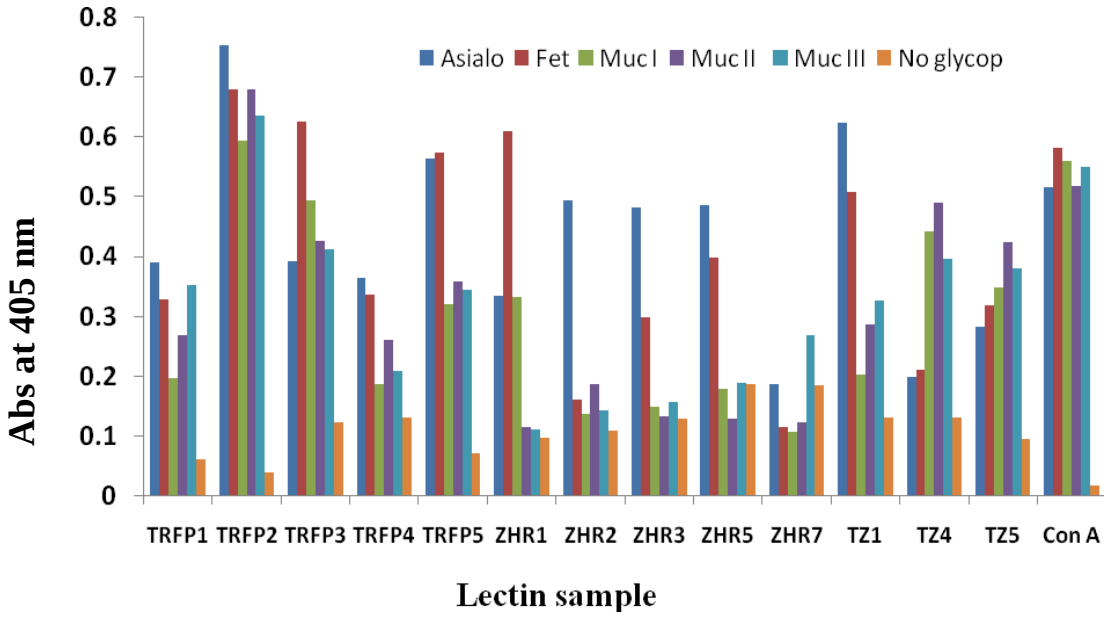

Figure 5: Glycoprotein specificities of mushroom lectin extracts. (■) asialofetuin; (■) fetuin; (■) mucin type I; (■) mucin type II; (■) mucin type III; (■) no glycoprotein.

4.3 Stabilities of selected mushroom lectin extracts.

4.3.1 Thermal stabilities on selected lectin extracts

Thermal stabilities of four selected mushroom lectin crude extracts, that is, TZ1, ZHR1, TRFP1 and TRFP2 were assessed. Loss of activity was observed in all extracts when they were incubated at 95˚C. While lectin extracts retained their activities after 180 minutes of incubation at room temperature, TRFP1 lost 50% of its activity 60 minutes after being incubated at room temperature. ZHR1 was stable after incubation at room temperature; however, the extract lost 100% of its activity when treated at both 60˚C and 95˚C (Figure 9). TZ1 was the most stable lectin extract, which retained 100% of its activity after 180 minutes of incubation at 25˚C and 60˚C. The extract then, lost only 50% of its activity 10 minutes after incubation at 95˚C (Figure 6).

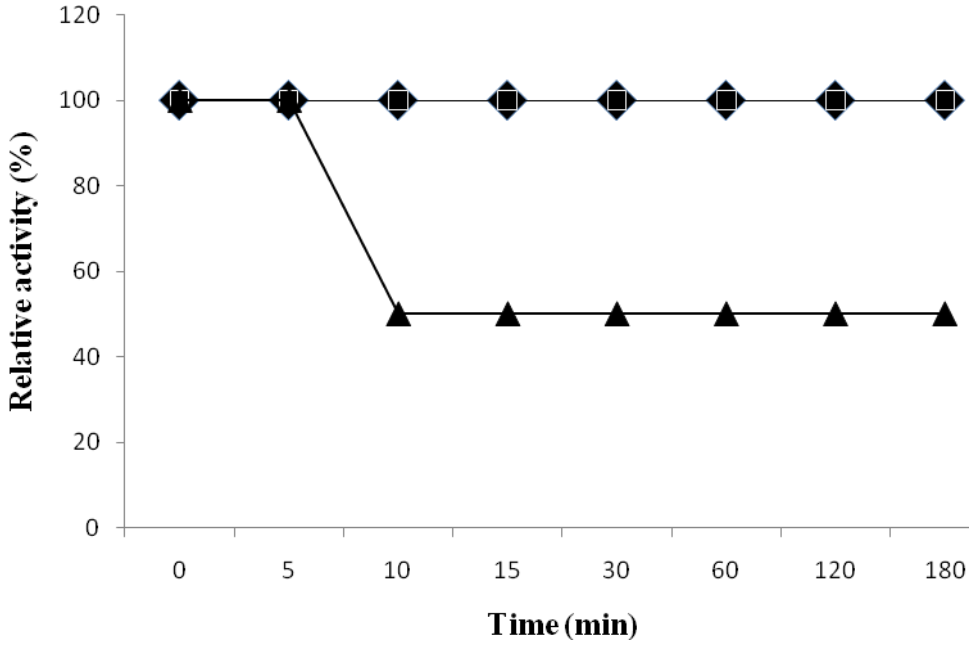

Figure 6: Thermal stability of TZ1 at 25˚C (■), 60˚C (◆) and 95˚C (▲).

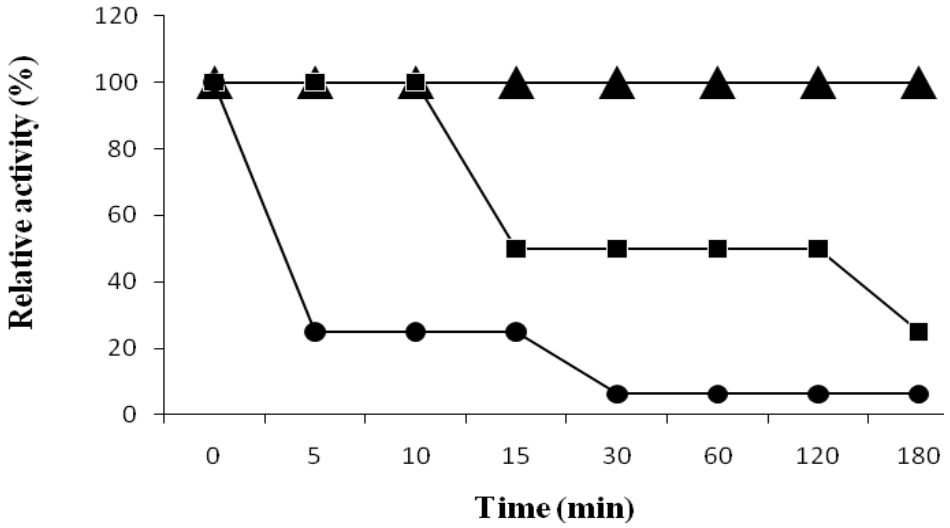

Figure 7: Thermal stability of TRFP2 at 25˚C (▲), 60˚C (■) and 95˚C (●).

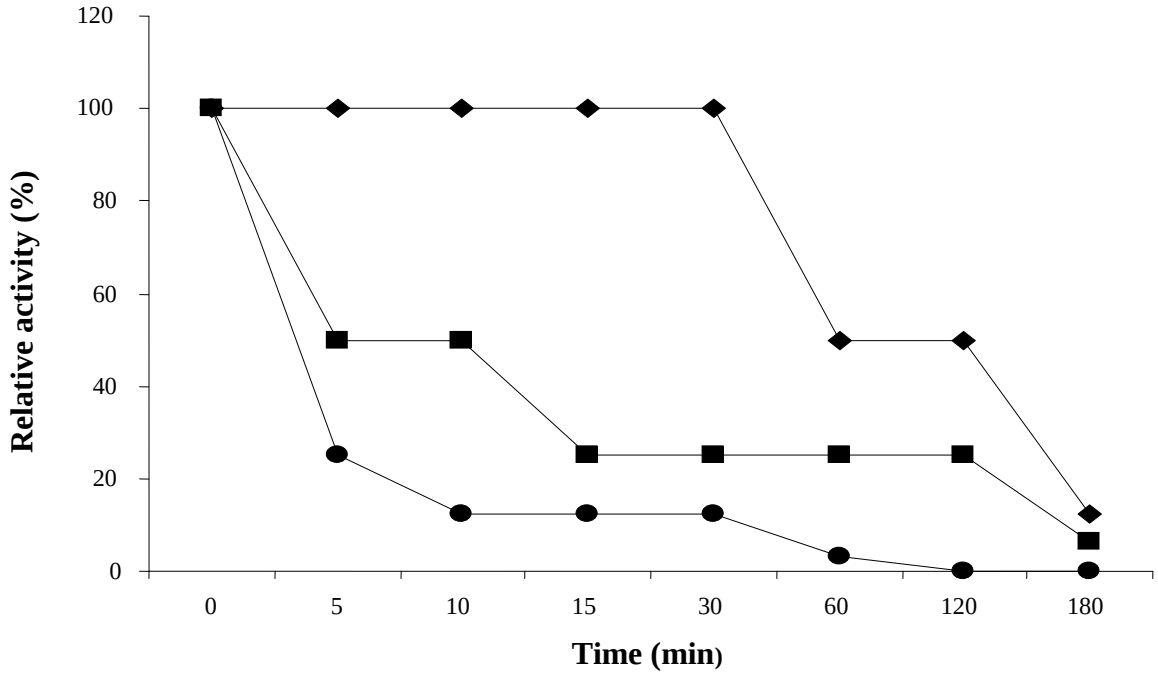

Figure 8: Thermal stability of TRFP1 at 25˚C (◆), 60˚C (■) and 95˚C (●).

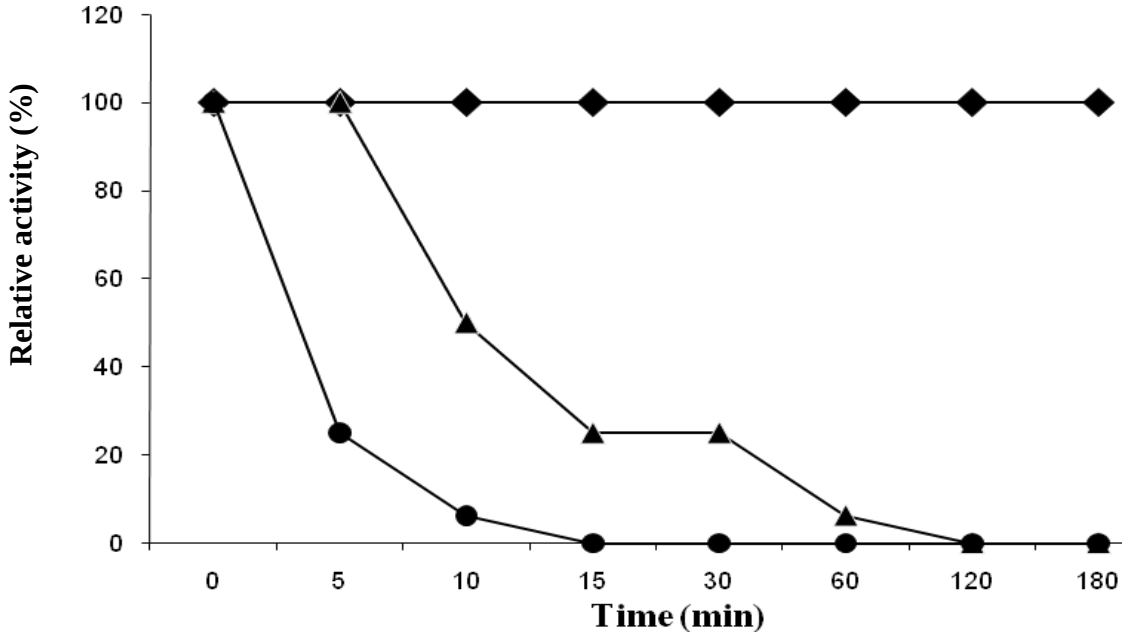

Figure 9: Thermal stability of ZHR1 at 25˚C (◆), 60˚C (▲) and 95˚C (●).

4.3.2 Proteolytic degradation on selected lectin extracts

Susceptibility of four lectin extracts to proteolysis was performed to assess the degradation of lectins by trypsin in a trypsin-N-isopropylacrylamide (NIPAAM) conjugate. Loss of activity was observed on three lectin extracts, of which ZHR1 was completely inactivated 2 hours after it was treated with the conjugate. However, TRFP2 was found to be the only extract that retained 100% of its activity even after three hours of treatment with the conjugate (Figure 11).

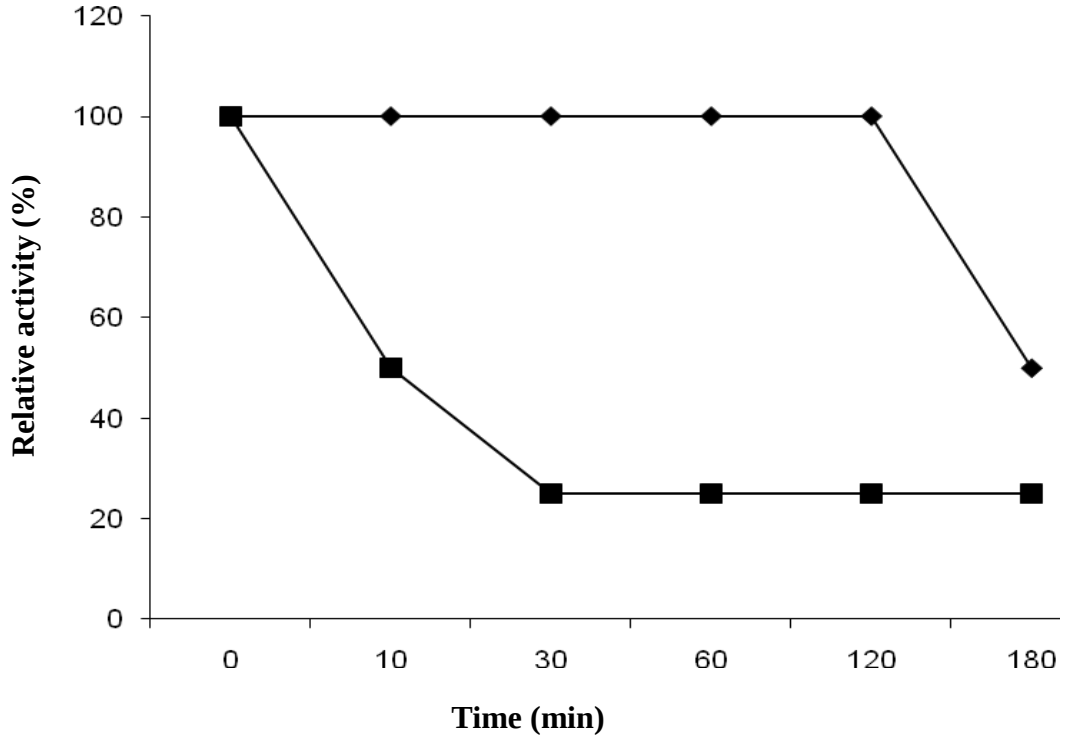

Figure 10: Proteolytic inactivation of TZ1 after treatment with trypsin-NIPAAM conjugate. Untreated TZ1 (◆) and TZ1 treated with trypsin-NIPAAM (■).

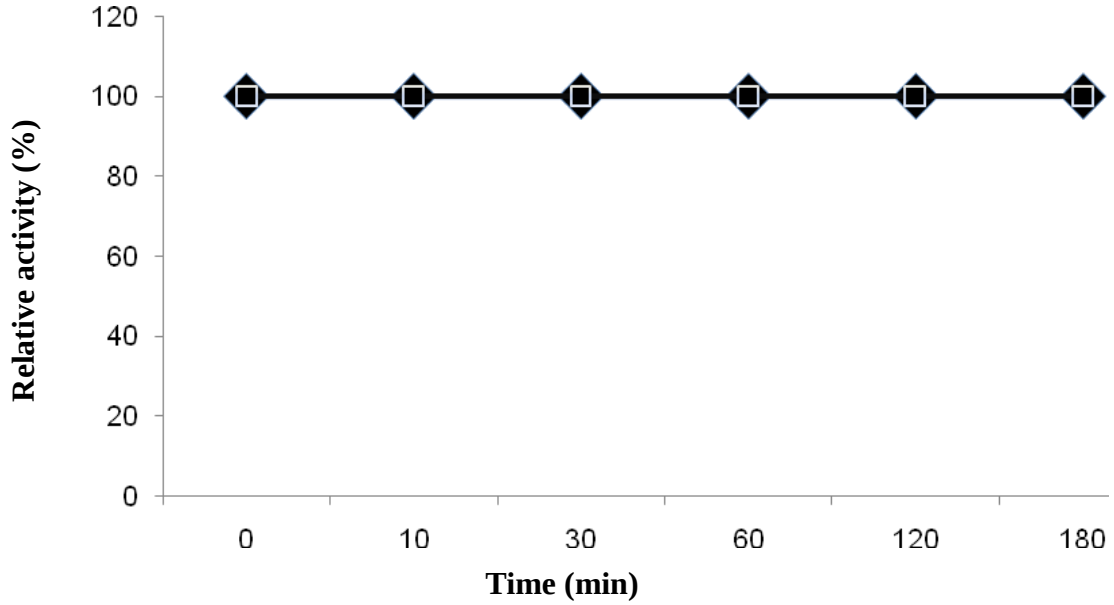

Figure 11: Proteolytic inactivation of TRFP2 after treatment with trypsin-NIPAAM conjugate. Untreated TRFP2 (■) and TRFP2 treated with trypsin-NIPAAM (◆).

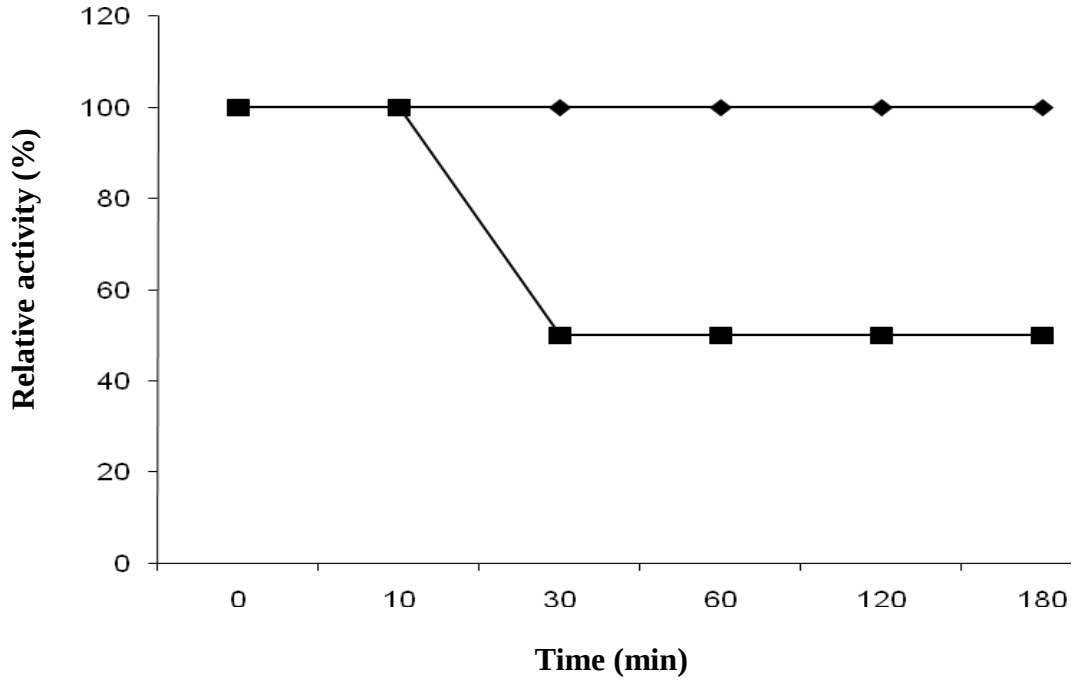

Figure 12: Proteolytic inactivation of TRFP1 after treatment with trypsin-NIPAAM conjugate. Untreated TRFP1 (◆) and TRFP1 treated with trypsin-NIPAAM (■).

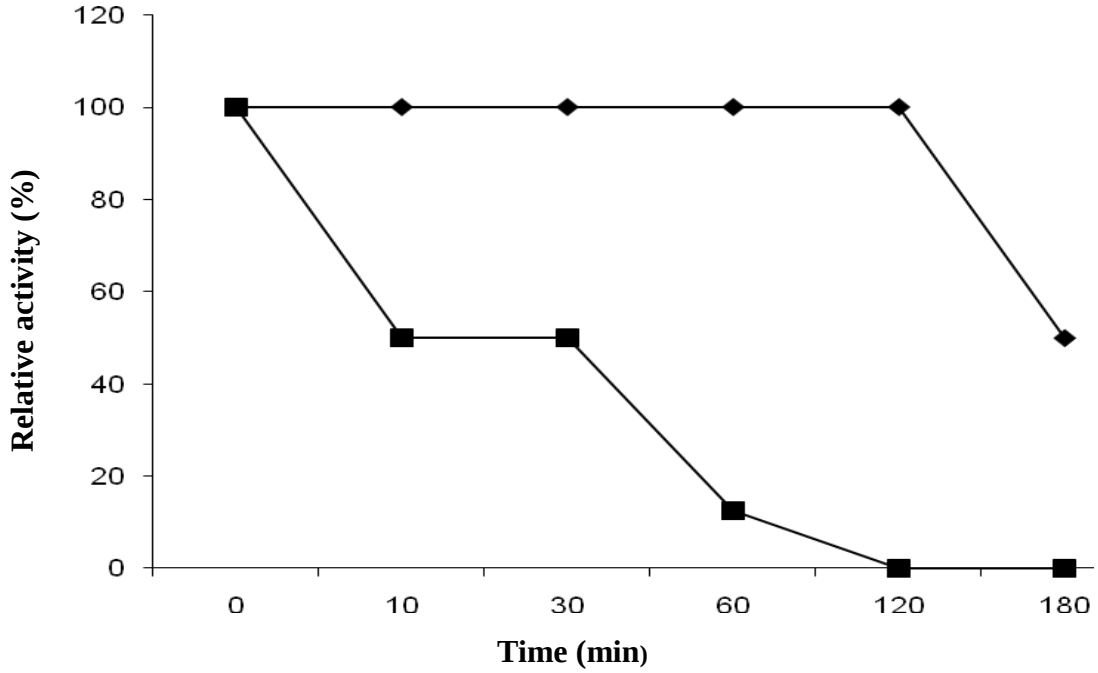

Figure 13: Proteolytic inactivation of ZHR1 after treatment with trypsin-NIPAAM conjugate. Untreated ZHR1 (◆) and ZHR1 treated with trypsin-NIPAAM (■).

4.4 Purification of *S. commune* lectin from ZHR1 crude extract

4.4.1 Determination of the most inhibiting sugar/glycoprotein

ZHR1 exhibited lectin properties which included haemagglutination activities, having binding affinity for some of the sugars and glycoproteins. Furthermore, haemagglutination activity of the lectin extract was destroyed by boiling and treatment with trypsin-NIPAAM conjugate. ZHR1 was hence, selected for identification and further characterizations. The mushroom sample, ZHR1 was identified by the centre of Applied Mycological Studies, University of Pretoria, South Africa, as *Schizophyllum commune*.

The glycoprotein and carbohydrates to which *S. commune* crude extract is most specific to were found to be fetuin and both galactose and lactose, respectively. This was crucial in selecting the column which was most suitable for purification of the lectin from the crude extract as well as in selecting the carbohydrate which was to be used for elution of the lectin. Fetuin inhibited haemagglutination activity of ScL up to the lowest concentration of less than 0.0006 mg/mL. The extract was highly specific to lactose and galactose, which inhibited its activity up to the lowest concentration of 8 mM each (Table 3).

Table 3: Glycoproteins and sugars which *S. commune* crude extract is most specific to.

Sugar/glycoprotein	Lowest concentration inhibiting lectin activity
Lactose	8 mM
Fructose	62.5 mM
Galactose	8 mM
Mucin type I	0.078 mg/mL
Fetuin	< 0.0006 mg/mL

4.4.2 Purification of *S. commune* lectin (ScL) using affinity and gel filtration chromatographies

ScL was purified using a two-step procedure which firstly involved affinity chromatography on a fetuin-agarose column (Figure 14). As revealed on 15% SDS-PAGE gels (Figure 15), the lectin was eluted with some impurities which then suggested further purification. Gel filtration chromatography on Bio-gel P100 column was then used to further purify the lectin (Figure 15).

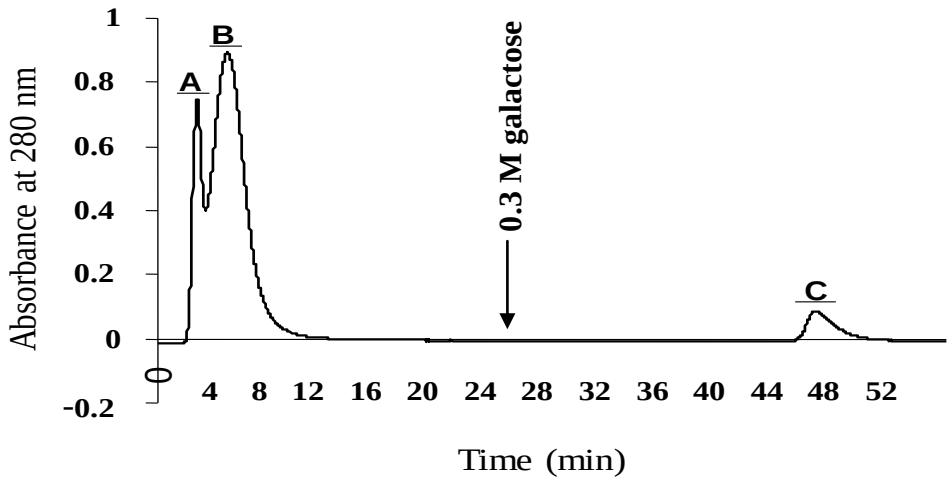

Figure 14: Elution profile of ScL using affinity chromatography on fetuin-agarose column. The bound lectin was eluted using 0.3 M galactose at a flow rate of 0.5 mL/min. A: unbound peak 1; B: unbound peak 2; C: bound peak.

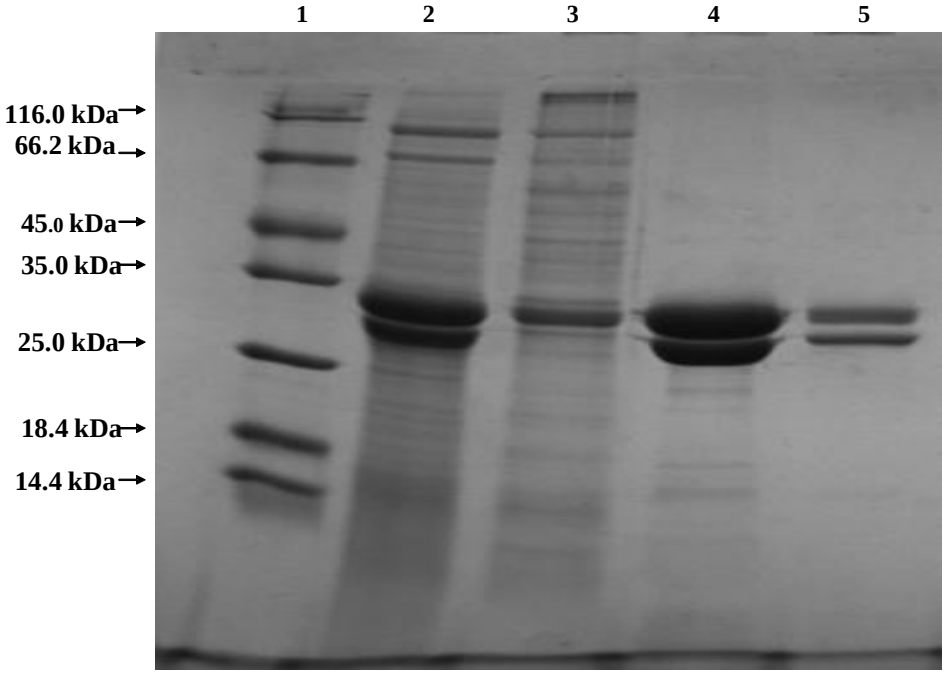

Figure 15: SDS-PAGE gel of *S. commune* lectin. Lane 1: Protein molecular weight marker; lane 2: Crude extract; lane 3: Unbound peak B, lane 4: *S. commune* lectin from fetuin-agarose column; lane 5: lectin from Bio-gel P100 column.

4.4.3 Assessment of purification of ScL using both, fetuin-agarose and mucin-agarose columns.

ScL was purified using both, fetuin-agarose and mucin-agarose columns to determine the most efficient column which is best suitable for purification of ScL. Both, fetuin and mucin inhibit agglutination activities of *S.commune* crude extract. As indicated earlier on, the extract is however, more specific to fetuin than mucin (Table 3). The chromatograms obtained when purifying ScL using the two columns are shown on Figure 16. Fractions collected from each column were analyzed on 15% SDS-PAGE gels. The gels reveal that ScL can be purified using affinity chromatography on both mucin-agarose and fetuin-agarose columns (Figure 17). In contrast to a mucin-agarose column, the use of a fetuin-agarose column requires further purification of the lectin as to get rid of impurities that get eluted with the lectin.

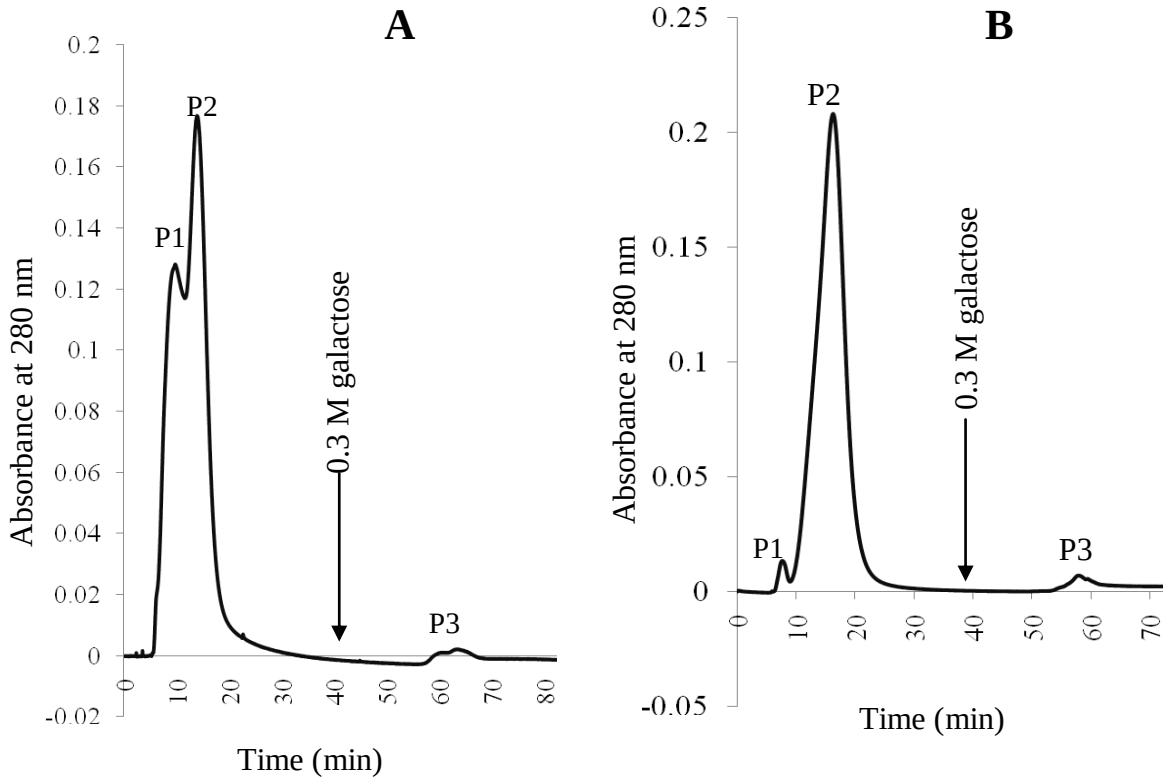

Figure 16: Elution profile of ScL on fetuin-agarose column (A) and mucin-agarose column (B). Bound protein was eluted using 0.3 M galactose at a flow rate of 0.5 mL/min. P1: first unbound peak; P2: second unbound peak; P3: bound peak.

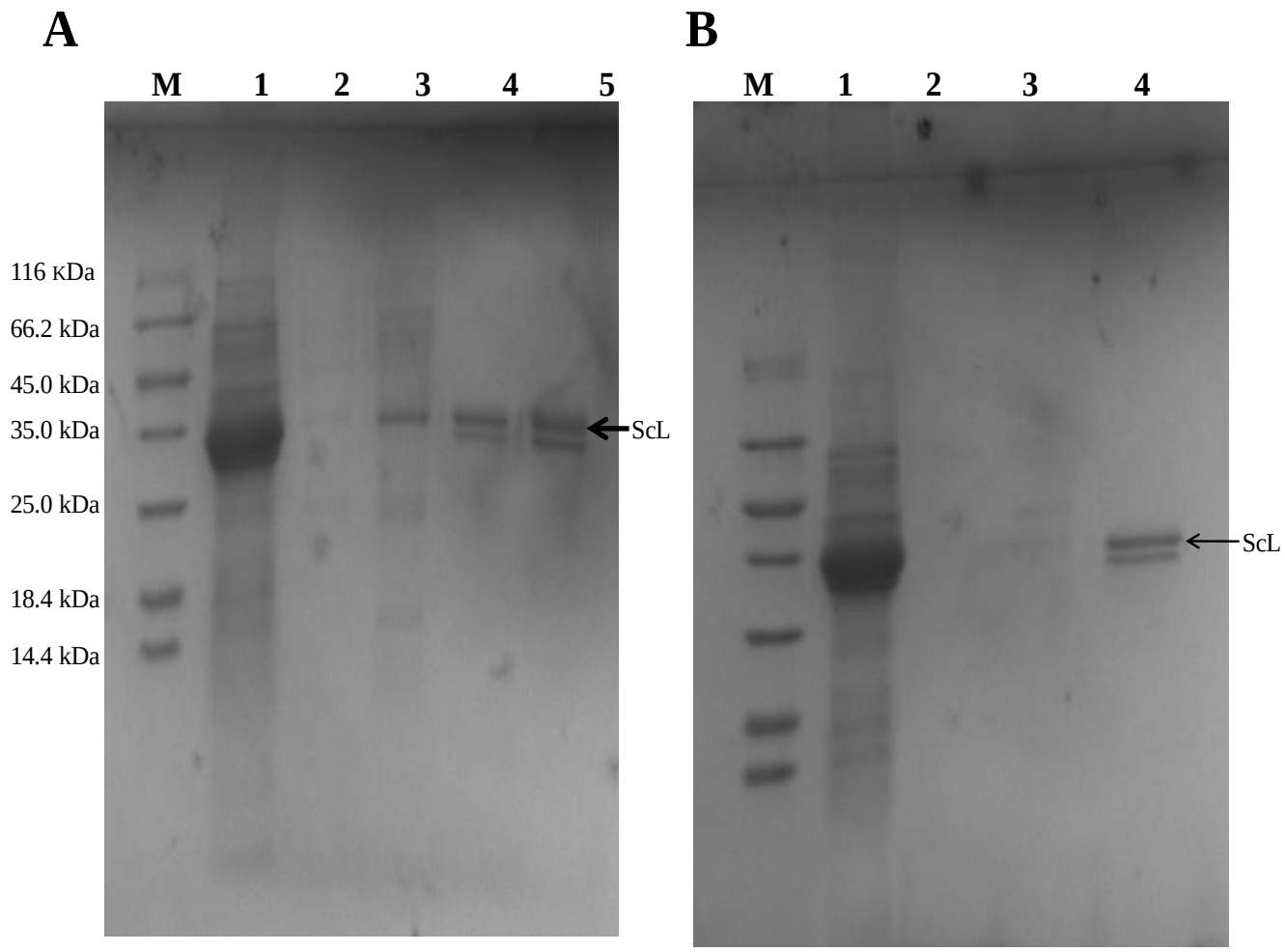

Figure 17: SDS-PAGE gel of ScL purified using a fetuin-agarose column (A) and a mucin-agarose column (B). Lane M: Molecular weight marker; Lane 1: Crude extract; Lane 2: fractions from the first unbound peak; Lane 3: fractions from the second unbound peak; Lane 4: fractions from bound peaks.

4.5 Characterization of *S. commune* lectin

4.5.1 Glycosylation of ScL

Biotin labelled Con A was used in the glycosylation assay on ScL purified using a fetuin-agarose column. Protein bands were firstly separated on 15% SDS-PAGE gels and transferred to a nitrocellulose membrane for blotting with biotin labelled Con A. Following addition of the substrate, it was revealed that ScL is a glycosylated lectin (Figure 16). As expected, fetuin, ovalbumin and some of the protein molecular weight markers were also glycosylated.

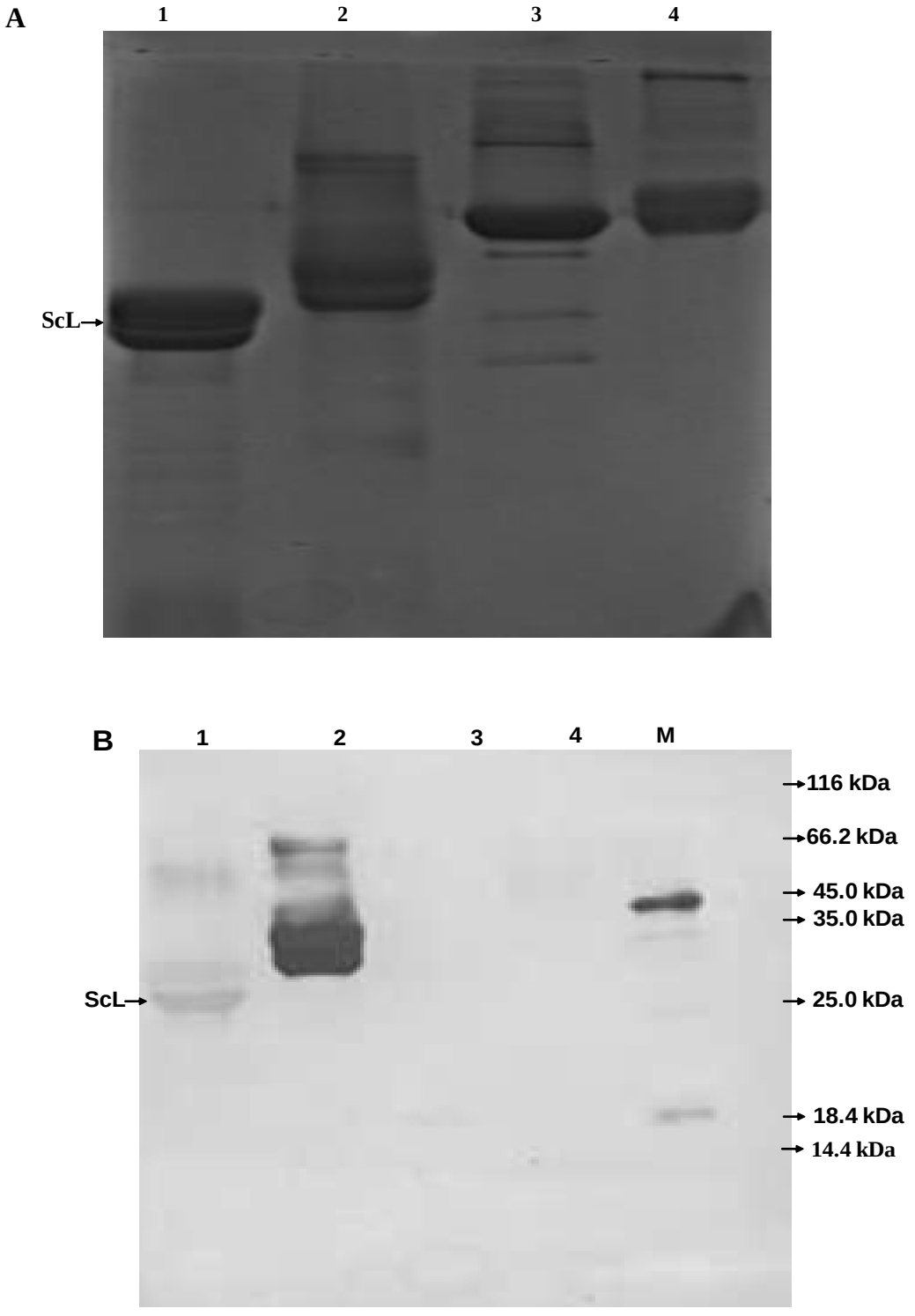

Figure 18: SDS-PAGE gel of ScL on 15% SDS-PAGE gels (A). Lectin blot analysis (B). Lane 1: ScL; lane 2: fetuin; lane 3: ovalbumin; lane 4: BSA and lane M: molecular weight markers.

4.5.2 Stability of ScL

4.5.2.1 Thermal stability

The effect of heat treatment on haemagglutination activity of ScL was determined, where ScL was found to be a heat labile lectin. The lectin lost 50% of its activity after being incubated at 60˚C for 5 minutes, whereas, 100% of its activity was lost after 30 minutes of heating at 80˚C (Figure 19). The lectin however, remained stable at room temperature (Figure 19).

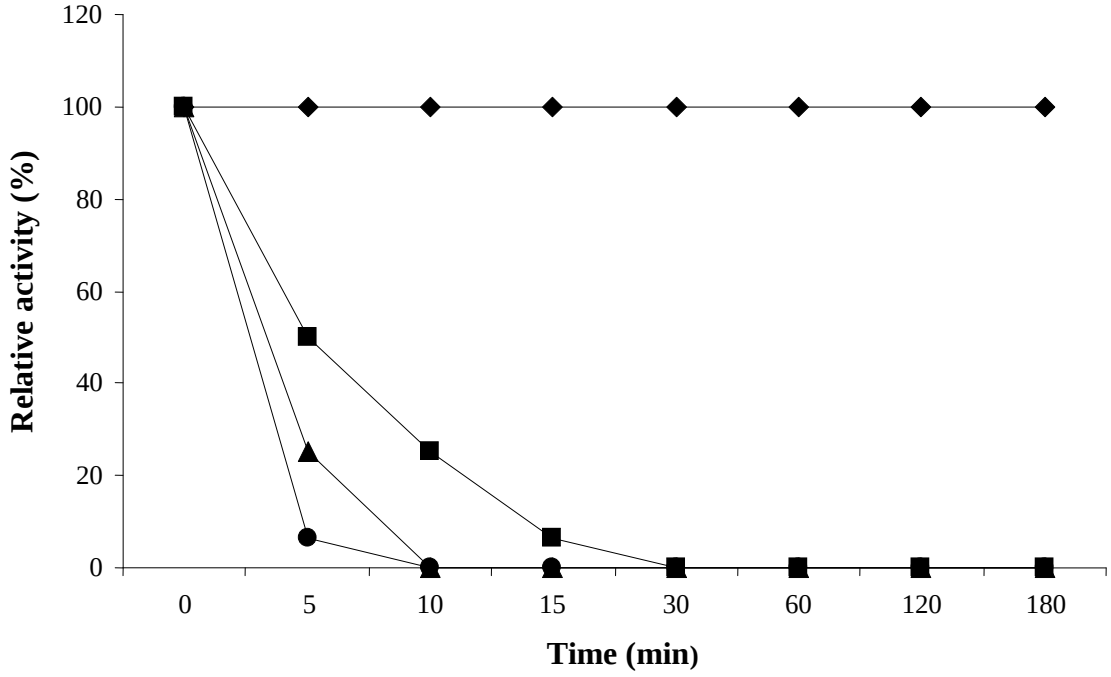

Figure 19: Thermal stability of ScL at 25˚C (◆), 60˚C (■), 80˚C (▲) and 95˚C (●).

4.5.2.2 Assessment of proteolytic degradation on ScL

The effect of proteolytic degradation on haemagglutination activity of ScL was also assessed. The extract was treated with trypsin-NIPAAM conjugate for 180 minutes. The activity of the lectin was completely lost after 30 minutes of treatment with the conjugate (Figure 20).

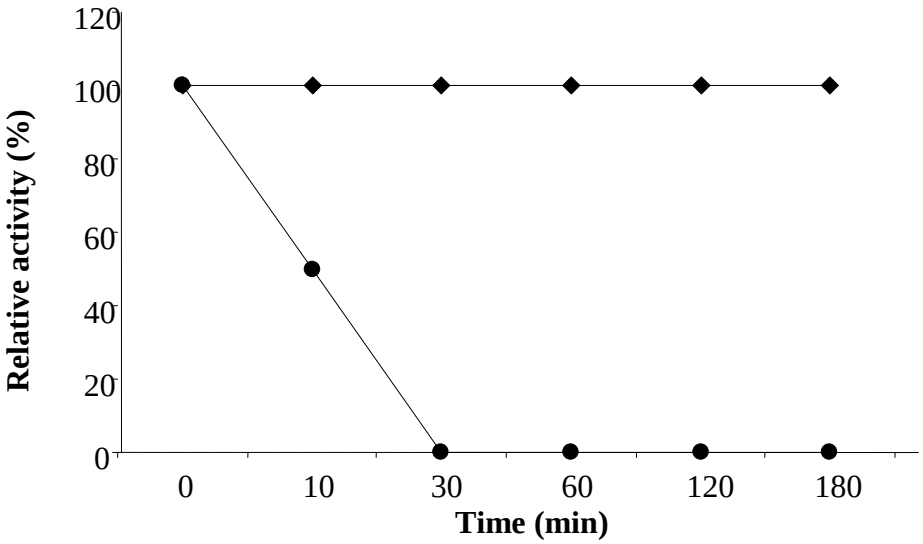

Figure 20: Proteolytic inactivation of ScL by trypsin-NIPAAM conjugate. Untreated ScL (◆) and ScL treated with trypsin-NIPAAM (●).

4.5.3 Immunochemical characterization of ScL

4.5.3.1 Reactivity of antibodies to ScL

Ouchterlony double immunodiffusion assay was performed to detect the presence of ScL antibodies produced. Two precipitin lines observed between (a) the antibody and the lectin crude extract and (b) the antibody and ScL confirmed the interaction between the proteins and the antibody and hence successful production of anti-ScL (Figure 21).

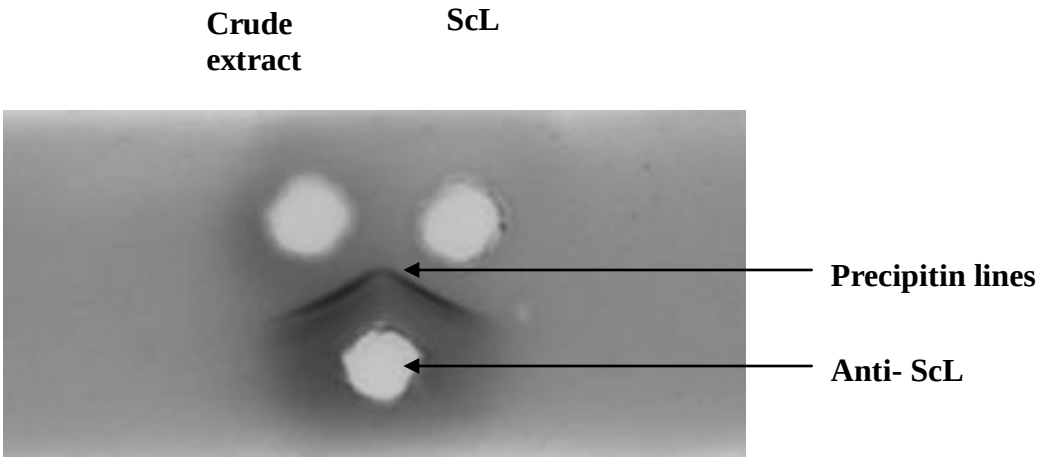

Figure 21: Double immunodiffusion detection of anti-ScL using *S. commune* crude extract and ScL on 1% agarose gel.

4.5.3.2 Purification of anti-ScL from antiserum by chromatographic methods

Anti-ScL was purified using affinity chromatography on an ScL-Agarose column. After elution with 0.5 M acetic acid (figure 22), fractions which were most likely to contain the purified antibody were pooled and blotted on the nitrocellulose membrane to test for the presence of the antibody. Following addition of the substrate, it was revealed that fractions from 132 to 145 minutes contained the antibody (Figure 23).

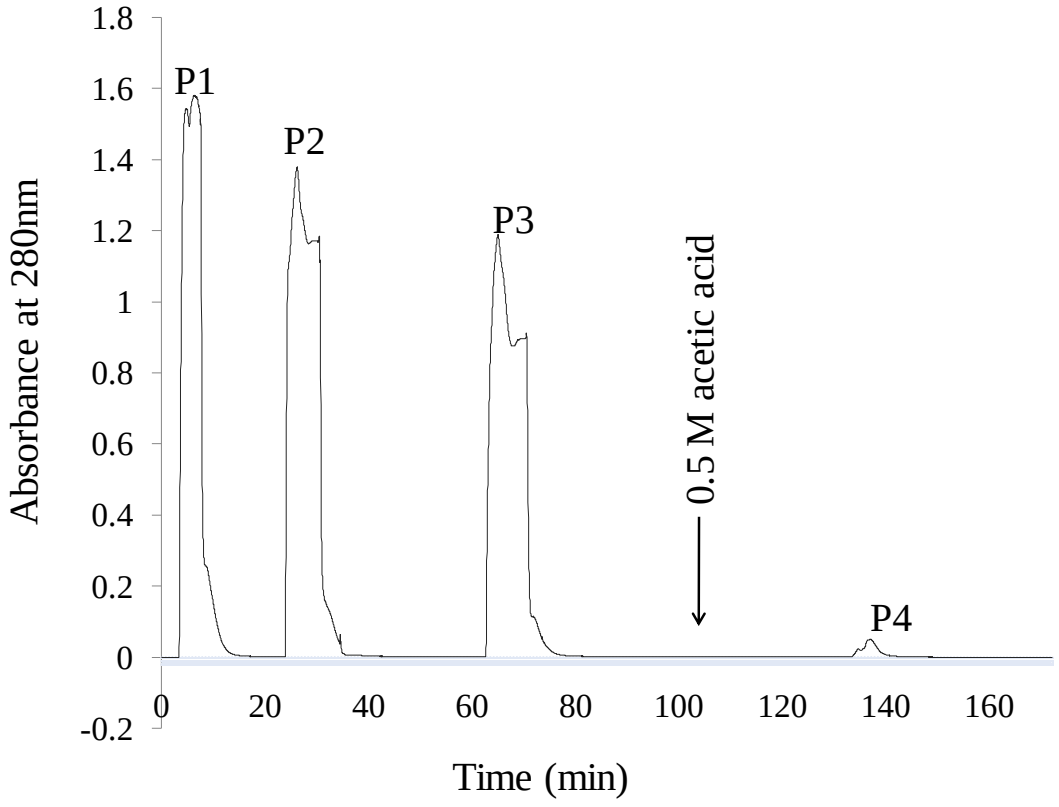

Figure 22: Elution profile of anti-ScL using affinity chromatography on ScL-agarose column. Bound protein was eluted using 0.5 M acetic acid at a flow rate of 0.5 mL/min. P1: unbound peak from the first loading; P2: unbound peak from first reload; P3: Third unbound peak from second reload; P4; Bound peak.

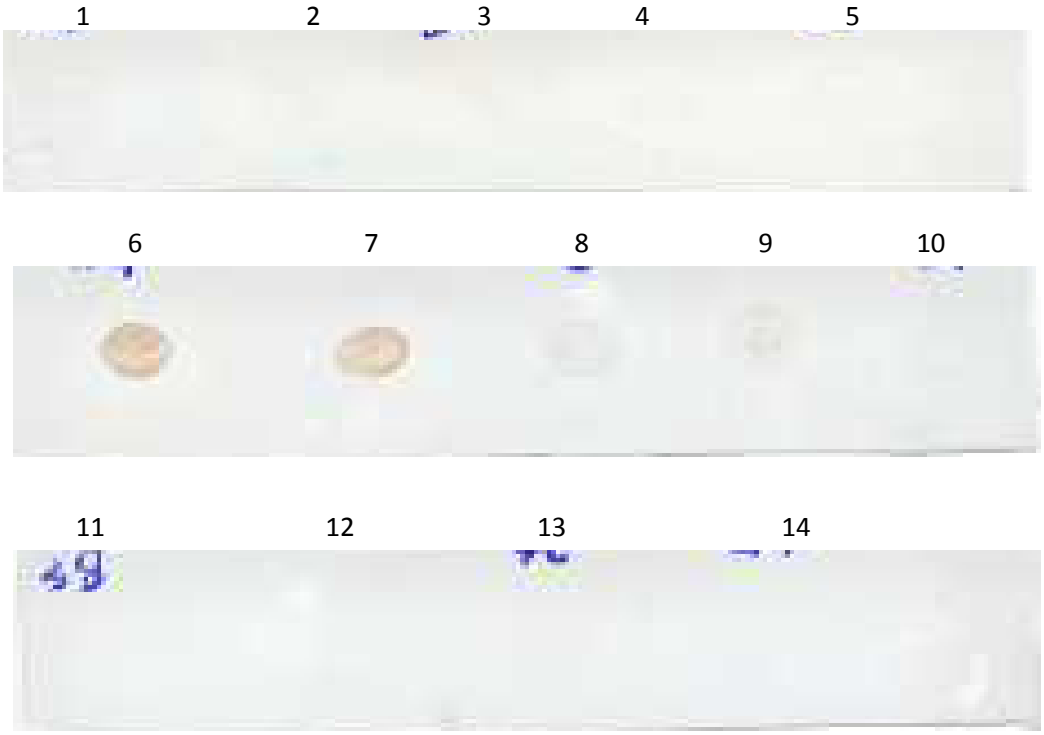

Figure 23: Detection of anti-ScL containing fractions by dot blot assay. 1;2;3;4;5: fractions from time, 120 to 130 minutes; 6;7;8;9: fractions from time, 132 to 145 minutes; 10;11;12;13;14: fractions from time, 146 to 160 minutes.

4.5.3.3 Western blot

Western blot confirmed the interaction of anti-ScL and ScL. The blot also revealed that anti-ScL binds specifically to ScL (Figure 24).

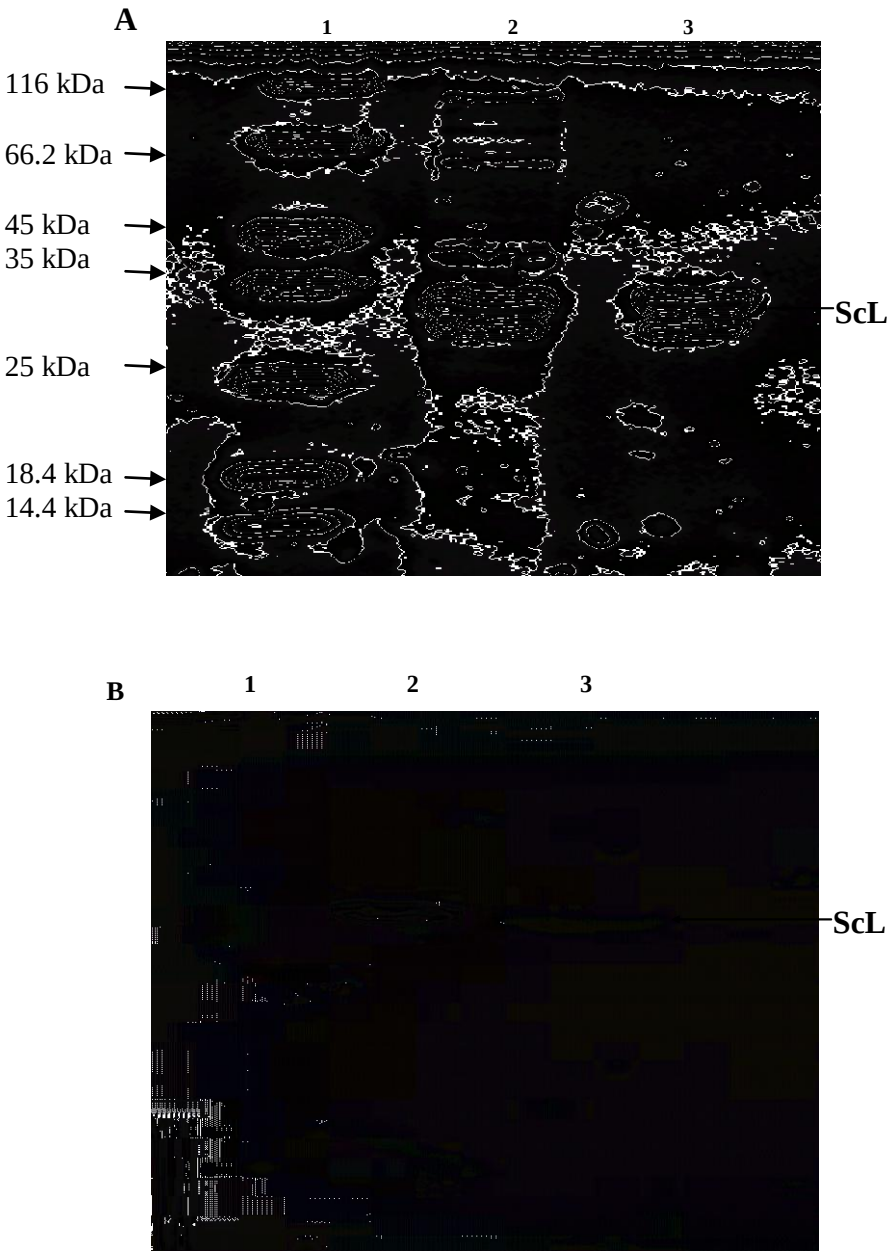

Figure 24: SDS-PAGE gel of ScL (A). Western blot analysis of ScL using rabbit anti ScL antiserum (B) Lane 1: Molecular weight marker; Lane 2: Crude extract; Lane 3: ScL.

CHAPTER 5

DISCUSSION

5.1 Lectin activities in crude mushroom extracts

Haemagglutination of lectins by red blood cells is a major attribute of these proteins (lectins) and it is therefore routinely used for their detection (Lis and Sharon, 1998). Fourteen mushroom lectin extracts revealed agglutination activities against rabbit red blood cells with titers ranging between 2 and 64 (Table 1). Some of the mushroom samples did not exhibit hemagglutination activities. Inability of the extracts to exhibit heamagglutination activities might be due to a number of factors. Firstly, some lectins, such as the monomeric mannose-binding lectin from orchids, contain a single carbohydrate-binding domain and are therefore, incapable of agglutinating red blood cells (Pusztai, 1991). On the other hand, the ability of certain lectins to cause agglutination of cells depends on particularly, the type of erythrocytes used. For example, a mushroom lectin from *Cordyceps militaris*, CML, exhibits agglutination activities towards the mouse and rat erythrocytes and not towards human erythrocytes (Jung *et al.*, 2007).

In a study conducted by Chumkhunthod (2004), lectins were detected by assaying agglutination activities of the samples on different types of red blood cells, that is, human, rat, goose and rabbit red blood cells. High heamagglutination activities were then observed when samples were assayed using rat erythrocytes. Hence, mushroom extracts might be having binding affinities for other types of red blood cells other than the rabbit red blood cells. It was also indicated that heamagglutination assays are not very effective and informative assays particularly for detection of lectins (Wang *et al*., 2009). Unlike the probe-binding assays, heamagglutination assays require multivalent binding between lectin and ligand and are hence applicable to only those lectins that are recognizable to glycans on blood cell surface (Matsumota *et al.* 2001).

Therefore, it could not be concluded as to whether lack of agglutination activities exhibited by some of the mushroom extracts was due to the total absence of lectins in the extracts. Mushroom extracts that did not agglutinate rabbit erythrocytes were therefore not included in further assays. Non-lectin compounds such as polyphenol compounds (florotannins) are

known to be capable of agglutinating erythrocytes (Perez-Lorenzo *et al*., 1998). Hence, to confirm that agglutination in mushroom crude extracts was due to the lectins; extracts were boiled for 60 minutes and assayed for agglutination activities. This resulted in complete loss of agglutination activities in all the extracts (Table 1).

For a long time, the main tool for the study of specificity of lectins and their affinity for ligands was inhibition of heamagglutination (Sharon and Lis, 2003). With regard to their binding affinities towards carbohydrates, lectins can be divided into two groups; those that recognize monosaccharides as well as oligosaccharides, and those that recognize oligosaccharides only (Wu *et al*., 2001). Most of the crude mushroom extracts in the present study form a group of lectins that recognize oligosaccharides only, that is, their heamagglutination activities were inhibited by glycoproteins only and not by carbohydrates. However, ZHR1, showed affinities towards both carbohydrates and glycoproteins (Table 2). Because most of the mushroom samples used in the study are wood-dwelling, it is then assumed that they form part of many microbial pathogens that exploit oligosaccharides displayed on the surface of host cells as receptors for toxin and adhesin (Paton *et al*, 2006).

To confirm glycoprotein binding affinities of mushroom crude extracts, enzyme linked glycoprotein assay (ELGA) was performed. The assay was also useful in differentiating the binding capabilities of lectin extracts among the mucins (mucin I, mucin II and mucin III) as well as between fetuin and asialofetuin. For example, TRFP3 showed high binding affinities towards fetuin and very low affinity towards Asialofetuin (Figure 5). If further investigated, the extract may be a source of the few mushroom lectins that bind specifically to sialic acid derivatives.

Initial screening resulted in selection of four mushroom extracts for further characterizations. TRFP1, TRFP2, ZHR1 and TZ1 were chosen based on their abilities to cause agglutination of rabbit red blood cells and also exhibiting higher titers. Extracts also exhibited binding affinities towards carbohydrates and glycoproteins.

5.2 Stabilities of mushroom crude extracts

Mushroom extracts were assessed for thermal stability and susceptibility to proteolytic degradation. TZ1 was considered the most stable extract which retained 100% of its agglutination activity even after 3 hours of incubation at 60˚C. The extract lost 50% its agglutination activity 3 hours of being subjected to 95˚C. At times, stability of protein crude samples when incubated at higher temperatures might be due to the non-lectin compounds which are able to cause agglutination of red blood cells. Such compounds include tannins, certain lipids, cationic substances and associated carbohydrates in carbohydrate-carbohydrate interactions (Rudiger and Gabius, 2001). However, mushroom lectins that are heat stable, some of which were found to be possessing important biological activities, have been reported. In a study conducted by Chang *et al.* (2007), an immunomodulatory lectin from *Agaricus bisporus* mushroom retained more than 70% of its TNF-α promoting activity as well as its ability to induce nitrogen oxide generation by cells 15 minutes after autoclaving at 121˚C.

While most of mushroom extracts were stable at 25˚C, agglutination activity of TRFP1 was lost at temperatures as low as 25˚C. Loss of activity in the lectin extract could have been due to the presence of proteolytic enzymes which are released into the buffer environment during cellular disruption. Proteases degrade the desired protein leading to protein precipitation and hence, loss of activity (http: //www. opsdiagnostics. com/ notes/ ranpri/ rpproteinstability2. htm, visited on 09/03/11).

Trypsinization of lectin extracts was also studied. TRFP 2 was found to be a very stable extract whose hemagglutination activity was not affected by treatment with trypsin-NIPAAM conjugate. The sample retained 100% of its activity 3 hours after treatment with the conjugate. The high stability of the mushroom sample was also observed when assayed for thermal stability; the sample retained some of its agglutination activities even after 3 hours of exposure to high temperature (95˚C). One such a lectin was previously isolated from the ground bean, *Vigna sesquipedalis* (Wong and Ng, 2003). Similarly to TRFP 2, the 60 kDa ground bean lectin was resistant to trypsinization and its activity was unstable when treated at temperatures above 40˚C. In addition to that, it has been observed that some of the lectins remain active even after passing through the gastrointestinal tract (Woodley, 2000).

However, to date, there has not been any study reported on mushroom lectins that are resistant to proteolytic degradation. Trypsinization of mushroom samples, TRFP 1, ZHR 1 and TZ1 resulted in reduction of their activity. ZHR 1, which was also thermolabile, lost 100% of its activity 2 hours after being subjected to the conjugate.

5.3 Purification and characterization of the lectin, ScL

Of the four mushroom extracts screened, one sample was selected for purification and further characterizations. In addition to the fact that ZHR1 exhibited some interesting lectin characteristics, there was also high quantity of the mushroom fruiting bodies to purify and characterize. ZHR1 was identified as *Schizophyllum commune;* hence, the name *S. commune* was used instead of ZHR1.

Depending on carbohydrate binding specificity of lectins, affinity chromatography can be effectively used for purification of these proteins (Doyle and Slifkin, 1994). First attempt to purify the lectin from *S. commune* crude extract was done using affinity chromatography where a fetuin-agarose column was used. Following the washing off of unbound proteins using saline-azide solution, bound proteins were eluted using 0.3 M galactose in saline-azide solution. Lectin fractions eluted from fetuin-agarose column were purified further using gel-filtration chromatography on Biogel P-100 to remove remaining impurities eluted with a pure lectin from the first chromatographic separation. SDS-PAGE analysis of purified lectin yielded two adjacent bands. The purified lectin is hence, a dimer, composed of subunits with a molecular weight estimated to be approximately 32 and 33 kDa. The lectin was designated ScL. Some lectins consist of two or four subunits that are either identical (homomeric) or slightly different (heteromeric) (Sharon and Lis, 2003). In comparison to certain lectins e.g. those from plants of Viciae tribe, it can be assumed that dimerisation of ScL is as a result of the cleavage of its polypeptides, resulting in an N-terminal, heavy (β) chain and a C-terminal light (α) chain. ScL is hence referred to as a "two-chain lectin" which is distinguished from the one-chain, uncleaved lectins such as a peanut agglutinin (PNA) and soybean agglutinin (SBA) (Sharon and Lis, 2003).

Following concentration of proteins extracts (using the ultra-filtration devices); agglutination activities were assayed on both, the bound and unbound fractions. ScL, with a titer of 2612

units per milligram was able to agglutinate 4% rabbit red blood cells. Unbound fractions were unable to cause agglutination. The inability of unbound proteins to cause agglutination hence suggested that agglutination activity exhibited by the *S. commune* crude sample was entirely based on ScL.

Both galactose and lactose inhibited hemagglutination activity on the extract up to the lowest concentration of 8 mM. Fetuin was able to inhibit agglutination activities of the extract up to the lowest concentration of less than 0.0006 mg/mL. Previously, lectins from the mushroom, *S. commune* were isolated and studied. Lectins were however, purified using different columns; an N-acetyl-D-galactosamine specific lectin from *S. commune* was purified using Porcine Stomach Mucin (PSM)- sepharose column (Chumkhunthod *et al*., 2006) whereas purification of a lactose specific *S. commune* lectin involved different chromatographic methods, that is, the DEAE-cellulose column, CM-cellulose, Q-sepharose as well as Superdex G-75 column (Han *et al*., 2005). ScL used throughout the present study was purified using a fetuin-agarose column.

ScL was purified using the two columns; a fetuin-agarose and a mucin-agarose column to determine if either one of the two columns exhibited better purification of ScL. Protein fractions eluted from the two columns were then analyzed on 15% SDS-PAGE gels. The gels revealed that both columns can be used for purification of ScL from the crude extract. ScL can be purified through a one-step chromatographic process on a mucin-agarose column. The lectin may also be purified through a two-step process that involves affinity chromatography on a fetuin-agarose column and then further purification on Biogel P-100 (Figure 18). A mucin-agarose column, can hence, be considered the most effective column for purification of ScL.

The sensitivity of ScL to high temperatures corresponds with some of the previously isolated mushroom lectins, that is, a 31.4 kDa lectin isolated from ascomycete *Cordyceps militaris* as well as a dimeric lectin isolated from the oyster mushroom, *Pleurotus ostreatus*. The results on heat stability of ScL also correspond with what has been previously reported on heat stabilities of *S. commune* lectins; both the lactose-specific and N-acetyl-D-galactosamine-specific *S.commune* lectins were reported as heat labile lectins. Their agglutination activities were affected when incubated at temperatures of more than 40˚C (Han *et al*., 2005; Chumkhunthod *et al,* 2006). In the current study, ScL lost 100% of its hemagglutination

activity 10 minutes after incubation at 80˚C and 95˚C, whereas 100% of its activity was lost 30 minutes after being subjected to trypsin-NIPAAM conjugate. ScL is thus characterized as a less thermostable lectin.

5.4 Development of anti-ScL antibodies

ScL was used to develop antibodies in the rabbits. Anti-ScL antibodies will be used in future for determination of immunomodulatory activities of ScL and for the detection of ScL in the samples. Ouchterlony double immunodiffusion assay was performed to detect the presence of the specific antibody in the antisera. Precipitin lines observed between (i) the antisera and the crude extract and (ii) antisera and a pure lectin, confirmed that anti-ScL was successfully produced. The antibody was then purified from the antisera using affinity chromatography on ScL-agarose column. The pure antibody was then designated anti-ScL. A single precipitin line that was observed between the antisera and the crude extract (from Ouchterlony double immunodiffusion assay, Figure 21), demonstrates that the antisera recognizes one protein in the crude extract. Western blot was also done to confirm the specificity of the antisera towards ScL. Two bands (an upper strong band and a lower faint band) observed on nitrocellulose membrane after addition of the substrate confirmed that the antisera binds specifically to ScL and not any other protein in the crude extract (Figure 2).

CHAPTER 6

CONCLUSIONS

Three mushroom samples, TRFP1, TRFP2 and TZ1 exhibit some promising lectin activities with varying stabilities towards tryptic ingestion and heat treatment.

ZHR1 was identified as *Schizophyllum commune,* from which the lectin, ScL can be purified.

ScL is characterized as a heat labile, glycosylated dimeric lectin, composed of subunits with a molecular weight of 32 and 33 kDa.

ScL can be purified using affinity chromatography on either a fetuin-agarose column or a mucin-agarose column with further purification using gel filtration chromatography on Biogel P-100 column.

ScL has high binding specificity towards galactose and N-acetyl-D-galactosamine.

ScL is immunogenic; hence, anti-ScL for use in determination of immunomodulatory and other important biological activities of ScL can be developed in rabbits.

CHAPTER 7

RECOMMENDATIONS

Work that needs to be done in future includes:

1. Perform hemagglutination assays on all mushroom samples using red blood cells collected from various sources that is, rabbits, human, rats, cow etc.
2. Identification of TZ1, TRFP1 and TRFP2 as well as purification of the lectins from their crude extracts.
3. Aseptic culturing of mushroom samples using suitable media and substrates to avoid lack of the samples to work on.
4. Characterize ScL by determining its amino acid sequence and hence comparing its profile with other mushroom lectins that are known.
5. Determine biological activities of ScL such as immunomodulatory, antifungal and antibacterial activities.

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